CHARACTERIZATION OF ALTERNATIVE NADH DEHYDROGENASES IN THE RESPIRATORY CHAIN OF *TOXOPLASMA GONDII* AS A NOVEL DRUG TARGETS

Dissertation

zur Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultäten

der Georg-August-Universität zu Göttingen.

vorgelegt von

AHMAD MAHMAOUD HASAN SALEH

Aus

SAIDA – PALÄSTINA

GÖTTINGEN, 2006

D 7

Referent: Prof. Dr. Uwe Gross

Korreferent: Prof. Dr. Gerhard Braus

Tag der mündlichen Prüfung: 31.10-01.11.2006

CHARACTERIZATION OF ALTERNATIVE NADH DEHYDROGENASES IN THE RESPIRATORY CHAIN OF *TOXOPLASMA GONDII* AS A NOVEL DRUG TARGETS

Dissertation

Submitted for the acquisition of Doctorate degree in Biology

Faculty of Biological Sciences

School of Natural Sciences and Mathematics

George August University

Göttingen, Germany

Submitted by

AHMAD MAHMAOUD HASAN SALEH

From

SAIDA – PALESTINE

GÖTTINGEN, 2006

The experimental part of this PhD thesis was done under the supervision of

Prof. Dr. UWE GROSS and Dr. WOLFGANG BOHNE in the

Institute of Hygiene and Medical Microbiology

Georg-August-University of Göttingen

Göttingen – Germany.

2006

TO MY FAMILY, SPECIALLY MY PARENTS

TO MY TEACHERS AND FRIENDS

I hereby declare that this submission entitled "Characterization of alternative NADH dehydrogenases in the respiratory chain of *Toxoplasma gondii* as a novel drug targets" is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher education, except where due acknowledgment has been made in the text.

Göttingen, 18.09.2006 **.....**

AHMAD M. SALEH

LIST OF PUBLICATIONS

1. ORIGINAL PUBLICATIONS

1. **Saleh, A.; Friesen, J., Gross, U.; and Bohne, W.** (2006) Growth inhibition of *Toxoplasma gondii* and *Plasmodium falciparum* by HDQ: a high affinity inhibitor of alternative (type II) NADH dehydrogenases. *Anti. Microb. Chem*. (In press, accepted)

2. **Saleh, A.; Gross, U.; and Bohne, W.** (2006) Identification of two isoforms of alternative (type II) NADH dehydrogenases in *Toxoplasm gondii*. Mol. Biochem. Parasit. (In press, submitted)

2. ABSTRACTS

1. **Saleh, A.**; **Fleige, T., Gross, U., and Bohne, W.** Characterization of the respiratory chain components in *Toxoplasma gondii*. Tagungsband zum 2. Gemeinsamen Kongress der DGHM und VAAM, Göttingen, 25-28.09.2005. *Biospektrum*. p. 155.

2. **Saleh, A.**; **Fleige, T., Gross, U., and Bohne, W.** Characterization of the respiratory chain components in *Toxoplasma gondii*. 2nd Short Course for Young Parasitologists. Vienna, February 20-22nd, 2006. p. 25.

3. **Saleh, A.**; **Fleige, T., Gross, U., and Bohne, W.** Identification and expression analysis of respiratory chain components in *Toxoplasm gondii*. 22. Jahrestagung der Deutschen Gesellschaft für Parasitologie e. V. Wien-22.02 – 25.02.2006. p. 172.

4. **Saleh, A.**; **Fleige, T., Gross, U., and Bohne, W.** Characterization of two isoforms of alternative NADH dehydrogenases (NADH:ubiquinone oxidoreductase) in *Toxoplasma gondii*. Apicomplexan Biology in the Post-Genomic Era. COST Action 857, 3rd Annual Workshop, Dresden, Germany, May 17th -20th , p. 88.

ACKNOWLEDGMENTS

I wish to acknowledge and express my great thanks to Prof. Dr. med. **Uwe Gross** for giving me the opportunity to conduct my PhD thesis at the Department of Medical Microbiology of Georg-August-University of Göttingen. Thank you Prof. Gross for being my 1st referee of this PhD thesis and for being my examiner in Microbiology.

I am especially grateful to Dr. **Wolfgang Bohne**, my direct supervisor, for the design of an interesting project, which I got the opportunity to work on. I am grateful for his patient supervision in the experimental work, his continuous guidance, encouragement, advice and extensive support.

I would also like to thank Prof. Dr. Gerhard **Braus** from the Faculty of Biology for accepting to be the 2nd referee of this PhD thesis, and to be my examiner in Genetics. Many thanks also to Prof. Dr. Ivo Feussner, for accepting to be my examiner in Biochemistry and a member of my thesis committee. Thank you Prof. Dr. Wolfgang **Liebl**, Prof. Dr. Ralf **Ficner**, Prof. Dr. Jörg **Stülke**, and Prof. Dr. Christina **Gatz** for accepting to join my PhD thesis committee.

My special thanks and gratitude go to the Deutscher Akademischer Austauschdienst (**DAAD**) for financial support (DAAD-Scholarship) which gave me the chance to be in Germany and to do my PhD.

Great thanks for my whole family, specially my dear mom and sister Maryam, as well as my dear friends Johannes F., Bashar L., Albert B., Javid D., Muayad M., Sueki M. and Rahhal B. for their consequent and warm assistance and support, without which I would have never made it so far.

Last but not least, I would like to thank all the people in the lab and in the Department of Medical Microbiology for their consequent support, the nice atmosphere, and the good advice. Thank you Tobias F., Karin R., Nicole M., Sandra M., Andrea G., Diana H., Philine K., Polya V., Anja, Frieder S., Maja R., Dr. Wolfgang B., Dr. Raimond L., and Dr. Carsten L.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

ABBREVIATIONS

Ab.	Absorbance
ABM-N	Antibody-Multiplier normal
ABM-S	Antibody-Multiplier special
ADP	Adenosine dinucleotide
AIDS	Acquired immunodifficieny syndrome
Amp	Ampicillin
AP	Alkaline phosphatase
ATP	Adenosine tri-phosphate
ATPase	ATP synthase
ATV	Atovaquone
bag	Bradyzoite antigen
BCA	Bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolylphosphate
BLAST	Basic Local Alignment Search Tool
Ble	Bleomycine
Bp	Base pairs
Brady.	Bradyzoite
BSA	Bovine serum albumin
BSA	bovine serum albumin
BSR4	Bradyzoite surface antigen
CAT	Chloramphenicol acetyltransferase
CCCP	Carbonyl cyanide m-chloro phenyl hydrazone
cDNA	Complementary DNA
CoQ	Coenzyme Q
Cp	Crossing point
CWA	Cell wall antigen
Cy2	Carbocyanin
Cy3	Indocarbocyanin
DHFR	Dihydrofolate reductase
DHFR-TS	Dihydrofolate reductase-thymidylate synthase
DHOR	Dihydroorotate dehydrogenase
DHPS	Dihydropteroate synthase

DMEM	Dubbelco minimal essential medium
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
dNTP	Dinucleotide phosphate
EDTA	Ethylenediaminetetraacetic acid
ENO	Enolase
ER	Endoplasmic reticulum
EST	Expressed sequence taq
EtBr	Ethidium bromide
ETC	Electron transport chain
ETF	Electron transport flavoprotein
FAD	Flavine adenine dinucleotide
FCS	Fetal calf serum
FIC	Fractional inhibitory concentration
FMN	Flavine mononucleotide
Fp	Flavoprotein
FQR	Fumarate quinone reductase
FRD	Fumarate reductase
g	force of gravity
GAPDH	Glyceraldehyde dehydrogenase
gDNA	Genomic DNA
GSH	Glutathione
HDQ	1-hydroxy-2-dodecyl-4(1H)quinolone
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFF	Human foreskin fibroplasts
HPLC	High Performance Liquid Chromatography
HXGPRT	Hypoxanthine-xanthine-guanine phosphoribosyltransferase
I50	Inhibitory 50%
IC50	Inhibitory concentration 50%
IFN-γ	interferon-gamma
Ip	Iron-sulfur protein
IPTG	isopropyl beta-D-thiogalactopyranoside
Kb	Kilo base pair
kDa	Kilo Dalton

lacZ	B-galactosidase
LB	Luria broth
LDH	Lactate dehydrogenase
MOPS	N-morpholinopropane- sulfonicacid
MQDH	Malate quinone dehydrogenase
mRNA	Messenger ribonucleic acid
NAD	Nicotineamide dinucleotide
NBT	Nitroblue tetrazolium chloride
NCBI	National Center for Biotechnology Information
NDB	External NADH dehydrogenase
NDH2	Type II NADH dehydrogenase
Ni-NTA	Nickel-nitrilotriacetic acid
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gelelectrophoresis
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PDH	Pyruvate dehydrogenase
PFAM	Protein family
PV	Parasitophroous vacuole
PYR	Pyrimethamine
Q	Quinone
RE	Restriction enzyme
RNA	Ribonucleic acid
Rpm	Round per minute
RPMI	Roswell Park Memorial Institute Media
rRNA	Ribosomal RNA
RT	Room temperature
RT-PCR	Reverse transcriptase-Polymerase chain reaction
RT-PCR	Reverse transcription PCR
SAG	Surface antigen
SDH	Succinate dehydrogenase
SDS	Sodiumdodecylsulfate
Spp.	Species

SQR	Succinate quinone reductase
Tachy.	Tachyzoite
TAE	Tris Acetate EDTA
TAO	Trypansome alternative oxidase
TATi	Transactivator
TCA	Tricarboxylic acid cycle
TEMED	N,N,N,N –Tetramethyl-Ethylenediamine
TES	N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic Acid
Tet	Tetracyclin
TFB	Transformation buffer
TK	Thymidine kinase
TMHMM	TransMembrane prediction using Hidden Markov Models
tRNA	Transport RNA
Trp	Tryptophan
TTR	Temperature transition rate
U	Unit
UPRT	Uracil phosphoribosyltransferase
UV	Ultraviolet

SUMMARY

The single mitochondrion of *Toxoplasma gondii* generates a membrane potential in the proliferative tachyzoite stage. However, its contribution to energy metabolism in dormant bradyzoites is less clear.

The steady state mRNA level of 11 nuclear genes coding for key subunits of the electron transport chain was quantified by quantitative real-time RT-PCR and compared in tachyzoite versus bradyzoites. No significant difference on the transcript levels between both stages was found when normalized to the expression of β-tubulin.

To facilitate expression studies of energy-associated components, the open reading frame of the following genes was determined and verified by RT-PCR: (i) alternative NADH dehydrogenase I and II; (ii) flavoprotein subunit of succinate dehydrogenase; (iii) cytochrome c1; and (iv) β-subunit of ATP synthase. These genes display highest similarities to orthologs in Plasmodium. They are single copy, nuclear encoded genes with their coding sequence interrupted by multiple introns. The N-terminus of the precursor proteins has characteristics of a mitochondrial targeting pre-sequence. The open reading frame of the five genes was fused with a c-myc tag and brought under control of a tetracycline regulatable promoter. These plasmids were stably transfected into the *T. gondii* TATi-1 line. Co-localization studies showed that the encoded proteins are expressed and transported correctly into the mitochondrion. Expression and localization of TgNDH2-I and TgATP-β were confirmed by mouse antiserum raised against their recombinant proteins. The over-expression of these proteins has no influence on the growth rate of the parasites.

The *T. gondii* genome predicts the presence of typical components of the respiratory chain, except complex I (NADH:Q reductase). Instead, two isoforms of a single subunit, non-proton pumping alternative NADH dehydrogenases, TgNDH2-I and TgNDH2-II, have been identified. The total DNA sequences encompass 2793 and 7686 base pairs and contain open reading frames of 1875 and 1974 base pairs, respectively. The respective genes code for a precursor polypeptides of 618 and 657 amino acid residues with a calculated MW of 67 and 72 KDa, respectively. The open reading frames encoding the isoforms show 58% nucleotide sequence identity, and their gene products share 43% amino acid sequence identity.

The fact that alternative (type II) NADH dehydrogenases of *T. gondii* and *P. falciparum* are absent in mammalian cells makes them promising antimicrobial drug targets. The quinolone-like compound 1-hydroxy-2-dodecyl-4(1)quinolone (HDQ) was recently described as a high affinity inhibitor of fungal alternative NADH-dehydrogenases in enzymatic assays, probably by interfering with the ubiquinol binding site of the enzyme.

In this study, HDQ effectively inhibited the replication rate of *T. gondii* in tissue culture. The IC50 of HDQ was determined for *T. gondii* with two independeant types of growth assays at ~2-8 nM. An important feature of the HDQ structure is the length of the alkyl site chain at position 2. Derivatives with alkyl site chains of C_6, C_8, C_{12} (HDQ) and C_{14} all displayed excellent anti-*T. gondii* activity, while a C_5 derivative completely failed to inhibit parasite replication. Due to the structural similarities, it is most likely that 1-hydroxy-2-alkyl-4(1)quinolones compete with ubiquinones for the same binding site in alternative NADH dehydrogenases. HDQ treatment of *T. gondii*-infected cultures resulted in an induction of bradyzoite differentiation. This effect is well known from other compounds which also inhibit parasite replication and simultaneously induce the expression of bradyzoite specific genes in *T. gondii*. A combined treatment of *T. gondii*-infected cells with HDQ and the antimalarial agent atovaquone, which blocks the ubiquinol oxidation site of cytochrom b in complex III, resulted in a strong synergism. An interference of the mitochondrial ubiquinone/ubiquinol cycle at two different locations appears thus to be a highly effective strategy to inhibit parasite replication. HDQ and derivatives represent particularly in combination with atovaquone promising compounds with high potential for anti-malarial and anti-toxoplasmal therapy.

In the background of the regulatable additional copies, the endogenous genes were targeted for disruption by homologous recombination to generate inducible knock-out mutants. Depletion of *TgNDH2-I* in a conditional knock-out mutant has neither influence on the replication rate nor on the *in vitro* stage conversion, suggesting that TgNDH2-II activity is sufficient for intracellular development. However, slight upregulation of *TgNDH2-II* has been shown on the steady state mRNA in the knock-out mutants compared to the wild type parasites. Moreover, TgNDH2-I depleted parasites display a strongly increased susceptibility for HDQ treatment, where 1 nM HDQ was sufficient to completely inhibit parasite replication. Taken together, we tempt to speculate that the over-expression of the second isoform i.e. TgNDH2-II compensates the loss of TgNDH2-I activity.

INTRODUCTION

1.1 *Toxoplasma gondii*

1.1.1 History, taxonomy, and ultrastructure of *Toxoplasma gondii*

Toxoplasma gondii is an obligate intracellular protozoan pathogen that was first described by Nicole and Manceaux (1908, 1909), working in Tunisia, in the rodent *Ctenodactylus gondi* and by Splendore (1908), working in Brazil, in a rabbit. The species designation originated from the name of the North African rodent (*Ctenodactylus gondi*) from which this parasite was isolated. The genus name *Toxoplasma* is derived from the Greek words *toxon*, meaning "bow or arc" and refers to the crescent shape of the tachyzoite and *plasma*, meaning "form". In 1923, Janku described the congenital form of the disease in an infant with hydrocephalus and microphthalmia (REMINGTON et al., 2001).

Toxoplasma gondii is a member of the superkingdom *Eukaryota*, the kingdom *Protista* and the phylum *Apicomplexa*. Apicomplexans are characterized by their intracellular parasitic lifestyle and the presence of conserved organelles, structures and a complex cytoskeleton at the apical end of the parasite, forming the machinery for host attachment and invasion (DUBEY et al., 1998). Many other protozoan parasites of medical and veterinary or economical importance exist within the phylum *Apicomplexa*, with varying degrees of biological similarity to *T. gondii.* The further taxonomic classification of *Toxoplasma gondii* is as follows: class *Coccidia*, order *Eimeriida*, and family Sarcocystidae (PETERSEN and DUBEY, 2005).

Other members of this phylum include human pathogens (*Plasmodium*: the cause of malaria, *Cryptosporidium:* an animal parasite and an opportunistic pathogen of humans, *Babesia, Cyclospora*, *Isospora*), and animal pathogens (*Eimeria*: the causative agents of chicken coccidiosis*, Theileria*: tick-borne parasites of cattle in Africa, *Neospora,* and *Sarcocystis*)

There are three infectious stages of *T. gondii*: the tachyzoite (the rapidly dividing form) in tissues, the bradyzoite (the slowly dividing form) inside cysts in tissues, and the sporozoites in the oocysts of cat faeces (PETERSEN and DUBEY, 2005). These stages are linked in a complex life cycle.

The term "tachyzoite" (tachos = speed in Greek) was coined by Frenkel (1973) to describe the stage that rapidly multiplied in any cell of the intermediate host and in nonintestinal epithelial cells of the definitive host. The tachyzoite is often crescent shaped, approximately 2 by 6 μm (SMITH, 1995), with a pointed anterior (conoidal) end and a rounded posterior end (**Fig. 1.1**). Ultrastructurally, the tachyzoite consists of various organelles and inclusion bodies including a pellicle (outer covering), apical rings, polar rings, conoid, rhoptries, micronemes, dense granules, micropore, mitochondrion, subpellicular microtubules, endoplasmic reticulum, Golgi complex, ribosomes, rough and smooth endoplasmic reticula, micropore, nucleus, amylopectin granules (which may be absent), and a multiple-membrane-bound plastid-like organelle, the apicoplast. The nucleus is usually situated towards the central area of the cell and contains clumps of chromatin and a centrally-located nucleolus (**Fig. 1.1**).

The conoid defines the apical end of the parasite and is thought to be associated with the penetration of the host cell. Micronemes, rhoptries and dense granules are the three major secretory organelles, found predominately at the apical end of the parasite. Microneme proteins are released very early in the invasion process, facilitating host-cell binding and gliding motility. Rhoptry proteins are also released during invasion, and can be detected within the lumen and membrane of the newly generated parasitophorous vacuole (PV). Dense-granule proteins are released during and after the formation of the PV, modifying the PV environment for intracellular survival and replication of the parasite.

The apicoplast is a plastid-like four-membrane organelle containing a 35 kb circular DNA. Most of the proteins functioning within the organelle are encoded by the nucleus, and are specifically targeted to the apicoplast. This targeting involves the secretory pathway, including the rough endoplasmic reticulum (ER) and a Golgi body situated immediately apical to the nucleus. Targeted proteins have a bipartite N-terminal extension, consisting of an ER signal sequence followed by a plastid transit peptide. *T. gondii* cells have a single nucleus and a single mitochondrion. It is hypothesized that reliance on the mitochondrion for cellular metabolism differs according to the life-cycle stage of the parasite (AJIOKA et al., 2001; DUBEY et al., 1998).

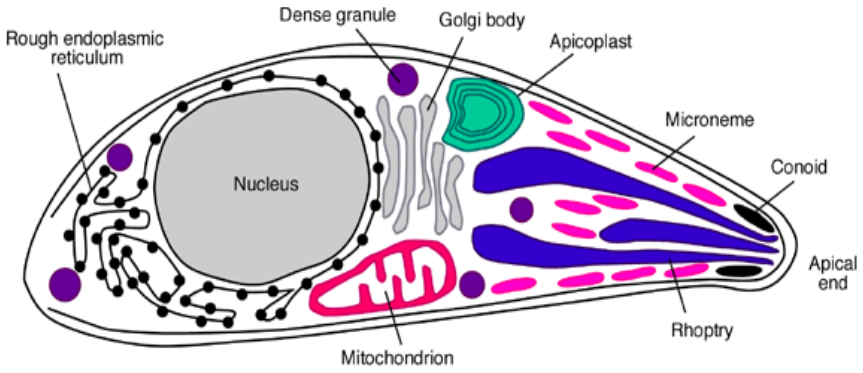


Figure 1.1: Ultrastrcucture of *Toxoplasma gondii* tachyzoite (AJOKA et al., 2001)

1.1.2 Life cycle of *Toxoplasma gondii*

The obligate intracellular pathogen *T. gondii* is unique among apicomplexans because it can invade and multiply in the nucleated cells of virtually all warm-blooded animals (DUBEY et al., 1998; WONG et al., 1993). Its life cycle is divided between feline and nonfeline infections, which are correlated with sexual and asexual replication, respectively (**Fig. 1.2**).

The asexual component consists of two distinct stages of growth depending on whether the infection is in the acute or chronic phase. The tachyzoite stage defines the rapidly growing form of the parasite found during the acute phase of toxoplasmosis. Sequential release of proteins from the three major secretory organelles of tachyzoites, namely the micronemes, rhoptries and dense granules (CARRUTHERS and SIBLEY, 1997), appears to facilitate host cell attachment, invasion and generation of the parasitophorous vacuole (COPPENS and JOINER, 2001). The parasitophorous vacuole provides a safe environment for the tachyzoites to multiply, because it is resistant to acidification and lysosomal fusion. Generation time of tachyzoites is 6 to 8 h (in vitro) parasites exit the cell, usually after 64 to 128 parasites have accumulated per cell (RADKE and WHITE, 1998).

In the infected animal, tachyzoites differentiate into bradyzoites (brady = slow in Greek) and form tissue cysts that first appear 7 to 10 days post-infection. These cysts are found predominantly in the central nervous system and muscle tissue, where they may reside for the life of the host. The development of tissue cysts throughout the body defines the chronic stage of the asexual cycle. Cysts that are ingested through eating of infected tissue are ruptured as they pass through the digestive tract, causing

bradyzoite release. These bradyzoites can then infect the epithelium of the intestinal lumen, where they differentiate back to the rapidly dividing tachyzoite stage for dissemination throughout the body, thereby completing the asexual cycle (DUBEY et al., 1998).

At some frequency within the host, there is apparently a low rate of spontaneous reactivation whereby bradyzoites differentiate back to tachyzoites. Normally, the immune response efficiently prevents the dissemination of these tachyzoites. In immunocompromised hosts, however, such reactivation may be unchecked and/or more frequent, leading to the hypothesis that the parasites might actively detect a lowered immunity against them (GROSS and POHL, 1996). The result, in either case, can be a massive and potentially fatal recrudescence.

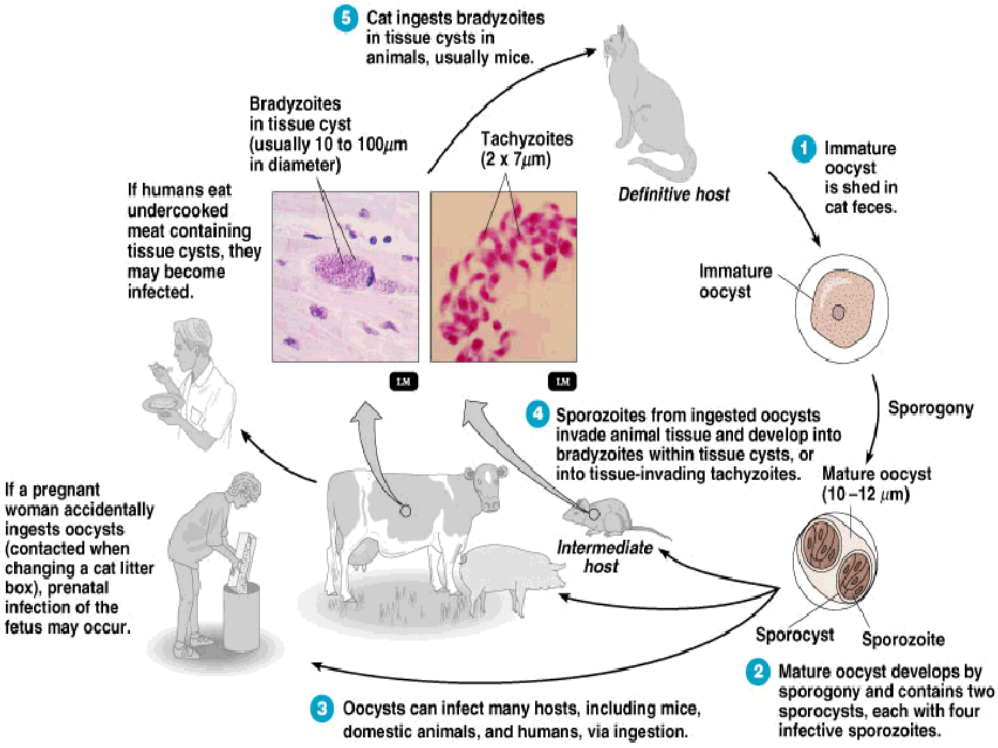


Figure 1.2 Life cycle of *Toxoplasma gondii* (JONES et al., 2003)

1.1.3 Medical importance of *Toxoplasma gondii*

Toxoplasma gondii infection is widespread in humans, although its prevalence varies widely from place to place. In the United States and the United Kingdom, it is estimated that 16 – 40 % of the population are infected, whereas in Central and South America and continental Europe, estimates of infection range from 50 to 90 %,

it is thought that the high difference in the infection rate may be due to differences in the consumption of rare or undercooked meat (DUBEY and BEATTIE, 1988).

T. gondii is transmitted to humans by three principal routes as shown in Figure 1.2 First, humans can acquire *T. gondii* by eating raw or inadequately cooked infected meat, especially pork, and mutton, or uncooked foods that have come in contact with infected meat. Second, humans can inadvertently ingest oocysts that cats have passed in their feces, either from a litter box or from soil (e.g., soil from gardening, on unwashed fruits or vegetables, or in unfiltered water). Third, women can transmit the infection transplacentally to their unborn fetus. In adults, the incubation period for *T. gondii* infection ranges from 10 to 23 days after the ingestion of undercooked meat and from five to 20 days after the ingestion of oocysts from cat feces (LYNFIELD and GUERINA, 1997).

Most infections in humans are asymptomatic but at times the parasite can produce devastating disease. Infection may be congenitally or post-natally acquired. Congenital infection occurs only when a woman becomes infected during pregnancy. Congenital infections acquired during the first trimester are more severe than those acquired in the second and third trimester (REMINGTON et al., 2001). While the mother rarely has symptoms of infection, she does have a temporary parasitemia. Focal lesions develop in the placenta and the fetus may become infected. At first there is generalized infection in the fetus. Later, infection is cleared from the visceral tissues and may localize in the central nervous system. A wide spectrum of clinical diseases occurs in congenitally infected children (DESMONTS and COUVREUR, 1974). Mild disease may consist of slightly diminished vision, whereas severely diseased children may have the full tetrad of signs: retinochoroiditis, hydrocephalus, convulsions and intracerebral calcification. Of these, hydrocephalus is the least common, but most dramatic, lesion of toxoplasmosis. By far the most common sequel of congenital toxoplasmosis is ocular disease (REMINGTON et al., 1995).

Postnatally acquired infection may be localized or generalized. Humans become infected by ingesting tissue cysts in undercooked or uncooked meat or by ingesting food and water contaminated with oocysts from infected cat faeces. Oocyst-transmitted infections may be more severe than tissue cyst-induced infections (SMITH, 1993). Enlarged lymph nodes are the most frequently observed clinical form of

toxoplasmosis in humans. Lymphadenopathy may be associated with fever, fatigue, muscle pain, sore throat and headache.

Encephalitis is the most important manifestation of toxoplasmosis in immunosupressed patients as it causes the most severe damage to the patient (DUBEY and BEATTIE, 1988). Infection may occur in any organ. Patients may have headache, disorientation, drowsiness, hemiparesis, reflex changes and convulsions, and many become comatose. Encephalitis caused by *T. gondii* is now recognized with considerable frequency in patients treated with immunosuppressive agents.

Toxoplasmosis ranked high on the list of diseases which led to death in patients with acquired immunodeficiency syndrome (AIDS); approximately 10% of AIDS patients in the USA and up to 30% in Europe were estimated to die from toxoplasmosis (LUFT and REMINGTON, 1992) Although in AIDS patients any organ may be involved, including the testis, dermis and the spinal cord, infection of the brain is most frequently reported. Most AIDS patients suffering from toxoplasmosis have bilateral, severe and persistent headache which responds poorly to analgesics. As the disease progresses, the headache may give way to a condition characterized by confusion, lethargy, ataxia and coma. The predominant lesion in the brain is necrosis, especially of the thalamus (RENOLD et al., 1992)

Currently, the combination of pyrimethamine and sulphadiazine is the treatment of choice for noncongenital toxoplasmosis, however neither is effective against *T. gondii* cysts. Pyrimethamine targets dihydrofolate reductase enzyme (DHFR) and sulphadiazine inhibits dihydropteroate synthase (DHPS). Unfortunately, owing to the toxic side effects and general low efficacy of these and other known parasiticidal drugs, new chemotherapeutic agents are urgently required (GEORGIEV, 1994).

1.2 Stage conversion of *Toxoplasma gondii*

Fast replicating tachyzoites differentiate into dormant bradyzoites to allow *T. gondii* to persist livelong in the brain tissue of its hosts. Parasite morphology and physiology is significantly altered during this stage conversion (DUBEY et al., 1998). Bradyzoites are characterized by a cyst wall and possess a strongly decreased replication rate compared to tachyzoites. Moreover, they are resistant to chemotherapy and to the immune system.

The development of *in vitro* systems to study bradyzoite-tachyzoite interconversion has opened doors to analyse the precise mechanisms of differentiation. Modifications of the environmental pH, shifting the temperature, IFN-γ treatment, or the inhibition of the mitochondrial respiratory chain induces transition from the tachyzoite to the bradyzoite stage (TOMAVO, 2001)

A significant change of the parasitic gene expression during stage differentiation has been demonstrated in a variety of studies including the monitoring of stage specific antigen expression, transcriptional profile analysis by RT-PCR and array analysis, and comparative EST database analysis (BOHNE et al., 1996; CLEARY et al., 2002; COPPIN et al., 2003; KNOLL et al., 1998; MANGER et al., 1998; MATRAJT et al., 2002; SINGH et al., 2002; SOETE et al., 1993). In contrast to replicative tachyzoites, metabolically dormant bradyzoites are characterized by numerous micronemes, a large number of amylopectin granules, and a nucleus that is located at the posterior end of the parasite (DUBEY et al., 1998). In addition, bradyzoites differ from tachyzoites by the stage-specific expression of certain antigenic components. The surface antigens SAG1 and SAG3 are exclusively found on tachyzoites (GROSS et al., 1996). In contrast, SAG2 has recently been identified to exist as an antigen family which is expressed either on bradyzoites or on tachyzoites, respectively (LEKUTIS et al., 2000); SAG4 and BSR4/p36 are other surface proteins which are -like the cytosolic protein BAG1 - bradyzoite-specifically expressed (BOHNE et al., 1995; KNOLL et al., 1998; ÖDBERG-FERRAGUT et al., 1996).

It is evident that during stage conversion, *T. gondii* converts its metabolism as well as the ultrastructrual morphology. In accordance with this is the finding that important isoenzymes, which are involved in glycolysis, were identified to be stage-specifically expressed. One of these, lactate dehydrogenase (LDH) is a glycolytic enzyme that forms lactate from pyruvate. The two isoforms that are stage-specifically expressed are LDH1 in tachyzoites and LDH2 in bradyzoites. RT-PCR studies demonstrated that *LDH2* mRNA seems to be present only in the bradyzoite stage, whereas transcripts of *LDH1* are present in both stages. However, the LDH1 peptide is only expressed in tachyzoites (YANG and PARMLEY, 1995). Likewise, the enzyme enolase (ENO) is present in two developmentally regulated isoforms. This enzyme catalyses the conversion of 2-phosphoglycerate to phosphoenol pyruvate. In this case, detection of mRNA of *ENO1* was only possible in the bradyzoite stage and ENO2 transcripts were

only found in tachyzoites (DZIERSZINSKI et al., 1999). Finally, levels of the glycolytic enzyme glucose 6-phosphate isomerase were found to be significantly increased in bradyzoites. This enzyme catalyses the inter-conversion of glucose 6-phosphate to fructose 6-phosphate (DZIERSZINSKI et al., 1999)

In addition to proteins that are associated with the individual parasite stage, other antigens have been shown to be exclusively expressed in association with the cyst such as the cyst wall antigen, CWA (GROSS et al., 1995), and MAG1 (PARMLEY et al., 1994).

Besides these antigens, microneme, rhoptry, and dense granule proteins were investigated. However, they seem to be expressed in both parasite stages. Additional stage specifically regulated genes will be identified with the help of recent developments in molecular biology, such as, for example, promoter or gene trap strategies (KNOLL et al., 1998; ROOS et al., 1997)

1.3 Genetics of *Toxoplasma gondii*

1.3.1 Nuclear and organellar genomes of *Toxoplasma gondii*

Genomic research on apicomplexan parasites, including genomic sequencing has generated a wealth of information that will undoubtedly lead to new therapies for these diseases. The genomes of these parasites, like those of other eukaryotic cells, are composed of both nuclear and organellar DNA. *T. gondii* contains a nuclear genome of about 87 Mb, a 6 kb mitochondrial genome, and an episomal 35 kb apicoplast genome (WILSON and WILLIAMSON, 1997).

The nuclear genome is haploid for most of the parasite's life cycle, except for a brief diploid phase in the cat intestine before meiosis (PFEFFERKORN, 1980). The 11 chromosomes are designated by Roman numerals Ib, Ia, II to X, and range in size from approximately 1.8 Mb to >10 Mb (SIBLEY and BOOTHROYD, 1992).

Compared with other protozoans, including related apicomplexans such as *Plasmodium falciparum*, *T. gondii* maintains a remarkably conserved nuclear genome despite a wide host range and nonobligatory sexual cycle.

Current evidence suggests that gene expression in *T. gondii* is transcriptionally regulated (SOLDATI and BOOTHROYD, 1995). Unlike in other protozoan parasites such

as *Trypanosoma* spp. and *Leishmania* spp., polycistronic transcription and RNA editing have not been detected. Although conventional *cis*-acting eukaryotic promoters such as the TATA box or SP1 motif have not been observed, upstream sequence analysis of several genes has identified a common highly conserved T/AGAGACG heptanucleotide core element (SOLDATI and BOOTHROYD, 1995).

T. gondii also contains an extrachromosomal 35 kb (kilobase) circular DNA within an organelle with plastid-like properties. The limited coding capacity of the 35 kb organellar genome suggests that proteins responsible for organelle function(s) must be encoded by the nucleus (AJIOKA, 2001). Scrutiny of *T. gondii* nuclear cDNA sequences has uncovered a number of proteins thought to carry out essential roles in the organelle (e.g. fatty acid biosynthesis).

The mitochondrial genome of *T. gondii* consists of a tandemly repeated element of ~6–7 kb in size and encodes three mitochondrial proteins (subunits I and III of cytochrome *c* oxidase, cytochrome *b)*, a number of short fragments representing the small and the large subunit rRNAs, but no tRNAs. This makes it the shortest mitochondrial genome with the most limited coding capacity known. The great majority of its proteins are nuclear encoded and have to be imported. Proteins are generally targeted to their destination via appropriate targeting signals. (ESSEIVA et al., 2004)

Population genetic analysis of nucleotide polymorphisms has divided the species into three closely related clonal lineages (referred to as Types I, II and III, respectively), with corresponding phenotypic differences (HOWE and SIBLEY, 1995). Acute virulence in mice is restricted to Type I strains (SIBLEY and BOOTHROYD, 1992). Additionally, Type I strains do not readily produce tissue cysts or participate in the sexual cycle, whereas Type II and III strains maintain the ability to complete the entire life cycle.

1.3.2 Functional gene analysis of *Toxoplasma gondii*

Several important biological attributes make *Toxoplasma gondii* an attractive model for the study of intracellular parasitism, development, and functional gene analysis in comparison with other protozoan parasites. First, the parasite is easily manipulated in the laboratory, having the ability to grow productively in virtually any vertebrate cell line. Second, it is possible to produce mutants and to propagate clones indefinitely. Phenotypic analysis of the mutants is aided by the fact that replicative stages are

haploid. Third, molecular genetic studies are supported by DNA transformation using a variety of selectable markers (AJIOKA, 1997).

Within the past decade, studies of *Toxoplasma* have benefited greatly from the development of molecular genetics as a tool for studying intracellular parasitism. As a result, molecular tools for both forward and reverse genetics have been developed to manipulate the genome of *T. gondii*. Available tools for molecular genetic manipulation include a wide variety of selectable markers, integrating an episomal vectors, and high-efficiency transformation systems that permit gene knockouts, insertional mutagenesis, complementation cloning, antisense repression, inducible expression, etc. A variety of markers have been developed to specifically select for transformed parasites without killing the host cells. These markers include chloramphenicol acetyltransferase (CAT) (KIM et al., 1993), dihydrofolate reductase-thymidylate synthase (DHFR-TS), hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT), tryptophan synthase, and phleomycin resistance. These markers have been used in gene replacement strategies via homologous recombination as well as in random insertional mutagenesis.

1.3.2.1 Targeted gene disruption by homologous recombination

Toxoplasma gondii was the first obligate intracellular protozoan to be transformed with exogenous DNA by Soldati and Boothroyd in 1993 (DONALD and ROOS, 1993; KIM et al., 1993; SOLDATi and BOOTHROYD, 1993). They developed a method to transfect the parasites transiently with DNA plasmid including the chloramphenicol acetyltransferase gene (cat) as a reporter under the control of the *T. gondii* p30 promoter. After the electroporation of the tachyzoites with cat-vector, the expression of CAT protein was observed. These results enabled them to produce other plasmid constructs with different promoters from *T. gondii* (SOLDATI and BOOTHROYD, 1993; DONALD and ROOS, 1993). The strength of the transient CAT-expression was dependant on the promoter; the expression of CAT was higher when the tub1-promoter of α-tubuline gene was used instead of p30 promoter.

Other studies have shown that the expression chloramphenicol acetyltransferase, in addition to its reporter function, could be used as a selection marker for stablely transfected parasites. The stable transfection could be achieved through homologous or non-homologous recombination of the vector in the parasite genome. After transfection of the tachyzoites with CAT-expressing vector, which confers resistance

to chloramphenicol, addition of this antibiotic to the culture media will select for stablely transfected parasites which integrated the vector in the genomic DNA (KIM et al., 1993).

Stable transformation can result from either homologous or random integration, making gene disruption and stable expression of transgenes readily achievable. Although the obligatory growth of *T. gondii* in nucleated host cells has restricted the choice of selectable markers, there are sufficient numbers of markers available for selection of stable transfectants. Effective selection markers include chloramphenicol acetyltransferase (cat), dihydrofolate reductase (DHFR) which confers resistance for pyrimethamine, bleomycin (ble), and tryptophan (trp) (DONALD AND ROOS, 1993; KIM et al., 1993; SIBLEY et al., 1994; MESSINAR et al., 1995; SOLDATI et al., 1995; DONALD et al., 1996). Another very good established selectable marker represents the hxgprt gene from *T. gondii*. It codes for the hypoxanthine xanthine guanine phosphoribosyl transferase (HXGPRT), which could be used for positive selection of resistance against mycophenolic acid and xanthine. HXGPRT selection must be performed in mutants lacking HXGPRT. HXGPRT can also be used for negative selection by using 6-thixanthine (DONALD et al., 1996) as can uracil phosphoribosyltransferase (UPRT; DONALD AND ROOS, 1995) and thymidine kinase (TK; RADKE AND WHITE, 1998).

Establishment of such techniques for stable transfection has enabled the deletion of genes and generating mutants, which well known as ‘knockouts’, by homologous recombination. Generating of such mutants would help in studying the function of the target gene and its role for the development or pathogenicity of the parasites.

Generating knockout mutants in *T. gondii* is a difficult process because in most cases the targeting vector integrates randomly in the genome by non-homologous recombination. Therefore, the efficiency of generating a gene deleted mutant is very low. Another possibility studying the functional gene analysis is through the establishment of an inducible expression system, which is based on the elements of the tetracycline resistance operon in *E. coli* (MEISSNER et al., 2001). By tetracycline regulatble expression of the additional copies of target genes, it is possible to generate a conditional and reversible gene deletion mutants.

1.3.2.2 Silencing gene expression by RNA interference (RNAi)

In the last few years, new molecular techniques have been established in order to accomplish purposeful gene studies in different eukaryotic organisms. These are based on the employment of RNA molecules, such as ribozymes, anti-sense and also double stranded RNA.

Apart from the advantage that they are not time- and labor-consuming for production of "knockout" mutants, relatively simple and fast, it makes it also possible to study the analysis of genes essential for the organism. Therefore, the use of RNA techniques leads mainly to a reduction "knockdown" of the gene expression, which is reversible, ensuring the survival of the cells. Such methods could represent a good alternative to the classical methods applied for generation of gene deletion mutants in *Toxoplasma gondii*, since these are very inefficient due to the high rate of non-homologous recombinations (NEKKAR et al., 1999)

The use of antisense RNA for functional gene analysis and regulation of gene expression has already been established in different cell systems including bacteria, *Dictyostelium*, *Leishmania* , *Drosophila*, *Xenopus oocytes* , mammalian cells, and plants. The employment of endogenous anti-sense RNA seems to take place also under natural conditions in eukaryotic cell systems for the regulation of gene expression (Gordon, 2003). Different mechanisms were set up to explain how these RNA molecules can exercise gene regulation. On the one hand, interaction of the anti-sense RNA with complementary transcripts takes place within the nucleus whereby a further processing of these mRNAs and/or also mRNA transport in the cytoplasm is blocked. On the other hand, the formation of RNA duplexes induces the nuclear dsRNase activity which results in a degradation of these dsRNAs. Since the translation of the homologous mRNAs is affected in the cytoplasm by anti-sense RNA, the expression of the appropriate gene products is interrupted. (CORNELISSEN, 1989).

In 1999, the first success of reducing gene expression in *Toxoplasma gondii* by means of anti-sense RNA was achieved (NAKKAR et al., 1999). With the knowledge that an anti-sense-induced mRNA degradation in eukaryotes is predominantly taking place in the nucleus and that the polyadenylation at the 3' - end of mRNA promotes the export of the mature transcripts to the cytoplasm (ECKNER et al., 1991), an efficient method

was developed, where these facts are considered. Eckner et al. (ECKNER et al., 1991) have shown that substituting the normal polyadenylation signal with *cis*-acting ribozymes leads to the nuclear retention of the product RNAs by generating export-deficient transcripts.

1.4 The respiratory chain and oxidative phosphorylation

In aerobic eukaryotic cells, the generation of energy in the form of adenosine triphosphate (ATP) is mainly driven by the activity of the respiratory chain enzymes of the mitochondrial inner membrane (**Fig 1.4**). The respiratory chain, composed of enzyme complexes I to IV, ubiquinone, cytochrome c, and ATP synthase (complex V), transfer electrons from NADH and succinate at one end to molecular oxygen at the other.

1.4.1 Enzyme complexes I - V of the electron transport chain

NADH dehydrogenases (NDHs) constitute one of the electron entry points into the membrane bound respiratory chain, oxidizing NADH and generating ubiquinol. This class of enzymes is divided into two major subfamilies, which can be discriminated on the basis of cofactor content and sensitivity towards rotenone: (i) the type-I NDH, or complex I and (ii) type-II NDHs (NDH-IIs), described in section 1.4.2.

The proton-pumping (type I) NADH:ubiquinone oxidoreductase, also called respiratory complex I, is the first of the respiratory chain complexes providing the proton motive force required for energy consuming processes like the synthesis of ATP. This multisubunit complex (up to 46 subunits) catalyzes the electron transfer from NADH to ubiquinone linked with proton translocation across the membrane. Complex I is characterized by its prosthetic groups, namely one FMN and up to nine iron–sulfur (FeS) clusters, its large number of subunits, and its sensitivity to a variety of natural compounds like rotenone or piericidin A (MIYOSHI, 1998). Homologues of complex I exist in bacteria, archaea and eukarya (FRIEDRICH, 1995).

Complex II, also referred to as succinate ubiquinone oxidoreductase (SQR) in aerobic cells, is an exclusively mitochondrial marker enzyme located in the mitochondrial membrane, or cytoplasmic membrane in the case of bacteria, where it plays a unique role in mammalian type mitochondria as a component of the TCA cycle as well as electron transport chain (HAGERHALL, 1997). Complex II is generally composed of four polypeptides, with the all subunits encoded in the nucleus. The largest

flavoprotein (Fp) subunit and the iron-sulfur (Ip) subunit are hydrophilic and together form a catalytic portion which serves reducing equivalents from succinate for further transfer to quinine (succinate dehydrogenase; SDH) in succinate:quinone reductase (SQR), or those from quinol to fumarate (fumarate reductase; FRD) in fumarate: quinone reductase (QFR). The Fp/Ip portion is bound to the matrix side of the inner of the inner mitochondrial membrane via two small membrane anchoring proteins containing heme *b* (cytochrome b subunits; CybL and CybS). Furthermore, CybL/CybS subunits seem to be essential for electron transfer between the catalytic portion and quinone species (TAKO et al., 2000).

Ubiquinol produced by the action of membrane-bound dehydrogenases such as complexes I, II, and electron transfer flavoprotein-ubiquinone oxidoreductase (ETF Q-reductase) is oxidized by complex III (ubiquinol-cytochrome *c* oxidoreductase or cytochrome bc_1 complex). Complex III in mammalian mitochondria contains 11 subunits, which include a membrane-bound diheme cytochrome *b*, and a membrane-anchored cytochrome c_1 and [2Fe-2S]-containing Rieske iron-sulfur protein. The electrons from ubiquinol are transferred to cytochrome *c* and this reaction develops the proton motive Q cycle. Complex III is thus another of the mitochondrial respiratory complexes where energy is conserved.

The final member of the mitochondrial electron transport chain that generates a transmembrane proton gradient is the terminal cytochrome oxidase (complex IV). Complex IV is a member of a superfamily of heme-copper oxidases found in many bacteria as well as the mitochondrion. The mammalian enzyme contains 13 different subunits, 3 of which are mitochondrially encoded. Complex IV has four redox metal centers, Cu_A, heme *a*, heme a_3, and Cu_B, that are part of a pathway from the substrate cytochrome *c*. Electrons are first transferred from cytochrome *c* to the mixed valence copper center (Cu_A) in subunit II. The electrons are subsequently transferred to cytochrome *a* in subunit I and then to the a_3 Cu_B binuclear active site, also in subunit I, where they reduce oxygen to two water molecules (YOSHIKAWA et al., 2000; LUDWIG et al., 2001).

The final component of the oxidative phosphorylation system of mitochondria is the ATP synthase (complex V or F_1F_0 ATPase). This enzyme is functionally reversible; it can use the proton gradient generated by the electron transport system to synthesize ATP and it can also hydrolyze ATP and pump protons against the electrochemical

gradient. The *E. coli* F_1F_0 ATPase contains 8 different subunits, whereas the bovine enzyme contains 16 different proteins (LUTTER, 1993). Both the bacterial and mammalian enzymes have a proton channel in the F_0 portion, which is linked to the catalytic F_1 portion by a stalk that is necessary for the structural rotation of the F1 portion during catalysis (JUNGE et al., 2001).

1.4.2 Alternative (typ II) NADH dehydrogenases (NDH-IIs)

Alternative non-proton-pumping NAD[P]H dehydrogenases (or type II NADH dehydrogenases) are single polypeptide enzymes that oxidize NAD[P]H originating from either the cytosol [external enzymes] or the mitochondrial matrix [internal enzymes] and feed electrons into the respiratory chain in a rotenone-insensitive manner. These enzymes, using FAD or FMN as a cofactor, have been described in plants (RASMUSSON, 1999; MOLLER, 2001), protozoa (FANG AND BEATTIE, 2002), fungi (VIDEIRA and DUARTE, 2002; KERSCHER, 2000; DE VRIES, 1988), many eubacteria (BJÖRKLÖF et al., 2000, MATSUSHITA et al., 2001) and archaebacteria (GOMES et al., 2001; BANDEIRAS et al., 2002; BANDEIRAS et al., 2003), however they vary widely in number and substrate specificity.

Seven alternative (type II) NADH dehydrogenases have been described in Arabidopsis, three of them identified as internal enzymes, whereas the other four are external (ELHAFEZ et al., 2006). In *Saccharomyces cerevisiae* mitochondria, which lack complex I, one internal and two external enzymes have been described (OVERKAMP et al., 2000). Only a single external NADH dehydrogenase is present, in addition to complex I, in the inner mitochondrial membrane of the fungus *Yarrowia lipolytica* (KERSCHER, 2000), while a single internal NADH dehydrogenase is present in addition to the complext I in the inner mitochondrial membrane of the kinetoplastid *Trypanosoma brucei* (FANG AND BEATTIE, 2003). These and other alternative dehydrogenases have been described extensively and implicated in several physiological phenomena, such as bacterial redox state regulation (HOWITT et al., 1999), cold-stress (SVENSSON et al., 2002) and photosynthetic metabolism (SVENSSON, 2001). It was also suggested that they work as "overflow systems" that keep reducing equivalents at physiological levels and prevent the production of reactive oxygen species (MOLLER, 2001). Despite this, we are still far from understanding their

specific cell functions and the molecular mechanisms underlying their physiological role.

Sequence similarity between alternative NADH:ubiquinone oxidoreductases and lipoamide dehydrogenases from various bacterial sources suggest a common ancestry (BJÖRKLÖF et al., 2000). Both enzyme classes catalyse similar redox reactions, i.e. electron transfer from NADH to ubiquinone and from dihydrolipoamide to NADH, respectively, and contain one molecule of non-covalently bound FAD as redox prosthetic group. The major difference is the absence of a reactive cysteine pair in both classes, which is one of the hallmarks of the FAD dependent NAD(P)H (disulphide) oxidoreductase protein family. Interestingly, the genome of the intracellular parasite *Rickettsia prowazekii* (ANDERSSON et al., 1998), which is believed to represent the closest eubacterial relative of mitochondria, does not contain a gene for an alternative NADH:ubiquinone oxidoreductase. This suggests that alternative NADH:ubiquinone oxidoreductases were most likely contributed by the nuclear genome and not by the endosymbiont.

It was speculated that the eucaryotic alternative NADH:ubiquinone oxidoreductase initially had an external orientation. Species like *Y. lipolytica*, which has only one single external but no internal alternative NADH:ubiquinone oxidoreductase (KERSCHER et al., 1999), may have conserved this original setup.

Subsequently, gene duplication and acquisition of a mitochondrial targeting sequence led to the recruitment of internal alternative NADH:ubiquinone oxidoreductase, as found in all other fungi and plants studied so far. In *S. cerevisiae*, there is good evidence for two consecutive gene duplication events, since the two external enzymes are much more closely related to each other than to the internal enzyme. The first event, leading to the separation of external and internal enzymes, may even have preceded speciation of the ascomycetous fungi. By phylogenetic analysis using the PAUP programme (SWOFFORD, 1992), the external enzymes from *S. cerevisiae* have been grouped together with the external enzyme from *Y. lipolytica* and a putatively external enzyme from *Schizosaccharomyces pombe* (KERSCHER et al., 1999).

Two alternative NADH:ubiquinone oxidoreductases, NDB from *S. tuberosum* (RASMUSSON et al., 1999), which was described as an external enzyme and p64 from *N. crassa* (MELO et al., 1999), which was described as an internal enzyme, were found

to contain an insertion homologous to Ca^{2+} binding EF-hand motifs (MARSDEN et al., 1990). The fact that both insertions reside in similar positions within the proteins makes it most likely that they originated from one single evolutionary event, although a direct alignment of these two insertions fails to reveal significant sequence identity (KERSCHER, 2000). Apparently, the original insertion comprised two consecutive Ca^{2+} binding EF-hand motifs, of which only the first is well conserved in *S. tuberosum* NDB and only the second is well conserved in *N. crassa* p64. Under the assumption that acquisition of a mitochondrial import sequence and insertion of a Ca^{2+} binding EF-hand motif each occurred only once during evolution, the view that *N. crassa* p64 is an internal enzyme should be challenged. This hypothetical scenario for the evolution of alternative NADH:ubiquinone oxidoreductases is summarized in Figure 1.3 (KERSCHER, 2000)

Even some procaryotes, like the cyanobacterium *Synechocystis*, possess two gene copies for alternative NADH:ubiquinone oxidoreductases. One of these is more closely related to *Mycobacterium* homologues than to the other *Synechocystis* gene, suggesting that gene duplication and possibly functional specification occurred very early, probably even before the separation of the two species (RASMUSSON, 1999).

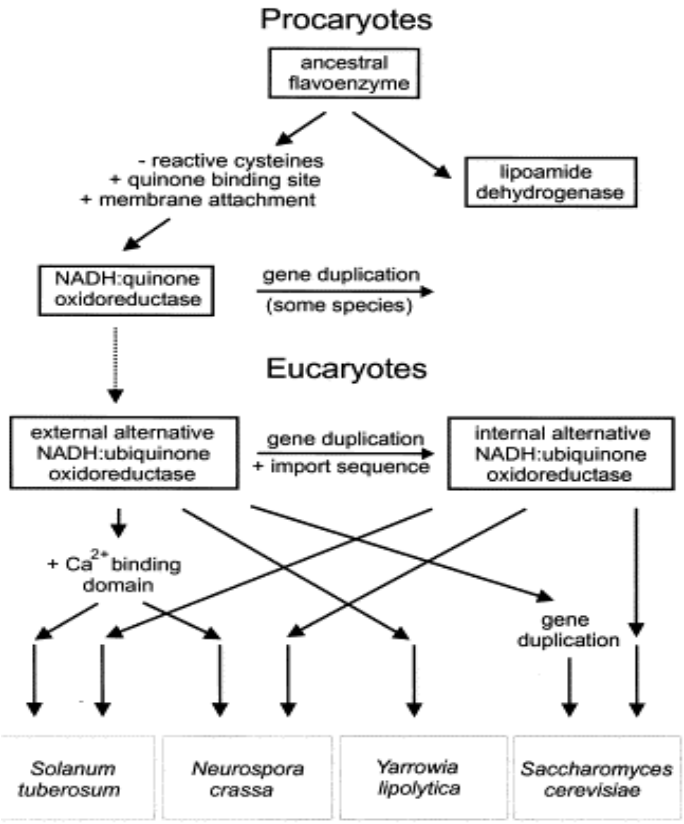


Figure 1.3: Hypothetical scenario for the evolution of alternative NADH: ubiquinone oxidoreductases (KERSCHER, 2000)

Recently, it was found that alternative NADH:ubiquinone oxidoreductase activity is also present in some archeae (GOMES et al., 2001). Evaluation of the question of common ancestry with eubacterial and eucaryotic enzymes will have to await the determination of its complete sequence.

All alternative NADH:ubiquinone oxidoreductase proteins examined so far possess two regions that meet most of the criteria for a dinucleotide binding βαβ fold domain (WIERENGA et al., 1985). One of these lies very close to the N-terminus, the second one lies about 130–145 amino acids further downstream. Both these regions may form the binding site for the non-covalently attached FAD cofactor or the substrate NADH. Their identity is at present unclear, but based on the finding that an N-terminal attachment of an oligo-histidine stretch to the *E. coli* NDH2 protein stabilised the binding of the FAD cofactor, it was speculated that the first βαβ fold domain represents the FAD binding site [BJÖRKLÖF et al., 2000]. This assumption is also supported by the homology of alternative NADH:ubiquinone oxidoreductases with lipoamide dehydrogenases. The structure of the latter enzyme with FAD bound to the first dinucleotide binding βαβ fold domain has been solved by X-ray crystallography.

Characteristic deviations from the βαβ fold consensus are found for both regions in some of the alternative NADH:ubiquinone oxidoreductase homologues. In the first region, the last of three highly conserved glycine residues is replaced by an alanine or serine residue in all the proteins from the ascomycetous fungi *S. cerevisiae*, *Y. lipolytica* and *N. crassa*, but not in the proteins from the plants *S. tuberosum* and *Arabidopsis thaliana*. A striking feature of this region of the *E. coli* NDH2 protein is the insertion of a highly basic stretch of amino acids (RKKKAK). A somewhat similar patch of basic residues is found immediately upstream from the first βαβ fold domain in all proteins from fungi and plants. Although the function of these basic amino acids is unclear, it was speculated that they might be necessary to stabilise the binding of the cofactor FAD (KERSCHER, 1999). The second βαβ fold domain includes an unusually large loop region. Again, the significance of this feature is unclear.

The mode of interaction with the hydrophobic substrate ubiquinone and the nature and localisation of the quinone binding site are unknown. Consensus patterns for quinone binding sites proposed by Rich and Fisher (RICH AND FISHER, 1999) cannot be found in alternative NADH:ubiquinone oxidoreductases. A tryptophan (W337 in the SCNDI1 precursor) that is conserved between all known alternative NADH:

ubiquinone oxidoreductases has, by analogy with the bacterial photoreaction centre (DEISENHOFER and MICHEl, 1989, 1991), been proposed to be involved in ubiquinone binding (DE VRIES, 1992).

Transmembrane helices have not been detected in the alternative NADH:ubiquinone oxidoreductases known so far. A segment of the *E. coli NDH2* protein was found to show a certain degree of homology to a segment of (*S*)-mandelate dehydrogenase from *P. putida* (FINEL, 1996) which, based on mutagenesis studies, had been suggested to form the membrane attachment domain of the latter enzyme (MITRA et al., 1993). However, since this segment is not well conserved among various alternative NADH:ubiquinone oxidoreductases, this suggestion was in the following deemed less likely by the same authors (BJÖRKLÖF et al., 2000). It is unclear, therefore, how these proteins interact with biomembranes .

Mitochondrial import of the SCNDI1 protein has been studied in detail (DE VRIES, 1992). It involves the cleavage of a 26 amino acid targeting sequence, probably by the matrix processing peptidase and is dependent on the membrane potential component of the proton-motive force. Processing also occurs during mitochondrial import of the internal NDA protein of *S. tuberosum*, although the cleavage site has not been determined (RASMUSSON, 1999). These results indicate that the import pathway of this internal alternative NADH:ubiquinone oxidoreductase is very similar to the import of subunits of respiratory chain complexes (HARTL et al., 1989). How and when the FAD redox prosthetic group is inserted, is unknown.

Much less is known about the targeting of external alternative NADH:ubiquinone oxidoreductases to the outer face of the inner mitochondrial membrane. Targeting of the external *S. tuberosum* NDB protein does not seem to involve removal of a presequence, since no size difference was observed between the precursor and the mature protein (RASMUSSON et al., 1999). On the other hand, preliminary data on the purification and N-terminal protein sequence of the SCNDE1 protein indicate that this external alternative NADH:ubiquinone oxidoreductase is processed by the removal of the first 41 amino acids (LUTTIK et al., 1998). This difference may be related to the fact that both external enzymes from *S. cerevisiae* possess N-terminal extension that exceed the length of the SCNDI1 presequence by 30 (SCNDE1) and 45 (SCNDE2) amino acid residues, respectively. No data are available regarding the targeting of

external alternative NADH:ubiquinone oxidoreductases from other sources, such as *Y. lipolytica* and *N. crassa*.

Association of alternative NADH:ubiquinone oxidoreductases with the mitochondrial inner membrane may involve the C-terminal part of the protein, which interestingly is not conserved between these enzymes and the water-soluble lipoamide dehydrogenases from bacteria. *S. tuberosum* NDA, expressed in *E. coli* with an N-terminally attached S tag, became unable to bind to biomembranes when the C-terminal part was lost by proteolytic degradation (RASMUSSON et al., 1999).

A different mode of membrane association may occur in the alternative NADH:ubiquinone oxidoreductases that contain Ca^{2+} binding EF-hand motifs. It has been proposed that Ca^{2+} binding facilitates association with the mitochondrial inner membrane, thereby regulating the activity of the enzyme (RASMUSSON et al., 1999). Interestingly, of the two alternative NADH:ubiquinone oxidoreductases containing Ca^{2+} binding EF-hand motifs known so far, *S. tuberosum* NDB was described as an external and *N. crassa* p64 as an internal enzyme.

Inhibitors for alternative NADH:ubiquinone oxidoreductases are rare and mostly unspecific. It is a well established fact that classical inhibitors of complex I do not inhibit the alternative enzyme. Flavone, which inhibits SCNDI1 with an I_{50} of 95 µM [DE VRIES, 1988], is known to inhibit other NADH dehydrogenases as well. A study on the inhibitory effect of hydroxyflavones on the external alternative NADH:ubiquinone oxidoreductase of plants (RAVANEL, 1990) revealed that platanetin (6-dimethylallyl-3,5,7,8-tetrahydroxyflavone) is a potent inhibitor of this enzyme (I_{50}=2 µM). It remains unclear, however, whether specificity for the external alternative enzyme is caused by selective affinity or by the inability of platanetin to permeate across the inner membrane of the intact potato tuber mitochondria used in this study. Thus, additional specific inhibitors would certainly be valuable tools for further studies, especially on the quinone binding sites of alternative NADH:ubiquinone oxidoreductases.

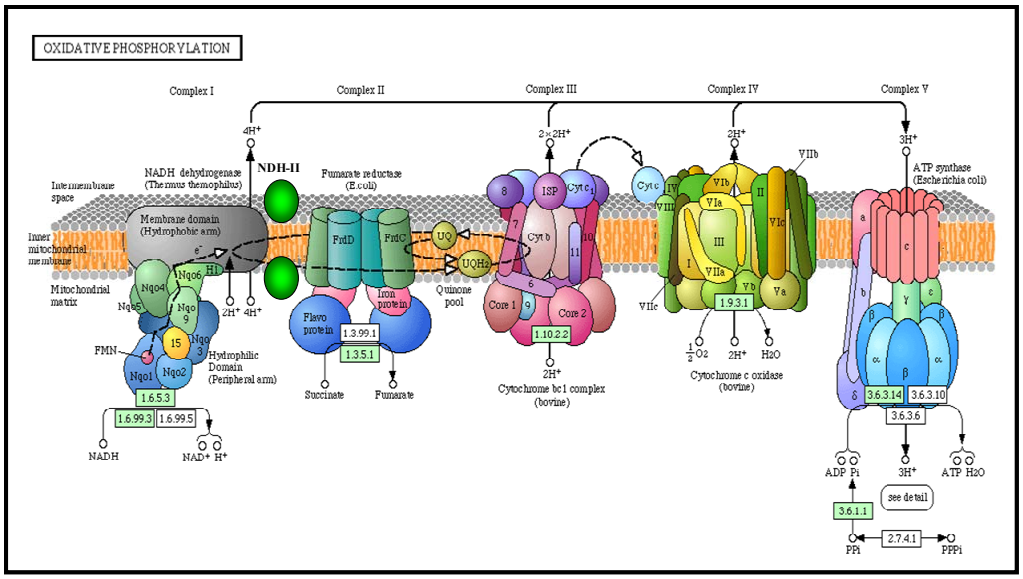


Figure 1.4: Schematic drawing of the mitochondrial respiratory chain components (http://www.genome.jp/kegg/pathway/map/map00190.html)

1.4.3 Carbohydrate metabolism in *Toxoplasma gondii*

Despite well-characterized roles in other organisms, very little is known about the mitochondrial functions of *Toxoplasma gondii*. So far, there is only one study in which enzymatic activities of TCA cycle components are compared between tachyzoites and bradyzoites. In cell homogenates of tachyzoite activities of succinate dehydrogenase (SDH) and NADP+ -dependent isocitrate dehydrogenase was detected, while no SDH activity could be found in bradyzoites. Moreover, high activities of phosphofructkinase, pyruvate kinase and lactate dehydrogenase were detected in both developmental stages, suggesting that energy metabolism in both forms may centre around a high glycolytic flux linked to lactate production (DENTON et al 1996). It was shown that oligomycin, an inhibitor of mitochondrial ATP synthesis, caused an increase in cytosolic Ca^{+2} levels in tachyzoites. This increase suggested a requirement for mitochondrial energy for the regulation of Ca^{+2} homeostasis in these parasites (MORENO and ZHONG, 1996).

Another study by Vercesi et al (1998) showed that phosphorylation of ADP in a digitonin permeabilized tachyzoites could not be obtained in the presence of pyruvate, 3-oxo-glutarate, glutamate, isocitrate, dihydroorotate, α-glycerophosphate, or endogenous substrates, but the rate of respiration was increased in the presence of succinate and malate, this activity was rotenone insensitive. They reasoned that malate might be stimulating ADP phosphorylation via conversion into succinate, where

malate can generate fumarate through the enzyme fumarase, and fumarate in turn could be converted into succinate through the enzyme NADH-fumarate reductase, this hypothesis was supported by detection of a low NADH-fumarate reductase activity in *T. gondii* mitochondrial extracts. Absence of rotenone inhibitory effect on the rate of respiration and on the extent of the mitochondrial membrane potential ($\Delta\Psi$) was taken as an indication that NADH-ubiquinone oxidoreductase (complex I) is absent or does not bind rotenone in *T. gondii* cells.

The inhibition of respiration and collapse of $\Delta\Psi$ by antimycin A and cyanide supported the presence of ubiquinone-cytochrome c oxidoreductase (complex III) and cytochrome c oxidase (complex IV) in the respiratory chain and the absence of alternative oxidase. The sensitivity of respiration and $\Delta\Psi$ to ADP and oligomycin suggested that the machinery for oxidative phosphorylation was similar to that observed in most vertebrate cells. Likewise, the sensitivity of $\Delta\Psi$ to the standard mitochondrial inhibitors and ionophores, such as CCCP and valinomycin, supported the notion that *T. gondii* mitochondria are also similar to vertebrate mitochondria in regard to the generation and utilization of electrochemical proton gradient, in contrary to an early study (TANABE and MURAKAMI, 1984) which failed to detect a membrane potential in *T. gondii* using rhodamine 123.

The inhibitory effect of atovaquone on respiration and mitochondrial membrane potential was taken as a further indication for its selectivity against parasite mitochondria and lead not only to pyrimidine biosynthesis inhibition (HAMMOND et al, 1985) but also to inhibition of ATP production. Taken together, these results indicated the ability of the single mitochondrion of *T. gondii* tachyzoites to carry out carry out energy linked functions such as respiration coupled with generation of a membrane potential sufficient for ATP generation by oxidative phosphorylation (VERCESI et al. 1998).

Similar investigations on bradyzoites are lacking and are indeed difficult to perform since the numbers of parasites which are needed for these kinds of experiments are difficult to obtain. During stage conversion of tachyzoites to bradyzoites, parasite morphology and physiology is significantly altered to adapt to the varying environmental changes during its life cycle (DUBEY et al. 1998). In particular, the carbohydrate metabolism appears to be different, where bradyzoites are believed to be metabolically less active than tachyzoites. Several stage specifically regulated glycolytic isoenzymes have been identified, among them two lactate dehydrogenases

and two enolases (TOMAVO, 2001). These isoenzymes have different biochemical properties and are most likely an adaptation to the specific requirements of metabolic fluxes in actively replicating and dormant parasites (DZIERSZINSKI et al., 2001). Moreover, treatment of tachyzoite-infected tissue cultures with respiratory chain inhibitors such as rotenone, antimycin, atovaquone or oligomycin is associated with the appearance of bradyzoite-specific marker proteins and the induction of bradyzoite differentiation (BOHNE et al. 1994; TOMAVO and BOOTHROYD 1995). Collectively, these observations suggest that mitochondrial oxidative phosphorylation is likely to be functionally impaired in encysted *T. gondii* bradyzoites whereas the tachyzoites probably use both mitochondrial and glycolytic functions to generate ATP (FULTON AND SPOONER, 1960; MELO et al., 1992; VERCESI et al., 1998; PFEFFERKORN et al., 1993, PFEFFERKORN and BOROTZ, 1994)

While the *T. gondii* genome predicts the presence of a complete glycolytic pathway, TCA cycle, and respiratory chain, experimental evidence of the contribution of the TCA cycle to energy metabolism is rare. Surprisingly, pyruvate dehydrogenase complex (PDH), the link between the cytoplasmic glycolytic pathway and TCA cycle, is found to be localized in the apicoplast rather than in the mitochondrion of *T. gondii*. Moreover, genes coding for multisubunit complex I of the respiratory chain are missing, instead genes coding for a single subunit, alternative NADH (type II) dehydrogenases (NDH2) and membrane bound, FAD-dependent malate quinone dehydrogenase (MQDH) are present.

1.5 Parasite mitochondria as a target for chemotherapy

Parasites have developed a wide variety of physiological functions to survive within the specialized environments of the host. Regarding energy metabolism, which represents an essential factor for survival, parasites adapt low oxygen tension in host mammals using metabolic systems that differ substantially from those of the host. Most parasites do not use free oxygen available within the host, but employ systems other than oxidative phosphorylation for ATP synthesis. Furthermore, parasites display marked changes in mitochondrial morphology and components during the life cycle, and these represent very interesting elements of biological processes such as developmental control and environmental adaptation. (KITA et al., 2003)

The enzymes in parasite-specific pathways offer potential targets for chemotherapy. Cyanide-insensitive trypanosome alternative oxidase (TAO) is the terminal oxidase of the respiratory chain of long slender bloodstream forms of the African trypanosome,

which causes sleeping sickness. Recently, the most potent inhibitor of TAO to date, ascofuranone, was isolated from the phytopathogenic fungus, *Ascochyta visiae*. The inhibitory mechanisms of ascofuranone have been revealed using recombinant enzyme (NIHEI et al., 2002).

Parasite-specific respiratory systems are also found in helminthes. The NADH-fumarate reductase system in mitochondria form a final step in the phosphoenolpyruvate carboxykinase (PEPCK)-succinate pathway, which plays an important role in anaerobic energy metabolism for the *Ascaris suum* adult. Enzymes in this system, such as NADH-rhodoquinone reductase (complex I) and rhodoquinol-fumarate reductase (complex II), form promising targets for chemotherapy. In fact, a specific inhibitor of nematode complex I, nafuredin, has been found in mass-screening using parasite mitochondria. (KITA et al., 2003)

Chalcones were evaluated for their antileishmanial and antimalarial activity *in vitro* and *in vivo*. Preliminary studies showed that these compounds destroyed the ultrastructure of *Leishmania* parasite mitochondria and inhibited the respiration and the activity of mitochondrial dehydrogenases of *Leishmania* parasites (CHEN et al., 1993). Licochalcone A inhibited the activity of fumarate reductase (FRD) in the permeabilized *Leishmania major* promastigote and in the parasite mitochondria, and it also inhibited solubilized FRD and a purified FRD from *L. donovani* (CHEN et al., 2001) Licochalcone A inhibited the bc_1 complex (ubiquinol-cytochrome *c* reductase) as well as complex II (succinate ubiquinone reductase, SQR) of *Plasmodium falciparum* mitochondria (MI-ICHE et al., 2005). In particular, licochalcone A inhibits bc_1 complex activity at very low concentrations. Because the property of the *P. falciparum* bc_1 complex is different from that of the mammalian host, chalcones would be a promising candidate for a new antimalarial drug (LI et al., 1995).

Targeting the mitochondrial ETC of the human malaria parasite and *Toxoplasma gondii* has already been shown to be a successful chemotherapeutic strategy. *P. falciparum* mitochondria use a different homolog of ubiquinone (CoQ_8) than their mammalian host, and several antimalarial drugs show specificity for parasite CoQ, including the hydroxynaphthoquinones (ELLIS, 1994). This strategy led to the development of atovaquone (2-[*trans*-4-(4′-chlorophenyl)cyclohexyl]-3-hydroxy-1,4-hydroxynaphtoquinone) (FRY AND PUDNEY, 1992), an inhibitor of complex III (or bc_1 complex), which has been successful clinically, especially in combination with

proguanil (Malarone), for the treatment of chloroquine-resistant infections (LOOAREESUWAN et al., 1999).

Atovaquone has been demonstrated to be effective against the agents of visceral leishmaniasis, *Leishmania donovani* (CROFT et al., 1992), *L. chagasi* (JERNIGAN et al.,1996) and *L. infantum* (CAUCHETIER et al., 2002). In *Plasmodium* and *Toxoplasma*, atovaquone acts as an inhibitor of the cytochrome bc_1 complex, competing with ubiquinol for the substrate binding site in cytochrome *b* (SYAFRUDDIN et al., 1999; MCFADDEN et al., 2000), and the same mechanism is assumed for *Leishmania*.

Competitive inhibitors of dihydrofolate reductase (DHFR), an associated enzyme with complex III (or bc_1 complex), are used in chemotherapy or prophylaxis of many microbial pathogens, including the eukaryotic parasites *Plasmodium falciparum* and *Toxoplasma gondii.* DHFR is a central enzyme in nucleic acid and amino acid synthesis in all cells, but the active sites of enzymes from different organisms show subtle differences that allow the identification of inhibitors specific for a particular species (BIAGINI et al., 2000). For example, pyrimethamine is a selective inhibitor that is effective in the nanomolar range against the DHFRs from *Plasmodium falciparum* and *Toxoplasma gondii*, but the human enzyme is relatively insensitive to the drug (PETERS W., 1987; CHIO et al., 1993). Thus, pyrimethamine has been used in malaria and toxoplasmosis therapy for many years.

1.6 Objectives of the study

Little is known about the significant role of the single mitochondrion of *Toxoplasma gondii* in the overall process of ATP generation in both stage forms of the parasite, tachyzoites and bradyzoites. Stage specific induction of a new subset of glycolytic isoenzymes with different metabolic activities indicates that anaerobic glycolysis plays a vital role in carbohydrate metabolism and ATP generation. Despite the findings that the antimalarial drug atovaquone has been shown to inhibit the electron transport chain and to collapse the mitochondrial membrane potential as well as the induction of stage conversion by different inhibitors of the respiratory chain, the significance of the TCA cycle and respiratory chain in ATP production by aerobic respiration and oxidative phosphorylation is not clear so far. Insensitivity of the respiratory chain to rotenone and the presence of a single nuclear gene coding for a membrane bound malate:quinone oxidoreductase indicate the respiratory chain in *Toxoplasma gondii* is significantly different from that of the host cells.

In this study, we seek to identify the respiratory chain components in *T. gondii*, to study their expression profile in tachyzoites and bradyzoites by quantitative real RT-PCR analysis, and to study their subcellular localization by fusing their proteins with c-myc tag and production of polyclonal antibodies. It was speculated that insensitivity of the respiratory chain to rotenone is due to the absence of canonical NADH:quinone oxidoreductase (complex I), we seek to confirm its absence and to identify the genes coding for alternative NADH dehydrogenases in the respiratory chain of *T. gondii*. Functional analysis of these respiratory chain components with the emphasis on alternative NADH dehydrogenases, by using a recently established tet-transactivator inducible system to generate conditional knockout mutants, and to investigate the phenotype of such mutants will be one major aim of this study.

A pathway which is well known as an effective drug target against Malaria and Toxoplasmosis is the mitochondrial electron transport chain (ETC). Atovaquone inhibits electron transport at the bc1 complex (complex III) by interfering with the ubiquinone oxidation site of cytochrom b (SRIVASTAKA et al., 1997; 1999; VERCESI et al., 1998). The absence of alternative NADH dehydrogenases in mammalian cells defines these enzymes as promising antimicrobial drug targets. A type II NADH:menaquinone oxidoreductase inhibitor was shown to interfere with *Mycobacterium tuberculosis* growth (WEINSTEIN et al., 2005; YANO et al., 2006). In *P. falciparum*, micromolar concentrations of low affinity flavin reagents were shown to inhibit PfNDH2 activity, to collapse the parasite´s mitochondrial membrane potential and to inhibit *P. falciparum* replication (BIAGINI et al., 2006).

Recently, the compound 1-hydroxy-2-dodecyl-4(1)quinolone (HDQ) was identified in enzymatic assays as a high affinity inhibitor of the alternative NADH-dehydrogenase from the fungus *Yarrowia lipolytica* (ESCHEMANN et al., 2005). In this study, we seek to study the potential of HDQ and HDQ derivatives, alone and in combination with atovaquone and pyrimethamine, on replication and differentioan of *T. gondii* by interfering with NADH dehydrogeanse activity and to validate this enzyme as a drug target.

MATERIALS AND METHODS

2.1 Materials

2.1.1 Instruments

Instrument	Model and Manufacturer
Agarose gel electrophoresis cells	Keutz Labortechnik, Reiskirchen
Balances	BP 221 S, Sartorius, Göttingen
	LP6200 S, Sartorius, Göttingen
Cell culture microscope	Modell DM IL, Leica, Heidelberg
Centrifuges	Megafuge 2.0 RS, Heraeus, Hanau
	Megafuge 2.0, Heraeus, Hanau
	Modell 5417 R, Eppendorf, Hamburg
	Modell 5417 C, Eppendorf, Hamburg
	RC-26 Plus, Sorvall-Kendro, Hanau
Incubators	Heraeus, Hanau
Fluorescense microscope	Modell DM R, Leica, Heidelberg
Gel documentation system	BioDoc II, Biometra, Göttingen
Gel electrophoresis cells for PAGE	Modell Mini Protean II, BioRad, München
Hybridization oven	Modell OV5, Biometra, Göttingen
Lightcycler	Roche, Basel, Schweiz
Magnet bars	RCT basic, IKA Labortechnik, Staufen
pH-Meter	Modell 766 Calimatic, Knick, Berlin
Pipettes	Modell Research, Eppendorf, Hamburg
Pipet-aid	Hirschmann Laborgeräte, Eberstadt
Photometer	Ultraspec 1000, Pharmacia Biotech, Freiburg
Power supply	EPS 600, Pharmacia Biotech, Freiburg
	Standard Power Pack P25, Biometra, Göttingen
Shaking incubator	SM-30 Control, Johanna Otto GmbH, Hechingen
Semi-Dry blotter	Sartorius, Göttingen
Thermocycler	Modell T3, Biometra, Göttingen
Thermomixer	Modell Compact, Eppendorf, Hamburg
UV-Table	TFX-20M, Gibco BRL, Eggenstein
UV-Crosslinker	UVC-500, Hoefer, San Francisco, CA, USA
Water bath	

2.1.2 Cell culture media and additives

2.1.2.1 Media and additives

- Roswell Park Memorial Institute (RPMI) 1640 Medium with 300 mg/l L–glutamine and 2 g/l $NaHCO_3$.

- Dulbecco's MEM (DMEM) Medium with 580 mg/l L–glutamine und 3.7 g/l $NaHCO_3$.

- DMEM Medium (10x) with 4.5 g/l D-glucose, 8 mg/l Phenol Red, without $NaHCO_3$, Na-pyruvate, L-glutamine.

- Penicillin / Streptomycin (Pen-Strep), 10.000 U / 10.000 µg/ml.

- Fetal Calf Serum (FCS); heat inactivated by incubation for 45 min at 56 ºC.

- EDTA (Versen) 1% in PBS without Ca^{2+}, Mg^{2+}.

- Trypsin (1:250) 0.25% in PBS without Ca^{2+}, Mg^{2+}.

- HEPES, 1 M.

- L–glutamine, 200 mM

- $NaHCO_3$ 7.5 % in PBS.

- PBS (phosphate buffered saline).

All media and additives were purchased from Biochrom, Berlin.

2.1.2.2 Disposable materials and plasticware

Disposable materials and plasticware used in the cell culture were purchased from **Nunc**, Roskilde, Denmark; **Falcon**, Becton – Dickinson, Heidelberg; **Corning Costar**, Bodenheim; Greiner, Frickenhausen; **Braun**, Braun-Melsungen, Melsungen.

2.1.2.3 Cell lines

Cell line (strain or clone)	Specification	Source
T. gondii	Prugniaud/hxgprt-	D. Soldati
	RH/lacZ+	W. Bohne
	RH/hxgprt-	W. Bohne
	RH-TATi	M. Meissner
	RH-TATi-ndh1/ndh1-cmyc	This study
	RH-TATi-ndh2/ndh2-cmyc	This study
	RH-TATi-sdh-fp/sdh-fp-cmyc	This study
	RH-TATi-cytc1/cytc1-cmyc	This study
	RH-TATi-atp-β/atp- β-cmyc	This study
	RH-TATi-Δndh1/ndh1-cmyc	This study
E. coli	DH5α	Invitrogen
	BL21(DE3)	Stratagene
	BL21-CodonPlus-RIL	Stratagene
	TOP10	Stratagene
	M15[pREP4]	Qiagen
Human foreskin fibroblasts (HFF)		

2.1.2.4 Bacterial culture media

Luria Broth (LB)

1% Bacto-Trypton

0.5% Bacto-yeast extract

0.5% NaCl

LB-Plates (~15 ml per plate)

0.5% Bacto-yeast extract

0.1% Bacto-Trypton

1% NaCl

1.5% Agar (Difco, Detroit, USA)

Ampicillin (100 µg/ ml) or

Tetracyclin (20 µg/ ml)

2.1.2.5 Chemicals and reagents

Unless stated otherwise, chemicals and solutions were purchased from Boehringer (Mannheim), Merck (Darmstadt), Roth (Karlsruhe), Calbiochem (Bad Soden), or Sigma (Deisenhofen). In general, solutions were prepared using double distilled water.

2.1.2.5.1 Drugs

HDQ (**Fig. 3.13**) and all other 1-hydroxy-2-alkyl-4(1)quinolone derivatives were kindly provided by Walter Oettmeier (Ruhr-Universität Bochum). The derivatives were dissolved in tissue culture grade DMSO at a concentration of 2.5 mM. All further dilutions were performed in tissue culture medium. Atovaquone was dissolved in DMSO at a concentration of 5 mM.

2.1.2.5.2 Antibodies

Rabbit-anti-Toxoplasma, Serum (polyclonal); W. Bohne.

Mouse-anti-His-Tag IgG (monoclonal); Qiagen, Hilden.

Mouse-anti-cmyc, 9E10 (monoclonal); Sigma.

Mouse-anti-AND1, Serum (polyclonal); this study.

Mouse-anti-ATP-β, Serum (polyclonal); this study.

Mouse anti-4F8, Serum (polyclonal); W. Bohne

Cy2- conjugated donkey-anti-rabbit IgG, F(ab')$_2$-Fragment; Dianova, Hamburg

Cy2- conjugated donkey-anti-mouse IgG, F(ab')$_2$-Fragment; Dianova, Hamburg

Cy3- conjugated donkey-anti-rabbit IgG, F(ab')$_2$-Fragment; Dianova, Hamburg

Cy3- conjugated donkey-anti-mouse IgG, F(ab')$_2$-Fragment; Dianova, Hamburg

AP- conjugated goat-anti-Digoxigenin IgG, F(ab')$_2$-Fragment; Roche, Mannheim

AP- conjugated goat-anti-mouse IgG; Dianova, Hamburg

AP: Alkaline Phosphatase

Cy2: Carbocyanin

Cy3: Indocarbocyanin

2.1.2.5.3 Membranes and filters

Nitrocellulose membrane	Hybond P (Amersham Biosciences)
Nylon membrane	Hybond P (Amersham Biosciences)

Falcon Cell Strainer, 70 µm Nylon

Corning Bottle Top Filter, 0.22 µm CA (Cellulose Acetate)

Corning 26 mm Syringe Filter, 0.20 µm Micron Membrane, 0.45 µm Micron Membrane

2.1.2.5.4 Enzymes

Quick T4 DNA Ligase	New England Biolabs, Schwalbach
Proteinase K	Roth, Karlsruhe
Pfu DNA-Polymerase	Promega, Mannheim
Taq DNA-Polymerase	Roche, Mannheim
Restriction endonucleases	New England Biolabs, Schwalbach
Shrimp Alkaline Posphatase	Boehringer Mannheim
RNase A	Sigma-Aldrich, Deisenhofen

2.1.2.5.5 Kits and reagents

RNA Isolation

GenElute Mammalian Total RNA Miniprep Kit	Sigma, Deisenhofen

Reverse Transcription

M-MLV (H minus) Reverse Transcriptase RNase, Point Mutant	Promega, Mannheim

DNA Cloning Kits

TOPO TA Cloning Kit	Invitrogen, Karlsruhe
QIAGEN PCR Cloning Kit	Qiagen, Hilden

Plasmid-DNA Isolation

GenElute Plasmid Mini Prep Kit	Sigma-Aldrich, Deisenhofen
GeneElute Plasmid Maxi Prep Kit	Sigma-Aldrich, Deisenhofen

DNA Isolation from Agarose Gels

QIAEX II Gel Extraction Kit	Qiagen, Hilden

DNA Purification

QIAquick PCR Purification Kit	Qiagen, Hilden

Genomic DNA Isolation

PEQLAB DNA Isolation Kit	Peqlab, Göttingen

Protein-His Tag Expression and Purification

QIAexpress	Qiagen, Hilden

Quantification of Proteins

Pierce BCA Protein Assay Kit	Pierce, Rockford, IL, USA

2.1.2.5.6 Molecular weight markers

DNA Standard

100 bp-DNA Ladder	MBI Fermentas, St. Leon-Rot
	1031, 900, 800, 700, 600, 500, 400, 300, 200, 100, 80 bp
1kb Ladder MBI	MBI Fermentas, St. Leon-Rot
	10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, 250 bp

Protein Standard

Pre-stained Protein Marker	New England Biolabs, Schwalbach
	175, 83, 62, 47.5, 32.5, 25, 16.5, 6.5 kDa

2.1.3 Vectors

2.1.3.1 Standard cloning vector pQE-30

The standard cloning vector pQE-30 was purchased from Qiagen (**Fig. 2.1**). N-termianl-6xHis-tag protein expression vector with a selection marker for ampicillin.

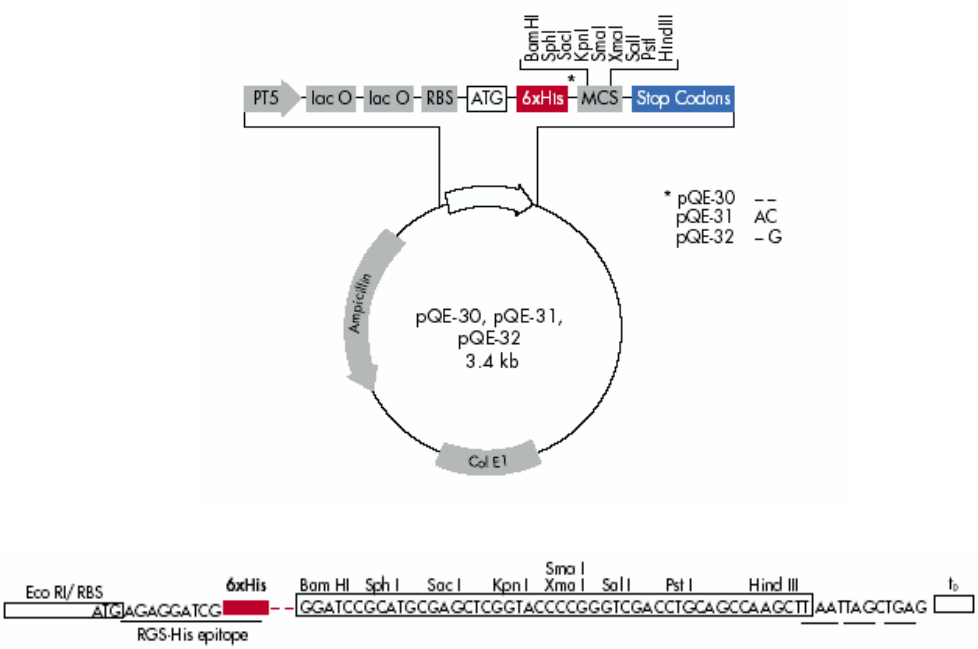


Figure 2.1 Standard cloning vector pQE 30

2.1.3.2 Standard cloning vector pCR 2.1-TOPO

The standard cloning vector pCR 2.1-TOPO was purchased from Invitrogen (**Fig. 2.2**). TA cloning vector used for sub-cloning and sequencing of DNA amplified by PCR with ampicillin and kanamycin as selectable markers.

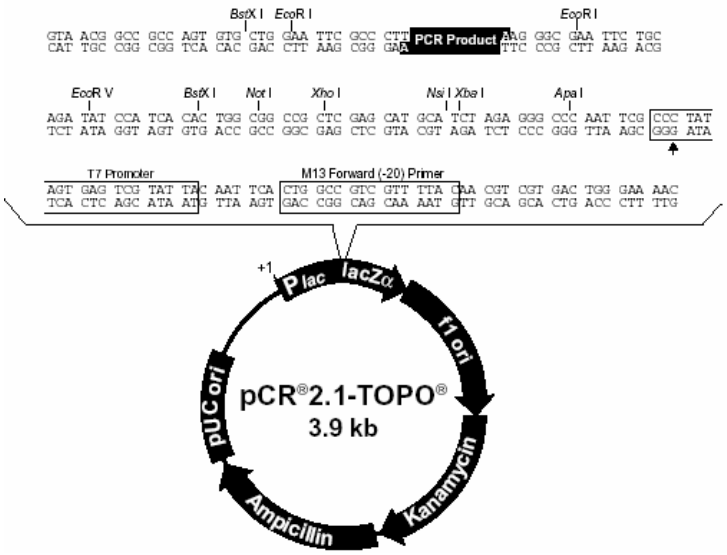


Figure 2.2 Standard cloning vector pCR 2.1-TOPO.

2.1.3.3 Standard cloning vector pDrive

The standard cloning vector pDrive was purchased from Qiagen (**Fig. 2.3**). It was used for sub-cloning and sequencing of DNA fragments, it has 2 selectable markers for ampicillin and kanamycin .

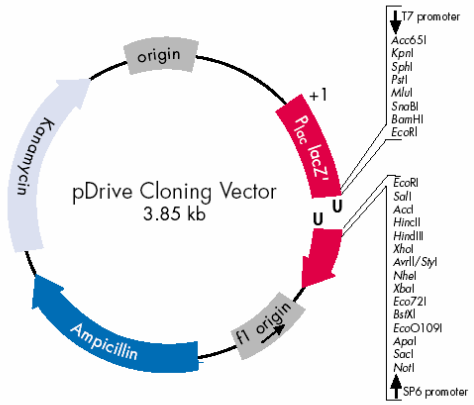


Figure 2.3 Standard cloning vector pDrive.

2.1.3.4 Targeting vectors

These vectors were designed in our lab by W. Bohne for generating targeting constructs for disrupting genes by single (pKS/CAT and pKS/HXGPRT) and double (pKS-CAT/HXGPRT) homologous recombination.

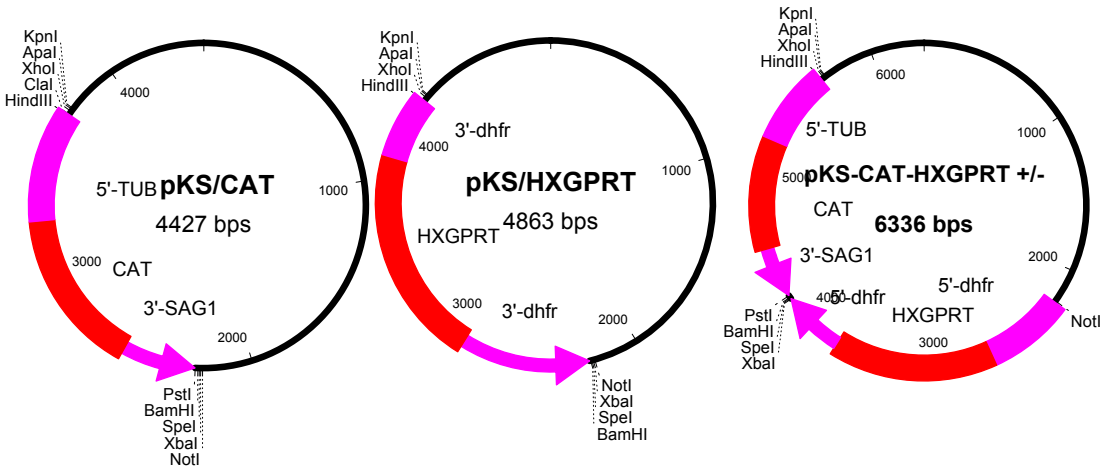


Figure 2.4 Vectors of homologous recombination

2.1.3.5 TATi expression vector (pTet7 Sag4-ACP-cmyc)

This vector was provided by D. Soldati and used for conditional expression of genes in *T. gondii* under the control of tetracycline inducible promoter. The ACP sequence is replaced by the open reading frame of the concerned gene fused with myc tag at C-terminal and its expression is put under the control of Tet7Sag4 promoter. It has the DHFR-TS selectable marker which confers resistance to pyrimethamine.

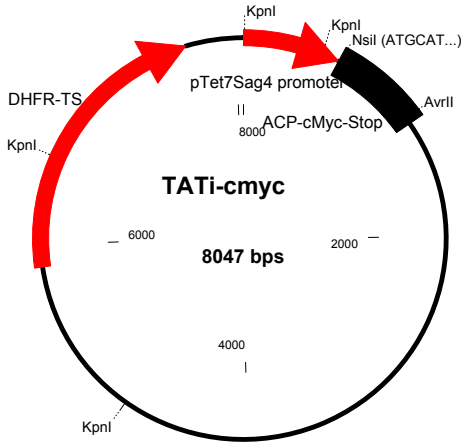


Figure 2.5 pTet7Sag4-ACP-cmyc vector

2.1.4 Oligonucleotide Primers

Oligonucleotide primers were purchased from Sigma-Genosys.

Table 2.1 PCR Oligonucleotide primers used to amplify the genomic DNA inserts to be cloned in the targeting constructs and primers for screening of knock-outs by PCR. Positions of primers are indicated according to the putative start codon of the complete gene sequence, (-): upstream, (+): downstream, (*): primers used for PCR screening.

Primer name	Sequence 5´→ 3´	Position	Size
BamHI-AND1/S1+	TAGGATCCGTGGTGGTTGTGGGGTCAGGCT	301-322	2186
XbaI-AND1/S2-	TATCTAGAGCCAAGTAGCGACCTGCCTGCTTC	2463-2486	
AND1/S3+*	CGTTCTCCGCCTTCTCCCCTGTCT	146-169	2457
AND1/S4-*	CGATGTCTGCAGCTGCTTGCCCAT	2579-2602	
KpnI-AND1/F1_1+	TAGGTACCGCGTCAAGGCCTCGAAACAAAT	-2032/-2010	3069
HindIII-AND1/F1_2-	TAAAGCTTCGACTCCGACGACCTGCGACTG	1015-1036	
AND1/F2_3+	TACAGCAGCAGTCGCCCGTAATCC	1189-1210	2831
AND1/F2_4-	TATCTCTTCCGCCGTCGCATCTTG	+1619/+1641	
SDH-Fp/1+	GCGCGAAGGGAGGCATTTAGAGG	3508-3530	3489
SDH-Fp /2-	CGCCGACGAGAGCCATCCACAC	6975-6996	

SDH-Fp /3+*	GAGAAAGAAGAAGGAACACCAATACG	3386-3411	3661
SDH-Fp /4-*	GCAGCAGCAAGACGGGGAAACCAT	7023-7046	
SDH-Fp /5+	GTCGCCTGGGCGAAAGACCTGCGG	1294-1317	3162
SDH-Fp /6-	CTCGAGCTGTCAGGCCTGTTCCCA	4432-4455	
KpnI-SDH-Fp/7+	TAGGATCCAGTTAAGGCTCCTGGGGTATC	4786-4807	3292
SpeI-SDH-Fp /8+	TAACTAGTGGCAACGTCCTTCATCATCTC	8056-8077	
SDH-Fp /9+*	AACGCAGCTCTCGGCAACATGACG	1229-1252	7022
SDH-Fp /10-*	GGATACGAGACAGCATGCATCTGT	8227-8250	
Cytc1/1+	GCCGACTTCTACCTCCCGACTGCTC	1065-1089	1832
Cytc1/2-	GACCCCGTTTTCCCCTTACCATCTGA	2872-2897	
Cytc1/3+*	AACAGAACTTTCCAGGCTCGCAACCA	945-970	2066*
Cytc1/4-*	TCACGGTATTCTCTTTCCTCGTCACT	2986-3011	
Cytc1/5+	CCTTTGCCCGGGCTCGTGTAGTGT	-623/-647	2248
Cytc1/6-	GTGGACGAAGGAGGCCCCGAGAGC	1602-1625	
Cytc1/7+	GCGTCCCGATCTCCAAAATGA	1785-1805	2292
Cytc1/8-	CGCGTCTCCGGTTCTACAGTG	+486/+507	
Cytc1/9+*	ACTCTGTCGGTCCACACAGCTGCG	-765/-732	4854
Cytc1/10-*	GCATGTAGGTGATATGTGTCAGTG	+644/+662	
XhoI-Cytc1/5+	TACTCGAGCCTTTGCCCGGGCTCGTGTAG	-623/-647	2248
HindIII-Cytc1/6-	TAAAGCTTGTGGACGAAGGAGGCCCCGAG	1602-1625	
SpeI-Cytc1/7+	TAACTAGTGCGTCCCGATCTCCAAAATGA	1785-1805	2292
XbaI-Cytc1/8-	TATCTAGACGCGTCTCCGGTTCTACAGTG	+486/+507	
ATP-β/1+	GTAATCGATGGGGAAGGGGGAGTC	468-491	2443
ATP-β/2-	CTGTGAACGGCTGGGAGAGGAATC	2887-2910	
ATP-β /3+*	ACTCTAATGCAGACAAAGAAAGGC	436-459	2525
ATP-β /4-*	TTCGGGCAGCACGACGAAGCGTCC	2937-2960	
ATP-β /5+	GGG GGAGACACCGCTGCAGATGGACT	-435/-409	2623
ATP-β /6-	CGACACCCGCAAACACAGAGAAACCT	2162-2187	
ATP-β /7+	GTGCGCGCGTCGCCCTGACTGCTC	2338-2361	3336
ATP-β /8-	AGCTGCATTTGGAAATCTGCGTCC	+974/+998	
SpeI-ATP-β /7+	TAACTAGTGTGCGCGCGTCGCCCTGACTG	2338-2358	3333
XbaI-ATP-β /8-	TATCTAGAAGCTGCATTTGGAAATCTGCG	+974/+995	
ATP-β /9+*	ATATGTGTGTGGGTGAACTCTCTA	-564/-540	4999
N-ATP-β /9+*	GAATCTTCATCCGCTCCAAATC	-824/-802	
ATP-β /10-*	ATTGTGTGGCCTCACTGTAACGTC	+1053/+1077	
T7 /-*	TAATACGACTCACTATAGGCCGAATTGGAG		
5'Tub/-*	GATTGGTGATCACTCCGCTTGATC		
Sag1+*	GCCACATGGGTCAAT ACACAAGAC		
3 DHFR+*	GACACAGTCGGTTGACAAGTGTT		

Table 2.2 HPLC purified oligonucleotide primers for gene expression analysis by quantitative real time RT-PCR. Positions of primers are indicated according to the putative start codon of the coding sequence (ORF) of the gene.

Primer Name	Sequence 5'→3'	Position	size
RT-AND1/1+	CTCGTCGACCAGCAAATGAAGG	1210-1231	352
RT-AND1/2-	TGAACGTCTGCGCCAAGTAGC	1541-1561	
RT-AND2/1+	GCCGCCAGGGTGGACATTTCAA	725-746	367
RT-AND2/2-	TACGTCCCCAGCAAACGGCTCC	1070-1091	
RT-SDH-Fp/1+	CTGCGAGAAGGCGTGGAGATG	1645-1674	328
RT-SDH-Fp/2-	CGTTGTCAAGAGGCTGCGAGAT	1951-1972	
RT-SDH-Ip/1+	ACGCCCGTACATGCAAAAGTTC	Not identified	355
RT-SDH-Ip/2-	TCATATGGGGCAGAGGCAAGAT	Not identified	
RT-CYTC1/1+	TCGGGGCCTCCTTCGTCCAC	401-420	458
RT-CYTC1/2-	GCCGCCAAGCAGAGCCATCA	839-858	
RT-ISP/1+	AACAGCGACTCTCATCCCGACTTC	592-615	382
RT-ISP/2-	CACCGATATTGATGAGCCACTGAG	950-973	
RT-CYTC/1+	AAATGCTCGCAATGCCACACCA	73-94	236
RT-CYTC/2-	GCGCGTTCCTTCTCCTTCTTGA	287-308	
RT-CYTC2a/1+	CTCTCCGTCCCGTCACCATCTG	474-495	351
RT-CYTC2a/2-	GCCGCATACACCGCTTCGTC	805-824	
RT-CYTC2b/1+	GGGATGCCGCGAGGAGAACT	78-97	285
RT-CYTC2b/2-	CCTACCCGGGATGGCGTCAG	344-363	
RT-ATP-α/1+	GCCCTGCTATCAACGTCGGTCTC	1241-1263	350
RT-ATP-α/2-	GTCCTGATGGTTCGCGTTGATGT	1568-1590	
RT-ATP-β/1+	ACTCCCGCCAATTCTCAACAGC	297-318	242
RT-ATP-β/2-	GAACCGGTCCACACTCATCCAC	517-538	
RT-Tub 3+	CGCCACGGCCGCTACCTGACT		
RT-Tub 4-	TACGCGCCTTCCTCTGCACCC		
RT-Bag 3+	GACCGGTCGCCTCTCAACAGC		
RT-Bag 4-	CGCGCAAAATAACCGGACACT		
RT-Actin 3+	CGTGCCGTCTTCCCGTCTATCGT		256
RT-Actin 4-	GGTTCGCCTTGGGGTTGAGTGG		
RT-GAPDH 1+	GGCTGTGGGCAAGATTATTCCT		
RT-GAPDH 2-	ATGATGCCGGCGTTGATGTC		

Table 2.3 Oligonucleotide primers used to amplify and to confirm the coding sequences (ORFs) of the target genes to be cloned in pTet7Sag4-cmcy (TATi) vector. Positions of primers are indicated according to the putative start codon of the coding sequence (ORF) of the gene.

Primer Name	Sequence 5'→3'	Position
AflII-AND1/ORF1+	TACTTAAGAAAATGGCAGGGCAGTGGCTG	1-18
AvrII-AND1/ORF2-	TACCTAGGCTTGCGTCGGTCGCCGTACACA	1833-1854
RT-AND1/ORF3+	AGTTCGCTGTCAGTCGACTCACGG	552-575
RT-AND1/ORF4-	GGACTCCAAAAGTGTTCACATCGG	638-661
RT-AND1/ORF5+	ATCCTCGTCGACCAGCAAATGAAG	1207-1230
RT-AND1/ORF6-	GTCTGCAGCAGTCTCCGAGCGCGT	1253-1276
NsiI-AND2/1+	TAATGCATAAAATGGCGATGCTCTTCTCCAGC	1-21
AvrII-AND2/2-	TACCTAGGGTGGTTGTAATATTCGTGATCC	1950-1971
AND2/SEQ1+	GCTCGTTGACCTACCGCAACGG	629-650
AND2/SEQ1-	TCCACATCTGTGCAGTGAGCCT	674-695
AND2/SEQ2+	GGCAAGCGGCGTGGGTGAAGGTG	1263-1284
AND2/SEQ2-	CGACGGGAAGGCCTCGAAGACG	1339-1360
RT-NsiI-SDH-Fp/1+	TAATGCATAAAATGCATGCGTCGGCATCCCTCA	1-22
RT-AvrII- SDH-Fp/2-	TACCTAGGGTACACGCGCTTCGCCGGCGGA	1986-2007
RT- SDH-Fp /3+	ATCGACCATGAGTATGATGCAATCGTC	208-234
RT- SDH-Fp /4-	GATGCTCCCGTCCTCCATGCACAT	757-780
RT- SDH-Fp /5+	TTTGCCCTGGATTTGATGATGAGC	694-717
RT- SDH-Fp /6-	ATTTTCGCGGTTTCGGTGATTC	1193-1214
RT- SDH-Fp /7+	CAATCGTGACCACATGCATTTG	1125-1146
RT- SDH-Fp /8-	CGAGAGCGATCGGTCCTTTACG	1710-1731
RT-NsiI-Cytc1/1+	TAATGCATAAAATGGGAGGCGGCGGAGGC	1-18
RT-AvrII-Cytc1/2-	TACCTAGGCAAATACTTCACGTTTCCGA	1169-1188
RT- Cytc1/5+	TCTCGCGAAACACCCACTTG	-59/-37
RT- Cytc1/6-	CGACAAGATGGCGGAAATGC	597-616
RT- Cytc1/7+	CTCTGTCCGACGTGGCTATGAAGT	531-554
RT- Cytc1/8-	CTGCGTCGCTCTCCAAGTGAAT	+63/+85
RT-NsiI-ATP-β/1+	TAATGCATAAAATGGCGTCTCCCGCACTC	1-18
RT-AvrII-AND1/2-	TACCTAGGCTTTCCGCTCGCCGCTTCCTG	1660-1680
RT- ATP-β /3+	GTCTCCCGCACTCCAAACTTG	6-26
RT- ATP-β /4-	ACTCGGCGACAGACAGAGCAG	941-961
RT- ATP-β /5+	GGAGTGATTAAGCGGAAGAAGTTG	823-846
RT- ATP-β /6-	TGTCCACAGGTGCAGCTTCTCAAT	+47/+71

Table 2.4 HPLC-purified oligonucleotide primers used to amplify the inserts to be cloned in the antisense (RNAi) vector. Positions of primers are indicated according to the putative start codon of the coding sequence (ORF) of the gene.

Primer Name	Sequence 5'→3'	Position	size
Bgl2-AND1/1+	TAAGATCTCGACGGCCAGGTGGAAGAAAAG	1071-1092	422
Bgl2-AND1/2-	TAAGATCTCGCCGCGTCGATGTCCTGAAGA	1473-1492	
Bgl2-AND2/1+	TAAGATCTCAAGCGGCGTGGGTGAGGTG	1265-1284	478
Bgl2-AND2/2-	TAAGATCTGCAGGCGCGTCGGCTTTTTC	1723-1742	
Bgl2-SDH-Fp/1+	TAAGATCTCTGGAGACGGCGGCGGGATGGT	866-887	466
Bgl2-SDH-Fp/2-	TAAGATCTTTAGAGCGGGAGGTGGTGAGCA	1310-1331	
Bgl2-CYTC1/1+	TAAGATCTTCTCGGGGCCTCCTTCGTCCAC	399-420	461
Bgl2-CYTC1/2-	TAAGATCTAGCCGCCAAGCAGAGCCATCAA	838-859	
Bgl2-ATP-β/1+	TAAGATCTCCCAGCGCCGTCGGATACCAG	1063-1083	433
Bgl2-ATP-β/2-	TAAGATCTCTGTGAACGGCTGGGAGAGGAA	1474-1495	

Table 2.5 Oligonucleotide primers used to amplify the ORFs and hydrophilic inserts to be cloned in the PQE30 vector. Positions of primers are indicated according to the putative start codon of the coding sequence (ORF) of the gene.

Primer Name	Sequence 5'→3'	Location	size
BamHI-AND1/ORF3+	TAGGATCCAAATGGCAGGGCAGTGGCTG	1-18	1857
HindIII-AND1/ORF4-	TAAAGCTTTCACTTGCGTCGGTCGCCGTAC	1836-1857	
BamHI-AND1/3+	TAGGATCCCGCACAAAGCGCGCCGACGGC	1057-1077	633
KpnI-AND1/4-	TAGGTACCGCCCAAGAACGTCCTCCATCCT	1668-1689	
BamHI-AND2/philic1+	TAGGATCCCCCTCGCGCAGAAGCCGATCCG	40-61	990
KpnI-AND2/philic2-	TAGGTACCCGGGAAGTACTTGCTCATGTCC	1008-1029	
BamHI-SDH-Fp/ORF3+	TAGGATCCATGCATGCGTCGGCATCCCTCA	1-22	2010
KpnI-SDH-Fp/ORF4-	TAGGTACCTCAGTACACGCGCTTCGCCGGC	1989-2010	
BamHI-SDH-Fp/3+	TAGGATCCCTCGGCAACATGACGGAG	367-384	786
KpnI-SDH-Fp/4-	TAGGTACCCAAATCCAAATGCATGTGGTCA	1132-1152	
BamHI-Cytc1/ORF3+	TAGGATCCATGGGAGGCGGCGGAGGCG	1-19	1191
HindIII-Cytc1/ORF4-	TAAAGCTTTCACAAATACTTCAGCTTTCCG	1170-1191	
BamHI-Cytc1/3+	TAGGATCCCAGCGTCGACCAGTTGCGTG	433-452	759
KpnI-Cytc1/4-	TAGGTACCTCACAAATACTTCAGCTTTCC	1171-1191	
KpnI-ATP-β/ORF3+	TAGGTACCATGGCGTCTCCCGCACTCCAAA	1-22	1683
HindIII-ATP-β/ORF4-	TAAAGCTTCTACTTTCCGCTCGCCGCTTCC	1662-1683	
BamHI-ATP-β/3+	TAGGATCCGGCGAGAGGACGCGCGAGG	772-790	389
KpnI-ATP-β/4-	TAGGTACCGACGGAGGTAATCGATCCCTTC	1137-1158	

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Cultivation and splitting of human foreskin fibroblasts (HFF)

Human foreskin fibroblasts (HFF) were for the cultivation of *Toxoplasma gondii* parasites *in vitro*. HFF cells were cultivated in 175 cm^2 (T175), 25 cm^2 (T25) cell culture flasks as well as in 6, 24, and 96 well cell culture plates in Dubellco´s minimal essential medium (DMEM) supplemented with 10%, heat inactivated fetal calf serum (FCS), and 1% Pen/Strep at 37°C in a 5% CO_2 and 85% humidity atmosphere. The HFFs in T175 flask grow to form a confluent monolayer after10 days of incubation. Afterwards, the HFFs were splitted into a new cell-culture flask by discarding the old media, washing the monolayer with 1% EDTA, detaching the cell monolayer with 10 ml Trypsin-EDTA, and re-suspending the HFF cells in 10% DMEM media (10% FCS, 1% FCS, 1% Pen/Strep). The cell suspension was then re-cultivated in T175 (50 ml/each), T25 (10 ml/each) cell culture flasks and 6 (5 ml/well), 24 (1.5 ml/well, with/out sterile glass cover slips), and 96 (150 µl/well) -well plates and incubated under the same conditions.

2.2.1.2 In vitro cultivation and maintenance of *T. gondii*

RH strain (the lab strain) of *Toxoplasma gondii* was mainly used in this study. Since *T. gondii* is an intracellular parasite, it needs a suitable host to grow inside. Therefore, tachyzoites were cultivated in cell culture flasks and plates with confluent monolayer of HFF and DMEM-media supplemented with 1% FCS, 1% Pen/Strep and 1% Amphotericin B (1% DMEM). After complete lysis of the host cells by the parasites about 2 to 3 days post infection and incubation at 37°C with5% CO_2, the tachyzoites were re-cultivated in T25 flasks with fresh confluent monolayers of HFF cells.

2.2.1.3 In vitro differentiation of tachyzoites to bradyzoites

Confluent HFF monolayers in T25 flasks were infected with 1 x 0^6 parasites in 1% DMEM medium and incubated for 3 hours at 37°C and 5% CO_2 to allow the parasites to infect the host cells. Afterwards, the old medium was replaced with pH shift medium (pH 8.2) and the cultures were incubated at 37°C without CO_2. The medium was replaced daily with a fresh pH shift medium to remove the extracellular parasites and

keep a constant pH of the culture. After 4 days of incubation with the pH shift medium, cells were detached and used for RNA isolation.

pH-Shift Medium

5 ml DMEM without $NaHCO_3$, Na-Pyruvate, L-Glutamine (Biochrom)

5 ml NaHCO3 (7.5%)

10 ml Tricin (1 M, pH 8,2 at 37 ºC)

5 ml Pen-Strep

5% FCS

5 ml L-Glutamine (200 mM, Biochrom)

pH 8.2 (at 37 ºC) with KOH

2.2.1.4 Freezing and thawing of *T. gondii* and HFF.

Suspension of detached HFF cells (alone or with intracellular *T. gondii*) in 40% DMEM was mixed with the same volume of 2x freezing solution (20% DMSO, 40 % FCS in DMEM) and aliquoted in 1 ml volumes in cryotubes and kept for few days at -80 and later stored in liquid nitrogen for long term storage.

For thawing the frozen cells, a cryotube is taken from -80ºC freezer or liquid nitrogen, thawed directly in a 37ºC water bath, cultured in a pre-warmed 10% DMEM medium and incubated at 37ºC with 5% CO_2. The medium is changed the second day with a fresh medium to remove the toxic effect of DMSO on the growth of cells.

2x Freezing solution

40% DMEM

40% FCS

20% Dimethylsulfoxide (DMSO)

2.2.1.5 Transfection and selection of *T. gondii*

Freshly lysed tachyzoites were collected, purified from the debris of host cells by filteration and low speed centrifugation (400 rpm), and counted microscopically. Cells (2.0×10^7) were washed once in cytomix and re-suspended in 400 µl of cytomix buffer (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM $K_2HPO_4/KH2PO_4$ pH 7.6, 25 mM HEPES pH 7.6, 2 mM EGTA, 5 mM $MgCl_2$) containing 2 mM ATP pH 7.6, 5 mM glutathione and 50 µg of a circular or linearized targeting construct. Electroporation was done in 2 mm gap cuvette using a BTX electro cell manipulator 600 (BTX, San Diego) set at a voltage of 2.5 kV cm^{-1}, 25 µF capacitance and 48 serial resistance (SOLDATI and BOOTHROYD,1993). Electroporated cells were immediately allowed to infect HFF

monolayer cells 1% DMEM in T25 flasks. Drug selection of the transfected parasites was applied either directly (chloramphenicol, pyrimethamine) or after 24 hours of electroporation (xanthine and mycophenolic acid).

In order to select for stable transformants, parasites transfected with the targeting constructs harboring the CAT, DHFR, and HXGPRT selectable markers, conferring resistance to chloramphenicol, pyrimethamine, and mycophenolic acid + xanthine were selected by a final concentration of 20 µM chloramphenicol, 1 µM pyrimethamine, and 1 µM mycophenolic acid + 1 µM xanthine that allowed no detectable growth of wild type parasites.

Cytomix	**Cytomix + GSH + ATP**
120 mM KCl	30 mg ATP
0,15 mM $CaCl_2$	38 mg Glutathione
10 mM K_2HPO_4/ KH_2PO_4, pH 7,6	in 25 ml Cytomix
25 mM HEPES	
2 mM EDTA	
5 mM $MgCl_2$	
pH 7.6 with KOH	

2.2.1.6 Cloning of *T. gondii*

Stably transfected parasites were first cloned by serial dilution in 24 well plates with confluent HFF monolayers under drug selection. Cultures were incubated at 37ºC and 5% CO_2 and kept for 5-6 days without disturbance. Afterwards, growth of *T. gondii* was checked microscopically and wells containing 1-10 plaque colonies were scratched and used to infect HFF monolayers in T 25 flasks. Parasites were collected after the complete lysis of the monolayers and used in genomic DNA isolation for PCR screening of the knock out parasites. Parasites which showed the expected PCR pattern of the knock out, were re-cloned with a serial dilution in 96 well plates with HFF monolayers and incubated with drug selection for 10-12 days without disturbance, single plaque colonies were cultured in T25 flasks, and confirmed for the knock out pattern by PCR.

2.2.1.7 Indirect immunofluorescence assay

For immunofluorescence staining of *T. gondii* parasites and their intracellular organells, a confluent monolayer of HFF cells cultivated on sterile cover slips in 24 well plates

were infteced with the desired strain of *T. gondii* (5 x 10^4 parasites/well) and incubated at 37ºC and 5% CO_2 for 24 hours. HFF monolayers with intracellular parasites were either fixed with 4% paraformaldehyde/1xPBS for 10 min and permeabilized with 0.25% Triton X-100/1x PBS for 15 min, or with ice-cold absolute methanol for staining of the outer membrane of the parasites. After blocking for 1h with PBS/1%BSA, they were incubated for 1h with a specific primary antibodies diluted in PBS/1% BSA followed by incubation for 1 h with a Cy3- or Cy2-conjugated secondary antibodies. Cover slips were washed and fixed on glass slides with Movoil for overnight. Antibodies used in immunofluorescence staining and their dilutions are shown in table 2.6

Blocking solution	**Moviol**
1% BSA	2.4 g Moviol 4-88, 6.0 g Glycerol
0.02% NaN3	6 ml dH_2O,
in 1x PBS	12 ml 0.2 M Tris/ HCl, pH 8,5
	at 4 ºC with rotation till clearance
	Incubation at50 ºC, 10 min
	Centrifugation for 15 min, 5000 g

Table 2.6 Antibodies used for used immunofluorescence staining.

Antibodies	Type	Dilution in 1% BSA
Rabbit anti-*T. gondii*	Primary	1:2000
Mouse anti-4F8	Primary	1:500
Mouse anti-AND1	Primary	1:20
Mouse anti-ATP-β	Primary	1:20
Mouse anti-myc 9E10	Primary	1:250
Cy3-conjucated anti-rabbit	Secondary	1:1000
Cy3-conjugated anti-mouse	Secondary	1:1000
Cy2-conjucated anti-rabbit	Secondary	1:500
Cy2-conjicated anti-mouse	Secondary	1:500

2.2.1.8 Replication rate determined by immunofluorescence microscopy

HFF monolayers were infected for 2 h with freshly lysed *T. gondii* (5 x 10^4 parasites/well), washed to remove extracellular parasites, and subsequently treated with adequate concentrations of drugs or a DMSO control. After 24 h of drug treatment, samples were fixed, permeabilized, and blocked as described in the above section, and

stained with a polyclonal rabbit anti-*T. gondii* antiserum (diluted 1:2000 in 1% BSA), followed by incubation with a cy3-conjugated anti-rabbit IgG (diluted 1:1000 in 1% BSA). The parasite numbers per vacuole were determined from at least 200 vacuoles by immunofluorescence microscopy.

2.2.1.9 Replication rate determined by β-Galactosidase activity.

The beta-Galactosidase activity of *T. gondii* which are stably transfected with the *E. coli lacZ* gene can be used to accurately quantify the parasite replication rate by using a colourimetric assay (MCFADDEN et al., 1997). Freshly lysed parasites of a beta-galactosidase expressing RH strain (BOHNE and ROOS, 1997) were used to infect the HFF monolayer ($1x10^4$ parasites/well) grown in 24 well plates in 1% DMEM media without phenol red for 4 hours at 37ºC and 5% CO_2. The infected monolayers were washed twice with 1% DMEM without phenol red media, and incubated with different concentrations of drugs (HDQ, HDQ derivatives, and pyrimethamine) in duplicate (1, 0.1, 0.01, and 0.001 µM) and a final concentration of 100 µM CPRG at 37ºC. Absorbance at 570 nm and 630 nm was measured using a spectrophotometer. Subtracted absorbance values (Abs. 570-630) were used for blotting with drug concentrations.

2.2.1.10 Host cell metabolic assay

The metabolic activity of the host cells was tested by using the AlamarBlue assay (Biosources International Inc., Camarillo, Calif.). Briefly, HFF were grown to confluence in 24-well plates, treated for 48 h with different concentrations of HDQ and processed according to the instructions provided by the manufacturer.

2.2.1.11 Calculation of synergism between atovaquone and HDQ

Potential synergy was evaluated as the sum of the fractional inhibitory concentrations (sum FIC) by the following equation: sum FIC = [(IC_{50} drug A in combination)/(IC_{50} drug A alone)] + [(IC_{50} drug B in combination)/(IC_{50} drug B alone)].

IC50-ATV = 8 nM, IC50 HDQ = 4 nM, IC50 = ATV+HDQ < 1 nM

Sum FIC of ATV and HDQ = [(1)/(8)] + [(1)/(4)] = 0.375

IC50- PYR = 750 nM, IC50 HDQ = 4 nM, IC50 PYR+HDQ < 1 nM

Sum FIC of PYR and HDQ = [(1)/(750)] + [(1)/(4)] = 0.251

Sum FIC-value < 0.5 represents synergism, values >1 antagonism and values=1 addition.

2.2.2 Modification of nucleic acids (DNA and RNA)

2.2.2.1 Isolation of genomic DNA and total RNA from *T. gondii*

For isolating genomic DNA from *T. gondii*, confluent HFF monolayers in T25 flasks were infected with ~10^6 parasites in 1% DMEM media and incubated for 3 days. After complete lysis of the host cells, parasites were collected by centrifugation at 2000 rpm for 10 min and used for isolating genomic DNA. DNA preparation was done using a DNA isolation kit (peqlab) in accordance to the manufacturer's instructions provided.

Total RNA from intracellular *T. gondii* infecting HFF host cells was extracted using a GenElute Mammalian Total RNA Miniprep Kit (Sigma) according to the manufacturer's recommendations. RNA isolation from intracellular tachyzoites was performed 24h after infection of confluent monolayers of HFF cells, and 4-5 days for intracellular bradyzoites. The isolation of RNA was combined with DNase digestion using the RNase-Free DNase Set (Sigma) for DNase digestion.

2.2.2.2 Quantification of DNA and RNA

DNA and RNA concentrations were determined by measuring the absorbance at 260 nm (A_{260}) in an Eppendorf spectrophotometer. DNA and RNA concentrations were quantified based on the formula that an absorbance of 1 unit at 260 nm corresponds to 50 µg DNA per ml (A_{260} = 1 ⇒ 50 µg/ml) and 40 µg RNA per ml (A_{260} = 1 ⇒ 40 µg/ml). To confirm the quantification of DNA and RNA, diluted aliquots (~ 10 µl) of each sample were migrated on agarose gel with ~ 10 µl 1 kb DNA ladder, the amount of DNA is estimated by comparing intensities of the bands on agarose between the sample and the different bands of the ladder.

2.2.2.3 Synthesis of cDNA (Reverse Transcription)

RNA was reverse transcribed to cDNA using the M-MLV reverse transcriptase (RNase H minus) from Sigma. Reaction mixtures containing ~ 5 µg of RNA were combined with 1x M-MLV RT buffer, 0.5 mM each dNTP, 1 µM Oligo(dT) primer, 5 Units M-MLV reverse transcriptase and RNase-free water in a total volume of 20 µl per sample. RNA was reverse transcribed at 37°C for 4 hours. A control sample without reverse transcriptase was incubated in parallel. The resulting cDNA was adjusted to 100 µl with H_2O and used for PCR or stored at -20°C.

2.2.2.4 Polymerase chain reaction (PCR)

T. gondii genomic DNA (gDNA) or complementary DNA (cDNA) were used as a template for amplification of the desired sequences, using oligonucleotide primers with or without introduced endonuclease restriction sites. A complete list of all used primers is described in Tables 2.1-2.4.

A standard PCR was performed in a final volume of 50 µl containing 5 µl gDNA or cDNA (corresponding to 20-50 ng), 0.2 mM dNTPs, 10x PCR-Mg^{2+} reaction buffer, 0.5 µM of forward and reverse primers and 1 U *Taq* or *Pfu* DNA polymerase. Amplification was performed in a heated led, T3 Thermocycler (Biometra) with the following cycling parameters: an initial denaturation step at 94°C for 3 min; 35 cycles of 95°C for 30 sec, annealing at 55-65°C for 60 sec, and extension at 72°C for 1 - 4 min; and an extension step of 72°C for 10 min. Different annealing temperatures were used based on the melting points of primer pairs, while elongation time was dependent on the length of the amplified region (1 min. for each 1 kb).

2.2.2.5 Real time PCR with Lightcycler

Comparable quantitative values for the expression of individual genes can be achieved by real-time PCR with the Lightcycler (Roche, Mannheim) (HIGUCHI et al., 1992). Fast start DNA SYBR Green I kit (Roche, Mannheim) was used for quantitative gene expression analysis of 11 genes in both tachyzoite and bradyzoite stages of *T. gondii*. Lightcycler hot-start PCR was performed in glass capillaries in triplicates and in a final volume of 20 µl (5 µl of cDNA template, 0.5 µM of each HPLC purified primer, 4 µl of the master mix and dH_2O). Capillaries were locked with covers and centrifuged at 3000 rpm for 30 sec. Samples were amplified using the following settings: 10 min at 95°C, followed by 50 cycles of 10 sec at 95°C, 15 sec at 55°C and 20 sec at 72°C, with a temperature transition rate (TTR) of 20°C/sec. The PCR was followed by a melting temperature analysis cycle comprising 95°C for 0 sec (TTR of 20°C/sec), 65°C for 15 s (TTR of 20°C/sec) and 95°C for 0 sec (TTR of 0.2°C/sec) to check the specificity of the PCR product. Each PCR included a negative control consisting of all components without template DNA to monitor possible contamination.

The obtained crossing points were used to calculate the relative amount of initial cDNA template for the examined genes and β-tubulin (housekeeping gene). Crossing points for examined genes were normalized for β-tubulin expression and the expression level of

each examined specific gene in tachyzoites and bradyzoites was calculated according to the following formula:

$$\frac{Exp_{Brady}}{Exp_{Tachy}} = \frac{2^{\Delta cp_{spez(tachy-brady)}}}{2^{\Delta cp_{Tub(tachy-brady)}}}$$

Exp: Expression

Tachy: tachyzoites

Bardy: Bradyzoites

Cp^{spec}: crossing point of the specific gene

Cp^{Tub}: crossing point of β-tubulin gene.

2.2.2.6 Agarose gel analysis of DNA

PCR products were mixed with 6x loading dye and resolved on 0.8-1.5 % agarose gels stained with ethidium bromide at a final concentration of 1 µg/ml. Gels were run at 120 V. DNA bands were visualized under UV-light and documented using a BioDoc II digital imaging system (Biometra).

TAE buffer	10x loading dye	TES
40 mM Tris	0.07% bromophenol blue	0.05 M Tris
1% acetic acid	33% glycerin	0.005 M EDTA
1 mM EDTA, pH 8.0	7% SDS	0.05 M NaCl
	in TES	pH 8.0

2.2.2.7 DNA extraction from agarose gels

DNA extraction from agarose gels was performed using the QIAEX II Gel Extraction Kit as described in the protocol. In short, desired DNA fragments were excised from the agarose gel, weighed and accordingly mixed with 3 volumes of buffer QX1 and 10 µl QIAEX II silica gel. The mixture was incubated at 50°C until the agarose was completely melted (10 min). The samples were further centrifuged and the pellet washed once with 500 µl of buffer QX1 and twice with 500 µl of buffer PE. The pellet was then air-dried for 10-15 min before re-suspended in 20-40 µl dH_20.

2.2.2.8 Restriction endonuclease digestion of DNA

PCR products with introduced endonuclease restriction sites were purified before digestion using a QIAquick PCR Purification Kit according to the manufacturer's recommendations. Purified PCR products and the cloning vectors were digested with specified restriction enzymes for subsequent ligation. A digestion reaction containing 5 µl purified PCR product or 10 ng of the cloning vector, 2 µl of the recommended 10x digestion buffer, 1 U of each restriction enzyme (RE), 2 µl 10% BSA (RE dependent), and dH_2O was set up. Digestion was done in a final volume of 50 µl at 37°C for 2 h. Digested PCR-products were checked on agarose gel, purified using Gel Extraction Kit and directly used for ligation.

2.2.2.9 Ligation of DNA

Ligation was performed in a final volume of 20µl containing digested vector and digested insert, 1µl of T4 DNA ligase (400 U/µl), 2µl of 10x ligation buffer and dH_2O. The ligation reaction mixture was incubated at RT for 20 min and 2-5 µl were used for subsequent transformation (see 2.2.2.11 Transformation of *E. coli*).

2.2.2.10 Preparation of chemically competent *E. coli* cells

A single fresh colony of *E. coli* cells (DH5α, TOP10, M15, BL21, and BL21+ strains) was used to inoculate 5 ml of LB medium. The culture was grown overnight at 37°C with vigorous shaking (220 rpm). 2 ml of the overnight culture was added to 100 ml pre-warmed LB medium and shaken until an OD_{600} of 0.5 was reached. The culture volume was transferred into 50 ml Falcon centrifuge tubes and cooled on ice for 5 min. *E. coli* cells were collected by centrifugation at 4000 x *g* for 5 min at 4°C. The cell pellet was gently resuspend in cold TFB1 buffer (40 ml for a 100 ml culture) containing 100 mM RbCl, 50 mM $MnCl_2$, 30 mM potassium acetate, 10 mM $CaCl_2$ and 15% glycerol at pH 5.8 and incubated on ice for 5 min. Cells were then pelleted by centrifugation as described above and resuspend in ice-cold TFB2 buffer (10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2$ and 15% glycerol at pH 6.8). After incubation on ice for additional 15 min, aliquots of 100 µl were frozen stored at –80°C.

2.2.2.11 Transformation of *E. coli*

An aliquot of frozen competent *E. coli* cells was thawed on ice and gently mixed with DNA to be transformed. After incubation on ice for 30 min, cells were heat shocked at 42°C for 30-45 sec and 900 µl LB medium was added. Cells were incubated at 37°C for 30 min before plated on selective plates (LB-agar containing 100 µg/ml ampicillin) or on LB-plates with 40 µl (40 mg/ml) for white-blue colony selection. Plates were incubated at 37°C overnight.

In case of expression cultures, transformed *E. coli* cells were not plated but used for direct inoculation of 10 ml overnight cultures with 1 M IPTG for inducing protein expression..

2.2.2.12 Colony picking

To screen for transformants containing the desired insert, single colonies were picked with a sterile pipette tip (Eppendorf) from a freshly grown selective plate and used to inoculate a 3 ml LB culture. The culture was grown overnight at 37°C and further used for plasmid preparation.

2.2.2.13 Plasmid preparation and transformant screening

Plasmid DNA was purified from *E. coli* cells using a plasmid purification kit (GenElute Plasmid Mini Prep Kit or GeneElute Plasmid Maxi Prep Kit) according to the manufacturer's instructions. Purified plasmid DNA was digested with specific restriction endonucleases to screen for plasmids containing the desired insert. The restriction digestion was carried out in a total volume of 20 µl containing plasmid DNA, restriction enzymes, restriction buffer and dH_2O as described above. The digestion was done at 37°C for 2 hours. Digested plasmid DNA was then analyzed by agarose gel electrophoresis.

2.2.2.14 DNA precipitation

Ethanol precipitation was used to concentrate DNA. 3 M sodium acetate, pH 5.2 (0.1 volume of the starting volume) and 100% ethanol (2.5 volumes the starting volume) were added to the aqueous DNA solution. After incubation at –20°C for at least 30 min, precipitated DNA was pelleted by centrifugation at 13,000 rpm for 15 min at 4°C. The supernatant was removed and the precipitated DNA was washed once with 70%

ethanol. The pellet was then air-dried and resuspend in an appropriate volume of dH_2O (50-100 µl).

2.2.3 Protein expression

2.2.3.1 Growth of standard *E. coli* expression cultures

10 ml of LB culture media containing antibiotics were inoculated with a positive colony and grown at 37°C overnight. 5 ml of the overnight culture was further used to inoculate 100 ml of prewarmed LB-medium with antibiotics. The culture was grown at 37°C with vigorous shaking until an OD_{600} of 0.6 was reached. Protein expression was induced by adding IPTG to a final concentration of 1 mM. Cultures were further incubated for additional 5 h at 37°C with vigorous shaking before cells were harvested by centrifugation at 4000 rpm for 20 min. Cell pellets were stored at –20°C.

A list of all *E. coli* strains used in this study can be found together with their antibiotic sensitivity in table 3.

Table 2.7 *E. coli* strains and their antibiotic sensitivity

Strain	antibiotic sensitivity	final concentration
BL21(DE3)	Ampicillin*	100 µg/ml
BL21-CodonPlus-RIL	Ampicillin*	100 µg/ml
	Chloramphenicol	25 µg/ml
M15	Ampicillin*	100 µg/ml
	Kanamycin	25 µg/ml

* The pQE-30 expression plasmid confers an ampicillin resistance.

2.2.3.2 Protein analysis by SDS-PAGE

Protein expression was analyzed by SDS-PAGE. Therefore, a pellet of harvested cells was resuspend in 8 M urea containing 0.1% NP40 and boiled at 95°C for 10 min to completely lyse the cells. 15 µl of the cell lysate was mixed with 5 µl 4x SDS-PAGE sample buffer (250 mM Tris-HCl, pH 6.8, 2% SDS, 20% mercaptoethanol, 40% glycerol and 1% bromophenol blue) and heated at 95°C for 5 min before loaded on a SDS polyacrylamid gel. A single SDS gel was run at 32 mV, two gels accordingly at 64

mV. SDS polyacrylamid gels were stained with Coomassie staining solution (0.025% Coomassie Brilliant Blue G, 30% methanol, 10% acetic acid) for 1h followed by a destaining procedure using a destaining solution containing 30% methanol and 10% acetic acid. To store Coomassie stained gels, they were sealed in cellophane and dried overnight.

10% Separating gel		**Final concentration**
944 µl	2 M Tris-HCl, pH 8.8	
100 µl	10 % SDS	0.375 M
1,670 µl	acrylamid/bisacrylamid 30 %	0.2%
2,265 µl	water (dH_2O)	10%
20 µl	APS 10 %	0.04%
10 µl	TEMED	0.2%

4.4% Stacking gel		**Final concentrations**
625 µl	0.5 M Tris-HCl, pH 6.8	
50 µl	10 % SDS	0.125 M
360 µl	acrylamid/bisacrylamid 30 %	0.2%
1.45 µl	water (dH_2O)	4.4%

2.2.3.3 Purification of 6xHis-tagged proteins under denaturing conditions

Insoluble 6xHis-tagged target proteins were purified under denaturing conditions in a batch purification procedure. A bacterial cell pellet of an expression culture (5-250 ml) was thawed on ice and resuspend in lysis buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 8.0). Cells were stirred for 60 min at RT for complete lysis, before centrifuged at 10,000 x *g* for 20 min to pellet the cellular debris. An aliquot of the supernatant was saved at –20°C for later analysis by SDS-PAGE. The cleared lysate was mixed with Ni-NTA beads (1 ml Ni-NTA slurry to 4 ml cleared lysate) and incubated on a rotary shaker (200 rpm) at RT for at least 60 min. The lysate Ni-NTA mixture was then loaded into a column and the column flow through was collected for later SDS-PAGE analysis. The column was washed twice with 4 ml wash buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 6.3) and wash fractions were saved. Recombinant protein was eluted with 4 times 0.5 ml elution buffer I (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 5.9) followed by 4 times with 0.5 ml elution

buffer II (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 4.5). Eluate fractions were collected and analyzed by SDS-PAGE.

2.2.3.4 Western blotting

Proteins separated by SDS-PAGE were transferred on nitrocellulose membranes (Hybond ECL) using a semidry blot system. For this, a blot sandwich was built up in the following order:

Anode (+): 6 Whatman filter papers soaked in 0.3 M Tris-HCl, pH 10.4 and 20% methanol → 3 Whatman filter papers soaked in 25 mM Tris-HCl, pH 10.4 and 20% methanol→ nitrocellulose membrane→ SDS-PAGE gel→ 9 Whatman filter papers soaked in 40 mM 6-aminocapronic acid, pH 7.6 and 20% methanol→ Cathode (-)

The proteins were transferred at 0.8 mA per cm2 gel size for 90 minutes.

The membrane was blocked over night at 4°C in blocking solution , after washing 5 minutes in washing solution, the membrane was incubated with primary antibodies (mouse α –His-tag, 1:2000; mouse α-AND1, 1:20; and mouse α-ATP-β) diluted in incubation solution for 120 minutes at room temperature or over night at 4°C. After washing three times 5 minutes each, the membrane was incubated for 90 minutes at room temperature with the appropriate AP-conjugated goat anti-mouse secondary antibody, diluted 1:5000 in incubation solution. The membrane was then washed once for 15 minutes and five times 5 minutes each in washing solution. Afterwards, the ECL-reagents were mixed 1:2 and the membrane was incubated for 1 minute. Superfluous solution was removed and the membrane was covered with plastic foil. Immunolabeling was then visualized by exposing Hyperfilm ECL to the membrane (Amersham Biosciences, Freiburg). NBT/BCIP coloring reagents were also used for development of membranes, where the nitroblue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) color reaction was performed in 20 mM Tris, pH 9.8, 500 mM NaCl, 0.5 mM $MgCl_2$.

Blocking solution:	**Washing solution**	**Incubation solution:**
5% dry skimmed milk	0.05% Tween-20	5% dry skimmed milk
0.2% Tween-20	in PBS, pH 7.4	0.05% Tween-20
02% NaN_3		in PBS, pH 7.4
in PBS, pH 7.4		

2.2.3.5 Quantification of proteins

Protein concentrations were determined using a BCA Protein Assay Kit in accordance to the manufacturer's recommendations. In brief, BSA standards were prepared in different concentrations as reference. Standards and unknown samples were incubated with 200 µl BCA working solution for 30 min at 60°C before the colorimetric detection was done by measuring the absorbance at 550 nm. Unknown samples were quantified with reference to the BSA standards used.

2.2.3.6 Immunization of mice and affinity purification of antibodies

Purified His-tagged recombinant proteins were concentrated in 1x PBS to a final concentration of 1mg/ml by using centriprep tubes (Millipore, Bedford, MA. USA) according to the to the manufacturer's instructions. For the initial immunization, 100 µg of each antigen (purified His-tagged protein) were mixed with the same volume (100 µl) of ABM-S complete adjuvant (Linaris, Betting, Germany) and injected at subcutaneous and intramascular sites of a BALB/c mice. Boosting was performed three times at 2 week intervals, each consisting of 50 µg of antigen mixed with the same volume of ABM-N incomplete adjuvant (Linaris, Betting, Germany). After the final boosting, mice were bled and the sera were separated from other blood components by centrifugation. To test for the presence and specificity of polyclonal antibodies, sera were used with different concentrations (1:50, 1:100, and 1:200) for immunofluorescence staining and western blot. Immunofluorescence staining showed a weak specificity of the polyclonal antibodies, while the western blot analysis using the His-tagged purified proteins and *T. gondii* lysate showed a high specificity. To get rid of other non-specific antibodies in the sera, we purified them by using affinity purification technique. Briefly, purified His-tagged proteins were run by SDS-PAGE, transferred to a nitrocellulose membrane, and the membrane strips containing the attached proteins were incubated over night with the corresponding sera, washed thoroughly, and the bound antibodies were eluted and stored at -20 C. The purified antibodies showed a high specificity by immunofluorescence staining.

2.2.3.7 Bioinformatic and computational analysis.

Preliminary genomic and/or cDNA sequence data of *T. gondii* were accessed via http://ToxoDB.org (version 3.0) and/or http://www.tigr.org/tdb/t_gondii/. Genomic data

were provided by The Institute for Genomic Research and by the Sanger Center (Welcome Trust). EST sequences were generated by Washington University.

The **B**asic **L**ocal **A**lignment **S**earch **T**ool (**BLAST**) at (http://www.ncbi.nlm.nih.gov/BLAST/) was used to search the GenBank database for nucleotide and protein sequences based on similarity to a query sequence as well as to align two sequences (bl2seq). DNAStar package was used to analyze nucleotide and amino acid sequences, select primers for PCR, predicting putative open reading frames size of putative proteins, and locating hydrophobic/hydrophilic domains in protein sequences.

The most popular online available bioinformatic programs used in this study to predict for subcellular localization of proteins are: MitoProtII 3.0 (http://ihg.gsf.de/ihg/ - mitoprot.html), SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/) , PSORT (http://psort.hgc.jp/form.html) , TargetP (http://www.cbs.dtu.dk/services/TargetP/) , PlasMit (http://gecco.org.chemie.uni-frankfurt.de/plasmit/index.html) , Predator (http://bioweb.pasteur.fr/seqanal/interfaces/predator-simple.html). Transmembrane helices predicting programs used are TMHMM (http://www.cbs.dtu.dk/services/ TMHMM-2.0/), SOSUI (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html), and TMPred (http://www.ch.embnet.org/software/TMPRED_form.html). MotifScan (http://myhits.isb-sib.ch/cgi-bin/motif_scan), InterPro Database (http://www.ebi.ac.uk/ interpro/databases.html) were used to predict for protein specific domains and motifs.

ClustalX (http://bips.u-strasbg.fr/fr/Documentation/ClustalX/), and ClustalW (http://www.ebi.ac.uk/clustalw/) were used to perform multiple sequence alignments, and aligned sequences were edited by BoxShade (http://www.ch.embnet.org/software/ BOX_form.html).

Genomes of other apicomplexan parasites were blasted for orthologs to *T. gondii* genes as *Theileria parva* at (http://www.tigr.org/tdb/e2k1/tpa1/), *Theileria hominis* at (http://www.sanger.ac.uk/Projects/T_annulata/), six *Plasmodium* species at (http://www.plasmodb.org/), *Cryptosporidium parvum* and *Cryptosporidium hominis* at (http://www.cryptodb.org/cryptodb/index.jsp).

PHYLIP, a Phylogeny Inference Package (http://evolution.genetics.washington.edu/ phylip.html) was used to draw a phylogenetic trees from multiple sequence alignment files created by ClustaW and ClustalX. Clone 5 Manager Software and Enhance Program (Sci Ed Software) were used to draw and edit plasmids sequence.

RESULTS

3.1 Characterization of the respiratory chain genes in *T. gondii*

3.1.1 Absence of complex I in the respiratory chain of *T. gondii*

Complex I (NADH:ubiquinone oxidoreductase; EC 1.6.5.3) is a membrane-bound, multisubunit enzyme. Complex I contains a common set of 14 subunits, the core subunits, which are considered sufficient for energy transduction. Seven of them are predominantly hydrophilic and encoded in the nucleus (the 75, 51, 49, 30 and 24 kDa subunits, TYKY and PSST, according to the nomenclature for bovine complex I) and seven are highly hydrophobic, predicted to be dominated by transmembrane helices and encoded by the mitochondrial genome in eukaryotes (subunits ND1–ND6 and ND4L). The seven nuclear genes are encoding the central, highly conserved subunits of complex I; these subunits contain the binding site for the FMN cofactor and the ligands of iron-sulfur clusters.

Extensive BLAST searching of the ToxoDB (http://ToxoDB.org) for a possible complex I subunits using the amino acid sequences encoded by the seven nuclear genes from bovine (*Bos Taurus*), yeast (*Yarrowia lipolytica*), and plant (*Arabidopsis thaliana*) as a query has shown no significant similarity in the *Toxoplasma gondii* genome using TBLASTN (protein vs. translated DNA) program and searching *T. gondii* all nucleotides (genomic and EST) database. Searching the online available genomes of other species of apicomplexa such as *Theileria parva*, *Theileria hominis*, six *Plasmodium* species, *Cryptosporidium parvum,* and *Cryptosporidium hominis* has also shown no significant similarity for any of the seven catalytic subunits of complex I, instead sequences with a high similarity to genes coding for a non-proton translocating, rotenone insensitive, single polypeptide alternative (type II) NADH dehydrogenase from bacteria, yeasts and plants has been identified. These findings indicate that *T. gondii* as well as other species of the phylum apicomplexa lack a conventional complex I, instead they have at least one alternative (type II) NAD(P)H dehydrogenase catalyzing the first step in the respiratory electron-transport chain in mitochondria, namely the reduction of ubiquinone (Q) by NADH.

3.1.2 Characterization of alternative NADH dehydrogenase genes of *T. gondii*

To identify genes coding for mitochondrial NADH dehydrogenases, we performed a BLAST search of the genomic database of *Toxoplasma gondii* Genome Sequencing Project at The Institute for Genomic Research and Sanger Center (http://ToxoDB.org and http://www.tigr.org/tdb/t_gondii/).

The putative amino acid sequence of the putative NADH dehydrogenase (PfNDH2) of *Plasmodium falciparum* 3D7 (accession number NP_704690) was used as the query sequence using TBLASTN (protein vs. translated DNA) program and searching *T. gondii* all nucleotides (genomic and EST) database.

Among other homologues, contigs TGG_994254 and TGG_994290 which are located on chromosomes Ib and IX, with high (P = 2.5e-71) and moderate similarity (P = 6.2e-37) to the putative NADH dehydrogenase of *P. falciparum,* contain the *T. gondii ndh2-I* and *ndh2-II* genes respectively. The preliminary coding sequences were determined by locating the regions of similarity (exons) of both query (PfNDH2) and subject (TGG_994254; TGG_994290) sequences. Determination of the putative start codon(s) of the first exon was/were identified by locating upstream, in-frame ATGs, confirmed by RT-PCR and sequencing of the 5' upstream region.

To confirm the sequence of the open reading frames (ORFs) and the exon-intron boundaries, the complete coding sequences of both genes were amplified by RT-PCR using specific primers designed to include the putative start and stop codons, cloned in TOPO TA cloning vector followed by sequencing of both DNA strands.

3.1.3 Sequence analysis of TgNDH2-I and TgNDH2-II

The *T. gondii ndh2-I* and *II* genes encode a precursor polypeptide of 619 and 657 amino acid residues (**Fig. 3.1**) with a predicted mass of 67.2 and 72.1 kDa respectively. In-frame stop codons are present 783 and 51 nt before the presumed initiation ATG codons of *ndh2-I* and *ndh2-II* respectively, the deduced amino acid sequence encoded by these regions have no other in-frame methionine residues and no putative conserved domains using NCBI BLAST. Other in-frame start codons are located 177 nt and 6 nt downstream the first one of *ndh2-I* and *-II* respectively. Polyadenylation signal of 6 residues (AACAAA) were found at positions 917 and 92 nt downstream the TGA stop codons for *ndh2-I* and *ndh2-II* respectively.

A. TgNDH2-I

```
  1 MAGQWLRLLA GASVPMLSLP ARCDSPASPS SPSSPKERVS ALLAQPPRPF SAFSPVSTWM
 61 STKWTSFRLR TGLLSPAAVA ASAVASASPA AREAPARRQK VVVVGSGWAA VSFLADLDMT
121 RYEPVVISPR DYFTFTPLLP SVCVGTLPAS ACMTGVRELL VRGGVPCGSF YEGRVAEICP
181 TEKKVRCQST HGKAQDAREW EESYDYLVVA AGADVNTFGV PGVKENAFFV KELEDARRLR
241 SALFDVIETA SVPGVSEEEK KKLLHFVVVG AGPTGVEVAA EIDDFFQAEG ATHFPQLRPL
301 VRITVVEMLP TVLAAYNNDV QAFAKRLLEE NPRVDLCLQS QVVGVGPDSV KVRTKRADGQ
361 VEEKEMPCGL LVWASGIKSP KVCLDLARKT AELREAQQQS PVILVDQQMK VRGCEGVYAL
421 GDCCRLSPPP LVQHADTLYE AATANGAAST DWLEREAPKL STVFPQLASS KYDFSQKPRQ
481 TQMTKEQFVK LLADIDAAYR APAPTAQNAK QAGRYLAQTF NAFPSVEEKR RAPAFVNQTR
541 GALVYLGHGQ AAADIEGWRT FLGGAATLLL WKAAYLQMQL TLHNAVACLG GWLRTSLVGR
601 AVCREHLDGE TVYGDRRK. 619
```

B. TgNDH2-II

```
  1 MAMLFSSSAA GSLPSRRSRS VAAKLAPFAS PIFSSPLLKQ GSDTAFGLRS PTPGLSVHPR
 61 FASRGPLTRS EEAGKAPFGF STARGEQTAT ETNAPRFGTS LCSSFPLSAT QKSHGSREKG
121 LTNERAFSTF SFQGFMKNVK SRNAKPYTGP PQKVVVLGTG WASVNFFRHL DPNIYDVTVI
181 SPRNYFTFTP LLPSVCAGTL SPLSCIEPVR SLTYRNGRKV ADFYEAHCTD VDFKNRIVAC
241 DSRQGGHFKV KYDYLVIAVG SESNTFGIKD VAANAFFLKE VEHAMAIRKK VMNNFELAAL
301 PQTSEKERDR LLHFVVVGGG PTGVESAAEF ADFIKEDMSK YFPQLIPHVS ISLIEGGSRL
361 LGTYPPDISA FAEKTLTEEL HVKLLLRSTV VGVDATSVRY VSNEPGASKE PKELLHGFVL
421 WASGVGEVPL VKKIIAENFP NVEGKPRLRG LPVDAQLRLL NQPNVYALGD CAAIAPPRLA
481 DAAQELFSKA GAAEPTPQWL GRHAPTLAQQ FPQLSPLKFN FAKLQSNEHL PADQFESFLA
541 EIDAAYRPPA PTAQNARQEG IYLAKVFNEC PHPEEKADAP AFQETWNGSL AYVGSGQAVA
601 HLPYFNIKGG FLSLPFWKAV YTQMQITWRS RTICLFDWLK TFFAGRDVGR DHEYYNH.
658
```

Figure 3.1: **Deduced primary structures of the TgNDH2-I and –II precursor polypeptides**. The putative cleavable mitochondrial targeting sequences as predicted by MitoProtII, as well as invariant three-G residues within conserved dinucleotide-binding motifs, are shown in bold. Pyridine nucleotide-disulphide oxidoreductase (PFAM PF07992.1) domain specific for binding of NAD(P)H is underlined. Residues of the putative quinone binding sites, type IA (LX_2HX_2T) (FISHER and ROCH, 2000) are grey-shaded.

The coding sequence of *ndh2-I* is interrupted by two introns, they are 450 and 483 bp long and start at position 1347 and 1931 after the translation start codon respectively; while *ndh2-II* is interrupted by 10 introns with different lengths (**Fig. 3.2**). The deduced primary structure of both proteins include an N-terminal mitochondrial-targeting sequences as predicted by MitoProtII and SignalP. The *T. gondii* NDH2-I and NDH2-II sequences like their plant, fungal, protozoan homologues have long amino terminal extensions with significant homology which is absent from bacterial homologues and have the longest amino terminals among all other apicomplexan NDH2s. The first 35 to 60 residues of the amino-terminal extensions of both proteins (**Fig. 3.3**) contain multiple residues with positively charged and hydroxylated side chains, characteristic of mitochondrial targeting sequences (SMALL and MCALISTER-HENN 1998, VON HEIJNE at al, 1989). However, correct transport and localization of both proteins in the mitochondria was further confirmed by *in vitro* expression of their

ORFs fused to c-myc tag within an expression vector (pTetO7Sag4-ACP-cmyc-DHFR) and detected by immunofluorescence staining (**Fig. 3.8**).

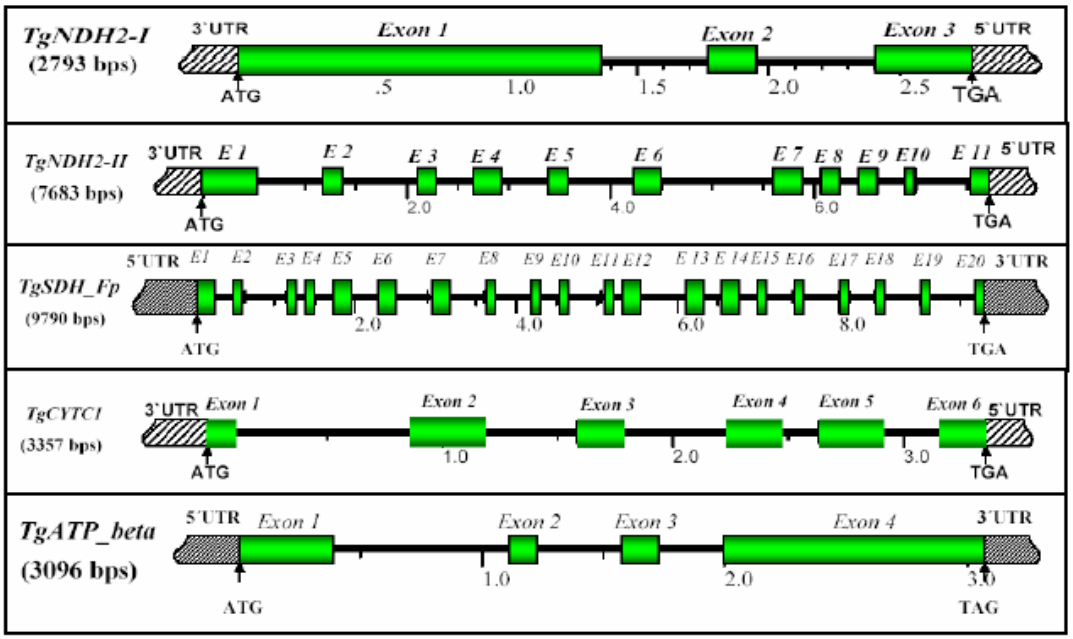


Figure 3.2: **Structure of the respiratory chain genes**. Schematic representation of gene structures of *T. gondii ndh2-I, ndh2-II, sdh-fp, cytc1*, and *ATP-β* showing. Size, number and position of the coding sequences (exons) are shown and represented by green boxes.

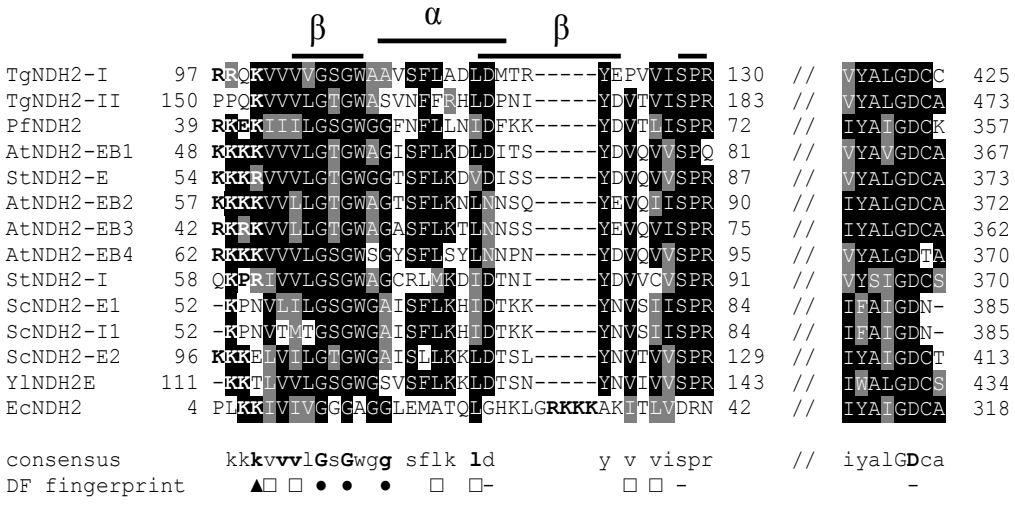
```
                                  +                       +                 +
TgNDH2-I      1 ------------MAGQWLRLLAGASVPMLS-------LPARCDSPASPSSPSS-----PK
TgNDH2-II     1 MAMLFSSSAAGSLPSRRSRSVAAKLAPFASPIFSSPLLKQGSDTAFGLRSPTPGLSVHPR
consensus     1 ............  ++ + .*.+  *  *.......*+   *.    +**. .....*+
                 +             +                    + +
TgNDH2-I     37 ERVSALLAQPP----RPFSAFSPVSTWMSTKWTSFRLRTGLLSPAAVAASAVASASP---
TgNDH2-II    61 FASRGPLTRSEEAGKAPFGFSTARGEQTATETNAPRFGTSLCSSFPLSATQKSHGSREKG
consensus    61   +. * +  ...+ **   . +     *     +  * * *   . *. +  .*+.+.
                                        +    ++ +
TgNDH2-I     90 ----------------------AAREAPARRQKVVVV 100
TgNDH2-II   121 LTNERAFSTFSFQGFMKNVKSRNAKPYTGPPQKVVVL 157
consensus   121 ....+..............+ *+   .  *+***.
```

Figure 3.3: Comparison of amino terminal sequences of *T. gondii* alternative NADH dehydrogenases. Black arrows indicate the cleavage sites of the mitochondrial targeting sequences as predicted by MitoProt II (cleavage sites/probability of export to mitochondria: 24/0.5466 for TgNDH2-I and 62/0.9917 for TgNDH2-II), Arginine (R) is at -1 position of the cleavage site. With SignalP, both have a prediction of signal peptide of 0.985 and 0.627 probabilities, max. cleavage sites are between positions 23-24 (Probability 0.803) and positions 34-35 (probability 0.132) of TgNDH2-I and II respectively (red arrows). Positively charged and hydroxylated residues in the first 35 to 60 residues of the amino-terminal extensions of both proteins are underlined and in bold.

Insights into the structure and function of both alternative NADH dehydrogenases were obtained by comparison with homologous proteins. Four prominent blocks of excellent sequence conservation that became apparent from the alignment of TgNDH2-I and TgNDH2-II with three NDH2 enzymes (isoforms) from *S. cerevisiae*, were also retained when two more distantly related NADH:ubiquinone oxidoreductases from *Solanum tuberosum* and *Arabidopsis thalina*, and the *E. coli* NDH protein were included in the alignment (**Fig. 3.4**). The possible functional significance of these motifs is discussed below.

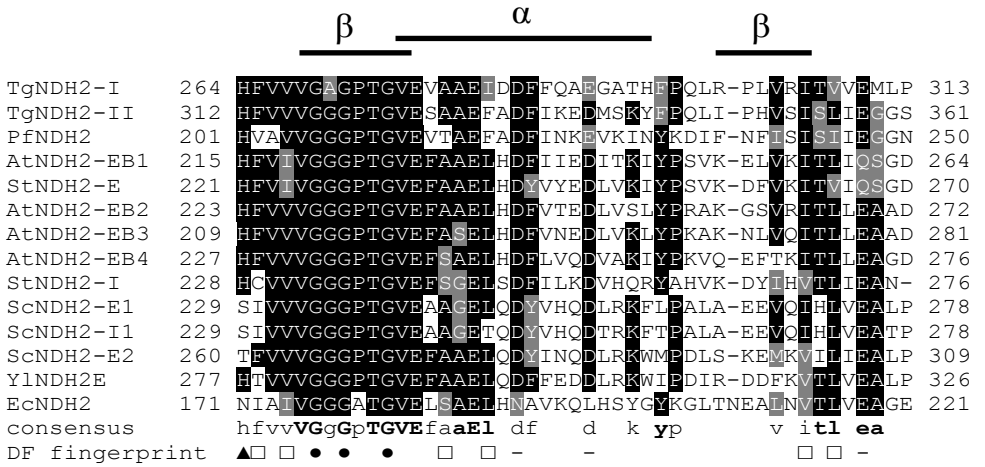
Dinucleotide fold I: This sequence conforms to most of the criteria for a dinucleotide binding βαβ fold (WIERENGA et al., 1985), which include a set of three glycines, spaced $GXGX_2G$, at the beginning, six regularly spaced hydrophobic residues, a conserved hydrophilic residue at the N terminus and a conserved acidic residue at the C terminus. This latter residue is aspartate in the *E. coli* protein, but is replaced by serine in all other cases. Also, the third, highly conserved but not invariant glycine residue of the $GXGX_2G$ motif in *T. gondii*, *P. falciparum*, and all four yeast proteins is replaced by other amino acids with small side chains, namely serine or alanine. In *E. coli*, this dinucleotide fold I domain lies close to the N terminus of the mature protein, in which the initiating N-formylmethionine is removed. Another peculiar feature of the *E. coli* protein is the insertion of the highly basic sequence RKKKAK at position 30-35 (JAWOROWSKI et al., 1981). While this insertion is not found in any of the other protein sequences, there is a somewhat similar basic stretch at the beginning of dinucleotide fold I (**Fig. 3.4a**). It was speculated that these basic amino acids may serve a similar function, for example in stabilizing the binding of FAD or NADH (KERSCHER et al., 1999).

A. Dinucleotide fold I (FAD binding motif)



Dinucleotide fold II: This sequence also conforms to the criteria for a dinucleotide binding βαβ fold (WIERENGA et al., 1985), but includes an unusually large loop region (**Fig. 3.4b**). The corresponding part of the ScNDI1 protein has already been suggested to form the binding site for the non-covalently attached FAD cofactor or the substrate NADH (DE VRIES et al., 1992).

B. Dinucleotide fold II (NADH binding motif)



C. Apolar/aromatic

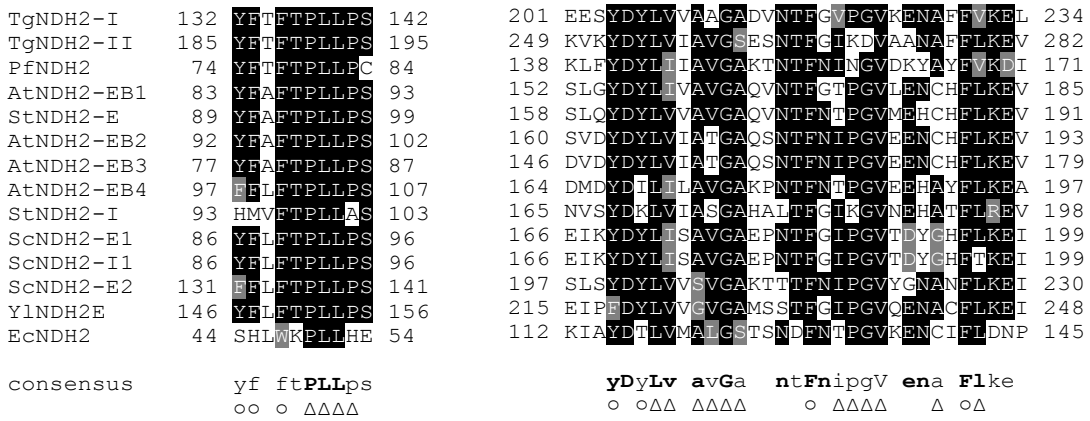


Figure 3.4: **Functional binding motifs of TgNDH2-I and TgNDH2-II.** Alignment of sequence motifs conserved between alternative NADH:ubiquinone oxidoreductases from *T. gondii*, P. *falciparum, A. thaliana, S. tuberosum, S. cerevisiae, Y. lipolytica* and *E. coli* by using ClustalW. Residues identical in at least seven of fourteen sequences are black shaded in the alignment and are indicated by one letter code in the consensus sequence, while residues similar to the consensus are gray shaded. In the consensus sequences, residues identical in all fourteen sequences are indicated in capital letter code and are printed in boldface, if this residue is present in the *E. coli* sequence. The consensus sequence of the dinucleotide fold motifs is compared with the dinucleotide fold fingerprint as defined in WIERENGA et al. (1985). In the fingerprints, conserved glycines are marked with filled circles ● and similar residues are indicated by the following symbols: hydrophobic residues by open squares □, hydrophilic residues (irrespective of charge) by closed triangles ▲, acidic residues by – symbols. Stretches of basic residues clustered at the N terminus of the first dinucleotide fold in the eukaryotic proteins and forming an insertion into this motif in the *E. coli* protein are shown in bold. Aromatic residues in the consensus of apolar/aromatic motifs are indicated by open circles ○, while apolar residues are indicated by open triangles Δ.

Apolar/aromatic: Two regions characterized by conserved apolar and aromatic residues can be identified (**Fig. 3.4c**). The first lies immediately after dinucleotide fold I and consists almost exclusively of apolar and aromatic residues. It is less well conserved in the *E. coli* sequence. The second is characterized by several conserved charged residues flanking stretches of apolar and aromatic residues. Both regions were speculated to form a pocket for the interaction of ubiquinone with the reactive moieties of FAD and NADH (KERSCHER et al., 1999) or to play a role in binding the proteins to membranes with amphipatic helices (FANG and BEATTIE, 2003).

Fisher and Rich proposed the existence of at least two types of quinone-binding sites (FISHER and RICH, 2000). TgNDH2-I and TgNDH2-II show the presence of type IA of Q-binding site (LX_2HX_2T) at positions 431-437 (**L**VQ**H**AD**T**) and 500-506 (**L**GR**H**AP**T**) respectively..

Identically positioned large insertions that correspond to calcium-binding motifs (EF-hand motif) of *N. crassa* NDE1 (MELO et al., 1999), *S. tuberosum* and *A. thaliana* NDB proteins (RASMUSSON et al., 1999, SVENSSON and RASMUSSON, 2001) are evident in NDH2s from *T. gondii, P. falciparum* and *P. yoelii* (**Fig. 3.5**). The EF hand motif is well conserved in all deduced Arabidopsis NDB sequences, while the EF motif of *N. crassa* NDE1 is not conserved in any of the plant and apicomplexan sequences. The corresponding insertion in apicomplexan sequences has no conserved residues representing the EF hand motif in *A. thalina, N. crassa* or any EF-motif containing proteins.

```
TgNDH2-I    385 RLSPPPLVQHADTLYEAATAN------GAASTDWLEREAPKLSTVFPQLASSKYDFSQKPRQTQMT---------------KEQFVKLLADIDAAYRA
TgNDH2-II   433 AIAPPRLADAAQELFSKAGA-------AEPTPQWLGRHAPTLAQQFPQLSPLKFNFAKLQSNEHLP---------------ADQFESFLAEIDAAYRP
PfNDH       319 KIQPKLLHEHTNEIIKILTG-------NKLTSEALKLKQSELTKTFPQLSISKWDYEK-NKKGEMT---------------PQQFHDYLFEIDKNYKS
PyNDH       366 QISPINSHEHVNEIINCLGN-------SKITSDVLKQKSKELSNIFPQLSDTKWDYNK-NKKSEMS---------------IKELQEYLFMIDKNYKS
AtNDEB1     332 SIAQRKILGDIANIFKAADAD----NSGTLTMEELEGVVDDIIVRYPQVELYLKSKHMRHINDLLADSEGNA----RKEVDIEAFKLALSEADSQMKT
StNDE       338 SVDQHKVMEDISTIFEAADKD----DSGTLSVEEFRDVLEDIIIRYPQVDLYLKNKHLLEAKDLFRDSEGNE----REEVDIEGFKLALSHVDSQMKS
AtNDEB2     339 TINQRKVMEDVSAIFSKADKD----KSGTLTLKEFQEAMDDICVRYPQVELYLKSKRMRGIADLLKEAETDDVSKNNIELKIEEFKSALSQVDSQVKF
AtNDEB3     325 TINQRKVMEDIAAIFKKADKE----NSGTLTMKEFHEVMSDICDRYPQVELYLKSKGMHGITDLLKQAQAENGSNKSVELDIEELKSALCQVDSQVKL
AtNDEB4     344 TINQRRVMEDIAAIFNKADKG----NTGTLKKKDFNSVVKDICQRYPQVELYLKKNKLKNIANLLKSANGED-----TQVNIEKFKQALSEVDSQMKN
NcNDE1      430 TIQNNVADHIITFLRNLAWKHGKDPESLELHFSDWRDVAQQIKKRFPQATAHLKR-----LDKLFEEYDKDQ----NGTLDFGELRELLKQIDSKLTS
                              * *     ** **  **                          # # ##        ##  ##
```

Figure 3.5: Conservation of the EF hand domain in different NDH2 proteins. The EF hand-carrying domain of potato NDB1 (RASMUSSON et al., 1999) was compared with the corresponding sequences in Arabidopsis NDB proteins, *N. crassa* NDE1 (MELO et al., 1999), an identically positioned large insert in homologs found in both isoforms of *Toxoplasma gondii* (ABA44355 and ABB17192), *Plasmodium falciparum* (CAD51833) and *Plasmodium yoelii* (EAA22988). The potato NDB1 Ca2+ binding EF hand motif pattern D-x-[DNS]-{ILVFYW} - [DENSTG]- [DNQGHRK] - {GP} - [LIVMC] - [DENQSTAGC] - x(2) - [DE] - [LIVMFYW] as defined in the PROSITE database are indicated by (*) which is conserved in all deduced Arabidopsis NDB sequences, with the exception of two positions in NDB4. In comparison, the *N. crassa* NDE1 EF hand motif (#) is not conserved in any of the plant and apicomplexa proteins. The *T. gondii, P. falciparum* and *P. yoelii* sequences contain no EF hand motifs. Conserved residues are shaded in black and amino acid position is given at the beginning of each sequence.

In consistent with other alternative NADH dehydrogenases, membrane-spanning helices have not been detected in the predicted amino acid sequences of both isoforms by using the public transmembrane predicting programs TMHMM 2.0, TMPred, and SOSUI. Therefore, it is proposed that these enzymes are not directly involved in mitochondrial transmembrane proton pumping. Rather, the enzyme provides reducing equivalents for downstream proton-pumping enzyme complexes (YAGI et al., 2004)

3.1.4 Phylogenetic analysis of the TgNDH2-I and TgNDH2-II

The overall amino acid sequence identity between both isoforms of *T. gondii* NDH2s and other alternative NDH2s reveals high homology to the alternative NADH dehydrogenases from plants (**Table 3.1a**). The highest amino acid identity of TgNDH2-I and TgNDH2-II were scored with the NDH2s from *A. thalina* (At-NDH2-EB3; 34%/37%), *S. tuberosum* (St-NDH2-E; 29%/32%), and *Oryza sativa* (Os-NDH2; 27%/29%) followed by *S. cerevisiae* (Sc-NDH2-E1; 28%/30%), *N. crassa* (Nc-NDH2-E2; 29%/31%) and *Y. lipolytica* (Yl-NDH2-E, 26%/29%), indicating a close relationship between plant and *T. gondii* enzymes. A ClustalW alignment (THOMPSON et al., 1994) of alternative NADH dehydrogenases from bacteria, fungi, plant and protozoa was then used as the basis for the generation of a phylogenetic tree using the PHYLIP program. Distance and maximum parasimony phylogenetic approaches were tested, substantially giving identical results. The results (**Fig. 3.6)** suggest that the apicomplexan enzymes share a most common ancestor and support that the phylum apicomplexa is an early branching eukaryotes. Moreover, kinetoplastid, fungal and plant alternative NADH dehydrogenases are the closest out groups to the apicomplexan sequences suggesting that the apicomplexan NDH2s have evolved closely with the external NDH2s of plants as supported by the phylogenetic tree generated by Michalecka (MICHALECKA et al 2003) and Uyemura (UYEMURA et al., 2004) for *P. falciparum* and *P. yoelii*. Phylogenetic trees by Fang (FANG et al 2003) and Michalecka have suggested that the 54 kDa NDH2 from trypanosome has evolved closely from the internal NDH2s of plants, which is also evident from the phylogenetic tree (**Fig 3.6)**.

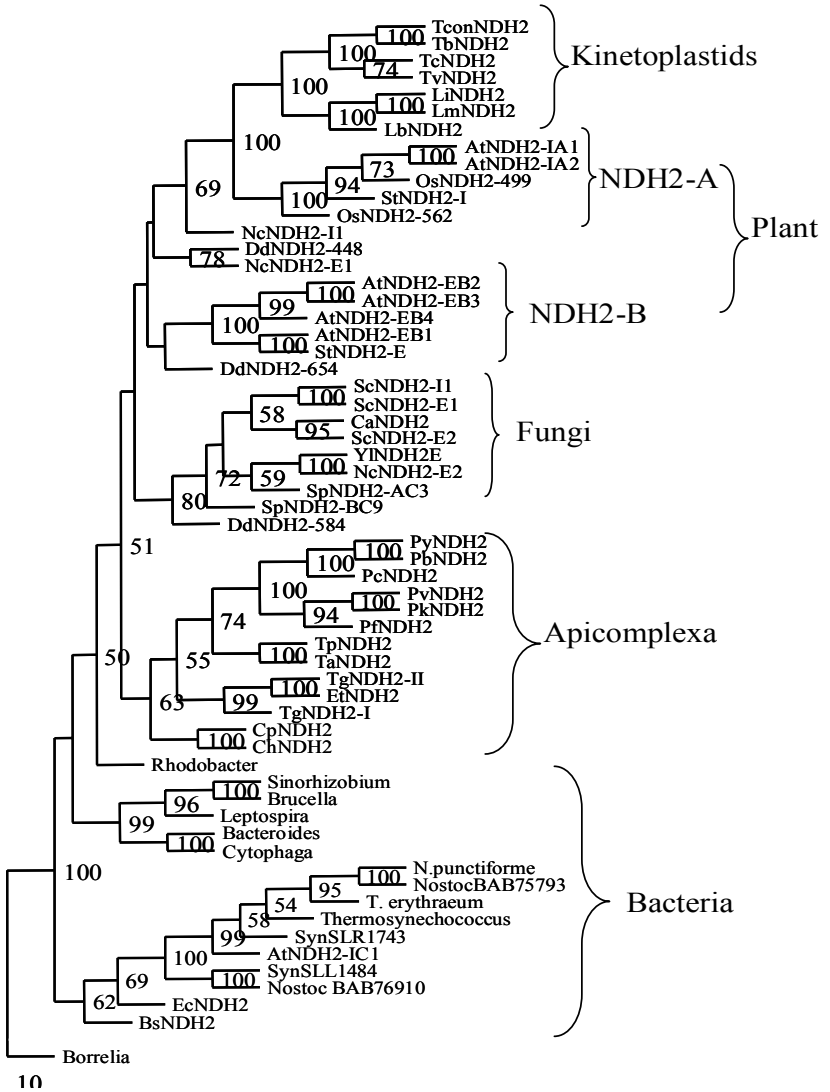


Figure 3.6: Phylogenetic distance-neighbor joining rooted tree of NDH2s. Phylogenetic analysis of bacterial, plant, fungal, and protist NAD(P)H dehydrogenase-like protein sequences. Sequences were aligned using ClustalW and ClustalX. Alignments were manually inspected and edited, assuring correct matches of conserved regions. The PHYLIP program was used for phylogenetic analyses using distance neighbor joining method. One hundred replicates with full searches were performed in a bootstrap test for the displayed tree. Bootstrap values are shown on the nodes. Plant proteins include the *Arabidopsis thalina* proteins: AtNDIA1 (NP_563783), AtNDIA2 (NP_180560), AtNDEB1 (NP_567801), AtNDEB2 (NP_567283), AtNDEB3 (CAB81256), AtNDEB4 (NP_179673), AtNDIC1 (NP_568205), *Solanum tuberosum*: StNDH2E (CAB52797), StNDH2I (CAB52796), *Oryza sativa* cv japonica: OsNDH2-499 (BAB68119), OsNDH2-562 (BAC15811). Protist proteins include the following apicomplexa organisms *Toxoplasma gondii* (ABA44355 and ABB17192), *Plasmodium falciparum* (CAD51833), *Plasmodium chabaudi* (XP_738714.1), *P. yoelii* (EAA22988), *P. berghei* (XP_673046.1), *P. knowleski* (PK9_1360c), *P. vivax* (Pv099180), *Theileria annulata* (CAI75759.1), *Theileria parva* (XP_763567.1), *C. hominis* (XP_668502.1), *C. parvum* (XP_628367.1), *T. brucei* (XP_823167), *T. congolense* (congo1180f06.p1k_8), *T. cruzi* (EAN89176), *T. Vivax* (Tviv501b02.q1k_4), *L. major* (CAJ09511), *L. infantum* (LinJ36.5870), *L. baraziliensis* (LbrM35.5100), *E. tenella* (Et_v1_Twnscn_Contig12641.tmp13). Fungal proteins include *N. crassa* proteins: NcNDE1(CAB41986), NcNDI1(S26704), *S. cerevisiae* proteins: ScNDE1 (CAA87359), ScNDE2(CAA98651), ScNDI(P32340), and *Y. lipolytica* (XP_505856.1). Bacterial proteins, *Nostoc* sp. (BAB75793), *N. punciforme* (ZP_00112108), *Synecchocystis* SLR1743, *Thermosynecchococcus* (BAC08688), *Synecchocystis* SLL1484, *Nostoc* sp. (BAB76910), *Rohodobacter* (ZP_00006015), *Leptospira* (AAN51595), *Bacteriodes* (AAO76644), *Cytophaga* (ZP_00119070), *Brucella* (AAL54028*), E. coli*; EcNDH2 (ZP_00723573), *B. subtillus*; BsNDH2 (ZP_01173397). I; internal, E; external; A:type A, B:type B, C: type C according to classification by MICHALECKA et al., 2003.

The genomes of other apicomplexan parasites such as Plasmodium, Theileria, Cryptosporidia, and Eimeria as well as the kinetoplastids like Trypanosoma and Leishmania show the presence of genes encoding the alternative (type II) NADH dehydrogenase. A high percentage of amino acid identity is shown among proteins from species of the same genus, with the highest identity (97%) between NDH2 from *C. hominis* and *C. parvum*, followed by *L. major* and *L. infantum* (96%) (**Table 3.2b**).

A.

	Organism	% Identity to				
		TgNDH2-I	TgNDH2-II	PfNDH2	PyNDH2	TbNDH2
PLANT	AtNDH2-IA1	26	28	25	23	**36**
	AtNDH2-IA2	24	28	24	23	**38**
	AtNDH2-EB1	**28**	**33**	**30**	**27**	31
	AtNDH2-EB2	**29**	**32**	**31**	**30**	32
	AtNDH2-EB3	**34**	**37**	**31**	**30**	31
	AtNDH2-EB4	**30**	**31**	**32**	**27**	33
	StNDH2-E	**29**	**32**	**29**	**26**	32
	StNDH2-I	27	30	24	24	**38**
	OsNDH2-499	27	28	26	24	**38**
	OsNDH2-562	24	29	22	20	**38**
FUNGI	ScNDH2-E1	28	30	29	31	25
	ScNDH2-E2	27	29	27	27	26
	ScNDH2-I1	26	29	28	30	23
	YlNDH2E	26	29	27	26	28
	NcNDH2-E2	29	31	27	24	29
	NcNDH2-E1	24	29	28	25	29
	NcNDH2-I1	26	27	24	23	31
	CaNDH2	27	28	29	25	25

B.

Organism	% Identity to																	
	Tg2	**Pf**	**Pc**	**Pb**	**Pk**	**Py**	**Pv**	**Ta**	**Tp**	**Ch**	**Cp**	**Tb**	**Tco**	**Tc**	**Tv**	**Lm**	**Li**	**Lb**	**Et**
T. gondii I	**38**	30	29	25	32	27	28	28	29	25	25	26	28	25	28	23	23	23	**36**
T. gondii II		**36**	34	30	36	33	28	35	34	29	29	29	28	26	30	28	28	27	**46**
P. falciparum			60	59	62	63	60	35	33	29	23	23	22	21	22	22	22	22	28
P. chabaudi				86	59	82	59	31	31	29	28	26	25	27	25	28	28	28	29
P. berghei					55	85	57	30	29	28	28	21	22	21	20	24	24	23	25
P. knowleki						59	81	34	32	29	29	23	23	22	23	22	23	22	26
P. yoelli							55	34	32	26	26	21	22	20	22	23	23	23	26
P. vivax								32	31	27	26	21	22	21	22	23	24	23	25
T. annulata									79	25	25	27	25	21	27	22	22	22	29
T. parva										25	25	26	26	21	28	22	22	23	29
C. hominis											**97**	22	21	19	23	21	21	20	20
C. parvum												21	21	19	23	21	21	20	20
T. brucei													76	67	68	57	57	57	27
T. congolense														62	66	54	54	53	28
T. cruzi															66	58	58	58	24
T. vivax																56	56	54	26
L. major																	**96**	84	23
L. infantum																		85	24
L. baraziliensis																			23

Table 3.1: **Amino acid sequence identity of TgNDH2-I and TgNDH2-II to other NDH2s** (**A**) Percentage of amino acid sequence identity of selected apicomplexan NDH2s with plant and fungal external and internal NDH2s. (**B**) Percentage of amino acid sequence identity of the putative NDH-2s of different protozoan parasites Highest identity percentages are shown in bold and grey-shaded.

Interestingly, Tg-NDH2-II has a higher amino acid identity to NDH2 from *E. tenella* (46%) than to TgNDH2-I (38%) and shares almost the same gene structure with 11 and 10 exons coding for TgNDH2-II and EtNDH2 respectively. Such finding raises the question of the evolutionary relationship between the two enzymes. As *T. gondii* is the only apicomplexan parasite which has two isoforms of NDH2 with significant amino acid identity (38%), it might be pointed to a gene duplication event. Based on the overall amino acid identity and phylogenetic analysis, it is tempting to speculate that NDH2s from apicomplexan and kinetoplastid protozoans are evolutionary distant.

3.1.5 Identification of genes coding for other respiratory chain proteins.

The putative amino acid sequences of the corresponding subunits of *Plasmodium falciparum* 3D7 (PfSDH-Fp, accession # NP_700807; PfCYTC1, accession # NP_702486, PfATP-β, accession # NP_701707) were used as query sequences using TBLASTN (protein vs. translated DNA) program and searching *T. gondii* all nucleotides (genomic and EST) database.

Among other homologues, contigs TGG_994577, TGG_994326, TGG_994270 which are located on chromosomes X and XII have the highest similarity (P = 9.3e-103, P = 3.9e-127, and P = 1.3e-187) to the query sequences from *Plasmodium falciparum* 3D7*,* contain the *T. gondii sdh-fp*, *cytc1,* and *ATP- β* genes, respectively.

To confirm the sequence of the open reading frames (ORF) and the exon/intron boundaries, the complete coding sequences were amplified by RT-PCR using specific primers designed to include the start and stop codons, and both DNA strands were sequenced from both directions after cloning in TOPO TA cloning vector.

The *T. gondii sdh-fp, cytc1* and *ATP- β* genes encode a precursor polypeptides of 669, 396, and 560 amino acid residues with a predicted mass of 72.75, 45.74, and 59.91 kDa, respectively (**Fig 3.6**). In-frame stop codons are present at 345, 162, 90 bp before the presumed initiation ATG codon of *sdh-fp, cytc1* and *ATP- β* respectively. The deduced amino acid sequences coded by these regions have no other in-frame methionine residues and no putative conserved domains checked by NCBI BLAST. Alternative in-frame start codons are located at 375, 87, and 102 bp downstream the first one, respectively. Polyadenylation signals of six residues (AAAAAA) are also found at positions 950, 553, and 15 downstream the stop codons, respectively.

The coding sequences of *sdh-fp, cytc1 and ATP-β* are interrupted by 19, 5, and 3 introns (**Fig. 3.2**). The deduced primary structures include an N-terminal

mitochondrial-targeting sequences for SDH-Fp and ATP-β but not for CYTC1 as predicted by MitoProt II and SignalP 3.0. However, correct transport and localization of proteins in the mitochondria were confirmed by immunofluorescence staining (**Fig. 3.8**).

Sequence analysis of their deduced amino acid sequences show that they keep conserved functional motifs and domains similar to their orthologs from other organisms. No transmembrane domains have been predicted to SDH-Fp and ATP- β, but 2 transmembrane helices have been detected at positions 126-144 and 356-378 for CYTC1 by using the TMHMM (**Fig. 3.7b**).

Two consensus Pfam motifs are identified for TgSDH-Fp by using InterProScan program , the PF00890.13 (FAD binding domain or fumarate reductase/succinate dehydrogenase flavoprotein, N-terminal) between residues 75 and 477 and the PF02910.9 (fumarate reductase/succinate dehydrogenase flavoprotein C-terminal domain) between residues 532-669 as binding sites for the ADP portion of FAD (**Fig. 3.7a**)

Cytochrome c1 of *T. gondii* has a predicted cytochrome c1 domain between residues 159-384 (PFAM PF02167.5) (**Fig. 3.6b**), while β-subunit of ATP synthase predicts 3 domains at positions 84-150 (PFAM PF02874.9, ATP synthase alpha/beta family, beta-barrel domain), 206-434 (PFAM PF00006.13, ATP synthase alpha/beta family, nucleotide binding domain), and 447-554 (PFAM PF00306.13, ATP synthase alpha/beta family, C terminal domain) **(Fig. 3.7c**). Multiple sequence alignments of the amino acid sequences of these proteins with their orthologes from bacteria, protists, fungi, plant and mammals show that they are highly conserved around the binding motifs and show the highest identity to their counterparts from apicomplexans, specially *P. Falciparum* (See Index, **Fig. 6.1, Fig. 6.2, Fig. 6.3**)

A. TgSDH-Fp

```
  1 MHASASLTQA LRPYARRPLS SALSRSSLSP ALAAGVARGL NAAQPQATGP SFVQQKRFFT
 61 NVKKPAYRVI DHEYDAIVVG AGGAGLRAAF GLASAGIKTA CLSKLFPTRS HTVAAQGGIN
121 AALGNMTEDD WRWHAYDTVK GSDWLGDQDA IQHMCREAPK VVRELESYGL PFSRTEDGRI
181 YQRAFGGQSL RFGKGGQAYR CAAAADRTGH AMLHSLYGQS LAHDCKFFVE YFALDLMMSG
241 ENDERKCHGV VAMCMEDGSI HRFAAKHTVL ATGGYGRAYQ SCTSAHTCTG DGGGMVSRAG
301 LPLEDLEFVQ FHPTGIFPAG CLITEGCRGE GGILRNGQGE AFMARYAPTA KDLASRDVVS
361 RSMTIEIREG RGCGPNRDHM HLDLTHLPPA TLHERLPGIT ETAKIFAGVN AEKEPIPVLP
421 TVHYNMGGIP TNWRAQVLTT SRSKAGGPDK IVQGLYAAGE AACASVHGAN RLGANSLLDL
481 VVFGREAART IADECKKDAS APSLPPNAGE ETLAMVDRLR HSNGPLTTAQ IRQRMQKTMQ
541 DHAAVFRTGA VLREGVEMMK DVAKSFADVG VKDRSLSWNT DLIETLELQN LLTQAVQTIV
601 GAEARKESRG AHAREDFKER DDKNWMKHTL SWQTHREVEK AEFPLTYRQV ISQPLDNEME
661 HIPPAKRVY. 669
```

B.TgCYTC1

```
  1 MGGGGGGALN KLFPGYKDKI WMKVPWRQQM IQHWNKSYEK QVYSESVALN RTFQARNQLV
 61 LDRLKPSGAY RLPAVDYKRQ LSRGTLVEGA DFYLPTAQEQ QRLARHFEPY SEQEQEERRK
121 FRFQSISVYL AVALGASFVH DYFYQRRPVA WCLEKEPPHP PSYPFWFKSL FHSHDIPSVR
181 RGYEVYRKVC ATCHSMEQLH FRHLVGEVLP EKRVKQIAAE YDVTDGPNDQ GEMYTRPGIL
241 GDAFPSPYPN EEAARYANGG AYPPDLSLIT AARHFGPDYL MALLGGYRDP PEGVELRPGL
301 YWNVWFPGNA IAMPPPLMDE MIDYEDGTPC NISQMSKDVV NFLTWATEPT ADERKLYGLK
361 CVSAIAIGTV LMTLWWRFYW AMYATRRIDF GKLKYL. 396
```

C.TgATP-β

```
  1 MASPALQTCW RNLARLSGAQ VRPSHFGAFS LGSRMSPFSS LLGARASPIA TGRAGLRFLS
 61 SAAPNPGKKP ASAAPPAGTN HGRITQVIGA VVDVHFDEQL PPILNSLEVQ GHTNRLVLEV
121 AQHLGENTVR TIAMDATEGL VRGQKVVDTG APIQVPVGVE TLGRIMNVIG EPVDECGPVP
181 AKKTYSIHRA APLFADQSTE PGLLQTGIKV VDLLAPYAKG GKIGLFGGAG VGKTVLIMEL
241 INNVANKHGG FSVFAGVGER TREGNDLYHE MMTTGVIKRK KLEDGKFDFT GSKAALVYGQ
301 MNEPPGARAR VALTALSVAE YFRDEQGQDV LLFIDNIYRF TQAGSEVSAL LGRIPSAVGY
361 QPTLATDLGQ LQERITTTKK GSITSVQAVY VPADDLTDPA PATTFAHLDA TTVLSRQIAE
421 LGIYPAVDPL DSTSRMLAPE IVGQEHYDTA RATQKLLQDY KSLQDIIAIL GMDELSEEDK
481 LVVSRARKIQ RFLSQPFTVA EVFTGKPGRF VELPETIKSA QTILRGECDD LPEMAFYMCG
541 GLEEVRSKAV KMAQEAASGK . 560
```

Figure 3.7: **Deduced primary structures of the TgSDH-Fp, TgCytc1, and TgATP-beta precursor polypeptides.** Predicted mitochondrial targeting sequences predicted by MitoProtII are underlined, predicted Pfam binding domains by InterProScan are bold, and predicted transmembrane domains by TMHMM are boxed.

3.1.6 GenBank submission of nucleotide sequences.

Sequence data for TgNDH2-I, TgNDH2-II, TgSDH-Fp, TgCYTC1, and TgATP-β have been submitted to the GenBank database under accession numbers DQ211932, DQ228957, DQ228958, DQ228959, and DQ228960 respectively.

3.1.7 Localization of the respiratory chain components in *T. gondii*

Apicomplexan parasites, including the human pathogens Toxoplasma and Plasmodium, contain a vestigial plastid, the apicoplast. This chloroplast-derived organelle is the remnant of a secondary endosymbiosis between an ancestral apicomplexan and a photosynthetic organism which is believed to be red or green algae (FUNES et al., 2002). As chloroplasts, the origin apicoplast, have a photosynthetic electron transport chain as well as mitochondria which have a respiratory electron transport chain, subcellular localization of these proteins is a necessary step to figure out their role in energy metabolism. The most popular subcellular prediction programs detect a mitochondrial signal sequences at the N-terminal of the amino acid sequences of both isoforms of TgNDH2, TgATP-β, and TgSDH-Fp but not for TgCYTC1 (**Table 3.2**)

Gene	Prediction scores %						
	MITOPROT	PSORT	PREDATOR	PLASMOAP	TARGETP	PLASMIT	SIGNALIP
TgANDI	M 54.6 %	M 42.4%	ER 42 %	-	M 33.5%	Non-mito 99	SP 98.5%
TgANDII	M 99.2 %	M 86.0%	M 23%	-	M 93.1%	Non-mito 99	SP 62.7%
TgSDH-Fp	M 99.6%	M 54.7%	M 33%	-	M 94.5%	Non-mito 99	SP 97.8%
TgCYTC1	Not predicted	-	None	-	-	Non-mito 99	No-SP
TgATP-beta	M 99%	M 54.2%	M 59%	-	M 91.8%	Non-mito 99	SP 46.9%

Table 3.2: Computational prediction of subcellular localization of respiratory chain components. Prediction of N-terminal targeting sequences was performed using SIGNALP(NIELSEN et al., 1997), TARGETP (EMANUELSSON et al., 2000), MITOPROT (CLAROS and VINCENS, 1996), PLASMIT (BENDER et al., 2003) and PLASMOAP (FOTH et al., 2003), PSORT, and Predator; M: Mitochondrial, SP: Signal Peptide.

To confirm the predicted mitochondrial localization of these proteins in *T. gondii*, their ORFs were amplified by RT-PCR and cloned into a TATi expression vector (pTetO7Sag4-ACP-cmyc-DHFR) fused to a c-myc tag at the C-terminal end. This vector allowed the expression of these ORFs under the control of an inducible regulatable promoter (transactivator responsive promoter) which is regulated by the tetracycline derivative anhydrotetracycline (Atc). After transfection and selection, additional copies of each gene were randomly integrated in the genome of parasites expressing the *T. gondii* specific transactivator TATi-1.

The localization of the expressed proteins was confirmed by immunofluorescence staining using primary antibodies for the c-myc tag (anti-myc mAb 9E10). To confirm the localization of the expressed proteins in the single mitochondrion of *T. gondii*, we used the S9-GFP expression vector which has a mitochondrial targeting sequence from *T. gondii* mitochondrial protein fused with GFP(DEROCHER et al., 2000). Stable parasites expressing the additional copies of genes of interest were co-transfected with this S9-GFP vector, and its transient expression was monitored by microscopy (**Fig. 3.8**). Co-localization with S9-GFP confirmed the expression, transport and localization of the additional (exogenous) copies of these genes and their encoded proteins in the single mitochondria of *T. gondii* but not in the apicoplast or any other organelles.

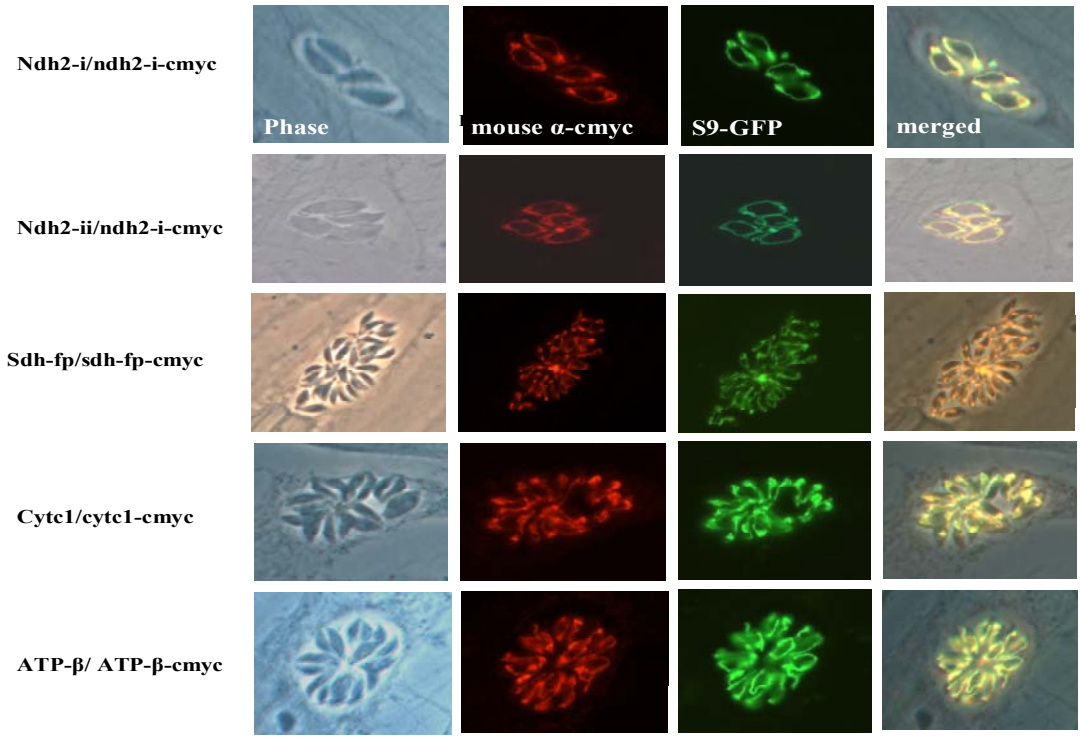


Figure 3.8: Mitochondrial targeting of the ectopic proteins. Tachyzoites of RH strain were transfected with TATi vectors containing cloned ORFs of *ndh2-I, ndh2-II, sgh-fp, cytc1*, and *ATP-β* , the expressed ectopic genes were detected by immunofluorescence microscopy using anti-myc mAb 9E10. Stabley transfected parasites expressing the ectopic genes were cotransfected with pCAT S9(33-159)-GFP, which encodes a mitochondrial targeted GFP fusion protein (DEROCHER et al., 2000). Co-localization of GFP fluorescence with c-myc immunofluorescence demonstrates a mitochondrial localization of proteins encoded by the ectopic genes.

3.1.8 Production of polyclonal antibodies for TgNDH-I and TgATP-β

Polyclonal antibodies raised against TgNDH2-I and TgATP-β were produced in mice to further confirm the mitochondrial localization of the endogenously expressed proteins, to study a possible stage regulation on the protein level, and to confirm the absence of the protein expression in the knock out mutants. The DNA sequences coding for hydrophilic regions of both proteins were amplified by *Pfu* DNA polymerase using cDNA from tachyzoites of RH strain as a template with upstream and downstream primers including BamH-I and Kpn-I restriction sites respectively (**Table 2.5**). The agarose gel extracted PCR products were sub-cloned in TOPO TA cloning vector, re-cloned in the standard expression vector pQE30 resulting in a fusion with N-6xHis tag (**Fig. 3.9a**), the accuracy of the cloned DNA sequences were confirmed by sequencing from both directions. The expression of the recombinant proteins in *E. coli* (BL21+ strain) was induced by 1 mM IPTG and analyzed by SDS

PAGE (**Fig. 3.9b**). The recombinant proteins were purified under denaturing conditions with Ni-NTA beads and analyzed by SDS PAGE (**Fig. 3.9c**) to check size and integrity of the purified proteins. Purified proteins were used to immunize mice and sera were collected after 4 immunizations with the recombinant proteins mixed with incomplete and complete adjuvants. Specificity of the obtained polyclonal antibodies in the sera was tested by immunoblotting with the purified recombinant and native TgNDH2-I and ATP synthase β subunit proteins from a *T. gondii* crude lysate by SDS-PAGE and western blotting (**Fig. 3.9d+e**), and by immunofluorescence staining (**Fig. 3.10**).

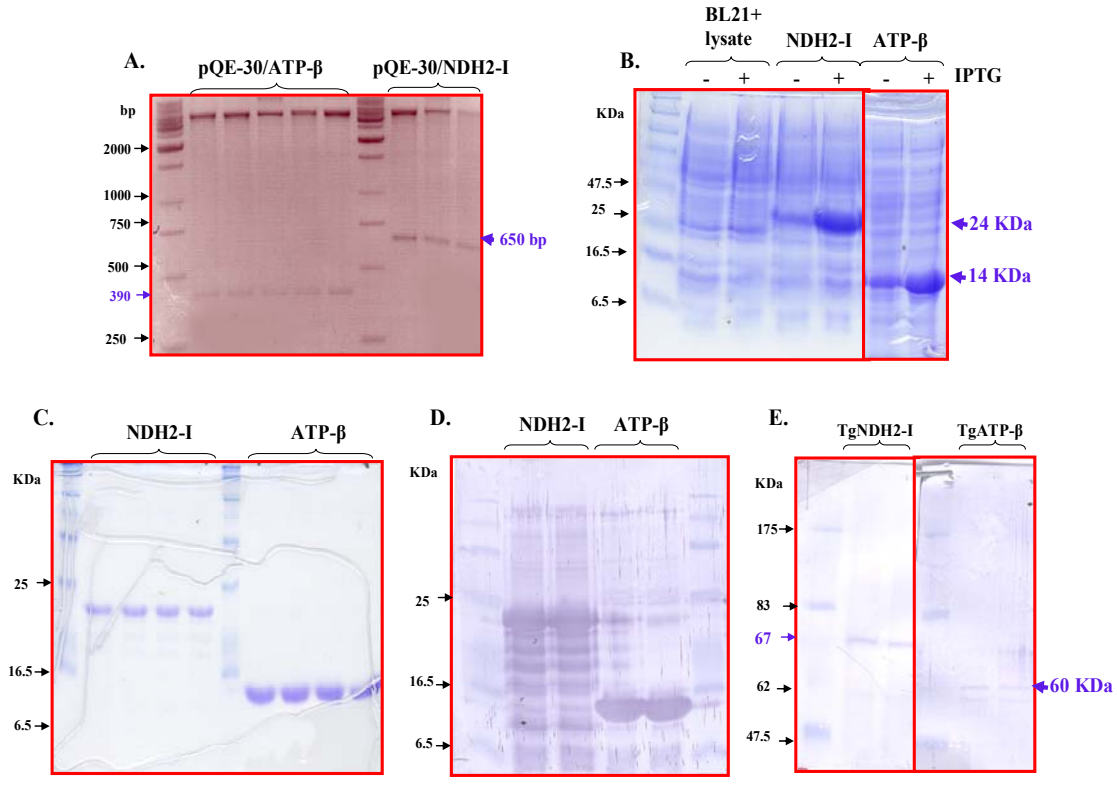


Figure 3.9: Production of polyclonal antibodies to NDH2-I and ATP-β. (**A**) Restriction analysis of the cloned hydrophilic regions of the ORFs of NDH2-I (390 bp) and ATP-beta (650 bp) in the expression vector PQE30, in BL21+ *E. coli* strain. (**B**) Expression of the recombinant protein in BL21+ *E. coli* strain by IPTG induction.(**C**) Purification of the recombinant proteins via 6xHis-tag for immunization in mice. (**D**) Western blot with the polyclonal serum against the His-tag purified recombinant proteins. (**E**) Western blot with purified monoclonal antibodies binding with NDH2-I and ATP-beta in *Toxoplasma gondii* lysate.

Immunofluorescence staining using polyclonal antibodies in the sera showed a cross reactivity with other cytosolic antigens of *T. gondii*. However, affinity purification of these antibodies from other components of the sera has shown a specific binding to the mitochondrial proteins (**Fig. 3.10**). This is a confirmation that endogenous TgNDH2-I and TgATP-β proteins are expressed and transported to the mitochondria of tachyzoites, and indicating for a functional respiratory chain in this stage.

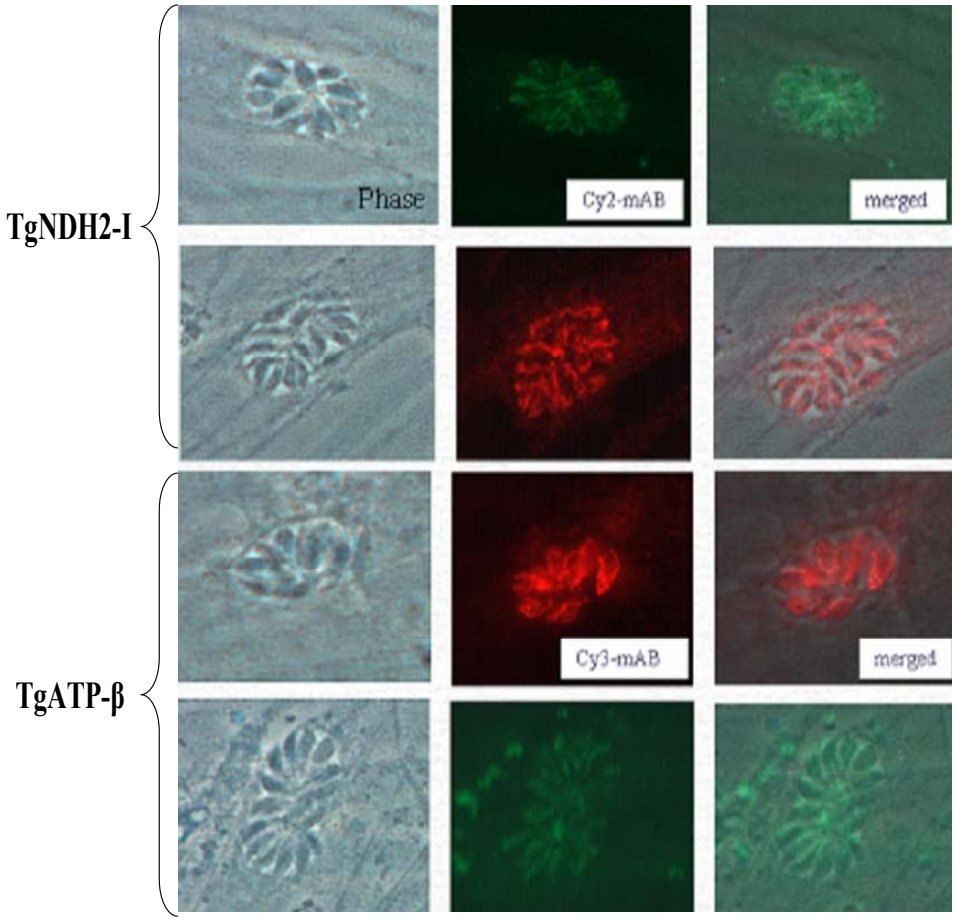


Figure 3.10: Immunofluorescence staining of endogenous NDH2-I and ATP-β by affinity purified polyclonal antibodies. Affinity purified mouse anti-TgNDH2-I and anti-ATP-β from polyclonal sera were used as a primary antibodies for immunofluorescence analysis for confirming the expression and localization of both subunits in the mitochondrial inner membrane of *T. gondii*. Cy3 (red) and cy2 (green) conjugated mouse antibodies were used as secondary antibodies.

3.1.9 Gene expression profile for the respiratory chain components in *T. gondii*

To study a possible differential expression of the respiratory chain components in both stages of *T. gondii*, we studied the steady-state levels for a single nuclear transcript of each the two alternative NADH dehydrogenases, cytochrome c1, and two nuclear transcripts for complexes II, III, IV and V (**Fig. 3.11, 3.12**). Quantitative real time RT-PCR experiments were performed by a Lightcycler (Roche) using single-stranded cDNA isolated from tachyzoites and *in vitro* differentiated bradyzoites (pH shift media) as a template with specific primer pairs. DNA polymerization was catalyzed by the Fast Start *Taq* DNA polymerase and SYBR green I was used as a reporter for detection. β-tubulin and actin were used as control housekeeping genes for normalization. Integrity, size, and specificity of the PCR amplified fragments were checked by agarose gel electrophoresis and DNA sequencing (**Fig. 3.11**).

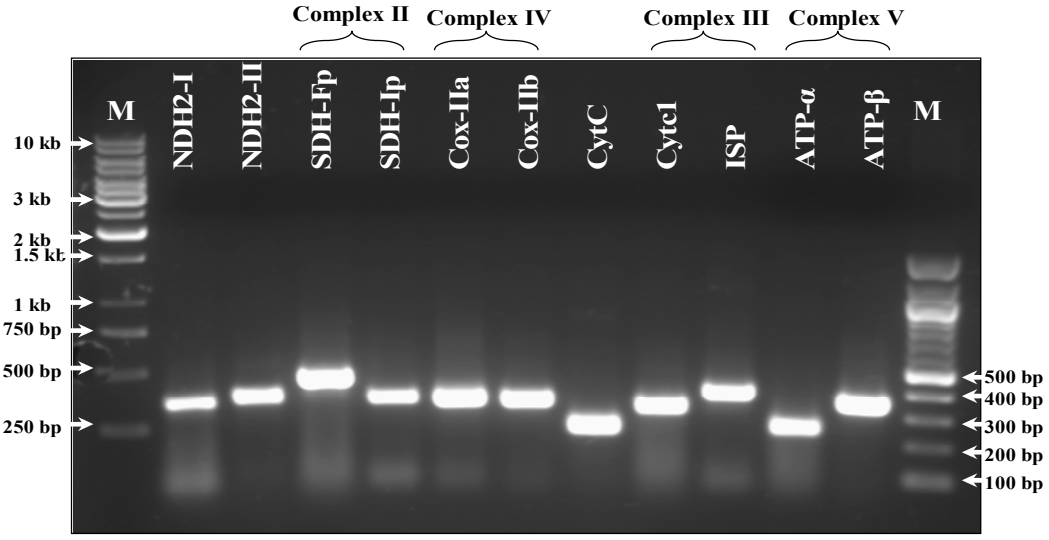


Figure 3.11: **Quantitative real time RT-PCR of the respiratory chain components**. The PCR products of real time RT-PCR for 11 genes coding for key subunits of the respiratory chain complexes of *T. gondii,* the size of the amplicons range between 350 and

Quantitative gene expression analysis, of the *in vitro* conversion of tachyzoites to bradyzoites, has revealed no significant difference in the steady-state mRNA level of the studied genes between tachyzoites and bradyzoites when normalized to the expression of β-tubulin and actin (**Table 3.3**, **Fig. 3.12**). Expression of bradyzoite marker *bag1* (BOHNE et al., 1995) was used as a positive control showing a very significant upregulation (more than 4000 folds) of *bag1* in bradyzoites when normalized with the expression of the housekeeping genes β-tubulin and actin.

No.	Gene	Subunit	Complex	Tach:Brad (tubulin)	Tach:Brad (actin)
1	*NDH2-I*	Alternative NADH dehydrogenase I	Single subnit	1:0.93	1:2.10
2	*NDH2-II*	Alternative NADH dehydrogenase II	Single subunit	1:1.03	1:2.37
3	*SDH-Fp*	Succinate dehydrogenase, flavoprotein subunit	Complex II	1:1.46	1:3.25
4	*SDH-Ip*	Succinate dehydrogenase, iron-sulfur subunit	Complex II	1:1.98	1:4.34
5	*Cytc1*	Cytochrome c1	Complex III	1:0.71	1:1.85
6	*ISP*	Iron sulfur protein (Reiske subunit)	Complex III	1:1.09	1:2.85
7	*Cytc*	Cytochrome C	Single subunit	1:0.39	1:1.02
8	*Cox-IIa*	Cytochrome c, subunit IIa	Complex IV	1:1.32	1:3.45
9	*Cox-IIb*	Cytochrome c, subunit IIb	Complex IV	1:0.44	1:1.14
10	*ATP-α*	ATP synthase, alpha subunit	Complex V	1:0.51	1:1.32
11	*ATP-β*	ATP synthase, beta subunit	Complex V	1:0.47	1:1.24

Table 3.3: Gene expression profile of the respiratory chain components.

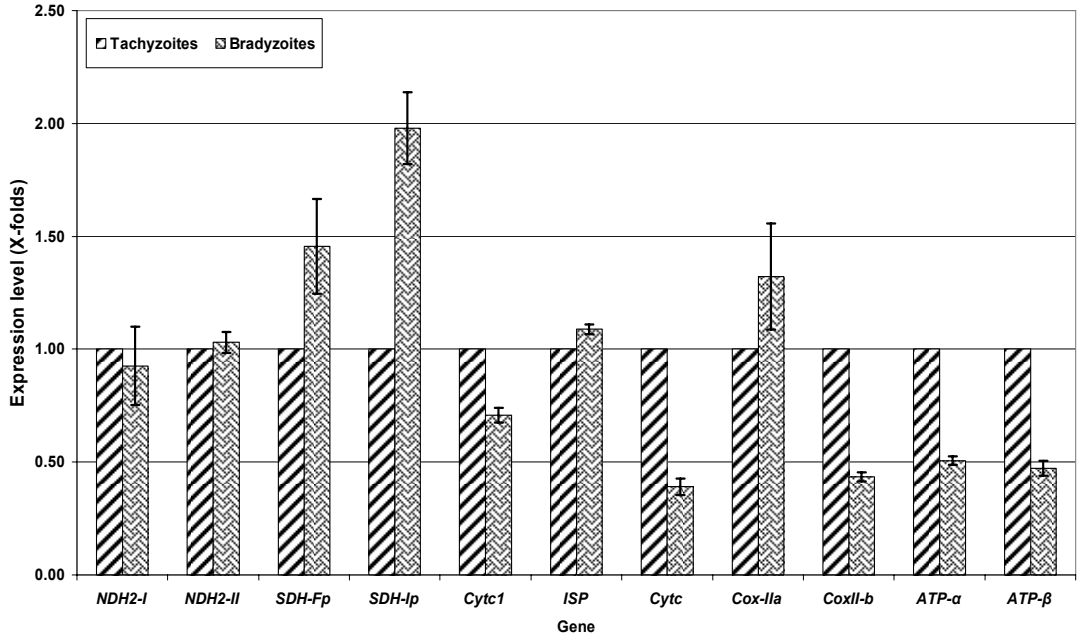


Figure 3.12: **Expression analysis of the respiratory chain components in *T. gondii***. Quantitative real time RT-PCR analysis of gene expression of both isoforms of type II NADH dehydrogenase, flavoprotein and iron-sulfur subunits of succinate dehydrogenase (complex II), cytochrome c1 and iron sulfur subunits of complex III, cytochrome c, cytochrome c-2a (cox IIa) and -2b (cox IIb) of complex IV, alpha and beta subunits of ATP synthase of complex V normalized to β-tubulin housekeeping gene in tachyzoites and bradyzoites. Relative mRNA levels are the amounts of mRNA for mitochondrial subunits over mRNA for β-tubulin.

3.2 Inhibitory effect of HDQ and its derivatives on *T. gondii*

3.2.1 HDQ effect on *T. gondii* replication.

The flavone compound 1-hydroxy-2-dodecyl-4(1)quinolone (HDQ) (**Fig. 3.13**) was recently shown to inhibit the activity of the mitochondrial alternative (type 2) NADH dehydrogenase of the fungus *Yarrowia lipolytica* in enzymatic assays (ESCHEMANN et al., 2005).

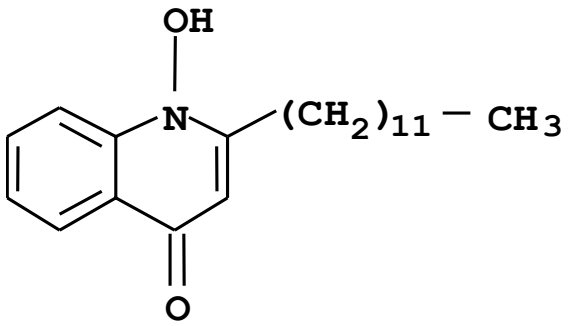


Figure 3.13: Structure of 1-hydroxy-2-dodecyl-4(1)quinolone (HDQ)

We tested the potential of this drug to inhibit the replication of the apicomplexan parasite *T. gondii*, which lacks the complex I associated (type I) NADH oxidoreductase, but instead encodes alternative NADH dehydrogenases. A confluent monolayer of HFF was infected with tachyzoites of the *T. gondii* RH-strain and treated with various concentrations of HDQ ranging from 1 nM to 10 µM. Two growth assays were applied to quantify the *T. gondii* replication rate. First, the average number of parasites per vacuole was determined 24 h post infection by microscopic analysis. In untreated cells, parasites completed up to four replication cycles, with 58% of vacuoles containing 8 parasites and 22% containing 16 parasites. In contrast, nanomolar concentrations of HDQ were found to effectively inhibit *T. gondii* replication with an 50% inhibitory concentration (IC50) of ~2 nM **(Fig. 3.14a)**. 90% of the parasites in samples treated with > 10 nM HDQ were located in vacuoles containing only a single parasite (**Fig. 3.14b)**, suggesting that the drug is immeadiately acting on the parasite. Moreover, immunofluorescence analysis of the intracellular *T. gondii* treated with 10 µM HDQ showed the presence of abnormal parasites with an altered morphology (**Fig. 3.19**). For the second, independant growth assay, a beta-galactosidase expressing *T. gondii* RH-strain (BOHNE and ROOS, 1997) was used to infect a HFF monolayer and the replication rate was determined 30 h post infection using a colourimetric assay **(Fig. 3.14c)**. The replication rate of *T. gondii*

was dose dependantly inhibited by HDQ in the nanomolar range with an IC50 of ~3 nM, which is in good agreement with the results from the microscopic growth assay.

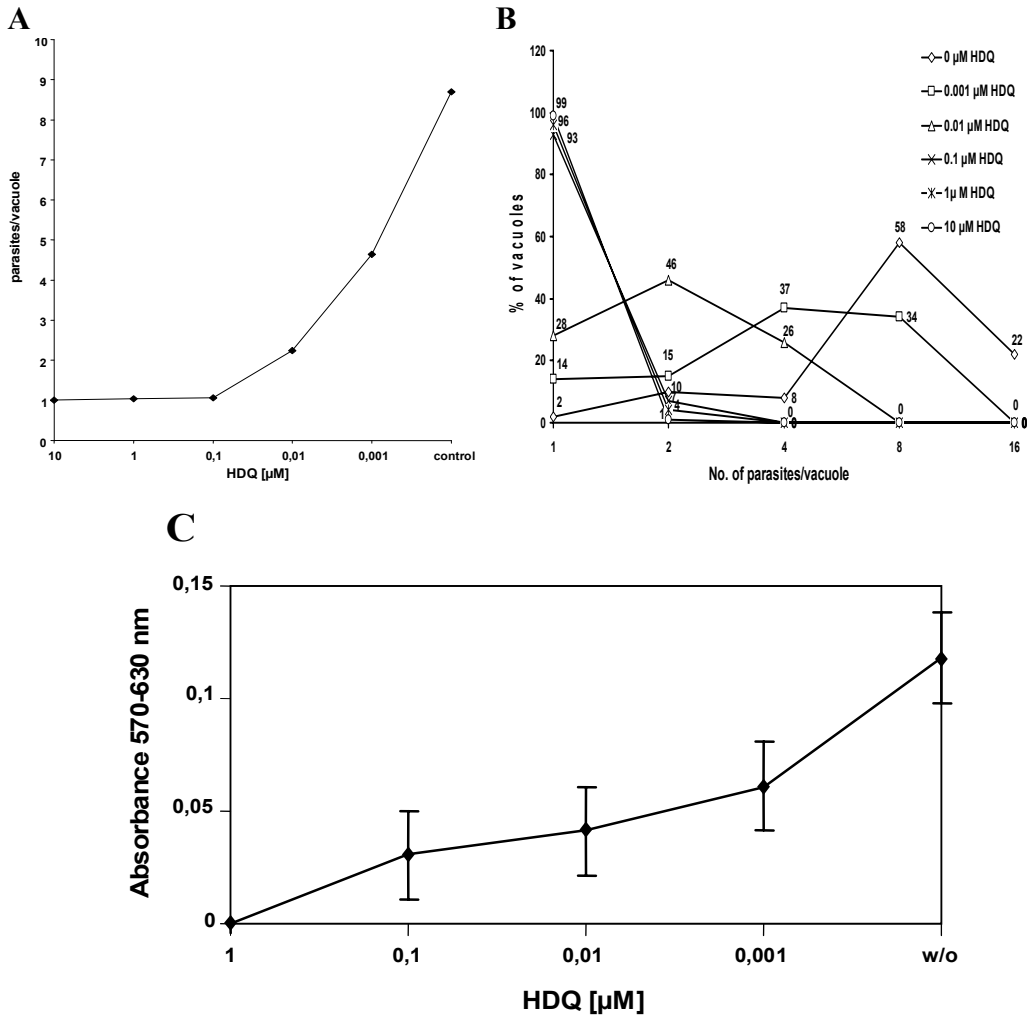


Figure 3.14: HDQ inhibits the *T. gondii* replication rate. (**A+B**) HFF monolayers were infected with *T. gondii* and treated with either the indicated concentrations of HDQ or a DMSO control. After 24 h of drug treatment, the average number of parasites per vacuole was determined by immunofluorescence microscopy **(C).** HFF monolayers were infected with a beta-galactosidase expressing *T. gondii* RH-strain (Bohne and Roos, 1997) and treated with either the indicated concentrations of HDQ or a DMSO control. Beta galactosidase activity was determined 30 h post infection using a colourimetric assay. The figure shows the means +/- s.d. of $OD_{570-630}$ from duplicates.

To analyze potential effects of HDQ on the host cell, we tested the metabolic activity of host cells with the AlamarBlue assay. No difference between HDQ treated and untreated cells was found, indicating that HDQ in the applied concentrations up to 10 µM has no inhibitory effect on the host cell metabolism.

3.2.2 Susceptibility of *T. gondii* to various HDQ derivatives.

Beside HDQ, which possesses a long hydrophobic $(CH_2)_{11}$-CH_3 (C_{12}) site chain at position 2 (**Fig. 3.13**), further 1-hydroxy-2-alkyl-4(1)quinolone derivatives with different alkyl site chain lengths were tested for their ability to inhibit *T. gondii* replication. Derivatives with alkyl site chains of $(CH_2)_5$-CH_3 (C_6), $(CH_2)_7$-CH_3 (C_8) and $(CH_2)_{13}$-CH_3 (C_{14}) inhibited as HDQ (C_{12}) the growth of *T. gondii* effectively at concentrations of 0.1 µM and 0.01 µM (**Fig. 3.15**). In contrast, a derivative with the alkyl site chain $(CH_2)_4$-CH_3 (C_5) did not inhibit *T. gondii* replication. This suggests, that the alkyl site chain at position 2 is essential for the inhibitory action of 1-hydroxy-2-alkyl-4(1)quinolone derivatives and needs to have a minimal length of –$(CH_2)_5$-CH_3 (C_6).

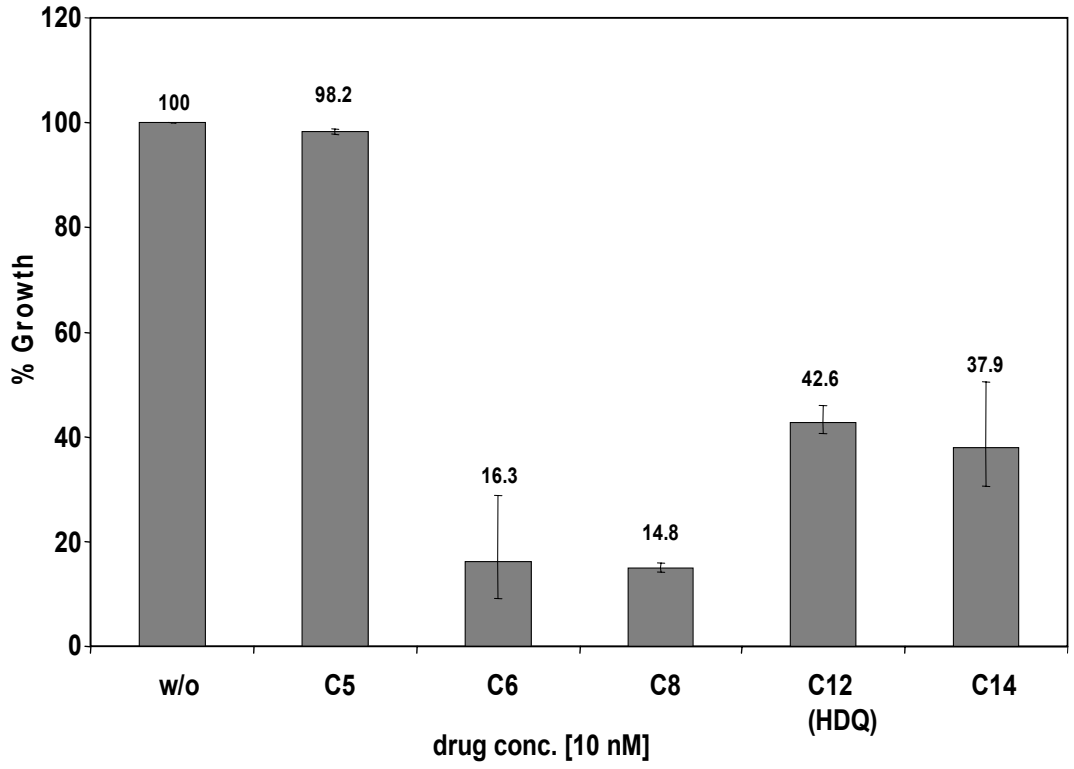


Figure 3.15: Susceptibility of *T. gondii* to various HDQ derivatives. HFF monolayers were infected with a beta-galactosidase expressing *T. gondii* RH-strain (BOHNE and ROOS, 1997) and treated with 10 nM 1-hydroxy-2-alkyl-4(1)quinolone derivatives with alkyl side chain lengths of C_5, C_6, C_8, C_{12} (HDQ) and C_{14}. Beta galactosidase activity was determined 30 h post infection using a colourimetric assay. Bars in the figure show the growth of drug treated samples as percentage of untreated controls. Means from two independent experiments +/- s.d. are given.

3.2.3 Recovery of parasite replication after 24 h of HDQ treatment

To further characterize the antiparasitic activity of the drug, we incubated *T. gondii* infected HFF for 24 h with either 0.1 or 1 µM HDQ, followed by cultivation of further 72 h without drug. The ability of the parasites to recover from the drug treatment was then determined by using the number of parasites per parasitophorous vacuole as a parameter for viability. A continuous treatment for 96 h with 0.1 and 1 µM HDQ resulted in an almost complete inhibition of parasite replication in which most parasitophorous vacuoles harbour 1-2 parasites, very rarely a maximum of 4 parasites can be observed, and none of the vacuoles contained 8 or more parasites. For the recovery experiment, a vacuole with 8 parasites was thus considered to contain replicating parasites (**Fig. 3.16**). In samples treated with 0.1 µM HDQ for 24 h, almost 50% of the vacuoles recovered from HDQ treatment. This number increased even to 65.8% when the 24h HDQ treatment started with a delay of 6 h post infection. However, when the HDQ concentration is increased to 1 µM, the recovery strongly decreased to less then 3%, suggesting that at this concentration, a 24 h HDQ treatment exerts a predominately parasiticidal activity.

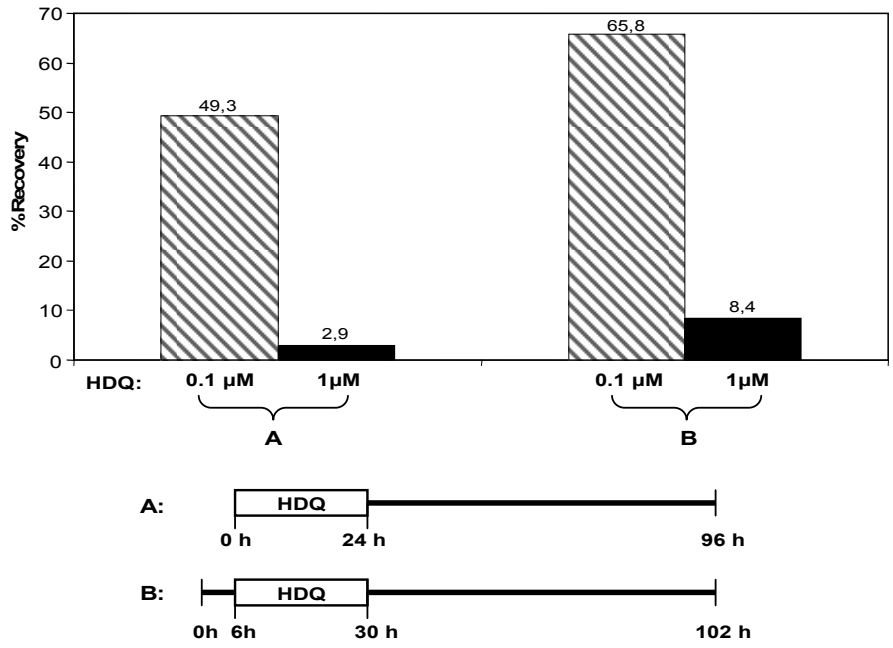


Figure 3.16: **Recovery of parasite growth after 24 h of HDQ treatment.** *T. gondii* (strain RH) infected HFF were treated for the indicated time periods (A and B) with either 0.1 or 1 µM HDQ, followed by cultivation of further 72 h without drug. The sizes of the parasitophorous vacuoles in the samples were determined by immunofluorescence microscopy. Parasitophorous vacuoles with 8 or more parasites were considered to contain viable parasites that recovered from drug inhibition. The figure indicates the fraction of vacuoles containing 8 or more parasites on the total number of vacuoles. In samples treated with 0.1 µM HDQ, ~50% of the vacuoles recovered from HDQ treatment. In contrast, the recovery rate strongly decreased to less then 3% after treatment with 1 µM HDQ.

3.2.4 Invasion assay of HDQ treated parasites.

To test whether the treatment of extracellular parasites with HDQ might alter their ability to invade the host cells, a defined number of fresh tachyzoites ($5x10^4$) were incubated with 5 different concentrations of the drug (10, 1, 0.1, 0.01, and 0.001 µM of HDQ) in 1% DMEM for 2 h at 37 C. Parasites were washed 3 times with 1% DMEM and used to infect HFF monolayers on cover slips. The number of vacuoles per field and intracellular parasites per vacuole were counted after 24h post-infection and compared with the numbers obtained from the untreated parasites (**Fig. 3.17**). No significant difference in the number of vacuoles per field (**Fig. 3.17a**) or average number of parasites per vacuole (**Fig. 3.17b**) has been observed, indicating that the drug has no effect on the ability of parasites to invade the host cells.

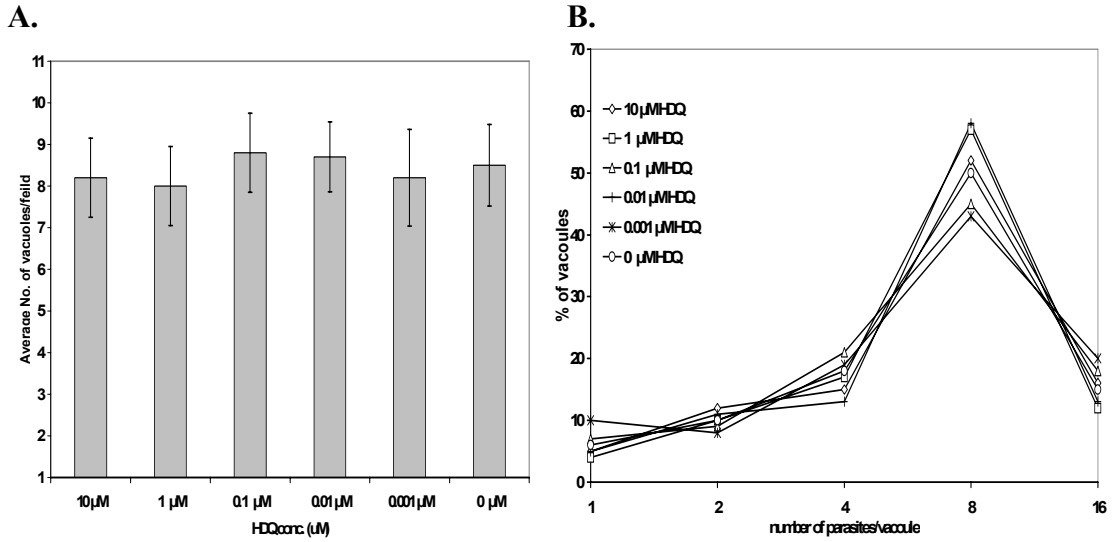
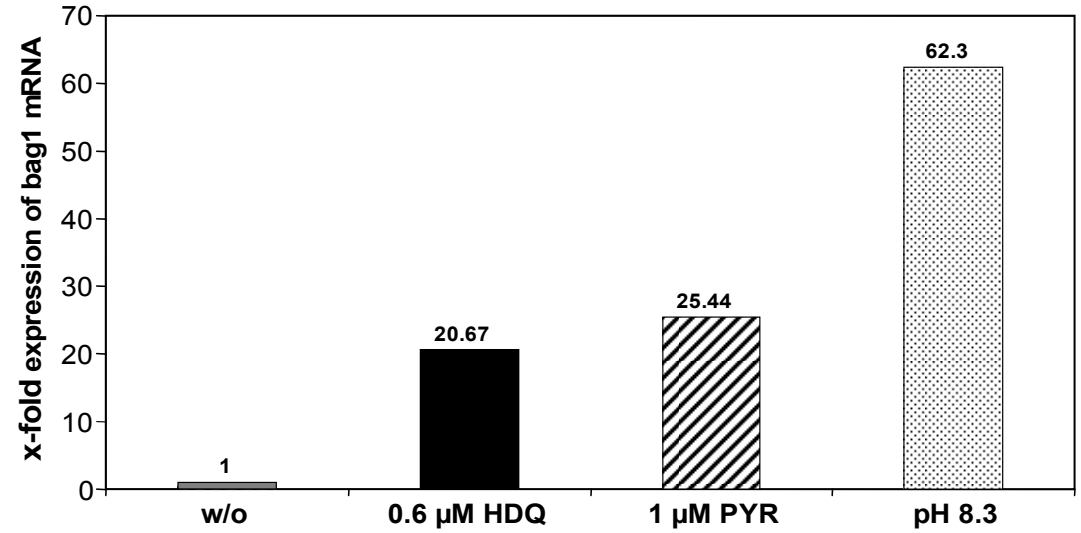


Figure 3.17: Invasion and replication assay of extracellular HDQ treated parasites. *T. gondii* (strain RH) parasites were treated for 2 h with the indicated concentrations of HDQ, followed by cultivation on HFF monolayers for further 24 h without drug. (**A**) Average number of the parasitophorous vacuoles per microscopic field and **(B)** average number of parasites per parasitophorous vacuole in the samples were determined by immunofluorescence microscopy.

3.2.5 HDQ induces bradyzoite differentiation in *T. gondii.*

An inhibition of the replication rate in *T. gondii* is often associated with the conversion of the parasite from tachyzoites to bradyzoites (SOETE et al., 1994; BOHNE et al., 1994; TOMAVO and BOOTHROYD, 1995). We thus tested the influence of HDQ treatment on bradyzoite specific gene expression and used as a differentiation marker the small heat shock gene *bag1* (BOHNE et al, 1995). Treatment with 0.6 µM HDQ for 5 days resulted in a ~20-fold increase of the *bag1* mRNA steady state level as measured by quantitative real-time RT-PCR (**Fig. 3.18**).

A.



B.

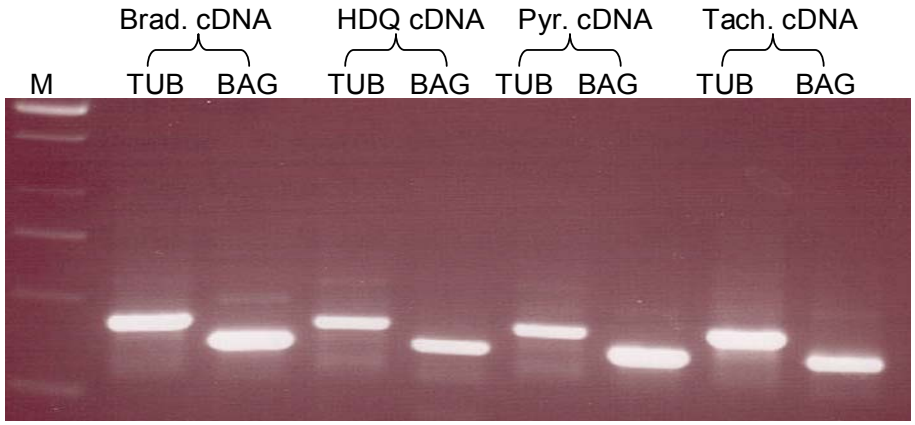


Figure 3.18: Quantitative real time RT-PCR analysis of *bag1* mRNA expression. *T. gondii* (strain RH) infected HFF were treated either with 0.6 µM HDQ-, 1 µM pyrimethamine, alkaline medium of pH 8.3 or left untreated. RNA from the untreated control was isolated 24 h post infection. RNA from HDQ-, pyrimethamine- and alkaline pH-treated samples was isolated 5 days post infection. (**A**) In a representative experiment, Lightcycler PCR was performed to amplify cDNAs of *bag1* and β-tubulin. Values represent the x-fold increase of *bag1* mRNA expression in HDQ-, pyrimethamine- and alkaline pH-treated samples compared to the untreated control after normalization for β-tubulin mRNA expression. (B) Agarose gel electrophoresis of the PCR products amplified by the Lighcycler.

Furthermore, induction of bradyzoite differentiation in HDQ treated parasites was confirmed for a second differentiation marker by immunofluorescence microscopy using the bradyzoite specific mAb 4F8 (**Fig. 3.19**). This mAb recognizes a so far uncharacterized bradyzoite-specific surface antigen (BOHNE et al.,1993).

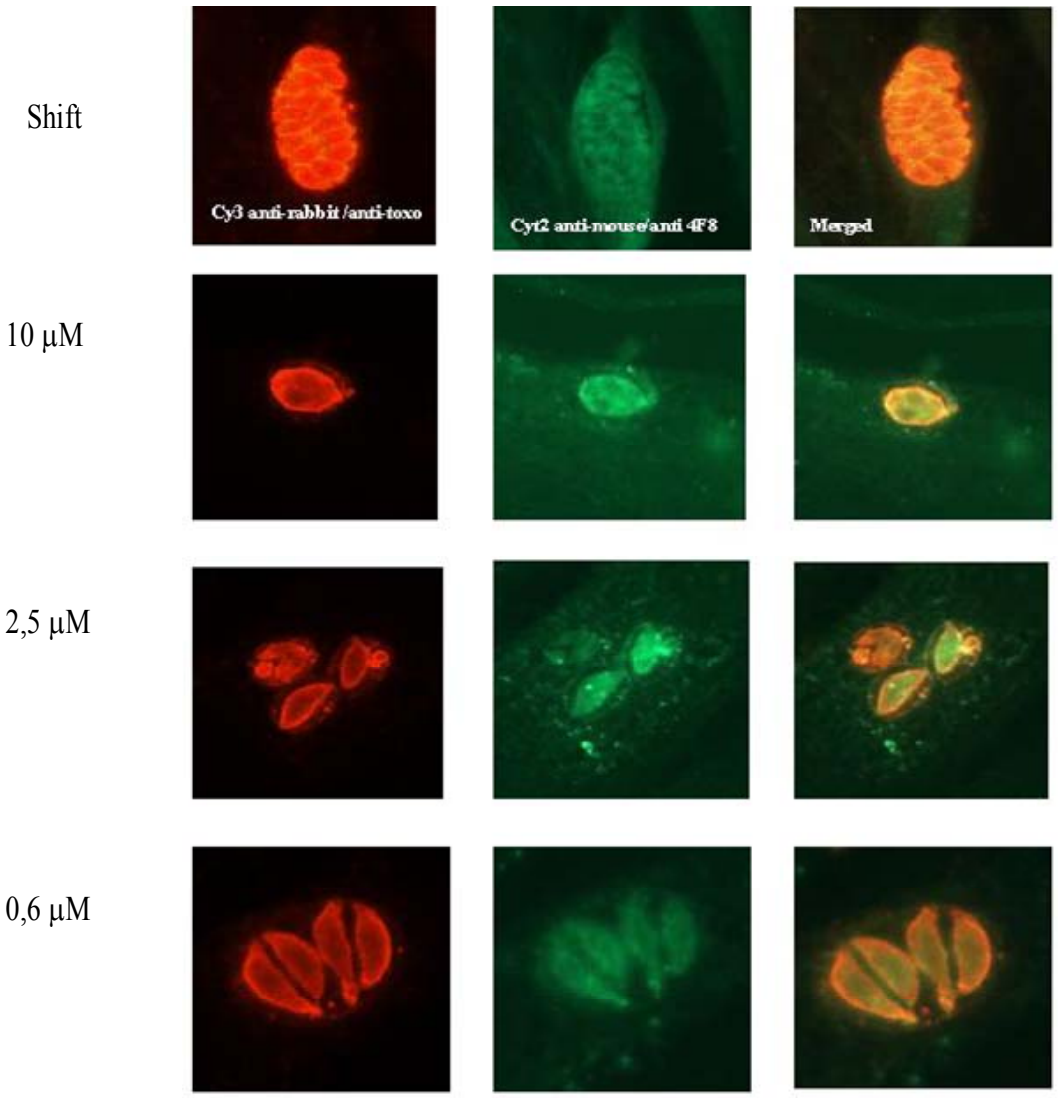
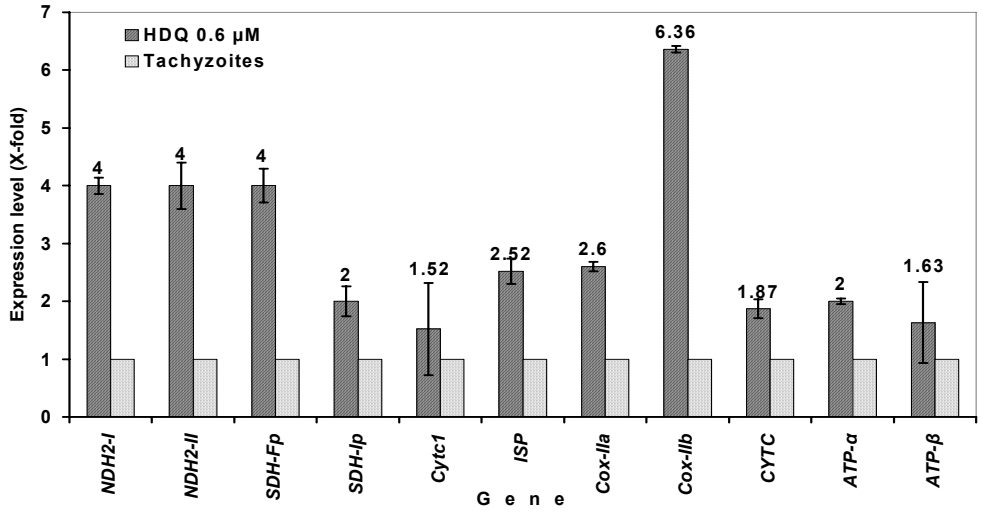


Figure 3.19: Expression of bradyzoite's marker 4F8 after HDQ treatment. HFF monolayers were infected with *T. gondii* and treated with the indicated concentrations of HDQ. After 5 days of continuous drug treatment, expression of the bradyzoite's marker 4F8 was detected by immunofluorescence microscopy using anti-4F8 polyclonal Abs.

3.2.6 Expression analysis of respiratory chain components after HDQ treatment.

To investigate a possible effect on the expression of the respiratory chain components due to the inhibition of the alternative NADH dehydrogenases by HDQ, the steady state mRNA level for 11 key subunits of the electron transport chain complexes was quantified by using a real-time RT-PCR in a Lightcycler and β-tubulin expression for normalization, the quantitative gene expression analysis of the drug treated parasites has shown a slight increase of their expression on the transcriptional level after drug treatment (**Fig. 3.20**).

A.



B.

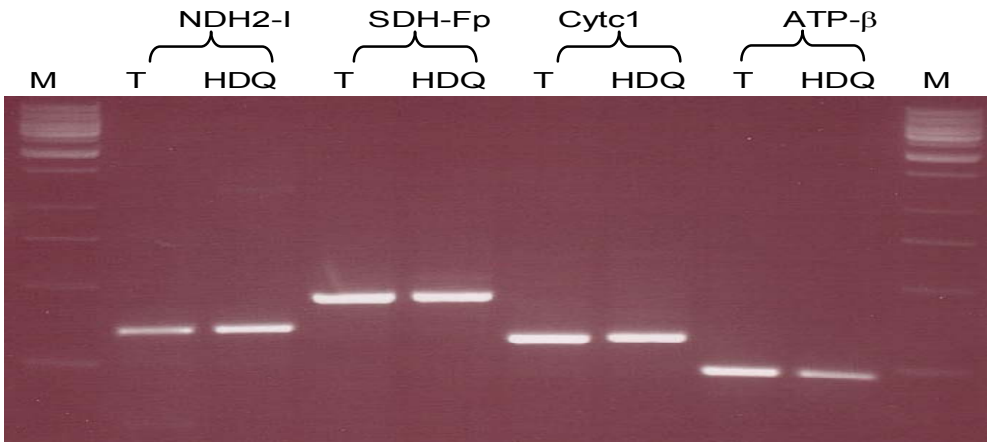


Figure 3.20: Expression analysis of respiratory chain components after HDQ treatment. *T. gondii* (strain RH) infected HFF were treated either with 0.6 µM HDQ or left untreated. RNA from the untreated control was isolated 24 h post infection. RNA from HDQ-treated samples was isolated 5 days post infection. (**A**)Lightcycler PCR was performed to amplify cDNAs of the indicated genes and β-tubulin. Values represent the x-fold increase of mRNA expression of the studied genes in HDQ-treated samples compared to the untreated control after normalization for β-tubulin mRNA expression. (**B**) Agarose gel electrophoresis of the PCR products for some studied genes.

3.2.7 Synergistic inhibitory effect of HDQ with atovaquone and pyrimethamine.

Pyrimethamine and atovaquone are well established drugs to treat Toxoplasmosis and Malaria. We investigated a potential synergistic effect of HDQ in combination with either pyrimethamin or atovaquone on the inhibition of *T. gondii* replication. A strong synergistic effect was found for the treatment of HDQ together with atovaquone **(Fig 3.21)**. The combination of HDQ and atovaquone, each at 1 nM, resulted in a stronger inhibition of parasite replication than a 10 nM treatment with the individual drugs. The sum fractional inhibitory concentration value for atovaquone and HDQ was 0.375 (see Materials and Methods), indicating a strong synergism between both drugs.

A weak, but reproducible synergistic effect was observed, when *T. gondii* infected HFF were treated with a combination of HDQ and pyrimethamine at 1 nM each.

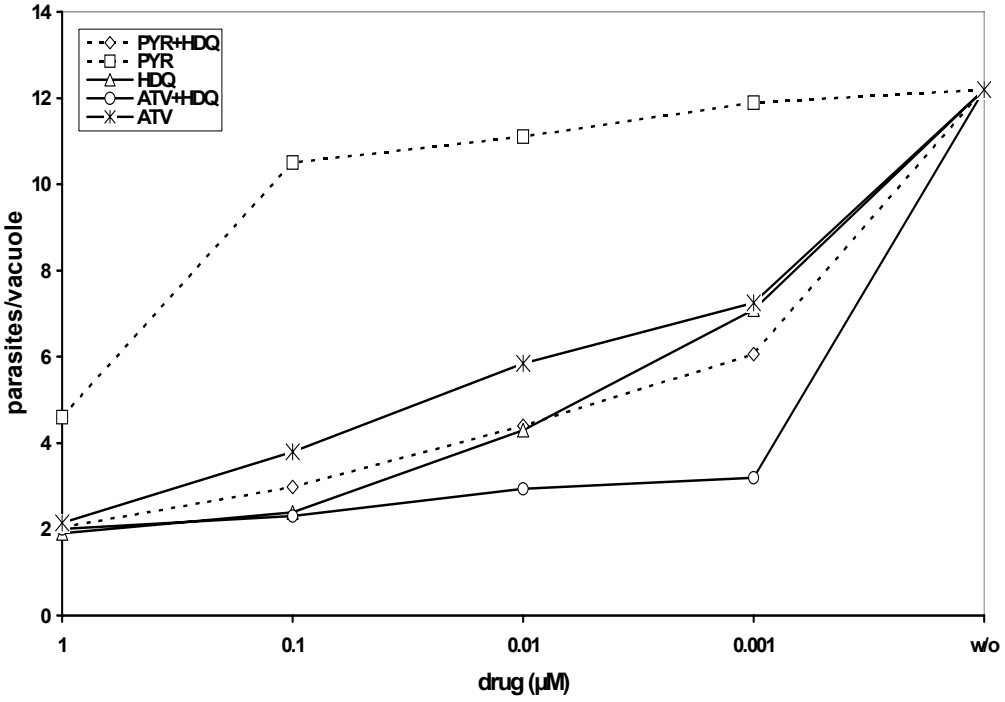


Figure 3.21: HDQ acts in synergism with atovaquone and pyrimethamine. An HFF monolayer was infected with tachyzoites of the *T. gondii* RH-strain and treated with the indicated concentrations of HDQ, atovaquone (ATV), and pyrimethamine (PYR) either alone or in combination. The average number of parasites per parasitophorous vacuole was determined 24 h post infection from at least 200 vacuoles by immunofluorescence microscopy.

3.3 Functional analysis of TgNDH2-I by targeted gene disruption.

3.3.1 Generation of conditional knock out mutants for *Tgndh2-I*

In order to study the link between carbohydrate metabolism and stage conversion as well as the role of respiratory chain components on the growth of the parasites, mutant parasites which are deficient in the following genes: (i) *ndh2-I*, (ii) *sdh-fp*, (ii) *cytc1*, and (iv) *ATP-β*, encoding for key subunits of the respiratory chain were seeked to be generated by targeted gene disruption.

Two strategies were applied for generation of these mutants. The first aims to generate a knock-out mutants by using a targeting construct harbouring 2 inserts flanking a CAT expression cassette as selectable marker (double homologous recombination) (**Fig. 3.22**) or by using targeting construct with single insert and CAT selectable marker (single homologous recombination) (**Fig. 3.23**). Parasites expressing the additional copies of the targeted genes, under the control of Tet-dependent transactivator promoter, were used to generate conditional knockout mutants (**Fig. 3.24**).

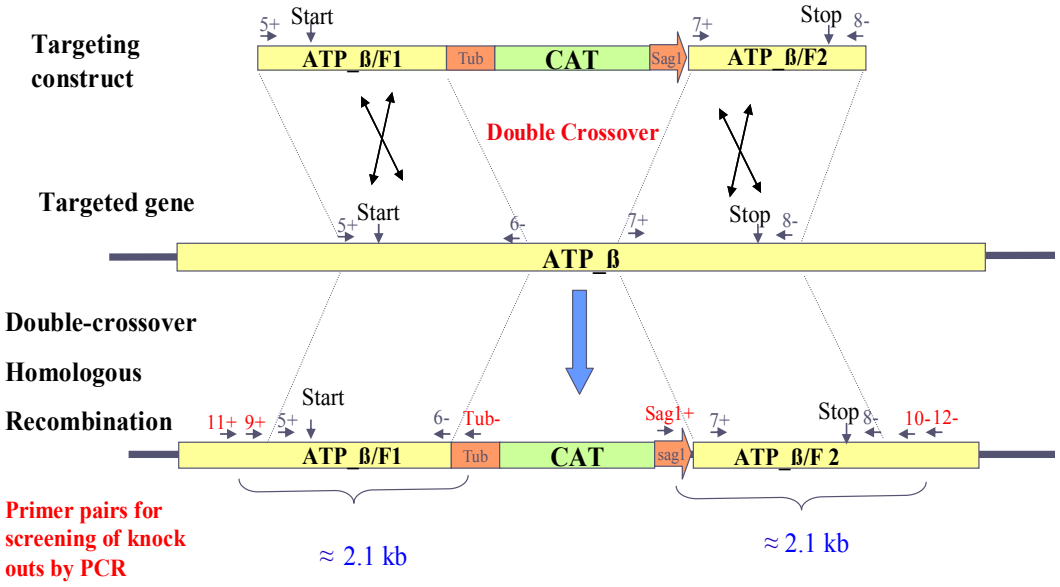


Figure 3.22: Targeting scheme for generation of a conditional knock-out mutants by double homologous recombination. Two fragments of the targeted gene of ~2.5 kb, about 100-200 bp away from each other, were cloned in pKS-CAT vector flanking the CAT expression cassette. A double cross-over event at the endogenous locus results in the integration of the targeting construct (F1-CAT-F2) into the targeted gene and generates a non-functional gene. Red arrows indicate the positions of primer sequences used for subsequent PCR analysis.

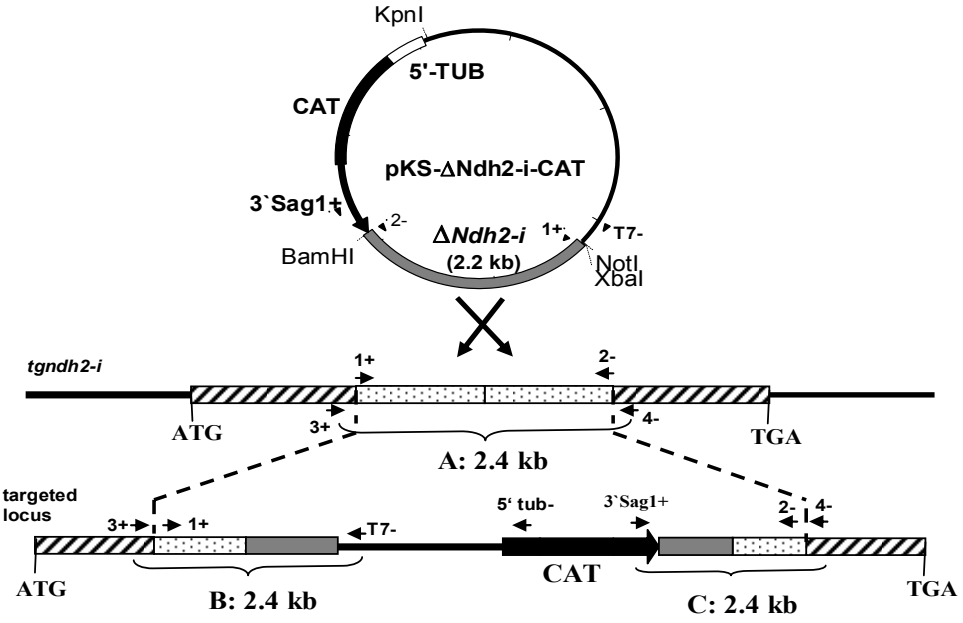


Figure 3.23: Generation of a conditional knock-out mutants by single homologous recombination. Parasites expressing exogenous copies of the targeted gene were transfected with a circular targeting vector, containing a 2.5 kb genomic fragment and a CAT expression cassette (TUB/CAT). The 2.5 kb genomic fragment encodes for a truncated, non-functional gene which lacks essential N- and C-terminal sequences of the coding region. A single cross-over event at the endogenous locus results in the integration of the targeting plasmid into the endogenous gene and generates two non-functional alleles. Arrows indicate the positions of primer sequences used for subsequent PCR analysis.

Targeting constructs and *T. gondii* clones expressing the exogenous copies of the targeted genes (designated; *ndh2-i/ndh2-i-cmyc, ndh2-ii/ndh2-ii, sdh-fp/sdh-fp-cmyc, cytc1/cytc1-cmyc,* and *ATP-β/ ATP-β-cmyc*) (**Fig 3.8)** were generated as described in chapter 2 (Materials and Methods). Clones which displayed a tight Atc-dependant regulation of the 5 target genes were chosen for deletion of the endogenous copies of these genes, the parasites were stably transfected with a targeting vectors containing single and double inserts to allow for the disruption the endogenous copies of the genes by homologous recombination. Individual clones were screened by PCR for loss of the wild type alleles and for integration of the targeting constructs by homologous recombination in the endogenous locus of the gene. All tissue cultures were performed without Atc, in order to induce the expression of the exogenous copies of the target genes. Unfortunately, the PCR screening for the stably transfected parasites with both strategies did not show disruption of the endogenous genes coding for TgNDH-II, TgSDH-Fp, TgCYTC1 and TgATP-β, most likely due to the high frequency of non-

specific integration of the targeting vectors by non-homologous recombination. Regarding the gene coding for TgNDH2-I, which was targeted by a construct with single DNA insert, one clone out of about 120 was identified to display the expected pattern of PCR products for the knock out (**Fig. 3.25a**). Nested PCR was used to confirm the specificity of the PCR products (**Fig. 3.25b**). RT-PCR analysis confirmed the absence of ndh2-I mRNA from the endogenous ndh2-I gene, demonstrating the functional disruption of the ndh2-I locus (**Fig. 3.25c**). The knock out parasites (Δndh2-i/ndh2-i-myc) were further used for phenotypic analysis.

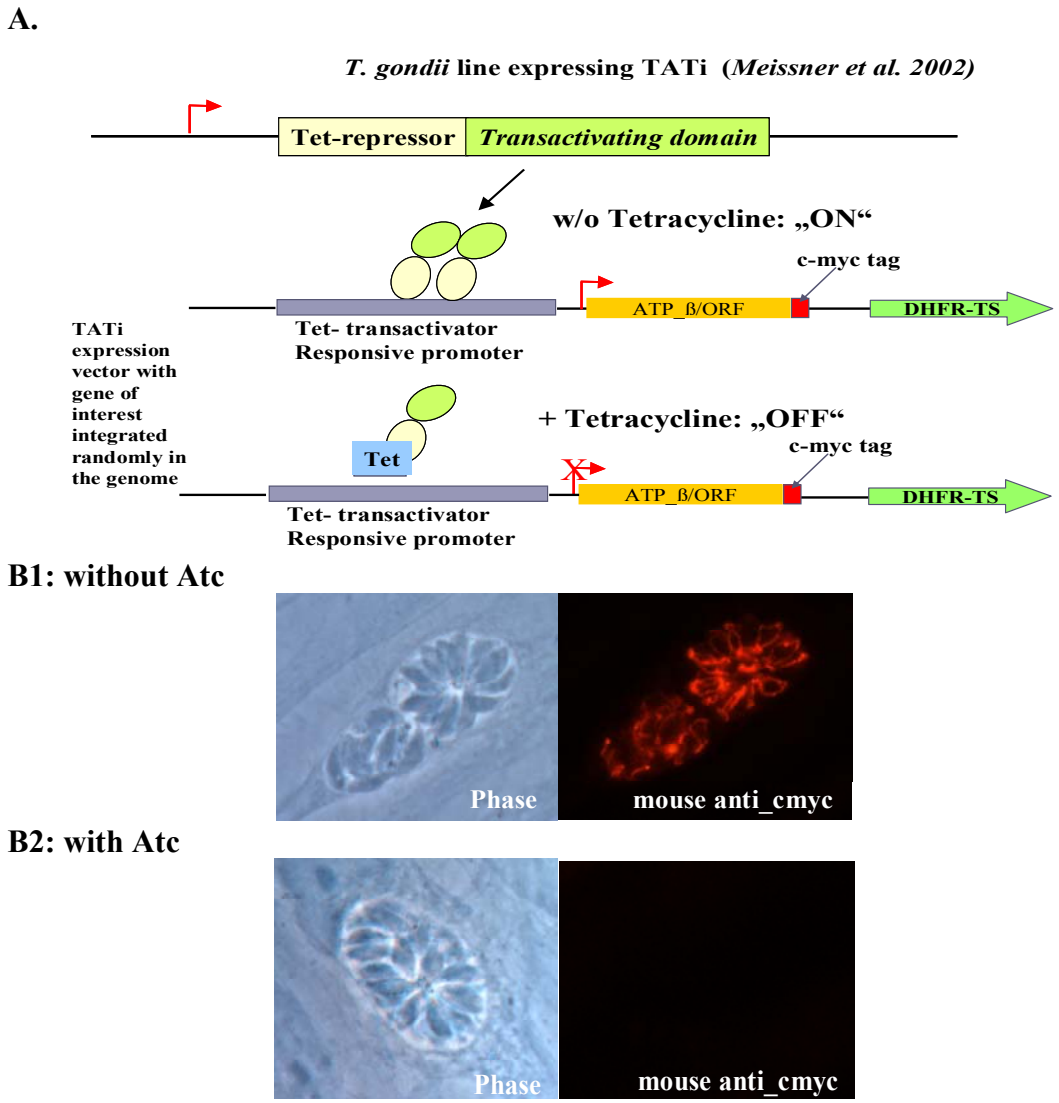


Figure 3.24: **Tetracycline inducible transactivator system** (**A**) Schematic representation of the tetracycline inducible transactivator system (tet off) *in T. gondii* expressing TATi. (**B**) Atc-regulated expression of AND1-myc**.** AND1-myc expression was detected by immunofluorescence microscopy in *andh1*/*Andh1*-myc parasites using anti-myc mAb 9E10. *Andh1*/*Andh1*-myc parasites were cultured one round before either in the absence or presence of Atc. AND1-myc expression was only detectable in the absence (**B1**), but not in the presence of Atc (**B2**).

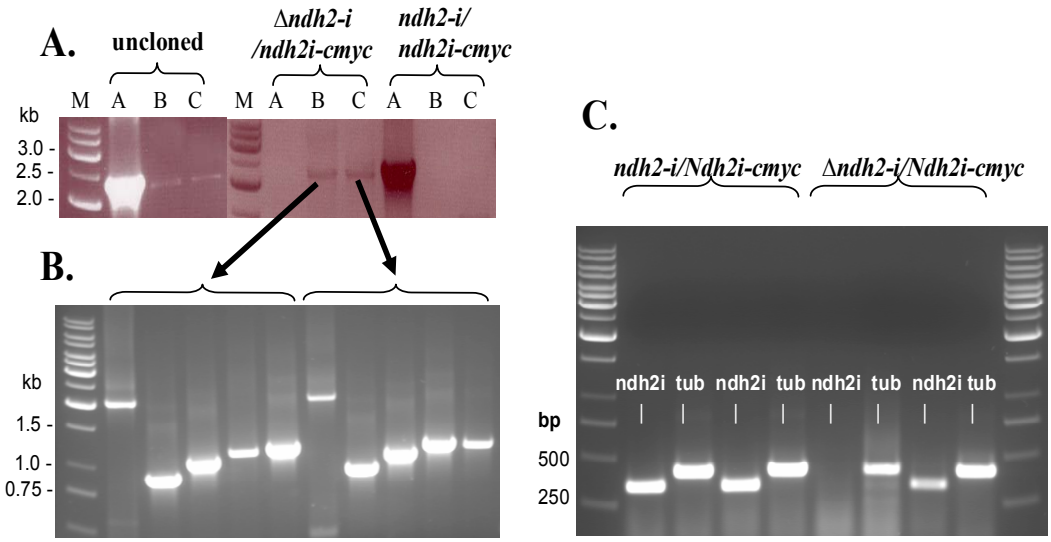


Figure 3.25: **Conditional knock out of TgNDH2-I.** (**A**) Confirmation of *Δandh1*/*Andh1-myc* identity by PCR. Primer pairs, which are specific for the intact wild type allele (A) and for the two non-functional *and1* allels (B and C) were used for PCR analysis on genomic DNA of (i) an uncloned, stabely transfected population, (ii) a single *Δandh1*/*Andh1-myc* clone and (iii) parental strain (*andh1*/*Andh1*-myc) parasites. Primers used for amplification of fragment "A" were: AND1_ 3+/AND1_4-; for fragment "B": AND1_ 3+/T7-; for fragment "C": SAG1+/AND1_ 4-. *Δandh1*/*Andh1-myc* parasites displayed the expected absence of the wild-type specific band and the presence of the two non-functional allels. (**B**) The specificity of PCR products "B" and "C" was confirmed by nested PCR using various internal primer combinations (see Materials and Methods). (**C**) RT-PCR analysis on *Δandh1*/*Andh1-myc* parasites for *andh1* mRNA expression. Parental strain (*ndh2-I*/*Ndh2-I*-myc) and *Δndh2-I*/*Ndh2-I-myc* parasites were cultured either with or without Atc for several rounds. cDNA of these parasites was amplified with a *ndh2-I* specific primer pair which amplifies both, the endogenous and the ectopic copy. Tubulin (tub) primers served as a control. The absence of a *ndh2-I* PCR product in *Δandh1*/*Andh1-myc* parasites cultured with Atc demonstrates both, the lack of *ndh2-I* mRNA derived from the endogenous gene and the successful repression of the ectopic copy.

3.3.2 Phenotypic analysis of knock out parasites (*Δandh1*/*Andh1-myc*)

3.3.2.1 TgNDH2-I is not essential for *T. gondii* replication

The growth rate *of* Δ*ndh2-i/ndh2-i-myc* and *ndh2-i/ndh2-i-myc* parasites was compared in the absence and presence of Atc (**Fig. 3.26**). The *ndh2-i/ndh2-i-myc* parasites showed no difference in the replication rate compared to the parental RH-TATi strain under both conditions, with and without Atc. The depletion of NDH2-I in Δ *ndh2-i/ndh2-i-myc* parasites did not result in a reduced intracellular replication rate, suggesting that NDH2-I is not essential for parasite growth. Continuous passage of Δ*ndh2-i/ndh2-i-myc* parasites in the presence of Atc also did not decrease growth rate, excluding the possibility that an insufficient dilution of formerly expressed enzyme prevents an effect. Moreover, depletion of NDH2-I in Δ *ndh2-i/ndh2-i-myc* parasites did not have an effect on the ability of these parasites to invade HFF host cells as monitored by microscopy, number of vacuoles per field were 14.75 for Δ*ndh2-i/ndh2-i-myc*+Atc, 12.75 for *ndh2-i/ndh2-i-myc* with or without Atc.

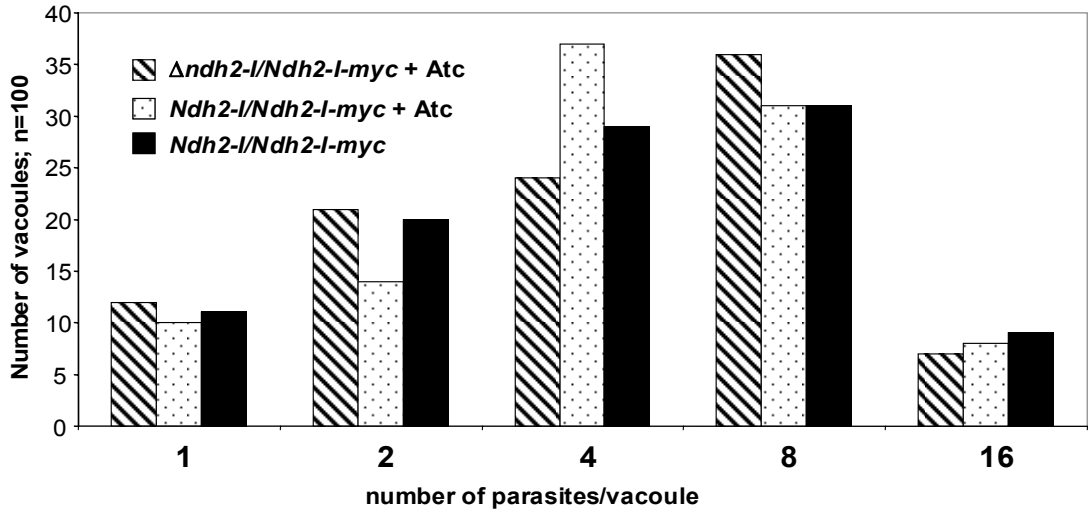


Figure 3.26: NDH2-I depletion does not result in a decreased growth rate. The growth rate of *Δandh1*/*Andh1-myc* parasites in the presence of Atc was compared with those from parental *andh1*/*Andh1*-myc parasites in the absence or presence of Atc. Parasites were cultured at least one round before in the absence or presence of Atc. The number of parasites per parasitophorous vacuole was determined 24 h post infection from 100 vacuoles by immunofluorescence microscopy. The distribution of vacuole sizes is shown in the figure. The average number of parasites/vacuole was 5.50 for *Δandh1*/*Andh1-myc*/+Atc, 5.62 for *andh1*/*Andh1-myc*/+Atc, 5.59 for *andh1*/*Andh1-myc*/-Atc parasites.

3.3.2.2 Expression of *NDH2-II* in knockout parasites (Δ*ndh2-i/ndh2-i-myc*)

Since there is another mitochondrial isoform of alternative NADH dehydrogenase (TgNDH2-II) in *T. gondii*, which is believed to have same function as the depleted isoform, we studied its expression in the tachyzoites of Δ*ndh2-i/ndh2-i-myc*+Atc**.** Quantitative real time RT-PCR has shown a slight upregulation of the gene expression of TgNDH2-II in the knock out parasites (Δ*ndh2-i/ndh2-i-myc*+Atc) in comparison to the wild type parasites (*ndh2-i/ndh2-i-myc*+Atc) when normalized to the expression of both house keeping genes β-tubulin and GAPDH (**Fig. 3.27**). This slight upregulation in the level of mRNA transcripts of *ndh2-II* might contribute to compensate the loss of NDH2-I activity leading to an absence of effect on the growth rate of knock out parasites (Δ*ndh2-i/ndh2-i-myc*) in comparison to wild type (*ndh2-i/ndh2-i-myc*) parasites.

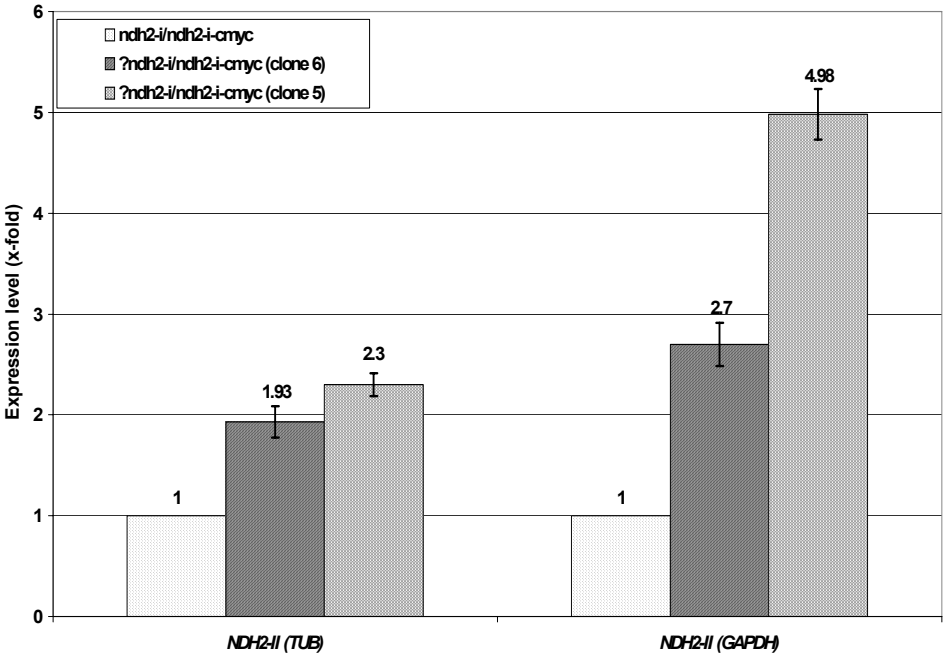


Figure 3.27: Quantitative real time RT-PCR analysis of *ndh2-II* mRNA expression. Tachyzoites of clones 5 and 6 of the mutant (Δ*ndh2-i/ndh2-i-myc*) and parental (*ndh2-i/ndh2-i-myc*) strains were allowed to infect HFF monolayers and followed by cultivation for 24 h in the presence of Atc. RNA was isolated 24 h post infection. Lightcycler PCR was performed to amplify cDNAs of *tgndh2-II*, β-tubulin, and GAPDH. Values represent the percentage of *bag1* mRNA expression in the knock out (Δ*ndh2-i/ndh2-i-myc*) compared to the parental (*ndh2-i/ndh2-i-myc*) strains after normalization for β-tubulin and GAPDH mRNA expression.

3.3.2.3 In vitro differentiation of knock out parasites (Δ*ndh2-i/ndh2-i-myc*)

To study the effect of NDH2-I disruption on the ability of these parasites to differentiate in vitro from tachyzoite to bradyzoites, we compared the expression profile of the bradyzoite marker *bag1* (Bohne, 1995) normalized to the housekeeping gene tubulin. Quantitative real time RT-PCR analysis by a Lightcycler has shown no significant difference in the expression of *bag1* gene in both knock out (Δ*ndh2-i/ndh2-i-myc*) and parental (*ndh2-i/ndh2-i-myc*) parasites (**Fig. 3.28**)

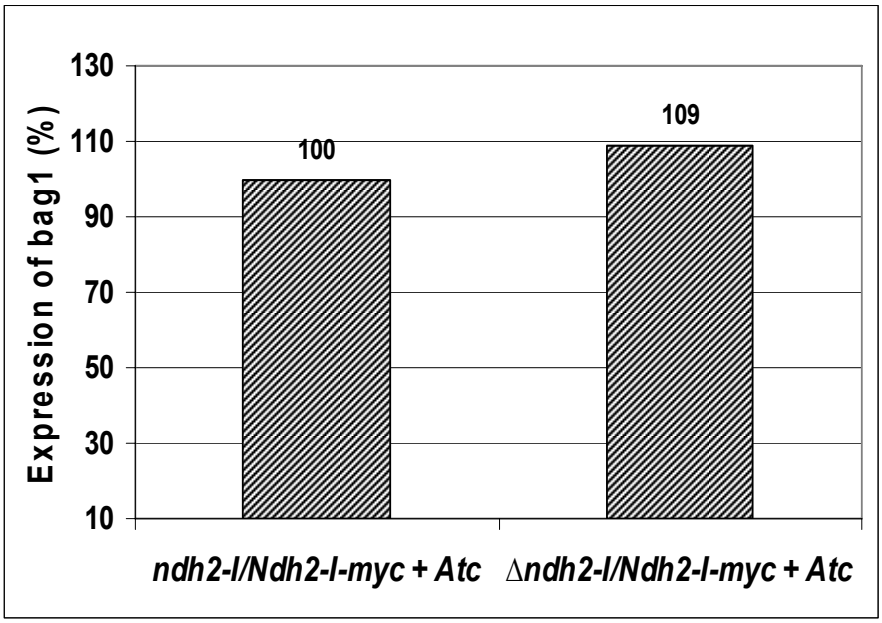


Figure 3.28: Quantitative real time RT-PCR analysis of *bag1* mRNA expression. Tachyzoites of both mutant (Δ*ndh2-i/ndh2-i-myc*) and parental (*ndh2-i/ndh2-i-myc*) strains were allowed to infect HFF monolayers, followed by cultivation in pH shift media (pH 8.3) with Atc. RNA was isolated 4 days post infection. Light cycler PCR was performed to amplify cDNAs of *bag1* and β-tubulin. Values represent the percentage of *bag1* mRNA expression in the knock out (Δ*ndh2-i/ndh2-i-myc*) compared to the parental (*ndh2-i/ndh2-i-myc*) strains after normalization for β-tubulin mRNA expression.

3.3.2.4 NDH2-I depletion leads to HDQ hypersensitivity

The susceptibility of *Δndh2-i*/*ndh2i-myc* and *ndh2-i*/*ndh2i-myc* parasites to HDQ was compared in the absence and presence of Atc **(Fig. 3.29)**. *Δandh1*/*Andh1-myc* parasites displayed in the presence of Atc (ectopic *Andh1* copy "OFF") a significant higher sensitivity to HDQ. The IC_{50} in NDH2-I depleted parasites is in the pM range. In contrast, if *Δandh1*/*Andh1-myc* parasites were cultured without Atc (ectopic *Andh1* copy "ON"), the phenotype was restored and parasites revealed the same sensitivity to HDQ as the parental RH-TATi strain with an IC_{50} of ~ 2-3 nM. The additional expression of the ectopic *Andh1* in *andh1*/*Andh1*-myc parasites had no influence on the IC_{50} of HDQ.

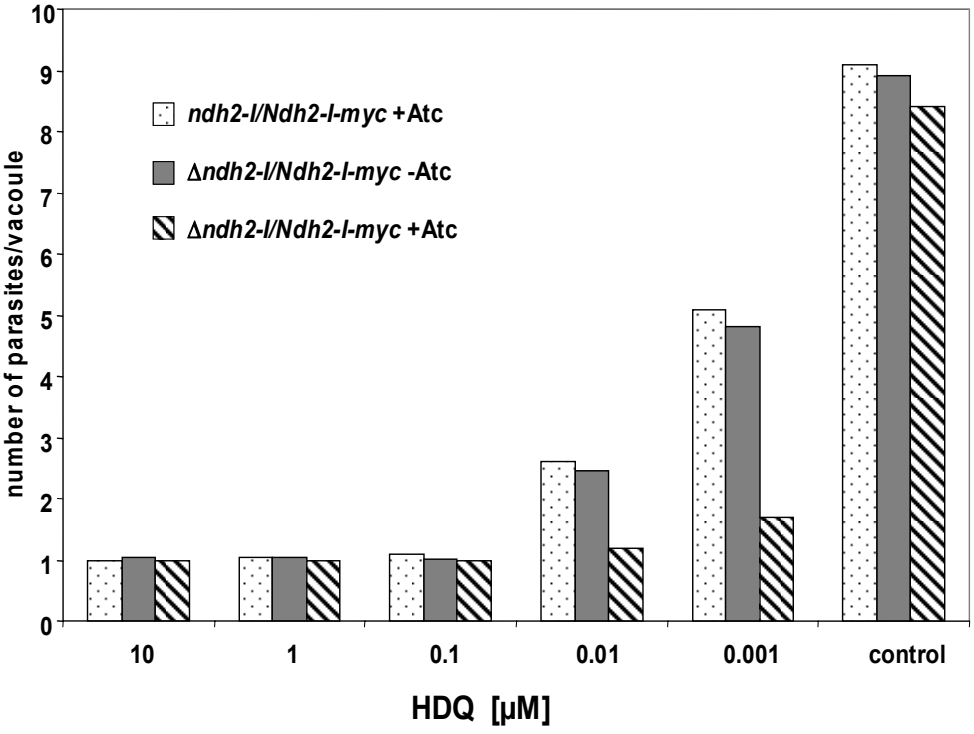


Figure 3.29: NDH2-I depletion results in HDQ hypersensitivity. The HDQ sensitivity of *Δandh1*/*Andh1-myc* parasites in the presence or absence of Atc was compared with parental *andh1*/*Andh1*-myc parasites +/- Atc. The average number of parasites per parasitophorous vacuole was determined 24 h post infection by immunofluorescence microscopy. NDH2-I depletion in *Δndh2-I*/*ndh2-II-my*/+Atc parasites results in a strongly increaed HDQ susceptibility with a complete inhibition of parasite replication at 1 nM.

DISCUSSION

4.1 The alternative NADH dehydrogenases in *T. gondii*

Based on BLAST search homologies, the genome of *T. gondii* predicts the presence of conventional respiratory chain components, with the exception of a multi-subunit, proton translocating, rotenone sensitive NADH dehydrogenase (complex I). Extensive searching of the *T. gondii* nuclear genome failed to uncover any complex I homologues. Furthermore, several complex I subunits that are almost always encoded on the mitochondrial genome of organisms containing complex I (e.g. *nad1, nad4 and nad5*; LANG et al., 1999) are absent on the *T. gondii* mitochondrial genome. Instead, nuclear genes coding for two isoforms of a single polypeptide, non-proton translocating, rotenone insensitive NADH dehydrogenases have been identified on chromosomes Ib and IX., with 619 and 657 amino acids long and a predicted size of 67 and 72 kDa respectively. It is clear that this phenomenon is common in most if not all protozoans of the phylum apicomplexa, where genomes of Plasmodium, Theileria, Cryptosporidium, Eimeria and Babesia lack genes encoding for complex I, but instead they have genes which encode for type II NADH dehydrogenase. Some previous biochemical studies have speculated the absence of complex I in *T. gondii* (VERCESI et al., 1998), *P. yoelii* (FRY and BEESLY, 1991; SRIVASTAVA 1997, Uyemura), and recently in *P. falciparum* (BIAGINI et al., 2006). Other protozoans of the phylum kinetoplastida like Trypanosoma (FANG et al, 2002; 2003) and Leishmania are expressing these enzymes in addition to complex I in their mitochondria.

4.2 Motifs and phylogenity of TgNDH2-I and TgNDH2-II

Both isoforms of type II NADH dehydrogenase of *T. gondii* (accession #: TgNDH2-I, DQ211932; TgNDH2-II, DQ228957) share the same amino acid sequence characteristics with other single polypeptide (type II) NADH dehydrogenases from bacteria, protists, fungi and plants. Analysis of the two putative TgNDH2 sequences revealed that they contain NADH- and FAD-binding domains. The first domain, the putative FAD-binding site, is close to the N-terminus of the protein, while the second domain, the putative NADH-binding site, lies about 130-145 amino acid further downstream. Both domains consist of a β sheet-α helix-β sheet structure which contains (i) a glycine-rich phosphate binding consensus sequence ($GXGX_2G$); (ii) a conserved negatively charged residue (D or E) at the end of the second β sheet; and (iii) six positions typically occupied by small hydrophobic residues. Similar to the NDH2 sequences from *S. cerevisiae*, *Y. lipolytica*, and

N. crassa, the last of three highly conserved glycine residues in the first dinucleotide binding site of *T. gondii* NDH2s is replaced by alanine or serine. In NADPH binding proteins, the third G in the $GXGX_2G$ motif associated with NADPH binding is generally replaced by S, A, or P, and the negative charge at the end of the second β sheet is missing (BELLAMACINA 1996). As these features are absent in the TgNDH2 sequences, it is likely that both enzymes in *T. gondii* bind NADH and not NADPH. However, we can not rule out that these enzymes could oxidize NADPH as we have no experimental evidence.

Both TgNDH2s contain the conserved nucleotide binding domain for FAD and also a second motif downstream from this that is characteristic of FAD binding motifs. This motif contains a conserved aspartate that forms a hydrogen bond with the ribitol moiety of FAD (EGGINK et al., 1990). Thus, both enzymes have the potential to bind both NADH and FAD, consistent with the expectation for functional NDH2 proteins. A similar patch of basic residues with unknown function is found immediately upstream from the first dinucleotide fold in TgNDH2 sequences and in all proteins from fungi and plants, it was speculated that these basic amino acids may serve a similar function in stabilizing the binding of FAD or NADH (KERSCHER et al., 1999).

Two regions characterized by conserved apolar and aromatic residues are located immediately after dinucleotide fold I, both regions may form a pocket for the interaction of ubiquinone with the reactive moieties of FAD and NADH (KERSCHER et al., 1999). Within the proposed ubiquinone binding site (Ser43-Gly75) of NDH2 from *E. coli*, His51 (Tyr in some species) and Glu52 (Gln in some species) are present in bacterial NDH-2. In eukaryotic NDH-2 including TgNDH2s, the corresponding residues are Pro and Ser, suggesting that the Ser residue may form a hydrogen bond to ubiquinone (SCHMID and GERLOFF, 2004). Moreover, both TgNDH2 proteins show the presence of putative quinone binding sites (QBS) of type IA with consensus LX_2HX_2T (FISHER and RICH, 2000). Unlike most NDH2 sequences from different species where the predicted quinone binding sites are located between the two dinucleotide binding regions (MELO et al., 2004), they are located downstream the second dinucleotide region in *T. gondii* NDH2 sequences. To achieve efficient electron transfer, the electron acceptor (ubiquinone) and therefore its binding site has to be in close proximity of the cofactor FAD, and the isoalloxazine ring of FAD is sandwiched between the nicotinamide of NADH and ubiquinone (**Fig. 4.1**) (MELO et al., 2004). Based on this model, His51 as a potential hydrogen bond donator for ubiquinone is located at <5 Å distance from the FAD-isoalloxazine. Taken together, it is

most likely that the highly conserved region between the dinucleotide regions but not putative quinone binding sites at C-terminal in both TgNDH2 sequences contains the quinone binding site.

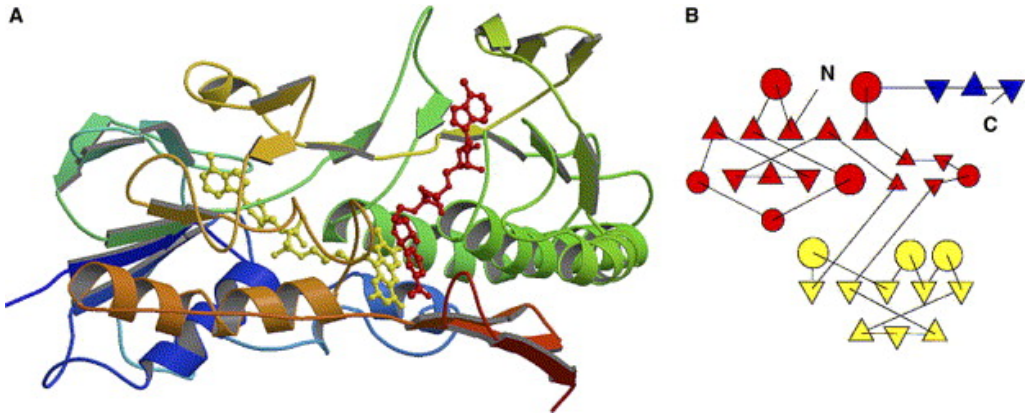


Figure 4.1: (A) Ribbon representation of modeled alternative NADH:ubiquinone dehydrogenase from *E. coli* (NDH-2). The ribbon is color-coded from N-terminus (blue) to C-terminus (red). FAD and NADH are displayed as ball and stick models colored in yellow and red, respectively. The residue identified as potentially involved in quinone binding, His51, forms part of the light blue helix behind the FAD molecule. (B) TOPS-Topology cartoon of the model. Circles and triangles represent α-helices and β-strands, respectively. The FAD-domain (Thr1–Asn129, Gly274–Gly350), NADH-domain (Thr130–Ala273) and the C-terminal domain (Lys351–Met385) are colored in red, yellow and blue, respectively. (MELO et al., 2004)

Ubiquinone is a lipid-like, hydrophobic compound present primarily in the mitochondrial membrane, therefore its binding site on NDH2 would also be expected close to the membrane.

In general, protein binding to membranes is mediated by hydrophobic interactions through transmembrane α-helices or β-strands pass through the bilayer or α-helices parallel to the plane of the membrane, which are in some times amphipathic with predominantly polar residues along one side of the helix and nonpolar side chains on the remainder opposite of the helical structure. Association of alternative (type II) NADH dehydrogenases with the mitochondrial inner membrane remain controversial, since transmembrane helices are not commonly present in NDH2 proteins. This binding may involve the C-terminal part of the protein, which is not conserved between these enzymes and the water soluble lipoamide dehydrogenases from bacteria (KERSCHER, 2000) or the Ca^{2+} binding EF-hand motifs in alternative NADH dehydrogenases which contain these motifs (RASMUSSON et al., 1999). TgNDH2 sequences were submitted to 9 different TM-prediction servers and analysed the results. Four methods [TMHMM (KROGH et al., 2001), PRED-TMR (PASQUIER et al., 1999), ALOM2 (NAKAI and KANEHISA, 1992), SOSUI (HIROKAWA et al., 1998)] predicted no TM-helices at all. DAS (CSERZO et al., 1997), TMAP (MILPETZ et al., 1995), TMPred

(HOFMANN and STOFFEL, 1993), TM-Finder (DEBER et al., 2001), and HMMTOP (TUSNADY and SIMON, 2001) predicted one or several TM-helices, but these methods produce a higher proportion of false positive results (typically from 11% for HMMTOP to 55% for TMPred by contrast to <2% for TMHMM (MOLLER et al., 2001)). The absence of transmembrane helices and the observation that hydrophobic and hydrophilic amino acids are located on opposite sides in some of the predicted α-helices suggest a membrane-protein interaction through the hydrophobic face of these amphipathic α-helices.

In organisms where type II alternative NADH dehydrogenases are the sole NADH oxidizing enzymes, their main function is respiratory chain linked NADH turnover, with the concomitant production of ATP. Where these proteins coexist with the other ion-gradient generating NADH dehydrogenases (complex I), they are likely to play a role in keeping the cell [NADH]/ [NAD^+] balance. In addition to metabolic functions, they might be involved in situations of NAD(P)H stress, play a regulatory role in response to the quinone pool redox state (HOWITT et al., 1999), or to the oxygen concentrations (BERTSOVA et al., 2001), and in metabolic adaptation of the organism to different carbon sources (DUARTE et al., 2003).

The role which might *T. gondii* NADH dehydrogenases have in the electron transport chain with the absence of canonical complex I is still unclear. In Plasmodium, O_2 consumption studies in *P. berghei* show that addition of exogenous NADH can not stimulate O_2 consumption (UYEMURA et al., 2000). However, a very similar assay system in *P. yoelii* found that the simultaneous addition of NADH and NADPH did stimulate O_2 consumption (UYEMURA et al., 2004). However, a complex III activity assay in *P. falciparum* found that NADH could stimulate electron transport (FRY & BEESLEY, 1991). It was shown recently (BIAGINI et al., 2006) that *P. falciparum* expresses type II NADH dehydrogenase which has a high, rotenone insensitive, NADH oxidation activity measured by enzymatic assay of the parasites extract. Moreover, inhibiting the PfNDH2 collapses the mitochondrial transmembrane potential, leading to parasite death. The substrate specificity of Plasmodium NAD(P)H dehydrogenase, thus, requires further confirmation. NADH and NADPH are considered impermeable to the inner mitochondrial membrane, the stimulation of electron transport by addition of exogenous substrate and since the intra-erythrocytic parasites are believed to have a rather inactive TCA cycle (FREY AND BEESLY, 1991; FRY et al., 1990, and VAIDYA 2004) would indicate Plasmodium NAD(P)H dehydrogenase is an external enzyme, lying on the inter-membrane space side of the inner

membrane. Such a scenario requires experimental confirmation, but would suggest this enzyme functions in oxidizing NAD(P)H produced from cytosolic reactions. If this scenario is correct, there would be no obvious enzyme capable of feeding electrons from NAD(P)H produced in the tricarboxylic acid cycle into the electron transport chain. In *T. gondii*, it is more complicated to predict their role in the overall cellular metabolism because of the presence of two isoforms, where both could feed electrons from the cytosol (external) or from the mitochondrial matrix (internal) into the electron transport chain for ATP generation, or one external and the other is internal. As the *T. gondii* lysate shows NADH oxidation activity with the addition of exogenous NADH, this is an evidence that at least one isoform is external and facing the inter-membrane space. Other evidences for a putative orientation of these enzymes could be taken from their high amino acid sequence identity with external enzymes from plants, phylogenetic analysis, presence of long N-terminal sequences, and presence of unusual or probably an incomplete TCA cycle. Only *T. gondii* among apicomplexan parasites has two isoforms of NDH2 which have 38% identity and show the highest amino acid sequence identity to the uncharacterized NDH2 from *Eimeria tenella* (36% to TgNDH2-I, 46% to TgNDH2-II) suggesting a gene duplication event.

As shown in Table 3.1a, NDH2s from apicomplexan parasites have the highest identity to the external NDH2s from plants, which is also evident from the phylogenetic tree. Kerscher (2000) has proposed that external alternative NADH dehydrogenases evolved from lipoamide dehydrogenases followed by gene duplication and the acquisition of a mitochondrial targeting sequence to form the internal alternative NADH dehydrogenases. In parallel, some genes encoding for external enzymes acquisitioned an insertion for EF-hand motif for binding Ca^{2+} as in *A. thaliana* and *S. tuberosum*. Both TgNDH2 sequences and other NDH2 sequences from apicomplexan parasites show a large insertion positioned to the EF- hand motif in external NDH2 sequences from plants. The apicomplexan inserts do not show the conserved fingerprint of EF-hand motif from plant or any other EF-hand motif proteins. The fact that both insertions reside in similar positions within the external plant proteins and *T. gondii* makes it most likely that they originated from one single evolutionary event, although a direct alignment of these insertions fails to reveal significant sequence identity.

Small et al has shown that internal alternative NADH dehydrogenase from *S. cerevisiae* have a short N-terminal sequence (25 residues) compared to the external enzymes which possess N-terminal extension that exceed the length of ScNDI1 presequence by 30

(ScNDE1) and 45 (ScNDE2) amino acid residues, respectively. (SMALL et al, 1998). The N-terminal sequences of both isoforms of *T. gondii* have the longest N-terminal sequences among other external and internal enzymes suggesting their external orientation.

The results of the phylogenetic tree suggest that the apicomplexan enzymes share a most common ancestor. Moreover, kinetoplastid, fungal and plant alternative NADH dehydrogenases are the closest out groups to the apicomplexan sequences suggesting that the apicomplexan NDH2 proteins have evolved closely with the external NDH2 proteins of plants as supported by the phylogenetic tree of Michalecka et al (2003) and Uyemura et al (2003) for NDH2 from *P. falciparum* and *P. yoelii*.

4.3 Structure and motifs of further components of the respiratory chain.

4.3.1 Succinate dehydrogenase, flavoprotein subunit

Succinate dehydrogenase, also referred to as succinate-ubiquinone oxidoreductase (SQR) or complex II, is a mitochondrial marker enzyme located in the cytoplasmic membrane of bacteria and the inner mitochondrial membrane of eukaryotes. It plays a unique role as a component of the TCA cycle (oxidizing succinate to fumarate and reducing FAD) as well as the electron transport chain (passes electrons from FADH2 to coenzyme Q), a direct link between major systems for energy metabolism. Anaerobic cells respiring with fumarate as the terminal electron acceptor also contain complex II, which catalyzes the reverse reaction of SQR as quinol-fumarate reductase (QFR) (**Fig. 4.2**).

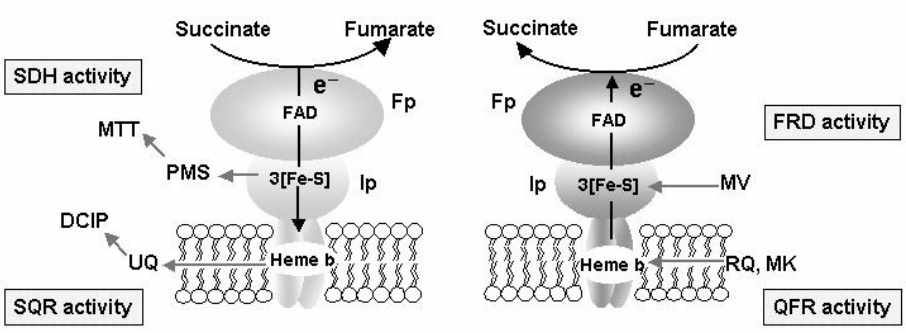


Figure 4.2: Subunit structure, electron transfer, and enzyme activities of two complex IIs. Complex II functions as succinate-ubiquinone reductase (left) and as quinol-fumarate reductase (right). Fp, flavoprotein subunit; Ip, iron-sulfur protein subunit; CybL and CybS, large and small subunits of cytochrome *b*; UQ, ubiquinone; RQ, rhodoquinone; MK, menaquinone; PMS, phenazine methosulfate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2,4-tetrazolium bromide; DCIP, 2,6-dichlorophenol-indophenol; MV, methyl-viologen. (MI-ICHI et al., 2003)

Complex II is generally composed of four polypeptides, with all subunits encoded in the nucleus. The largest flavoprotein (Fp) subunit and iron-sulfur (Ip) subunit are hydrophilic and together form the a catalytic portion which serves reducing equivalents from succinate transfer to quinone in SQR, or those from quinol to fumarate in QFR. The Fp/Ip portion is bound to the matrix side of the inner mitochondrial membrane via two small membrane-anchoring proteins containing heme *b* (cytochrome *b* subunits, CybL and CybS). Furthermore, CybL/CybS subunits seem to be essential for the electron transfer between the catalytic portion and quinone species (TAKEO et al., 2000).

The genome of *T. gondii* predicts 2 genes coding for the two major subunits of succinate dehydrogenase. In this study, the coding sequence of the gene coding for the flavoprotein subunit was identified (DQ228958), while ToxoDB predicts the gene coding for the iron-sulfur subunit (TgTwinScan_1272). In contrast to *P. falciparum* where *SDH-Fp* and *SDH-Ip* genes contain a single open reading frame and the genes are located on different chromosomes (KITA et al., 2003), both genes in *T. gondii* have multiple exons (20 exons in *SDH-Fp*, and 7 exons in *SDH-Ip*) and are located on chromosome X. The expression of these genes in tachyzoite and bradyzoite stages of *T. gondii* was confirmed by quantitative real time RT-PCR. The *SDH-Fp* and *SDH-Ip* genes encode proteins of 669 (Fp) and 342 (Ip) amino acids, with predicted molecular masses of 72.7 and 38.6 kDa, respectively. Most parts of the amino acid sequences of *T. gondii* Fp and Ip are highly similar to those from other species. A mitochondrial presequence essential for the import of the mitochondrial proteins encoded by nuclear DNA, along with conservation of the known active site and other functional residues suggests that the *T. gondii* complex II is actually functioning, the histidine for covalent binding to cofactor FAD, the substrate binding active site domains on Fp, and the cysteines constituting three iron sulfur clusters on Ip. The *T. gondii* and *P. falciparum* specific, similarly positioned extra sequences and a unicellular specific deletion site may cause structural and functional changes to the parasite enzymes, although the physiological significance of such changes is not clear (TAKEO et al., 2000). Interestingly, we confirmed that SDH-Ip sequence from *T. gondii* has a large insertion of 40 residues (Asn126-Pro 165) which is not found in any other SDH-Ip proteins, even that of *P. falciparum*. The significance of such a relatively large species specific insertion in SDH-Ip of *T. gondii* is still unknown. Despite the fact that Fp and Ip are well conserved subunits, the structure of both subunits in the malaria parasite (KITA et al., 2002) and *T. gondii* clearly differ considerably from that of the host.

In contrast to the Fp and Ip subunits, the primary structure is conserved, the cytochrome *b* exhibits species-specific characteristics, however the functionally important amino acid residues and orientation within the membrane are conserved (HIRAWAKE et al., 1997).

The functional significance of complex II in *T. gondii* and *P. falciparum* remains controversial. Only two early studies on activity of complex II in *T. gondii* are available so far. In cell homogenates of tachyzoites, succinate dehydrogenase (SDH) activity was detected, while no SDH activity could be found in bradyzoites, which was taken as an evidence for the presence of a TCA cycle associated with a respiratory chain in tachyzoites and of its absence in bradyzoites (DENTON et al, 1996). Denton et al (1998) showed that addition of succinate and malate to digitonin permeabilized tachyzoites was followed by a discrete increase in the rate of oxygen uptake and ADP phosphorylation, malate stimulation was rotenone intensive. In contrast, glutamate, oxoglutarate, pyruvate, isocitrate, and glycerol 3-phosphate were unable to stimulate ADP phosphorylation in permeabilized tachyzoites. However, a relatively high succinate dehydrogenase activity was detected in *T. gondii* mitochondrial extracts upon addition of succinate or malate, while moderate NADH-fumarate reductase activity was detected upon addition of fumarate under similar conditions (VERCESI et al., 1998). Taken together, the presence of succinate dehydrogenase/fumarate reductase system was proposed.

In a direct enzyme assay, Takashima and colleagues (TAKASHIMA et al., 2001) found succinate dehydrogenase activity in partially purified mitochondria from *P. falciparum*. The addition of succinate has furthermore been shown to promote O_2 consumption, to contribute to $\Delta\Psi$ across the inner membrane, and to stimulate complex III activity (FRY & BEESLEY, 1991; UYEMURA et al., 2000, 2004). Succinate dehydrogenase has been purified from asexual stage *P. falciparum* (SURAVERATUM et al., 2000), supporting a functional role of this enzyme during this period of the life cycle. Studies have found that the addition of fumarate can inhibit dihydroorotate- and NADH-linked electron transport (FRY & BEESLEY, 1991; TAKASHIMA et al., 2001), suggesting that succinate dehydrogenase may function in the reverse reaction to reduce fumarate to succinate, with complex II thus acting as a terminal oxidase for electrons from coenzyme Q.

4.3.2 Cytochrome bc1 complex, cytochrome c1 subunit

Electrons donated along the electron transport chain are sourced from several biochemical reactions. However, all converge at coenzyme Q (also known as ubiquinol or ubiquinone), a hydrophobic, isoprenoid-containing molecule embedded in the inner mitochondrial membrane. Reduced coenzyme Q donates electrons to complex III (also known as the cytochrome bc_1 complex or cytochrome c reductase). Electrons donated by coenzyme Q are transferred through several complex III proteins to reduce cytochrome *c* ultimately (CROFTS and BERRY, 1998). All three of the core catalytic subunits of complex III have homologues in the *T. gondii* genome. These are cytochrome *b* (encoded on the mitochondrial genome), the Rieske iron sulphur protein (TgTwinScan_2256) and cytochrome c_1 (DQ228959). Electrons from coenzyme Q are passed to either the Rieske iron sulphur protein or to the haem prosthetic group of cytochrome b (CROFTS, 2004). Electrons from the Rieske protein are donated via the haem group of cytochrome c1 to the haem of the peripheral cytochrome c. In *T. gondii*, the identified *CYTC1* has a sequence of 3357 nucleotides, interrupted by 5 introns, and located on chromosome XII. The identified open reading frame of 1197 nucleotides encodes a protein of 398 amino acids, with a predicted molecular mass of 46 kDa. . The expression of *CYTC1* in tachyzoite and bradyzoite stages of *T. gondii* was confirmed by quantitative real time RT-PCR. Amino acid sequence of *T. gondii* CYTC1 is highly similar to those from other species.

The cytochrome c_1 subunit of the cytochrome *c* reductase complex of most eukaryotic organisms is synthesized as a preprotein with a long (60-80 amino acids) amino-terminal extension that has been shown to direct the protein along a conservative sorting pathway; the first part of the presequence directs the protein into the mitochondrial matrix, and the second part of the signal causes the protein to be sorted into or through the inner mitochondrial membrane to its final destination (JENSEN et al., 1992). Arnold et al (1998) has shown that the carboxyl-terminal region of the yeast cytochrome c_1 protein contains a previously unrecognized targeting signal that acts in a membrane potential-dependent fashion to insert the α-helical membrane anchor of the protein into the inner membrane so that the mature protein has the proper N_{out}-C_{in} orientation (ARNOLD et al., 1998). Apparently, both the amino- and the carboxyl-terminal targeting sequences are required by the yeast system for proper membrane insertion and orientation.

Interestingly, a long N-terminal extension of 80 residues, beyond the 61-amino acid presequence of yeast protein that is required for mitochondrial protein import, is present in

CYTC1 from *T. gondii* and other apicomplexan parasites as well. However, a mitochondrial presequence characteristics in this N-terminal extension are missing and thus non of the subcellular predicting servers was able to predict a signal peptide. More interestingly, the sequence of 70 residues which comes next to the 80 residues N-terminal extension has a high similarity with the 61 amino acid mitochondrial presequence of CYTC1 from yeast, where both possess the characteristics of a typical conservative sorting signal.

The *T. gondii* protein also has a region near the carboxyl terminus that is predicted to form a membrane-spanning α-helix (amino acids 351-378 indicated by double lined boxed). An α-helical plot of the 18 residues that follows the transmembrane domain (amino acids 379-395 indicated in triple lined boxed) shows that this region may form an amphipathic α-helix with the positively charged residues and the hydrophobic residues localized on opposite sides of the structure. The membrane spanning-helix and the amphipathic α-helix identified in the yeast (ARNOLD et al., 1998) and trypanosome (PRIEST and HAJDUK, 2003) proteins align with the predicted helices in the *T. gondii* protein. In the yeast protein, these carboxyl-terminal regions have been implicated as a secondary signal for the membrane potential-dependent insertion of cytochrome c_1 into the inner mitochondrial membrane, which could be the case for *T. gondii* cytochrome c_1.

Several lines of evidence point to a functional complex III in *T. gondii* parasites. Complex III activity has been detected in *T. gondii*, and typical inhibitors of complex III proteins, such as antimycin, have been shown to collapse the mitochondrial membrane potential ($\Delta\Psi$) and to inhibit respiration by inhibiting the consumption of oxygen, which functions as the terminal acceptor of electrons in the electron transport chain. Moreover, the same effect was detected by the antimalarial compound atovaquone (PFEFFERKORN et al., 1993; VERCESI et al., 1998). It was further shown that mutations in the coenzyme Q binding site of cytochrome *b* in *T. gondii* correlate with resistance to atovaquone, strongly suggesting that this complex is the major target for atovaquone (MCFADDEN et al., 2000, KESSL et al., 2006). Consistent with the role of cytochrome *b* in contributing to the inner membrane proton gradient, atovaquone and other complex III inhibitors were shown to collapse membrane potential in *T. gondii*, supporting the role of a functional, proton-translocating complex III in *T. gondii* (VERCESI et al., 1998).

4.3.3 F_0F_1-ATP synthase, β-subunit

ATP synthesis at the mitochondrial inner membrane is catalyzed by an intricate complex called ATP synthase. This membrane-bound complex catalyzes the phosphorylation of ADP by inorganic phosphate using the proton motive force generated by the electron transport chain. ATP synthase consists of two multi-subunit containing components that can function in both ATP synthesis and ATP hydrolysis. The F_0 base is embedded in the inner membrane and functions during ATP synthesis to direct protons down their concentration gradient (CAPALDI and AGGELER, 2002). This movement of protons is coupled to activity of the matrix localized F_1 knob, which is attached to the base via stalk proteins. The F_1 component functions as a rotary motor, binding ADP and Pi and reacting with these substrates to form ATP before release of the ATP (CAPALDI and AGGELER, 2002). ATP is then transported out of the mitochondrion via an ADP/ATP carrier protein. F_1 consists of five major subunits, all of which have well conserved homologues in the *T. gon*dii genome. These include the α (TgTwinScan_3877), β (DQ228960), γ (TgTwinScan_5820), δ (TgTwinScan_3455), and ε (TgTwinScan_4986) subunits. No homologues of the F_0 base are apparent in the genome. It is possible that amino acid conservation of these subunits is insufficient to enable their bioinformatic identification. The three dimensional structure of the mitochondrial bovine heart F_1-ATP synthase determined by Abrahams et al. (ABRAHAMS et al., 1994) shows that *β*-subunits alternate with *α*-subunits in a sphere-like structure, building a catalytic core that rotates during catalysis (SABBERT et al., 1996) (**Fig. 4.3**).

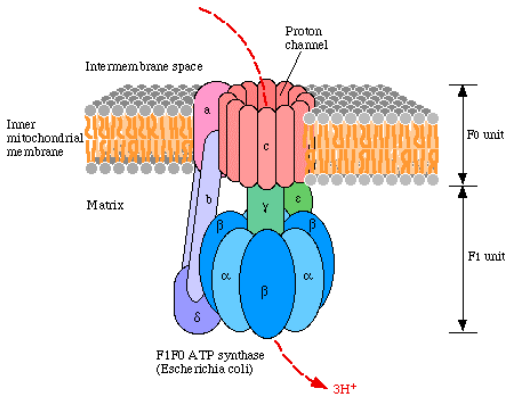


Figure 4.3: Schematic drawing of F_0F_1-ATP synthase

We identified the gene coding for β subunits of F_1 knob of F_0F_1-ATP synthase (*ATP-β*) in *T. gondii*, which has a sequence of 3098 nucleotides, interrupted by 3 introns, and located on chromosome XII. The identified open reading frame of 1683 nucleotides encodes a protein of 560 amino acids, with a predicted molecular mass of 60 kDa. . The expression *ATP-β* in tachyzoite and bradyzoite stages of *T. gondii* was confirmed by quantitative real time RT-PCR. Alignment of amino acid sequences of ATP- β from *T. gondii* and other species show that these proteins are extremely well conserved (**Fig. 6.4**). The N-terminal extension of ATP- β protein of *T. gondii* contain multiple residues with positively charged and hydroxylated side chains, characteristic of mitochondrial targeting sequences (**Fig. 6.4**). A short apicomplexan specific insertion of 9 residues (Lys281-Phe289, KLEDGKFDF) with unknown function, is located 33 residues downstream the start of the nucleotide binding domain.

It was demonstrated that O_2 consumption was inhibited by the ATP synthase F_1 inhibitor oligomycin (VERCESI et al., 1998) and caused an increase in cytosolic $Ca+^2$ levels in tachyzoites suggesting a requirement for mitochondrial energy for the regulation of Ca+2 homeostasis in these parasites (MORENO and ZHONG, 1996). Moreover, oligomycin and ADP affected ΔΨ, suggesting that mitochondrial ATP synthase uses the proton gradient generated by the electron transport chain to synthesize ATP (VERCESI et al., 1998). However, direct evidence for the activity of ATP synthase in T. gondii is lacking, and the importance of mitochondrial ATP synthase in contributing to the cellular ATP pool in both developmental stages of *T. gondii* is not yet clear.

4.4 Mitochondrial localization of the respiratory chain proteins.

Most of the mitochondrial proteins are encoded within the nucleus, synthesized on cytosolic ribosomes, and directed to their final mitochondrial destination by cleavable amino-terminal presequences (VOOS et al., 1999). Many of the imported mitochondrial proteins have a bipartite, amino-terminal presequence that directs the proteins along a conservative sorting pathway; the first part of the presequence directs the protein into the mitochondrial matrix, and the second part of the signal causes the protein to be sorted into or through the inner mitochondrial membrane to its final destination (MOKRANJAC and NEUPERT, 2005).

There are several other mechanisms by which proteins can target to the mitochondrion. These include transmembrane domains, such as tail-anchor sequences, which typically

target proteins to the outer mitochondrial membrane, and ill-defined internal signals that can target proteins to the inner membrane (EGAN et al., 1999; PFANNER and GEISSLER, 2001). Proteins with a presequence may also contain additional sequences to target them to subcompartments of the mitochondrion. All proteins targeted beyond the mitochondrial outer membrane must traffic across membranes via a series of well-defined protein complexes.

As discussed previously, the respiratory chain proteins (NDH2-I, NDH2-II, SDH-Fp, CYTC1, and ATP-β) are characterized with long N-terminal sequences, with mitochondrial presequence characteristics. We submitted the amino acid sequences of these proteins to 7 different subcellular localization prediction servers, four methods (MitProtII, Psort, Predator, and TargetP) predicted a mitochondrial localization for 4 of the respiratory chain proteins with different probability values, moreover SignalP predicted a signal peptide for the same proteins. None of the used methods predicted a signal peptide or a mitochondrial presequence for CYTC1. Plasmodium specific subcellular localization methods PlasmoAP and PlasMit, which predict for apicoplast and mitochondrion proteins respectively, failed to predict any subcellular localization of *T. gondii* respiratory chain proteins.

By fusing the presequence of mitochondrial proteins to a green fluorescent reporter protein, it has been shown that such presequences can be sufficient to target proteins to the mitochondrion in *T. gondii* and Plasmodium species (DEROCHER 2000, SATO et al., 2003; TONKIN et al., 2004). Therefore, we fused the open reading frames with c-myc tag in pTetO7Sag4-ACP-cmyc expression vector, and their expression was brought under the control of an inducible regulatable promoter (transactivator responsive promoter), which is regulated by a tetracycline derivative anhydrotetracycline (Atc) in *T. gondii* specific transactivator TATi-1 line.

The localization of the expressed proteins was confirmed by immunofluorescence staining using primary antibodies for the c-myc tag (anti-myc mAb 9E10) and confirmed by co-localization with S9-GFP expression vector (DEROCHER 2000) which has a mitochondrial targeting sequence from *T. gondii* mitochondrial protein fused with GFP. Immunofluorescence microscopy has shown that expressed proteins were transported and localized correctly to the single mitochondrion of *T. gondii.* Further confirmation of the expression and mitochondrial localization of the endogenous proteins was achieved by producing polyclonal antibodies. Polyclonal antibodies raised against the hydrophilic regions of NDH2-I and ATP-β proteins *of T. gondii* were generated by mice immunization,

and further used to confirm the expression and the mitochondrial localization of the endogenous proteins by immunoblotting and immunofluorescence staining.

4.5 mRNA expression analysis of the respiratory chain components.

During its developmental transition from tachyzoites to bradyzoites, *T. gondii* modifies its morphology and metabolism. It has been documented that the parasite expresses three stage-specific glycolytic isoenzymes: lactate dehydrogenase (YANG and PARMLEY, 1995; 1997), glucose 6-phosphate isomerase (DZIERSZINSKI et al., 1999) and enolase (TOMAVO et al., 1991). Genes coding for lactate dehydrogenase from tachyzoites *LDH1* and bradyzoites *LDH2* were found to be developmentally regulated during the parasite's life cycle. Using RT-PCR, Yang and Parmley (1997) demonstrated that mRNA of *LDH2* was only detected in the bradyzoite stage while transcript of *LDH1* was equally abundant in both bradyzoite and tachyzoite stages, while the LDH1 enzyme is only detected in tachyzoites when two-dimensional PAGE was performed. Another glycolytic enzyme, enolase was found to be expressed differentially between both stages. Semi-quantitative RT-PCR analysis of cDNA isolated from tachyzoite and bradyzoite stages of *T. gondii* was performed in order to investigate the differential expression of *ENO1* and *ENO2* genes. These studies revealed that the mRNA of *ENO1* was only detected in the bradyzoite stage, while that of the *ENO2* mRNA was exclusively found in tachyzoites (DZIERSZINSKI et al., 2001). Based on the nearly constant level of the housekeeping β-tubulin transcript amplified in both tachyzoite and bradyzoite forms, the RT-PCR results were consistent with the existence of a developmentally controlled transcription of *ENO1* and *ENO2* genes.

The Glucose 6-phosphate isomerase mRNA is readily detected in both bradyzoite and tachyzoite stages. However, the levels of G6-PI transcript is increased 10-fold in the encysted bradyzoites, suggesting that G-PI gene is subjected to transcriptional control in encysted bradyzoites (DZIERSZINSKI et al., 1999).

Taking the advantage of establishment of *in vitro* stage conversion techniques (BOHNE et al., 1994; SOETE et al., 1994; WEISS et al. 1995), we studied the steady state mRNA transcript levels of 11 nuclear genes coding for key subunits of the respiratory chain in tachyzoite and bradyzoite stages of *T. gondii*. Quantitative real time RT-PCR was performed using specific primers designed to amplify short (300-500 bp) cDNA fragments of the nuclear genes coding for 11 subunits.

Based on the nearly constant level of the housekeeping β-tubulin and actin transcripts amplified in both tachyzoite and bradyzoite stages, the RT-PCR results show no significant developmentally controlled transcription of the tested genes. It is evident that both tachyzoites and bradyzoites have a similar mRNA level of the studied genes, however we can not rule out the presence of translational or post-translational differential regulation of these genes which is found to be the case for LDH1. mRNA of *LDH1* was found to be equally abundant in both bradyzoite and tachyzoite stages, while the LDH1 enzyme was only detected in tachyzoites when two-dimensional PAGE was performed (YANG and PARMLEY, 1997)

4.6 HDQ: A high affinity inhibitor of alternative NADH dehydrogenases

The mitochondria of different parasites have been validated as a drug target. Well-established antimalarial drugs, such as sulphadoxine/pyrmethamine, chloroquine derevatives (atovaqoune) and artemisinin are the most common treatments for malaria through out the world, are interfering with the functions of the mitochondrion in away or another. The antimalarial drug atovaqoune has been shown to inhibit the electron transport at the bc1 complex (FRY et al., 1992) and to collapse the mitochondrial membrane potential in *P. yoelii* (SRIVASRAVA et al., 1997), the respiratory chain of *T. gondii* and *P. falciparum* by interfering with the ubiquinone cycle, it binds with the (ubiquinol binding pocket) coenzyme Q binding site of the cytochrome *b* of *bc1* complex (KESSL et al., 2003; 2005; and 2006) . Pyrimethamine inshibits the DHFR enzyme in the purine synthesis pathway in the mitochondrion. Flavines inhibit the DHOR enzyme in the pyrmidine synthesis pathyway which sinks electrons to the respiratory chain at site of complex III. Other respiratory chain inhibitors like oligomycin show a collapse in the membrane potential (VERCESI et al., 1998) and inhibits growth of *T. gondii* and *P. falciparum*. Inhibitors of complex II like chalcone show antimalarial (CHEN et al., 2001) and antileishmanial (MI-ICHE et al., 2005) effects.

Due to their absence in mammalian cells alternative NADH dehydrogenases are recognized as important antimicrobial drug targets(WEINSTEIN et al., 2005; BIAGINI et al., 2006). Of particular interest is a recent study on the effects of the antimalarial drug artemisinin in a model yeast system (LI et al., 2005). Up-regulation of yeast single-subunit NAD(P)H dehydrogenases was found to increase sensitivity of yeast to artemisinin, whereas ablation of expression lowered the sensitivity. Expression *of P. falciparum* NADH

dehydrogenase in yeast cells where native NAD(P)H dehydrogenase had been ablated restored sensitivity to artemisinin. This suggests that mitochondrial electron transport, and the functions of the NAD(P)H dehydrogenase in particular, may be crucial in activating and mediating the effects of this important antimalarial drug.
The compound HDQ was recently described as the first high affinity inhibitor of alternative NADH dehydrogenases with an IC50 in enzymatic assays of 200 nM (ESCHEMANN et al., 2005). In this study, HDQ was found to able to inhibit replication of the apicomplexan *T. gondii* in nanomolar ranges. *T. gondii* parasites lack a conventional complex I in their electron transport chain and instead possess alternative NADH:dehydrogenases.

Although it can not completely ruled out that HDQ has additional effects on *T. gondii* which is unrelated to alternative NADH-dehydrogenase inhibition, the observed synergism between HDQ and the complex III inhibitor atovaquone suggests, that HDQ as atovaquone affects the mitochondrial electron transport chain. This synergism is most likely due to the inhibition of the ubiquinone/ubiquinol cycle at two different locations, which is the reduction site (alternative NADH dehydrogenase) for HDQ and the oxidation site (complex III) for atovaquone. A synergism of atovaquone with low affinity inhibitors of alternative NADH dehydrogenases was recently shown for *Plasmodium*, demonstrating that simultaneous inhibition of the ubiquinone/ubiquionol cycle at different points has a huge pharmacological potential (BIAGINI et al., 2006). However, the general flavines used in the previous study as diphenylene iodonium chloride have an IC50 which is ~500-fold higher compared to HDQ and are most likely unsuitable for clinical studies (ESCHEMANN et al., 2005, BIAGINI et al., 2006).

In contrast, the IC50 of HDQ in the low nanomolar range makes HDQ a highly attractive candidate for further studies, particularly since we did not observe any negative effects on the human host cells at the applied concentrations. HDQ and derivatives represent thus promising compounds with high potential for anti-malarial and anti-toxoplasmal therapy. In addition to *Toxoplasma* and *Plasmodium*, HDQ might be effective against a broader spectrum of pathogens/parasites including other apicomplexan parasites with a mitochondrial respiratory chain, as well as some kinetoplastids like *Trypanosoma* which express type II NADH dehydrogenases in addition to a conventional complex I (FANG and BEATTIE, 2002; 2003).

A critical feature of the 1-hydroxy-2-alkyl-4(1)quinolone structure is the length of the alkyl site chain at position 2. While derivatives with alkyl site chains of C_6, C_8, C_{12} (HDQ) and C_{14} all displayed excellent anti-parasitic activities in nanomolar ranges, a C_5 derivative completely failed to inhibit *T. gondii* replication. A minmal alkyl site chain length of C_6 appears thus to be required for drug action. Due to the structural similarities, it is most likely that 1-hydroxy-2-alkyl-4(1)quinolones compete with ubiquinones for the same binding site in alternative NADH dehydrogenases (ESCHEMANN et al., 2005). However, it has to be mentioned that steady-state inhibition kinetics of HDQ on the *Y. lipolytica* alternative NADH-dehydrogenase displayed a non-competitive pattern for the hydrophobic ubiquinone derivative DBQ, which the authors of this study used as an electron acceptor (ESCHEMANN et al., 2005). This unexpected finding was explained by a proposed ping-pong mechanism for the two substrate reaction of the enzyme (ESCHEMANN et al., 2005). Future biochemical analysis will reveal whether HDQ exhibits the same inhibition mode on the *T. gondii* and *P. falciparum* orthologs as on the *Y. lipolytica* enzyme.

HDQ treatment of *T. gondii* infected cultures results in an induction of bradyzoite differentiation. This effect is well known from other compounds which also inhibit parasite replication and simultaneously induce the expression of bradyzoite specific genes in *T. gondii*. Exposure to stress conditions as alkaline pH or nitric oxide (SOETE et al., 1994; BOHNE et al., 1994) and particularly the inhibition of the respiratory chain by inhibitors as oligomycin or atovaquone (BOHNE et al., 1994; TOMAVO and BOOTHROYD, 1995) appears to generate an adaptive response leading to stage conversion of the parasite.

4.7 Functional analysis of TgNDH2-I by conditional knockout mutants

4.7.1 Generation of conditional knock out mutants

To address the role of respiratory chain components on the overall cellular metabolism, we applied a reverse genetic approach recently developed in both *T. gondii* and *P. falciparum,* the anhydrotetracycline (Atc)-controlled inducible expression system (MEISSNER et al., 2001; 2002; 2005) which has been used successfully to generate conditional knock out mutants for essential genes (MITAL et al, 2005). Tetracycline-inducible expression systems are widely used for exogenous control of gene expression in eukaryotes. These systems are based on two regulatory elements derived from the tetracycline resistance operon of the *Escherichia coli* Tn10 transposon, being a tet repressor fused with the tet-dependent

transactivator (TetR) and the tet operator (tetO) DNA sequence. Interaction of dimeric TetR with tetO elements, which are placed in the vicinity of the transcriptional start site will promote the binding of RNA polymerase or auxiliary transcription factors and initiate transcription of a gene (BERENS and HILLEN, 2003). Addition of tetracycline causes conformational changes and dissociation of TetR from tetO elements, thereby blocking gene transcription. This system enables inducible expression of heterologous genes as well as functional studies of endogenous genes.

Because disrupting genes that play crucial functions in the mitochondrion by homologous recombination techniques accompanied with inducible knockout systems is essential in understanding both the role of that particular gene, and of the mitochondrion in cellular processes, we tried to disrupt the single nuclear genes coding for both isoforms of type II NADH dehydrogenases, flavoprotein subunit of succinate dehydrogenase (complex II), cytochrome c1 (complex III), and β-subunit of ATP synthase (complex V).

Parasites expressing an Atc-responsive transactivator protein (TATi line, which express only the endogenous genes) were transfected with a recombinant *myc*-tagged version of the previous genes under the control of a *SAG1*-based conditional promoter (MEISSNER et al., 2002). The addition of Atc to transactivator-expressing parasites resulted in a significant decrease in the activity of the conditional promoter as shown by immunofluorescence microscopy with antibodies directed against the myc tag. Immunofluorescence microscopy was used to identify clones (designated "*ndh2-I/ndh2-I-myc*","*ndh2-II/ndh2-II-myc*","*sdh-fpsdh-fp-myc*","*cytc1/cytc1-myc*", and " *ATP-β /ATP-β -myc*") that stably express Atc-regulated exogenous genes with a mitochondrial localization indistinguishable from endogenous genes. Quantitative real time RT-PCR of these clones grown without or with Atc showed an increase mRNA level of the cloned genes in the absence of Atc, however this increase in expression was not correlated with an increase in growth rate of these clones.

Targeting the endogenous, single copy genes (*ndh2-I, ndh2-II, sdh-fp, cytc1, and ATP- β*) in these clones with a targeting constructs based on single and double homologous recombinations with Atc inducible system has led to a disruption of the gene coding for the first isoform of type II NADH dehydrogenases (*ndh2-I*), but not any of other targeted genes probably due to a high efficiency of random, non-homologous integration of the targeting constructs in the genome of *T. gondii,* or due to lack of accessibility of these genes to the targeting constructs. Sequences at the extreme 5' and 3' end of *Tgndh2-II* that were excluded from the targeting construct allowed PCR-based screening for knockout

plasmid integration into the endogenous locus of *ndh2-I* in *ndh2-I/ndh2-I-cmyc* parasites, and specificity of the knock out PCR products were confirmed by nested PCR. RT-PCR showed that the knock out parasites (designated, Δ*ndh2-I/ndh2-I-cmyc*) do not express endogenous *Tgndh2-I* and express detectable *Tgndh2-I-myc* only in the absence of Atc.

4.7.2 Phenotypic analysis of the conditional knock out mutants

The analysis of the conditional *Tgndh2-I* knock-out mutant (Δ*ndh2-I/ndh2-I-cmyc*), provides important conclusions about the relative importance of both isoforms. Since the growth rate of NDH2-I (Δ*ndh2-I/ndh2-I-cmyc*) depleted parasites is identical to NDH2-I expressing parasites (*ndh2-I/ndh2-I-cmyc*), NDH2-I activity appears not to be essential for normal intracellular development of *T. gondii*. This means that NDH2-II activity under normal tissue culture conditions is sufficient for maintaining parasite metabolism.

Alternative (type II) NADH dehydrogenases can be orientated with their reactive site either inside (internal) or outside (external) of the mitochondrial matrix (KERSCHER, 2000; MELO et al., 2004), thus transferring electrons from cytosolic or mitochondrial matrix NADH into the electron transport chain. Although there is no direct evidence for the orientation of the two isoforms, the most likely scenario which is in agreement with the behaviour of the conditional NDH2-I knock out mutant (Δ*ndh2-I/ndh2-I-cmyc*) is, that both isoforms are located at the same side of the membrane, most probably external, thus enabling NDH2-II to compensate the loss of NDH2-I activity. Alternatively, if NDH2-I and NDH2-II are located at different sites of the membrane, the transfer of electrons from either cytosolic or mitochondrial NADH to ubiquinol could not be directly compensated by NDH2-II, but would require additional shuttle systems, which mediate the translocation of NADH.

To further analyze the effect of this gene disruption, we studied the gene expression of the second isoform *ndh2-II* on the transcrpitional level by quantitiative real time RT-PCR using the Lightcycler and the housekeeping gene β-tubulin for normalization. The obtained crossing points were used to calculate the relative amount of initial cDNA template for *ndh2-II* and β-tubulin in the conditional knock out mutants (Δ*ndh2-I/ndh2-I-cmyc*) and wilde type (*ndh2-I/ndh2-I-cmyc*), showing a slight upregulation of *ndh2-II* in the knock out parasites. This slight upregulation in the expression of *ndh2-II* in the *ndh2-I* depleted parasites could also explain the ineffectivity of gene disruption of *ndh2-I* on growth rate of the depleted parasites by compensating the lost activity of NDH2-I, specially if they are oriented to the same side of the inner mitochondrial membrane.

As tachyzoite to bradyszoite differentiation was shown to be induced by different inhibitors of the respiratory chain, we seeked to study stage conversion of parasites diffecient with NDH2-I, *in vitro* differentiation of the conditional knock out parasites (Δ*ndh2-I/ndh2-I-cmyc*) was performed using pH shift media (alkaline pH) in the presence of Atc. Quantitiative real time RT-PCR was used to investigate the bradyzoite specific gene expression and used the small heat shock gene *bag1* as a differentiation marker (BOHNE et al., 1995). As found for growth rate, no significant difference of the mRNA transcripts of *bag1* has been detected in bradyzoites of the knock out (Δ*ndh2-I/ndh2-I-cmyc*) and wild type (*ndh2-I/ndh2-I-cmyc*) parasites, indicating no effect of *ndh2-I* disruption on the ability of *in vitro* differentiation of the knock out parasites.

While no phenotype was obvious under normal culture conditions, NDH2-I depleted parasites were extremly susceptible to HDQ treatment. 1 nM HDQ was sufficient to completely inhibit parasite replication and to protect the HFF monolayer from lysis. This increased sensitivity could be the result of one of the following mechanisms or a combination of both.

(i) NDH2-I and NDH2-II can complement each other, but NDH2-II has a higher affinity for HDQ as NDH2-I. In NDH2-I depleted parasites the complementation leads to no obvious phenotype in the absence of drug, but in the presence of HDQ the lower IC50 of NDH2-II becomes dominant.

(ii) HDQ hypersensitivity in NDH2-I depleted parasites is not a matter of different HDQ affinities between NDH2-I and NDH2-II, but due to a reduced ubiquinol concentration as a result of lacking NDH2-I activity. The hydrophobic ubiquinol is likely to compete with the quinolone-like HDQ for the same binding site within the enzyme, thus if HDQ acts as a competitive inhibitor in NDH2-I, a reduced ubiquinol concentration would in turn lower the IC50 for HDQ. However, it has to be mentioned that steady-state inhibition kinetics of HDQ on the *Y. lipolytica* alternative NADH-dehydrogenase displayed a non-competitive pattern for the hydrophobic ubiquinone derivate DBQ, which was used in the enzymatic assay as an electron acceptor (ESCHEMANN et al., 2005). This unexpected finding was explained by a proposed ping-pong mechanism for the two substrate reaction of the enzyme (ESCHEMANN et al., 2005). Detailed biochemical studies on recombinant TgANDH2-I and TgANDH2-II will reveal the enzymatic characteristics of both *T. gondii* isoforms.

4.8 Proposed scheme for the electron transport chain in *T. gondii*

A model for the electron transport chain was recently proposed for Plasmodium (VAN DOOREN, 2006) (**Fig. 4.4**). The electron transport chain of *T. gondii* has most of the components proposed for that of Plasmodium. However, in T. gondii, two isoforms of alternative NADH dehydrogenase (TgNDH2-I and TgNDH2-II) have been identified instead of one isoform in Plasmodium (PfNDH2). Moreover, the mitochondrion of *T. gondii* has a matrix localized NAD-dependent malate dehydrogenase and a membrane bound, FAD-dependent malate:quinone oxidoreductase (FLEIGE, PhD thesis, 2006), while only FAD-dependent malate:quinone oxidoreductase have been identified in Plasmodium (VAN DOOREN et al., 2006)

Based on previous biochemical studies and the bioinformatic analysis of *T. gondii* genome, we can propose the following possible composition and functions for the electron transport chain in *T. gondii*.

Electrons can be donated to coenzyme Q from a variety of mitochondrial inner membrane dehydrogenases. As in Plasmodium (**Fig. 4.4**) five such enzymes exist in *T. gondii*, although the role of several of these is not yet clearly defined.

Beside the two isoforms of alternative NADH dehydrogenase and succinate dehydrogenase (complex II), the following enzymes are present in the electron transport chain of *T. gondii*:

(1). Malate is converted to oxaloacetate to complete the tricarboxylic acid cycle, a reaction that is typically catalyzed by NAD-dependent malate dehydrogenase. Instead, the genome of *T. gondii* reveals as in Plasmodium, the presence of a membrane bound malate-quinone oxidoreductase homologue (TgTwinScan_0081). Malate-quinone oxidoreductase is an enzyme found in some bacteria, and reduces FAD in the generation of oxaloacetate. Electrons from $FADH_2$ are then donated to coenzyme Q and the electron transport chain.

(2). Dihydroorotate dehydrogenase (DHODH), an enzyme involved in pyrimidine biosynthesis, which catalyzes the oxidation of dihydroorotate to orotate, donating electrons to coenzyme Q via a FAD cofactor. *T. gondii* dihydroorotate dehydrogenase (TgTwinScan_1012) localizes to the mitochondrion, most probably to the inner membrane (ASAI et al., 1983). In the biosynthesis of pyrimidines, the *T. gondii* electron transport chain functions as an electron sink.

(3). FAD-linked, or mitochondrial, glycerol-3-phosphate dehydrogenase forms part of the glycerol-3-phosphate shuttle. This shuttle essentially provides an alternative means of

directing electrons from cytosolic NADH to mitochondrial coenzyme Q. The first step in this shuttle involves cytosolic NAD^+-linked glycerol-3-phosphate dehydrogenase, which reduces dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate, oxidizing cytosolic NADH in the process. Glycerol-3-phosphate is then transported to the mitochondrial intermembrane space, where mitochondrial glycerol-3-phosphate dehydrogenase oxidizes glycerol-3-phosphate back to dihydroxyacetone phosphate, with the electron donated to its FAD cofactor. In turn, this electron is passed into the electron transport chain via coenzyme Q. The *T. gondii* genome has homologues of both NAD^+-linked (TgTwinScan_2421) and FAD-linked (TgTwinScan_7069) glycerol-3-phosphate dehydrogenases. However, glycerol 3-phosphate was not able to stimulate ADP phosphorylation in digitonin permeabilized tachyzoites of *T. gondii*, though it is often taken by the mitochondria (VERCESI et al., 1998).

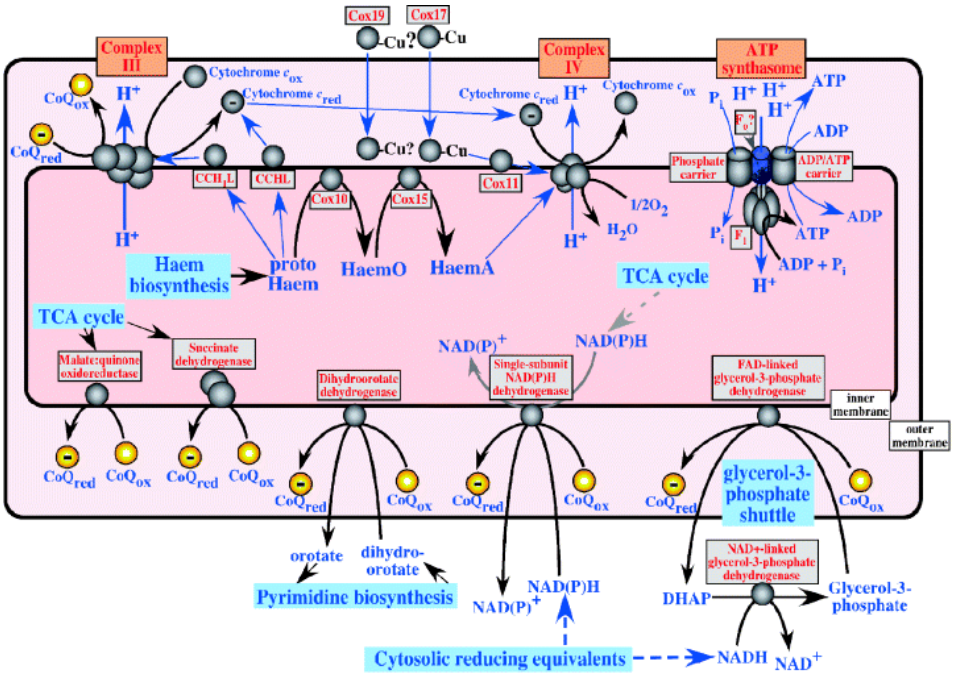


Figure 4.4: A putative model for electron transport through the inner membrane of the mitochondrion of Plasmodium. Several proteins and protein complexes localize to the inner membrane of mitochondria, where they function in the accepting and donating of electrons. Several of these enzymes (bottom of diagram) donate electrons to coenzyme Q (CoQ; yellow sphere). Those with clear homologues in T. gondii include succinate dehydrogenase, malate : quinone oxidoreducatase, dihydroorotate dehydrogenase, glycerol-3-phosphate dehydrogenase and NAD(P)H dehydrogenase. It is unclear whether NAD(P)H dehydrogenase oxidizes NAD(P)H derived from the cytosol or from the mitochondrial matrix. Electrons from coenyzme Q are donated to complex III (cytochrome *c* reductase; top of diagram), which passes electron through to cytochrome *c* and translocates protons (H^+) from the matrix into the intermembrane space. Cytochrome *c* is a soluble intermembrane space protein that donates electrons to complex IV (cytochrome *c* oxidase), with oxygen functioning as the terminal electron acceptor. Complex IV also translocates protons across the inner membrane. The proton gradient generated by complexes III and IV is harnessed by the F_0F_1 ATP synthase complex for the production of ATP. (VAN DOOREN et al., 2006)

In summary, bioinformatic and biochemical evidence suggests that electrons are donated to the Toxoplasma electron transport chain via the FAD-linked tricarboxylic acid cycle enzymes malate : quinone oxidoreductase and succinate dehydrogenase (complex II). Electrons are also donated during pyrimidine biosynthesis via DHODH. The contribution of electrons donated from cytosolic and mitochondrial NADH and NADPH to the electron transport chain requires further elucidation of the functions and localizations of NAD(P)H dehydrogenase and glycerol-3-phosphate dehydrogenases.

Based on previous biochemical studies and the bioinformatic analysis, we can propose several possible functions for the electron transport chain in *T. gondii*. It clearly functions as an electron sink for the dihydroorotate dehydrogenase reaction of the essential pyrimidine biosynthesis pathway (LOPEZ et al., 2006). It is clear that the electron transport chain of *T. gondii* functions in generating a $\Delta\Psi$ across the inner membrane. It remains to be determined whether $\Delta\Psi$ has a biologically important role in these parasites. By analogy with other systems, $\Delta\Psi$ may be required for protein import and in the transport of solutes (LALOI, 1999; PFANNER and GEISSLER, 2001). Interestingly, one study has shown that the addition oligomycin, an inhibitor of mitochondrial ATP synthase, caused an increase in cytosolic Ca^{2+} levels in tachyzoites. This increase suggested a requirement for mitochondrial energy for the regulation of cellular Ca^{2+} homeostasis in these parasites (MORENO and ZHONG, 1996).

The ATP proportion generated by glycolysis and oxidative phosphorylation in *T. gondii* remains an opened question. However, it is tempting to speculate that in tachyzoite stage most of the ATP is generated by glycolysis, a minimal amount of ATP is generated coupled to electrons donated from pyrimidine biosynthesis, and perhaps by the small amount of NADH generated during glycolysis via the glycerol-3-phosphate shuttle and/or an external NADH dehydrogenase(s).

REFERENCES

1. **Ajioka J.W.** (1997) Analysis of apicomplexan parasites. In Methods: A Companion to Methods in Enzymology. **13:**79-80, Academic Press, Orlando, FL, USA.

2. **Ajioka J.W., Fitzpatrick J.M., and Reitter C.P.** (2001). *Toxoplasma gondii* genomics: shedding light on pathogenesis and chemotherapy. *Exp Rev Mol* **6:**1.19.

3. **Andersson S.G., Zomorodipour A., Andersson J.O., Sicheritz-Pontén T., Alsmark U.C., Podowski R.M., Kurland C.G., Eriksson A.S., Winkler H.H. and Kurland C.G.** (1998). The genome sequence of *Rickettsia prowazekii* and the origin of mitochondria. *Nature* **396**:133–140.

4. **Arnold I., Folsch H., Neupert W., and Stuart R.A.** (1998). Two distinct and independent mitochondrial targeting signals function in the sorting of an inner membrane protein, cytochrome c1. *J Biol Chem*. **273:**1469-76.

5. **Asai T., O'Sullivan W.J., Kobayashi M., Gero A.M., Yokogawa M., and Tatibana M.** (1983). Enzymes of the de novo pyrimidine biosynthetic pathway in *Toxoplasma gondii*. *Mol Biochem Parasitol*. **7:**89-100.

6. **Bandeiras T.M., Salgueiro C., Kletzin A., Gomes C.M. and Teixeira M.** (2002). *Acidianus ambivalens* type-II NADH dehydrogenase: genetic characterisation and identification of the flavin moiety as FMN, *FEBS Lett.* **531**:273–277.

7. **Bandeiras T.M., Salgueiro C.A., Huber H., Gomes C.M. and Teixeira M.** (2003). The respiratory chain of the thermophilic archaeon *Sulfolobus metallicus*: studies on the type-II NADH dehydrogenase, *Biochim. Biophys. Acta* **1557**:13-19.

8. **Bender A., van Dooren G., Ralph S.A., McFadden G.I., and Schneider G.** Properties and prediction of mitochondrial transit peptides from *Plasmodium falciparum. Mol Biochem Parasitol*. **132:**59-66.

9. **Berens C., and Hillen W.** (2003). Gene regulation by tetracyclines. Constraints of resistance regulation in bacteria shape TetR for application in eukaryotes. *Eur J Biochem*. **270:**3109-21.

10. **Bertsova Y.V., Bogachev A.V., and Skulachev V.P.** (2001). Noncoupled NADH:ubiquinone oxidoreductase of *Azotobacter vinelandii* is required for diazotrophic growth at high oxygen concentrations. *J Bacteriol.* **183:**6869-74.

11. **Biagini G.A., Viriyavejakul P., O'neill P.M., Bray P.G., and Ward S.A.** (2006). Functional characterization and target validation of alternative complex I of *Plasmodium falciparum* mitochondria. *Antimicrob. Agents Chemother*. **50**:1841-51.

12. **Bjorklof K., Zickermann V. and Finel M.** (2000). Purification of the 45 kDa, membrane bound NADH dehydrogenase of *Escherichia coli* (NDH-2) and analysis of its interaction with ubiquinone analogues. *FEBS Lett.* **467**:105–110.

13. **Bohne W. and Roos D.S.** (1997). Stage-specific expression of a selectable marker in *Toxoplasma gondii* permits selective inhibition of either tachyzoites or bradyzoites. *Mol. Biochem. Parasitol*. **88:**115-26.

14. **Bohne W., Gross U., Ferguson D.J., and Heesemann J.** (1995). Cloning and characterization of a bradyzoite-specifically expressed gene (hsp30/bag1) of *Toxoplasma gondii*, related to small heat-shock proteins of plants. *Mol Microbiol* **16:**1221-30.

15. **Bohne W., Heesemann J., and Gross U. (**1993). Induction of bradyzoite-specific *Toxoplasma gondii* antigens in gamma interferon-treated mouse macrophages. *Infect. Immun*. **61:**1141-5.

16. **Bohne W., Heesemann J., and Gross U.** (1994). Reduced replication of *Toxoplasma gondii* is necessary for induction of bradyzoite-specific antigens: a possible role for nitric oxide in triggering stage conversion. *Infect. Immun*. **62:**1761-1767.

17. **Bohne W., Parmley S.F., Yang S., and Gross U.** (1996). Bradyzoite-specific genes in *Toxoplasma gondii*. *Curr. Top. Microbiol. Immunol*. **219**:81-91.

18. **Capaldi R.A., and Aggeler R.** (2002). Mechanism of the F(1)F(0)-type ATP synthase, a biological rotary motor. *Trends Biochem Sci*. **27:**154-60.

19. **Carneiro P., Duarte M., and Videira A.** (2004). The main external alternative NAD(P)H dehydrogenase of *Neurospora crassa* mitochondria. *Biochim Biophys Acta* **1608**: 45–52.

20. **Carruthers, V.B. and Sibley L.D.** (1997). Sequential protein secretion from three distinct organelles of *Toxoplasma gondii* accompanies invasion of human fibroblasts. *Eur J Cell Biol* **73:** 114-123.

21. **Cauchetier E., Loiseau P.M., Lehman J., Rivollet D., Fleury J., Astier A., Deniau M., and Paul M..** (2002). Characterization of atovaquone resistance in *Leishmania infantum* promastigotes. *Int. J. Parasitol*. **32:**1043-1051.

22. **Chen M., Christensen S.B., Blom J., Lemmich E., Nadelmann L., Fich K., Theander T.G., and Kharazmi A.** 1993. Licochalcone A, a novel antiparasitic agent with potent activity against human pathogenic protozoan species of Leishmania. *Antimicrob. Agents Chemother*. **37:**2550-2556.

23. **Chen M., Zhai L., Christensen S.B., Theander T.G., and Kharazmi A..** (2001). Inhibition of fumarate reductase in *Leishmania major* and *L. donovani* by chalcones. *Antimicrob. Agents Chemothe*r. **45:**2023-2029.

24. **Chio L.C., and Queener S.F.** (1993). Identification of highly potent and selective inhibitors of *Toxoplasma gondii* dihydrofolate reductase. *Antimicrob Agents Chemother*. **37**:1914–1923.

25. **Claros M.G., and Vincens P.** (1996). Computational method to predict mitochondrially imported proteins and their targeting sequences. *Eur J Biochem.* **241:**779-86.

26. **Cleary M.D., Singh U., Blader I.J., Brewer J.L., and Boothroyd J.C.** (2002). *Toxoplasma gondii* asexual development: identification of developmentally regulated genes and distinct patterns of gene expression. *Eukaryot Cell*. **1:**329-40.

27. **Coppens I. and Joiner K.A.** (2001). Parasite host cell interactions in toxoplasmosis: new avenues for intervention? *Exp Rev Mol Med* **2001:**1-20.

28. **Coppin A., Dzierszinski F., Legrand S., Mortuaire M., Ferguson D., and Tomavo S.** (2003). Developmentally regulated biosynthesis of carbohydrate and storage polysaccharide during differentiation and tissue cyst formation in *Toxoplasma gondii*. *Biochimie.* **85:** 353-61.

29. **Croft S.L., Hogg J., Gutteridge W.E., Hudson A.T., Randall A.W.**(1992) The activity of hidroxynaphthoquinones against *Leishmania donovani*. *J Antimicrob Chemother.* **30**:827–832.

30. **Cserzo M., Wallin E., Simon I., von Heijne G., and Elofsson A.** (1997). Prediction of transmembrane alpha-helices in prokaryotic membrane proteins: the dense alignment surface method. *Protein Eng.* **10:**673-6.

31. **de Melo E.J., de Carvalho T.U., and de Souza W.** (1992). Penetration of *Toxoplasma gondii* into host cells induces changes in the distribution of the mitochondria and the endoplasmic reticulum. *Cell Struct Funct*. **17:**311-7.

32. **de Vries S. and Grivell L.A.** (1988). Purification and characterization of a rotenone-insensitive NADH:Q6 oxidoreductase from mitochondria of *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **176**:377–384.

33. **de Vries S., van Witzenburg R., Grivell L.A. and Marres**. **C.A.** (1992). Primary structure and import pathway of the rotenone-insensitive NADH-ubiquinone oxidoreductase of mitochondria from Saccharomyces cerevisiae. *Eur. J. Biochem.* **203**:587–592.

34. **Deber C.M., Wang C., Liu L.P., Prior A.S., Agrawal S., Muskat B.L., and Cuticchia A.J.** (2001). TM Finder: a prediction program for transmembrane protein segments using a combination of hydrophobicity and nonpolar phase helicity scales. *Protein Sci.* **10:**212-9.

35. **Deisenhofer J. and H. Michel.** (1991). Structures of bacterial photosynthetic reaction centers. *Annu. Rev. Cell Biol.* **7**:1–23.

36. **Deisenhofer J. and Michel H.** (1989). The photosynthetic reaction center from the purple bacterium *rhodopseudomonas viridis*. *Science* **245**:1463–1473.

37. **Denton H., Roberts C.W., Alexander J., Thong K., and Coombs G.H.**. (1996). Enzymes of energy metabolim in the bradyzoites and tachyzoites of *Toxoplasma gondii*. *FEMS Microbiol. Letters* **137:** 103-108.

38. **DeRocher A., Hagen C.B., Froehlich J.E., Feagin J.E., and Parsons M.** (2000). Analysis of targeting sequences demonstrates that trafficking to the *Toxoplasma gondii* plastid branches off the secretory system. *J Cell Sci*. **113:**3969-77.

39. **Desmonts G., and Couvreur J.** (1974). Congenital toxoplasmosis. A prospective study of 378 pregnancies. *N Engl J Med* **290:** 1110–16.

40. **Donald R.G. and Roos D.S.** (1993). Stable molecular transformation of *Toxoplasma gondii*: a selectable dehydrofolate reductase-thymidylate synthase marker based on drug resistance in malaria *Proc. Natl Acad. Sci. USA*, **90**: 11703–11707.

41. **Donald R.G. and Roos D.S.** (1995) Insertional mutagenesis and marker rescue in a protozoan parasite: cloning the uracil phosphoribosyl-transferase locus from *Toxoplasma gondii Proc. Natl Acad. Sci. USA*, **92**: 5749–5753.

42. **Donald R.G., Carter D., Ullman B., and Roos D.S..** (1996). Insertional tagging, cloning, and expression of the *Toxoplasma gondii* hypoxanthine-xanthine-guanine phosphoribosyltransferase gene. Use as a selectable marker for stable transformation. *J. Biol. Chem*. **271:**14010-14019.

43. **Duarte M, Peters M, Schulte U, Videira A.** (2003). The internal alternative NADH dehydrogenase of *Neurospora crassa* mitochondria. *Biochem J.* 371:1005-11.

44. **Dubey J.P., and Beattie C.P.**: Toxoplasmosis of Animals and Man. CRC Press, Boca Raton, FL, 1988

45. **Dubey J.P., Lindsay D.S., and Speer C.A.** (1998). Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. *Clin. Microbiol. Rev.* **11:**267-299.

46. **Dzierszinski F., Mortuaire M., Dendouga N., Popescu O., and Tomavo S.** (2001). Differential expression of two plant-like enolases with distinct enzymatic and antigenic properties during stage conversion of the protozoan parasite *Toxoplasma gondii*. *J Mol Biol.* **309:**1017–1027.

47. **Dzierszinski F., Popescu O., Toursel C., Slomianny C., Yahiaoui B., and Tomavo S.** (1999). The protozoan parasite *Toxoplasma gondii* expresses two functional plant-like glycolytic enzymes. *J Biol Chem* **274:**34888-95.

48. **Egan B., Beilharz T., George R., Isenmann S., Gratzer S., Wattenberg B., and Lithgow T.** (1999). Targeting of tail-anchored proteins to yeast mitochondria *in vivo*. *FEBS Lett*. **451:**243-8.

49. **Eggink G., Engel H., Vriend G., Terpstra P., and Witholt B**. (1990). Rubredoxin reductase of *Pseudomonas oleovorans*. Structural relationship to other flavoprotein oxidoreductases based on one NAD and two FAD fingerprints. *J Mol Biol*. **212:**135-42.

50. **Elhafez D., Murcha M.W. , Clifton R., Soole K.L., Day D.A., and Whelan J.** (2006) Characterization of mitochondrial alternative NAD(P)H dehydrogenases in Arabidopsis: Intraorganelle location and expression. *Plant Cell Physiol.* **47:** 43-54.

51. **Ellis J.E.** (1994). Coenzyme Q homologs in parasitic protozoa as targets for chemotherapeutic attack. *Parasitol. Today*. **10:**296-301.

52. **Emanuelsson O., Nielsen H., Brunak S., and von Heijne G.** (2000). Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J Mol Biol.* **300:**1005-16.

53. **Eschemann A., Galkin A., Oettmeier W., Brandt U., and Kerscher S.** (2005). HDQ (1-hydroxy-2-dodecyl-4(1H)quinolone), a high affinity inhibitor for mitochondrial alternative NADH dehydrogenase: evidence for a ping-pong mechanism. *J. Biol. Chem*. **280:**3138-42.

54. **Esseiva A.C., Naguleswaran A., Hemphill A., and Schneider A..** (2004). Mitochondrial tRNA import in *Toxoplasma gondii*. *J. Biol. Chem*. **279:**42363–42368.

55. **Evans D.R. and Guy H.I.** (2004). Mammalian pyrimidine biosynthesis: fresh insights into an ancient pathway. *J Biol Chem* **279**: 33035–33038

56. **Fang J. and Beattie D.S.** (2002). Novel FMN-containing rotenone-insensitive NADH dehydrogenase from *Trypanosoma brucei* mitochondria: isolation and characterization. *Biochemistry* **41**:3065–3072.

57. **Fang J. and Beattie D.S.** (2003). Identification of a gene encoding a 54 kDa alternative NADH dehydrogenase in *Trypanosoma brucei*. *Mol Biochem Parasitol.* **127:**73-7.

58. **Finel M.** (1996). Genetic inactivation of the H(+)-translocating NADH:ubiquinone oxidoreductase of *Paracoccus denitrificans* is facilitated by insertion of the ndh gene from Escherichia coli. *FEBS Lett.* **393**:81–85.

59. **Fisher N., and Rich P.R.** (2000). A motif for quinone binding sites in respiratory and photosynthetic systems. *J Mol Biol*. **296:**1153-62.

60. **Fleige T.,** (2006). Compartmentalization of carbohydrate metabolism in *Toxoplasma gondii.* PhD thesis.

61. **Foth B.J., Ralph S.A., Tonkin C.J., Struck N.S., Fraunholz M., Roos D.S., Cowman A.F., and McFadden G.I.** (2003). Dissecting apicoplast targeting in the malaria parasite *Plasmodium falciparum*. *Science.* 2003 **299:**705-8.

62. **Frenkel J. K.** (1973). *Toxoplasma* in and around us. *BioScience* **23:**343-352.

63. **Friedrich T., Steinmüller K., and Weiss H.** (1995). The proton-pumping respiratory complex I of bacteria and mitochondria and its homologue in chloroplasts. *FEBS Lett.* **367**:107–111.

64. **Fry M., and Beesley J.E.** (1991). Mitochondria of mammalian Plasmodium spp. *Parasitology.* **102:**17-26.

65. **Fry M., and Pudney M.** (1992). Effect of mitochondrial inhibitors on adenosinetriphosphate levels in *Plasmodium falciparum*. *Biochem Pharmacol*. **43:**1545–1553.

66. **Fry M., Webb E., and Pudney M.** (1990). Effect of mitochondrial inhibitors on adenosinetriphosphate levels in *Plasmodium falciparum*. *Comp Biochem Physiol B*. **96:**775-82.

67. **Fulton J.D., and Spooner D.F**. (1960). Metabolic studies on *Toxoplasma gondii*. *Exp Parasitol*. **9**:293-301.

68. **Funes S., Davidson E., Reyes-Prieto A., Magallon S., Herion P., King M.P., and Gonzalez-Halphen D.A.** (2002). green algal apicoplast ancestor. *Science.* **298:**2155.

69. **Gardner M.J., Bishop R., Shah T., de Villiers E.P., Carlton J.M., Hall N., Ren Q., Paulsen I.T., Pain A., Berriman M., Wilson R.J., Sato S., Ralph S.A., Mann D.J., Xiong Z., Shallom S.J., Weidman J., Jiang L., Lynn J., Weaver B., Shoaibi A., Domingo A.R., Wasawo D., Crabtree J., Wortman J.R., Haas B., Angiuoli S.V., Creasy T.H., Lu C., Suh B., Silva J.C., Utterback T.R., Feldblyum T.V., Pertea M., Allen J., Nierman W.C., Taracha E.L., Salzberg S.L., White O.R., Fitzhugh H.A., Morzaria S., Venter J.C., Fraser C.M., Nene V.** (2002). Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature*. **419:** 498-511.

70. **Georgiev V.S**. (1994). Management of toxoplasmosis. *Drugs* **48**:179-188.

71. **Gomes C.M., Bandeiras T.M., and Teixeira M.** (2001). A new type-II NADH dehydrogenase from the archaeon *Acidianus ambivalens*: characterization and in vitro reconstitution of the respiratory chain. *J. Bioenerg. Biomembr.* **33:**1-8.

72. **Gross U., and Pohl F.** (1996). Influence of antimicrobial agents on replication and stage conversion of *Toxoplasma gondii*. *Curr. Top. Microbiol. Immunol.* **219:**235-245.

73. **Gross U., Bohne W., Soete M., and Dubremetz J.F.** (1996) Developmental differentiation between tachyzoites and bradyzoites of *Toxoplasma gondii*. *Parasitol Today* **12:**30-3.

74. **Gross U., Bormuth H., Gaissmaier C., Dietrich C., Krenn V., Bohne W., and Ferguson D.J.** (1995). Monoclonal rat antibodies directed against *Toxoplasma gondii* suitable for studying tachyzoite bradyzoite interconversion in vivo. *Clin Diagn Lab Immunol* **2:**542-8.

75. **Hagerhall C.** (1997). Succinate:quinone oxidoreductase. Variations on a conserved theme. *Biochem Biophys Acta* **1320:**107-41.

76. **Hammond D.J., Burchell J.R., and Pudney M.** (1985). Inhibition of pyrimidine biosynthesis de novo in *Plasmodium falciparum* by 2-(4-t-butylcyclohexyl)-3-hydroxy-1,4-naphthoquinone in vitro. *Mol Biochem Parasitol.* **14:**97-109.

77. **Hartl F.U., Pfanner N., Nicholson D.W. and Neupert W..** (1989) Mitochondrial protein import. *Biochim. Biophys. Acta* **988**:1–45.

78. **Higuchi R., Dollinger G., Walsh P.S., and Griffith R.** (1992). Simultaneous amplification and detection of specific DNA sequences. *Biotechnology.* **10:**413-7.

79. **Hirawake H., Taniwaki M., Tamura A., Kojima S., and Kita K.** (1997). Cytochrome b in human complex II (succinate-ubiquinone oxidoreductase): cDNA cloning of the components in liver mitochondria and chromosome assignment of the genes for the large (SDHC) and small (SDHD) subunits to 1q21 and 11q23. *Cytogenet Cell Genet*. **79:**132-8.

80. **Hirokawa T., Boon-Chieng S., and Mitaku S.** (1998). SOSUI: classification and secondary structure prediction system for membrane proteins. *Bioinformatics.* **14:**378-9.

81. **Hofmann K. and Stoffel W.** (1993). TMbase – A database of membrane spanning proteins segments. *Biol. Chem. Hoppe-Seyler* **374**:166.

82. **Howe D.K. and Sibley L.D.** (1995). *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. *J Infect Dis* **172:**1561-1566.

83. **Howitt C..A, Udall P.K., and Vermaas W.F.** (1999). Type 2 NADH dehydrogenases in the *cyanobacterium Synechocystis* sp. strain PCC 6803 are involved in regulation rather than respiration. *J Bacteriol.* **181:**3994-4003.

84. **Howitt C.A., Udall P.K. and Vermaas W.F.** (1999). Type 2 NADH dehydrogenases in the cyanobacterium *Synechocystis* sp. strain PCC 6803 are involved in regulation rather than respiration. *J. Bacteriol.* **181**: 3994–4003.

85. **Jaworowski A., Campbell H.D., Poulis M.I., and Young I.G.** (1981). Genetic identification and purification of the respiratory NADH dehydrogenase of *Escherichia coli*. *Biochemistry*. **20:**2041-7.

86. **Jensen R.E., Schmidt S., and Mark RJ.** (1992). Mutations in a 19-amino-acid hydrophobic region of the yeast cytochrome c1 presequence prevent sorting to the mitochondrial intermembrane space. *Mol Cell Biol*. **12:**4677-86.

87. **Jernigan J.A., Pearson R.D., Petri W.A., and Rogers M.D.** (1996). In vitro activity of atovaquone against *Leishmania chagasi* promastigotes. *Antimicrob Agents Chemother*. **40:** 1064.

88. **Jones J., Lopez A., and Wilson M.** (2003). Congenital toxoplasmosis. Am Fam Phys. **67**:2131–2138.

89. **Junge W., Pänke O., Cherepanov D.A., Gumbiowski K., Müller M., and Engelbrecht S.** (2001). Inter-subunit rotation and elastic power transmission in F0F1-ATPase. *FEBS Lett.* **504:**152**–**60.

90. **Kerscher S.J.** (2000) Diversity and origin of alternative NADH: ubiquinone oxidoreductases. *Biochim. Biophys. Acta* **1459:**274–283.

91. **Kerscher S.J., Okun J.G. and Brandt U.** (1999) A single external enzyme confers alternative NADH:ubiquinone oxidoreductase activity in *Yarrowia lipolytica*. *J. Cell Sci.* **112**:2347–2354.

92. **Kessl J.J., Ha K.H., Merritt A.K., Lange B.B., Hill P., Meunier B., Meshnick S.R., and Trumpower B.L.** (2005). Cytochrome b mutations that modify the ubiquinol-binding pocket of the cytochrome bc1 complex and confer anti-malarial drug resistance in *Saccharomyces cerevisiae*.*J Biol Chem*. **280:**17142-8.

93. **Kessl J.J., Ha K.H., Merritt A.K., Meshnick S.R., and Trumpower B.L.** (2006) Molecular basis of *Toxoplasma gondii* atovaquone resistance modeled in *Saccharomyces cerevisiae*. *Mol Biochem Parasitol*.**146:**255-8.

94. **Kessl J.J., Lange B.B., Merbitz-Zahradnik T., Zwicker K., Hill P., Meunier B., Palsdottir H., Hunte C., Meshnick S., and Trumpower B.L.** (2003). Molecular basis for atovaquone binding to the cytochrome bc1 complex. *J Biol Chem*. **278:**31312-8.

95. **Kim K., Soldati D. and Boothroyd J.C**. (1993). Gene replacement in *Toxoplasma gondii* with chloramphenicol acetyltransferase as selectable marker. *Science* **262:** 911-914.

96. **Kita K., Hirawake H., Miyadera H., Amino H., and Takeo S.** (2002). Role of complex II in anaerobic respiration of the parasite mitochondria from *Ascaris suum* and *Plasmodium falciparum*. *Biochim Biophys Acta*.**1553:**123-39.

97. **Kita K., Nihei C., and Tomitsuka E..** (2003). Parasite mitochondria as drug target: diversity and dynamic changes during the life cycle. *Curr med chem*. **10:**2535-48.

98. **Knoll L.J. and Boothroyd J.C.** (1998). Molecular biology's lesions about Toxoplasma development: Stage-specific homologs. *Parasitol. Today* **14:** 490-493.

99. **Knoll L.J., and Boothroyd J.C.** (1998). Isolation of developmentally regulated genes from *Toxoplasma gondii* by a gene trap with the positive and negative selectable marker hypoxanthine-xanthine-guanine phosphoribosyl-transferase. *Mol Cell Biol*. **18:**807-14.

100. **Knoll L.J., and Boothroyd J.C.** (1998). Isolation of developmentally regulated genes from *Toxoplasma gondii* by a gene trap with the positive and negative selectable marker hypoxanthine-xanthine-guanine phosphoribosyl-transferase. *Mol Cell Biol* **18:**807-14.

101. **Krogh A., Larsson B., von Heijne G., and Sonnhammer E.L.** (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol*. **305:**567-80.

102. **Laloi M**. (1999). Plant mitochondrial carriers: an overview. *Cell Mol Life Sci* **56**: 918–944.

103. **Lang B.F., Gray M.W., and Burger G.** (1999). Mitochondrial genome evolution and the origin of eukaryotes. *Annu Rev Genet*. **33:**351-97.

104. **Lekutis C., Ferguson D.J., and Boothroyd J.C.** (2000). *Toxoplasma gondii*: identification of a developmentally regulated family of genes related to SAG2. *Exp Parasitol*. **96:**89-96.

105. **Li R., Kenyon G.L., Cohen F.E., Chen X., Gong B., Dominguez J.N., Davidson E., Kurzban G., Miller R.E., Nuzum E.O., Rosenthal P.J., and McKerrow J.H.** (1995). In vitro antimalarial activity of chalcones and their derivatives. *J. Med. Chem*. **38:**5031-5037.

106. **Looareesuwan S., Chulay J.D., Canfield C.J., Hutchinson D.B.** (1999). Malarone (atovaquone and proguanil hydrochloride): a review of its clinical development for treatment of malaria. Malarone Clinical Trials Study Group. *Am J Trop Med Hyg***. 60:**533–541.

107. **Ludwig B., Bender E., Arnold S., Hüttemann M., Lee I., and Kadenbach B.** (2001). Cytochrome C oxidase and the regulation of oxidative phosphorylation**.** *Chembiochem.* **2:**392**–**403.

108. **Luft, B.J., and Remington J.S.** (1992). Toxoplasmic encephalitis in AIDS. *Clin. Infect. Dis*. **15:**211-222.

109. **Lutter R.** (1993). F1F0-ATP synthase from bovine heart mitochondria: development of the purification of a monodisperse oligomycin-sensitive ATPase. *Biochem. J.* **295:**799**–**806.

110. **Luttik M.A., Overkamp K.M., Kötter P., de Vries S., van Dijken P. and Pronk J.T.** (1998). The Saccharomyces cerevisiae NDE1 and NDE2 genes encode separate mitochondrial NADH dehydrogenases catalyzing the oxidation of cytosolic NADH. *J. Biol. Chem.* **273**:24529–24534.

111. **Lynfield R., and Guerina N.G.** (1997). Toxoplasmosis. Pediatr Rev **18:**75-83.

112. **Manger ID, Hehl A, Parmley S, Sibley LD, Marra M, Hillier L, Waterston R, and Boothroyd JC.** (1998). Expressed sequence tag analysis of the bradyzoite stage of *Toxoplasma gondii*: identification of developmentally regulated genes. *Infect Immun*. **66:** 1632-7.

113. **Marsden B.J., Shaw G.S. and Sykes B.D.** (1990) Calcium binding proteins. Elucidating the contributions to calcium affinity from an analysis of species variants and peptide fragments. *Biochem. Cell Biol.* **68**:587–601.

114. **Matrajt M., Donald R.G., Singh U., and Roos D.S.** (2002). Identification and characterization of differentiation mutants in the protozoan parasite *Toxoplasma gondii*. *Mol Microbiol*. **44:** 735-47.

115. **Matsushita K., Otofuji A., Iwahashi M., Toyama H. and Adachi O.** (2001). NADH dehydrogenase of *Corynebacterium glutamicum*. Purification of an NADH dehydrogenase II homolog able to oxidize NADPH, *FEMS Microbiol. Lett.* **204**: 271–276.

116. **McFadden D.C., Seeber F., and Boothroyd J.C.** (1997). Use of *Toxoplasma gondii* expressing beta-galactosidase for colorimetric assessment of drug activity in vitro. *Antimicrob Agents Chemother*. **41:**1849-53.

117. **McFadden D.C., Tomavo S., Berry E.A., and Boothroyd J.C.** (2000). Characterization of cytochrome b from *Toxoplasma gondii* and Q_o domain mutations as a mechanism of atovaquone resistance. *Mol. Biochem. Parasitol*. **108:**1-12.

118. **Meissner M., Brecht S., Bujard H., and Soldati D.** (2001). Modulation of myosin A expression by a newly established tetracycline repressor-based inducible system in *Toxoplasma gondii*. *Nucleic Acids Res*. **29:**E115.

119. **Meissner M., Krejany E., Gilson P.R., de Koning-Ward T.F., Soldati D., and Crabb B.S.** (2005). Tetracycline analogue-regulated transgene expression in *Plasmodium falciparum* blood stages using *Toxoplasma gondii* transactivators. *Proc Natl Acad Sci U S A*. **102:**2980-5.

120. **Meissner M., Schluter D., and Soldati D.** (2002). Role of *Toxoplasma gondii* myosin A in powering parasite gliding and host cell invasion. *Science.* **298:**837-40.

121. **Melo A.M, Bandeiras., T.M., and Teixeira M.**. (2004). New insights into type II NAD(P)H:quinone oxidoreductases. *Microbiol. Mol. Biol. Rev.* **68:**603-16.

122. **Melo A.M. Duarte M. and Videira A..** (1999). The 24-kDa iron-sulphur subunit of complex I is required for enzyme activity. *Biochim. Biophys. Acta* **1412**:282–287.

123. **Melo A.M., Duarte M., and Videira A.** (1999). Primary structure and characterisation of a 64 kDa NADH dehydrogenase from the inner membrane of *Neurospora crassa* mitochondria. *Biochim Biophys Acta.* **1412:**282-7.

124. **Messinar M., Niesman I.R., Mercier C., and Sibley L.D.** (1995). Stable DNA transformation of *Toxoplasma gondii* using phleomycin selection. *Gene.* **165:**213–217.

125. **Michalecka A.M., Svensson A.S., Johansson F.I., Agius S.C., Johanson U., Brennicke A., Binder S., and Rasmusson A.G.** (2003). Arabidopsis genes encoding mitochondrial type II NAD(P)H dehydrogenases have different evolutionary origin and show distinct responses to light. *Plant Physiol*. **133:**642-52.

126. **Mi-Ichi F., Miyadera H., Kobayashi T., Takamiya S., Waki S., Iwata S., Shibata S., and Kita K.** (2005). Parasite mitochondria as a target of chemotherapy: Inhibitory effect of Licochalcone A on the *Plasmodium falciparum* respiratory chain**.** *Ann NY Acad Sci* **1056:** 46-54.

127. **Mi-Ichi F., Takeo S., Takashima E., Kobayashi T., Kim H.S., Wataya Y., Matsuda A., Torrii M., Tsuboi T., and Kita K.** (2003). Unique properties of respiratory chain in *Plasmodium falciparum* mitochondria. *Adv Exp Med Biol*. **531:**117-33.

128. **Milpetz F., Argos P. and Persson B.** (1995). TMAP: a new email and WWW service for membrane-protein structural predictions. *Trends Biochem Sci*, **20:**204-205.

129. **Mital J., Meissner M., Soldati D., and Ward G.E.** (2005). Conditional expression of *Toxoplasma gondii* apical membrane antigen-1 (TgAMA1) demonstrates that TgAMA1 plays a critical role in host cell invasion. *Mol Biol Cell*. **16:**4341-9.

130. **Miyoshi H.** (1998). Structure-activity relationships of some complex I inhibitors. *Biochim. Biophys. Acta* **1364:**236–245.

131. **Mokranjac D., and Neupert W.** (2005). Protein import into mitochondria. *Biochem Soc Trans*. **33:**1019-23.

132. **Moller I.M.** (2001). Plant mitochondria and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive oxygen species. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52**: 561–591.

133. **Moller S., Croning M.D., and Apweiler R.** (2001). Evaluation of methods for the prediction of membrane spanning regions. *Bioinformatics.* **17:**646-53.

134. **Moreno S.N., and Zhong L.** (1996) Acidocalcisomes in *Toxoplasma gondii* tachyzoites. *Biochem. J.* **313:**655–659.

135. **Nakai K., and Kanehisa M.** (1992). A knowledge base for predicting protein localization sites in eukaryotic cells. *Genomics.* **14:**897-911.

136. **Nielsen H., Engelbrecht J., Brunak S., and von Heijne G.** (1997) A neural network method for identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Int J Neural Syst.* **8:**581-99.

137. **Nihei C., Fukai Y., and Kita K.** (2002). Trypanosome alternative oxidase as a target of chemotherapy, *Biochim Biophys Acta* **1587**:234–239.

138. **Ödberg-Ferragut C., Soete M., Engels A., Samyn B., Loyens A., van Beumen J., Camus D., and Dubremetz J.F.** (1996). Molecular cloning of the *Toxoplasma gondii* SAG4 gene encoding an 18 kDa specific surface protein. *Mol Biochem Parasitol*. **82:**237-44.

139. **Overkamp K.M., Bakker B.M., Kotter P., van Tuijl A., de Vries S., van Dijken J.P. and Pronk J.T.** (2000). In vivo analysis of the mechanisms for oxidation of cytosolic NADH by *Saccharomyces cerevisiae* mitochondria. *J. Bacteriol.* **182**:2823–2830.

140. **Parmley S.F., Yang S., Harth G., Sibley L.D., Sucharczuk A., and Remington J.S.** (1994). Molecular characterization of a 65-kilodalton *Toxoplasma gondii* antigen expressed abundantly in the matrix of tissue cysts. *Mol Biochem Parasitol* **66:**283-96.

141. **Pasquier C., Promponas V.J., Palaios G.A., Hamodrakas J.S., Hamodrakas S.J.** (1999). A novel method for predicting transmembrane segments in proteins based on a statistical analysis of the SwissProt database: the PRED-TMR algorithm. *Protein Eng.* **12:**381-5.

142. **Peters W.** Chemotherapy and drug resistance in malaria. 2nd ed. London, United Kingdom: Academic Press; 1987.

143. **Petersen E. and Dubey J.** - Biology of Toxoplasmosis. In: Toxoplasmosis: a comprehensive clinical guide. **Joynson, D.H. and Wreghitt, T.G.** 1. ed. Cambridge University Press, 2005. p. 1-43.

144. **Pfanner N. and Geissler A.** (2001). Versatility of the mitochondrial protein import machinery. *Nat Rev Mol Cell Biol* **2**: 339–349.

145. **Pfanner N., and Geissler A.** (2001). Versatility of the mitochondrial protein import machinery. *Nat Rev Mol Cell Biol*. **2:**339-49.

146. **Pfefferkorn E.R., Borotz S.E.** (1994). Comparison of mutants of *Toxoplasma gondii* selected for resistance to azithromycin, spiramycin, or clindamycin. *Antimicrob Agents Chemother*. **38:**31-7.

147. **Pfefferkorn E.R.**, **Borotz S.E.**, **Nothnagel R.F.** (1993). Mutants of Toxoplasma gondii resistant to atovaquone (566C80) or decoquinate. *J Parasitol.* **79:**559-64.

148. **Pfefferkorn L.C. and Pfefferkorn E.R.** (1980) *Toxoplasma gondii*: genetic recombination between drug resistant mutants. *Exp Parasitol* **50:** 305-316

149. **Priest J.W., and Hajduk S.L.** (2003). *Trypanosoma brucei* cytochrome c1 is imported into mitochondria along an unusual pathway. *J Biol Chem*. **278:**15084-94.

150. **Radke J.R., and White M.W.** (1998). A cell cycle model for the tachyzoite of *Toxoplasma gondii* using the *Herpes simplex* virus thymidine kinase. *Mol. Biochem. Parasitol.* **94:**237-247.

151. **Rasmusson A.G., Svensson A.S., Knoop V., Grohmann L., and Brennicke A.** (1999). Homologues of yeast and bacterial rotenone-insensitive NADH dehydrogenases in higher eukaryotes: two enzymes are present in potato mitochondria. *Plant J.* **20:**79-87.

152. **Rasmusson A.G., Svensson S., Knoop V., Grohmann L. and Brennicke A.** (1999). Homologues of yeast and bacterial rotenone-insensitive NADH dehydrogenases in higher eukaryotes: two enzymes are present in potato mitochondria. *Plant J.* **20**:79–87.

153. **Ravanel P., Creuzet S. and Tissut M.** (1990). Inhibitory effect of hydroxyflavones on the exogenous NADH dehydrogenase of plant mitochondrial inner membranes. *Phytochemistry* **29**:441–445.

154. **Remington J.S., McLeod R., and Desmonts G.** Toxoplasmosis. In: Infectious Disease of the Fetus and Newborn Infant. **Remington J.S., and Klein J.O.**, eds. Philadelphia: W.B. Saunders Company, **1995:** 140–267.

155. **Remington J.S., Mcleod R., Thulliez P., and Desmonts G.** - Toxoplasmosis. In: **Remington J.S. and Klein J.O.** Infectious diseases of the fetus and newborn infant. 5. ed. Philadelphia, W.B. Saunders, 2001. p. 205-346.

156. **Remington J.S., McLeod R., Thulliez P., and Desmonts G.** Toxoplasmosis. In: Infectious diseases of the fetus and newborn infant. **Remington J.S., and Klein J.O.**, 5th ed. Philadelphia: Saunders, **2001:**205-346.

157. **Renold C., Sugar A., and Chave J.P.** (1992). Toxoplasma encephalitis in patients with the acquired immunodeficiency syndrome. *Medicine* **71**: 224–39.

158. **Rich P. and Fisher N.** (1999). Generic features of quinone-binding sites. *Biochem. Soc. Trans.* **27**:561–565.

159. **Roos D.S., Sullivan W.J. Striepen B., Bohne W., and Donald R.G**. (1997). Tagging genes and trapping promoters in *Toxoplasma gondii* by insertional mutagenesis. *Biochem Methods* **13:**112-22.

160. **Sabbert D., Engelbrecht S., and Junge W.** (1996). Intersubunit rotation in active F-ATPase. *Nature.* **381:**623-5.

161. **Sato S., Rangachari K., and Wilson R.J.** (2003). Targeting GFP to the malarial mitochondrion. *Mol Biochem Parasitol*.**130:**155-8.

162. **Sibley L.D. and Boothroyd J.C.** (1992). Virulent strains of *Toxoplasma gondii* comprise a single clonal lineage. *Nature* **359:** 82-85.

163. **Sibley L.D., Messinar M., and Niesman I.R.** (1994). Stable DNA transformation in the obligate intracellular parasite *Toxoplasma gondii* by complementation of tryptophan auxotrophy. *Proc. Natl. Acad. Sci. USA* **91:**5508-5512.

164. **Singh U., Brewer J.L., and Boothroyd J.C.** (2002). Genetic analysis of tachyzoite to bradyzoite differentiation mutants in *Toxoplasma gondii* reveals a hierarchy of gene induction. *Mol Microbiol*. **44:** 721-33.

165. **Small W.C., and McAlister-Henn L.** (1998). Identification of a cytosolically directed NADH dehydrogenase in mitochondria of *Saccharomyces cerevisiae*. *J Bacteriol.* **180:**4051-5.

166. **Smith J.E.** (1995). A ubiquitous intracellular parasite: the cellular biology of *Toxoplasma gondii*. *Int. J. Parasitol.* **25:**1301-1309.

167. **Smith J.L.** (1993). Documented outbreaks of Toxoplasmosis: Transmission of *Toxoplasma gondii* to humans. *J Food Prot* **56**: 630–9.

168. **Soete M., Camus D., and Dubremetz J.F.** (1994). Experimental induction of bradyzoite-specific antigen expression and cyst formation by the RH strain of *Toxoplasma gondii* in vitro. *Exp Parasitol*. **78:**361-70.

169. **Soete M., Fortier B., Camus D. and Dubremetz J.F.** (1993). *Toxoplasma gondii*: kinetics of bradyzoite-tachyzoite interconversion in vitro. *Exp. Parasito*l., **76:** 259-264.

170. **Soldati D. and Boothroyd J.C.** (1993). Transient transfection and expression in the obligate intracellular parasite *Toxoplasma gondii*. *Science* **260:** 349–352.

171. **Soldati D., Kim K., Kampmeier J., Dubremetz J.F., and Boothroyd J.C.** (1995). Complementation of a *Toxoplasma gondii* ROP1 knock-out mutant using phleomycin selection. *Mol. Biochem. Parasitol.* **74:** 87-97.

172. **Soldati, D. and Boothroyd, J.C.** (1995). A selector of transcription initiation in the protozoan parasite *Toxoplasma gondii*. *Mol Cell Biol* **15:** 87-93.

173. **Srivastava I.K., Rottenberg H., and Vaidya A.B.** (1997). Atovaquone, a broad spectrum antiparasitic drug, collapses mitochondrial membrane potential in a malarial parasite. *J. Biol. Chem.* **272:**3961-6.

174. **Suraveratum N., Krungkrai S.R., Leangaramgul P., Prapunwattana P., and Krungkrai J.** (2000). Purification and characterization of *Plasmodium falciparum* succinate dehydrogenase. *Mol Biochem Parasitol*. **105:**215-22.

175. **Svensson A.A. and Rasmusson A.G.** (2001) Light-dependent gene expression for proteins in the respiratory chain of potato leaves. *Plant J.* **28**:73–82.

176. **Svensson A.S., and Rasmusson A.G.** (2001). Light-dependent gene expression for proteins in the respiratory chain of potato leaves. *Plant J.* **28:**73-82.

177. **Svensson A.S., Johansson F.I., Moller I.M. and Rasmusson A.G.** (2002). Cold stress decreases the capacity for respiratory NADH oxidation in potato leaves. *FEBS Lett.* **517**:79–82.

178. **Swofford D.L. and Begle D.P.** (1993). PAUP: Phylogenetic analysis using parsimony. Version 3.1.1. User's manual. Laboratory of Molecular Systematics, Smithsonian Institution, Washington, D.C.

179. **Syafruddin D., Siregar J.E., and Marzuki S.** (1999) Mutations in the Cytochrome *b* gene of *Plasmodium berghei* conferring resistance to atovaquone. *Mol. Biochem. Parasitol.* **104**:185–194.

180. **Takashima E., Takamiya S., Takeo S., Mi-ichi F., Amino H., and Kita K.** (2001). Isolation of mitochondria from *Plasmodium falciparum* showing dihydroorotate dependent respiration. *Parasitol Int.* **50:**273-8.

181. **Takeo S., Kokaze A., Ng C.S., Mizuchi D., Watanabe J., Tanabe K., Kojima S., and Kita K.** (2000) Succinate dehydrogenase in *Plasmodium falciparum* mitochondria: Molecular characterization of the SDHA and SDHB genes for the catalytic subunits, the flavoprotein (Fp) and iron-sulfur (Ip) subunits. *Mol. Biochem. Parasitol*. **107:**191-205.

182. **Tanabe K. and Murakami K.** (1984). Reduction in the mitochondrial membrane potential of *Toxoplasma gondii* after invasion of host cells.*J Cell Sci.* **70:**73-81.

183. **Thompson J.D., Higgins D.G., and Gibson T.J.** (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22:**4673-80.

184. **Tomavo S.** (2001). The differential expression of multiple isoenzymes forms during stage conversion of *Toxoplasma gondii*: an adaptive developmental strategy. *Int J Parasitol*. **31**:1023–1031.

185. **Tomavo S., Boothroyd J.C.** (1995). Interconnection between organellar functions, development and drug resistance in the protozoan parasite, *Toxoplasma gondii. Int J Parasitol*. **25:** 1293-9.

186. **Tonkin CJ, van Dooren GG, Spurck TP, Struck NS, Good RT, Handman E, Cowman AF, and McFadden GI.** (2004). Localization of organellar proteins in *Plasmodium falciparum* using a novel set of transfection vectors and a new immunofluorescence fixation method. *Mol Biochem Parasitol*. **137:**13-21.

187. **Tusnady G.E., and Simon I.** (2001). The HMMTOP transmembrane topology prediction server. *Bioinformatics.* **17:**849-50.

188. **Uyemura S.A., Luo S., Moreno S.N., and Docampo R.** (2000). Oxidative phosphorylation, Ca(2+) transport, and fatty acid-induced uncoupling in malaria parasites mitochondria. *J Biol Chem.* **275:**9709-15.

189. **Uyemura S.A., Luo S., Vieira M., Moreno S.N., and Docampo R.** (2004). Oxidative phosphorylation and rotenone-insensitive malate- and NADH-quinone oxidoreductases in *Plasmodium yoelii yoelii* mitochondria in situ. *J Biol Chem*. **279:**385-93.

190. **Uyemura S.A., Luo S., Vieira M., Moreno S.N., and Docampo R.** (2004). Oxidative phosphorylation and rotenone-insensitive malate- and NADH-quinone oxidoreductases in *Plasmodium yoelii yoelii* mitochondria in situ. *J Biol Chem*. **279:**385-93.

191. **Vaidya A.B. (**2004). Mitochondrial and plastid functions as antimalarial drug targets. *Curr. Drug Targets Infect. Disord.* **4:**11-23.

192. **van Dooren G.G., Stimmler L.M., and McFadden G.I.** (2006). Metabolic maps and functions of the Plasmodium mitochondrion. *FEMS Microbiol Rev*. **30:**596-630.

193. **Vercesi A.E., Rodrigues C.O., Uyemura S.A., Zhong L., and Moreno S.N.** (1998) Respiration and oxidative phosphorylation in the apicomplexan parasite *Toxoplasma gondii*. *J Biol Chem*. **273:**31040-7.

194. **Videira A. and Duarte M. (2002)**. From NADH to ubiquinone in *Neurospora* mitochondria. *Biochim. Biophys. Acta* **1555**:187–191.

195. **von Heijne G., Steppuhn J., and Herrmann R.G.** (1989). Domain structure of mitochondrial and chloroplast targeting peptides. *Eur J Biochem*. **180:**535-45.

196. **Voos W., Martin H., Krimmer T., and Pfanner N.** (1999). Mechanisms of protein translocation into mitochondria. *Biochim Biophys Acta*. **1422:**235-54.

197. **Weinstein E.A., Yano T., Li L.S., Avarbock D., Avarbock A., Helm D., McColm, Duncan K., Lonsdale J.T., and Rubin H. (**2005). Inhibitors of type II NADH:menaquinone oxidoreductase represent a class of antitubercular drugs. *Proc. Natl. Acad. Sci. U S A*. **102:**4548-53.

198. **Wierenga R.K., de Maeyer M.C. and Hol W.G.** (1985). The interaction of pyrophosphate moieties with α-helices in dinucleotide-binding proteins. *Biochemistry* **24**:1346–1357.

199. **Wilson R.J., and Williamson D.H.** (1997). Extrachromosomal DNA in the Apicomplexa. *Microbiol. Mol. Biol. Rev*. **61:**1-16.

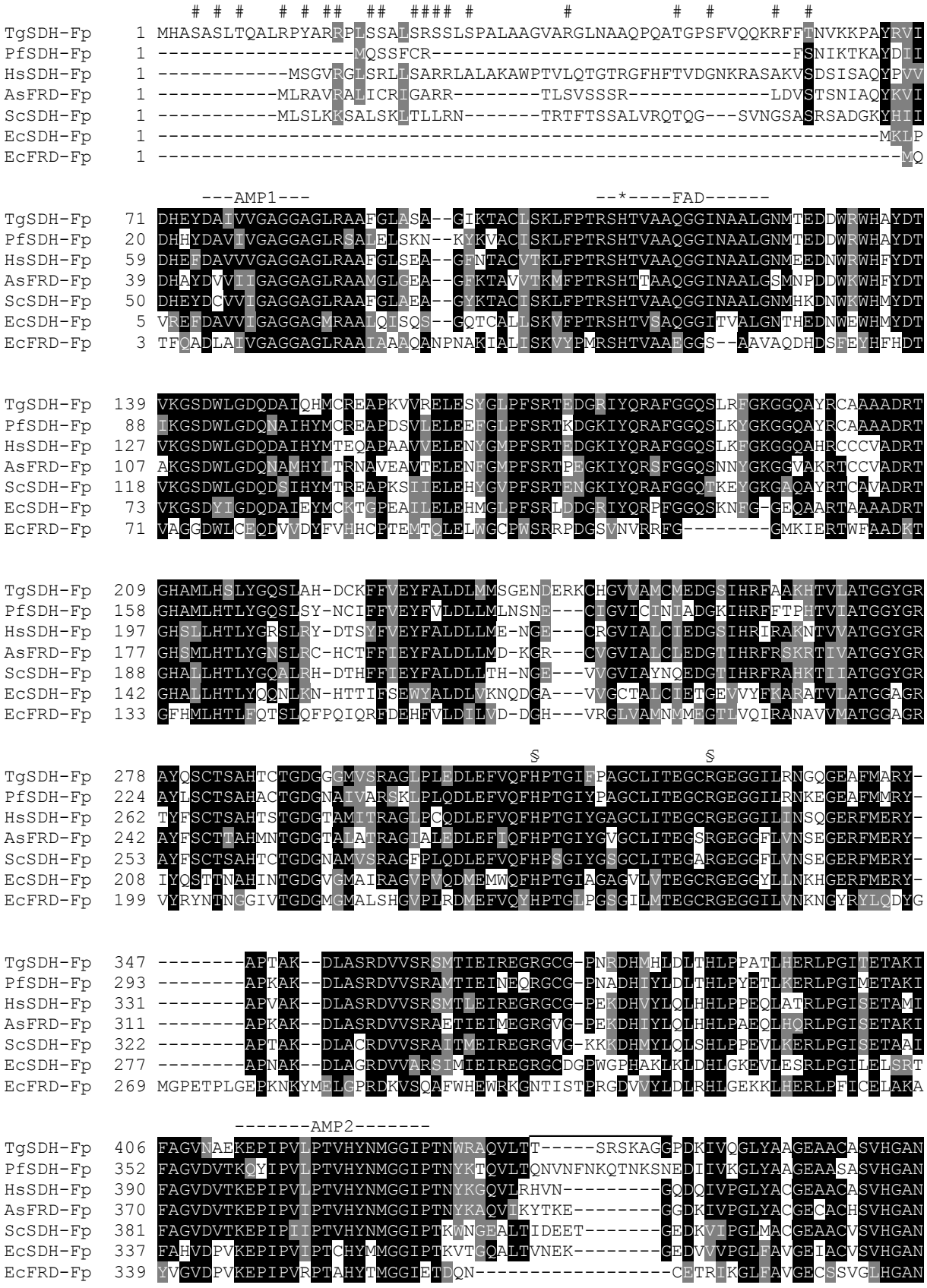
200. **Wong S.Y., and Remington J.S.** (1993). Biology of *Toxoplasma gondii*. *AIDS* **7:**299-316.

201. **Yang S., and Parmley S.F.** (1995). A bradyzoite-specifically expressed gene of *T. gondii* encodes a polypeptide homologous to lactate dehydrogenase. *Mol Biochem Parasitol.* **73:**291-4.

202. **Yang S., and Parmley S.F.** (1997). *Toxoplasma gondii* expresses two distinct lactate dehydrogenase homologous genes during its life cycle in intermediate hosts. *Gene.* **184:**1-12.

203. **Yoshikawa S., Shinzawa-Itoh K., and Tsukihara T.** (2000). X-ray structure and the reaction mechanism of bovine heart cytochrome c oxidase. *J. Inorg. Biochem.* **82:**1–7.

APPENDEX


TgSDH-Fp
PfSDH-Fp
HsSDH-Fp
AsFRD-Fp
ScSDH-Fp
EcSDH-Fp
EcFRD-Fp
---AMP1---
--*----FAD------
§
§
-------AMP2-------

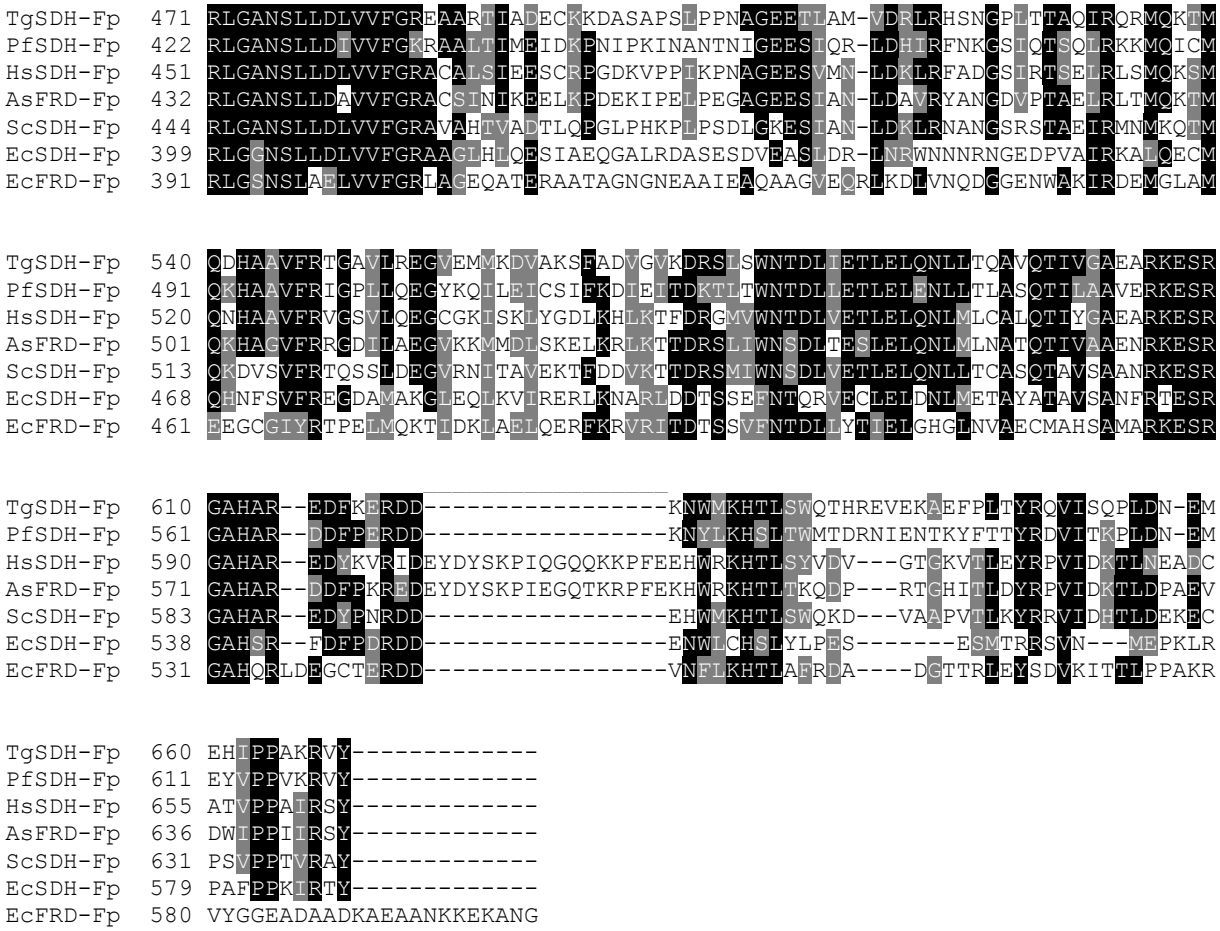


Figure 6.1: **Alignment of the amino acid sequences of complex II Fp subunits from various species.** The top line shows the sequence of *T. gondii* deduced from *SDH-Fp* gene (DQ228958).Dashes indicate gaps introduced to maximize similarity within the alignment. Numbers indicate the position of amino acids from the C-terminus for each organism. Basic and hydroxylated amino acids in the N-terminal regions are indicated by #. § Indicates conserved histidine (His312 in *T. gondii*) and arginine (Arg328) residues in the active site. Three segments interacting with FAD (AMP-1, FAD, and AMP-2) are overlined with dashed lines. * Indicates FAD-binding histidine (His111). Specific insertions or deletion sites are overlined with continuous lines. FRD: fumarate reductase, SDH: succinate dehydrogenase, Fp: flavoprotein. Tg, *T. gondii* (ABB17193.1), Pf*, P. falciparum* (BAA13119.1); Hs, *H. sapiens* (P31040), As; *A. suum* (BAA21636.1); Sc, *S. cerevisiae* (S34793_S), Ec, *E. coli* (SDH, BAA35390.1; FRD, AAC77114.1).

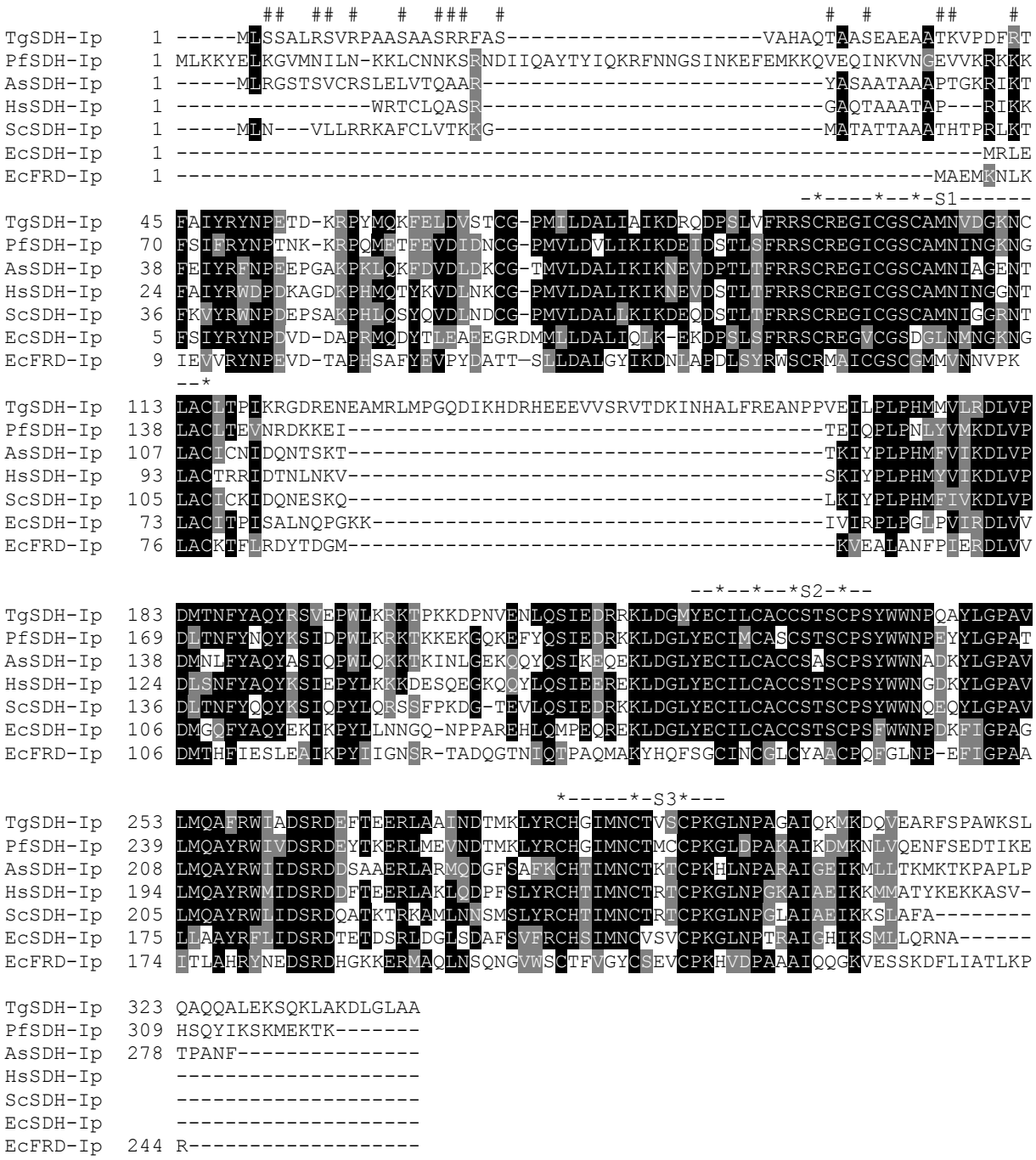
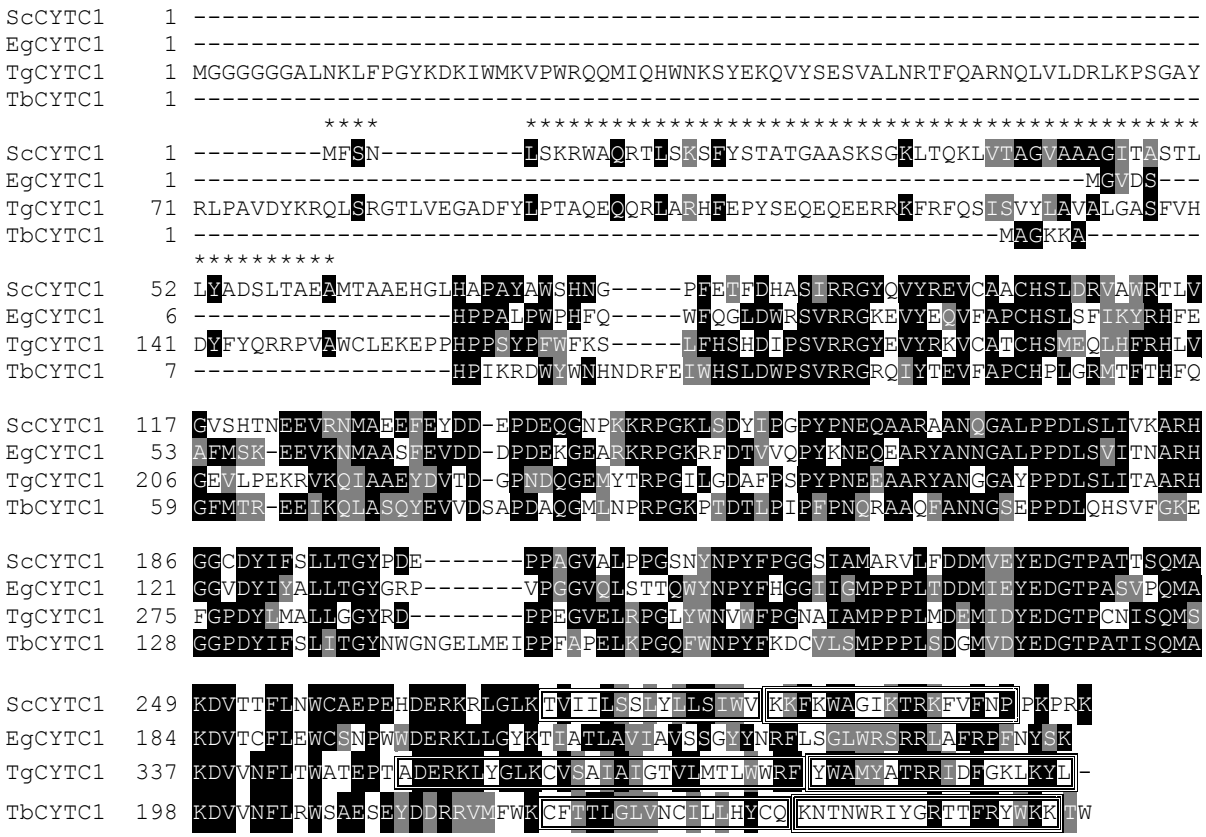


Figure 6.2: Alignment of the amino acid sequences of complex II Ip subunits from various species. The top line shows the sequence of *T. gondii* deduced from *SDH-Ip* gene (TgTwinScan_1272). Dashes indicate gaps introduced to maximize similarity within the alignment. Numbers indicate the position of amino acids from the C-terminus for each organism. Basic and hydroxylated amino acids in the N-terminal regions are indicated by #. * Indicates conserved cysteine residues in three clusters (S1, S2, and S3; overlined) comprising the iron sulfur centers (Cys95, 100, 103, and 115 in S2; Cys227, 230, 232, and 237 in S2; and Cys284, 290 and 294 in S3 in *T. gondii*). FRD: fumarate reductase, SDH: succinate dehydrogenase, Fp: flavoprotein. Tg, *T. gondii* (TgTwinScan_1272), Pf, *P. falciparum* (AAN36215); Hs, *H. sapiens* (BAA01089.1), As; *A. suum* (BAA23716.1); Sc, *S. cerevisiae* (AAA35021.1), Ec, *E. coli* (SDH, AAG55048.1; FRD, AAA23438.1).

A.



B.

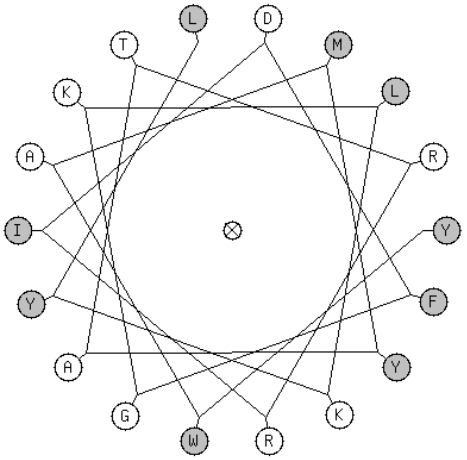
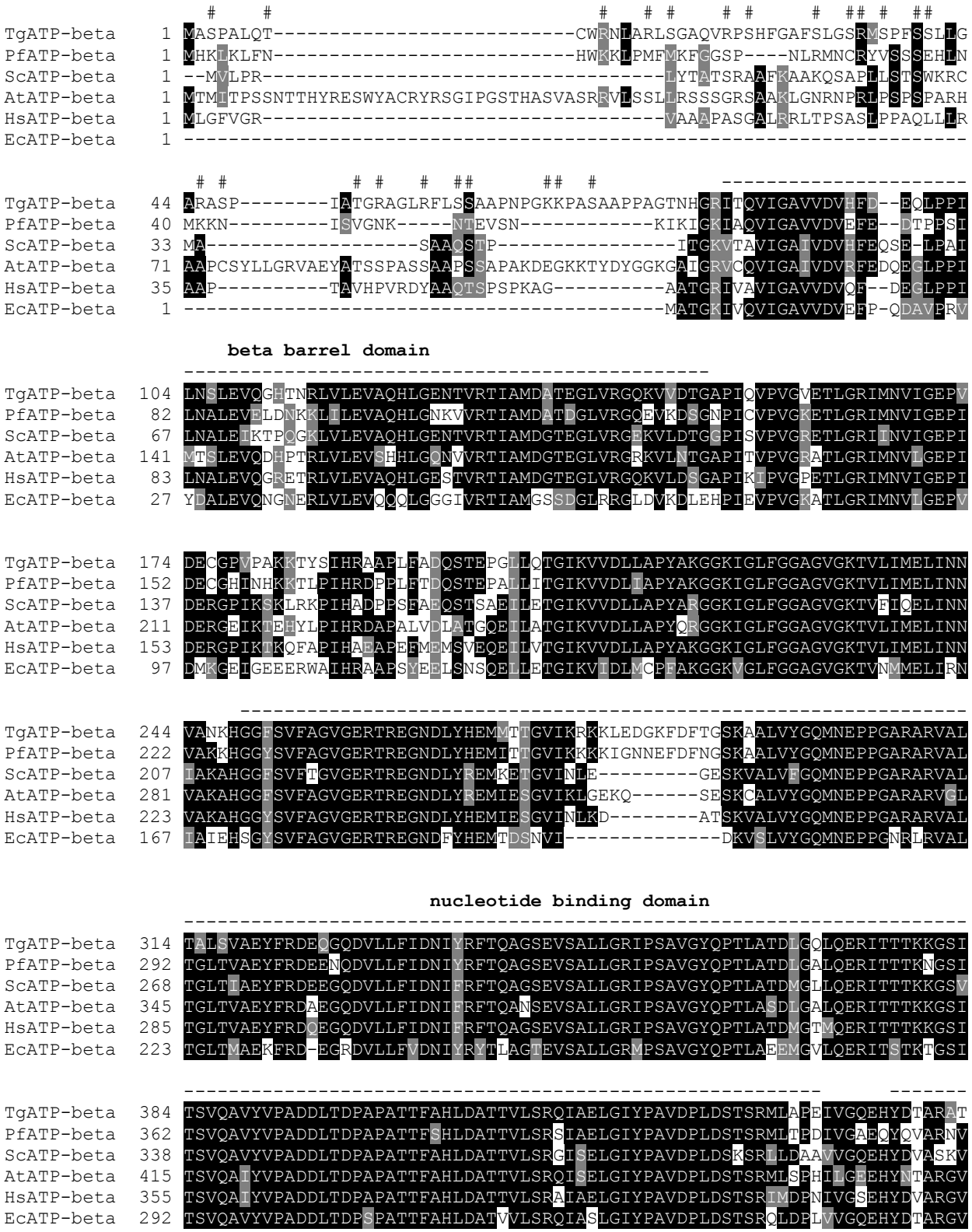


Figure 6.3: Alignment of the amino acid sequences of cytochrome c1 subunits from various species. (A) the translated amino acid sequence of *T. gondii* (*Tg*) cytochrome c_1 from DNA sequence DQ228959 was aligned with the *S. cerevisiae* (*Sc*) cytochrome c_1 protein sequence (CAA25375), *T. brucei* (*Tb*) (AAZ12956) and with the *E. gracilis* (*Eg.*) protein sequence (JQ0021) by using the ClustalW program. Amino acids in T. gondii, yeast, and trypanosome sequences that are predicted to span the lipid bilayer are indicated by double line boxes, and sequences that may form an amphipathic α-helix are indicated in triple line boxes. *B*, α-helical plot of residues 379-395 of *T. gondii* cytochrome c_1. Positively charged residues are R, T and hydrophobic residues are grey circled.


beta barrel domain
nucleotide binding domain

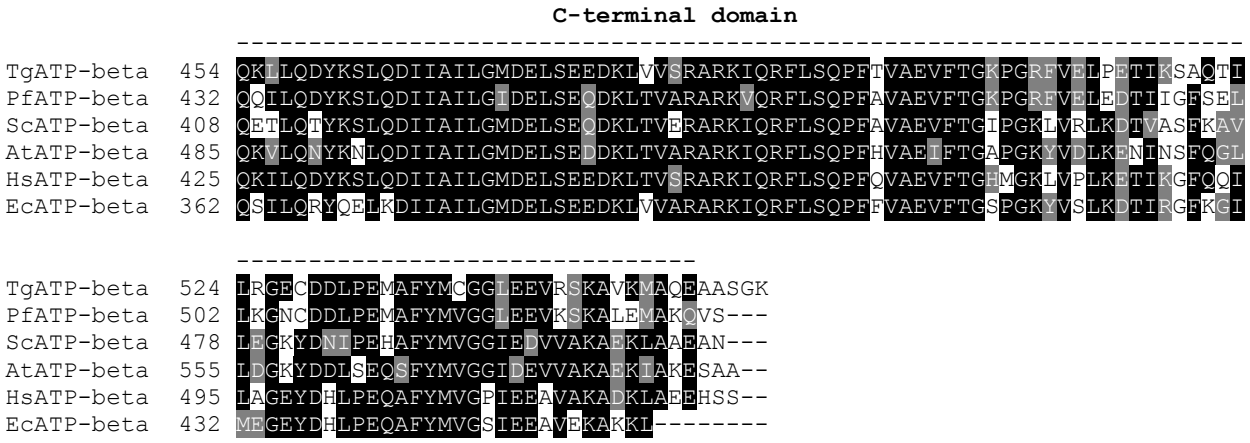


Figure 6.4: Alignment of the amino acid sequences of ATP synthase, β subunits from various species. The translated amino acid sequence of *T. gondii* (*Tg*) ATP synthase, β subunit from DNA sequence DQ228959 was aligned with the *S. cerevisiae* (*Sc*) ATP synthase, β subunit protein sequence (NP_012655.1), *P. falciparum* (*Pf*) (NP_701707.1), *H. sapiens* (P06576), *E. coli* (AAA24737.1), and with the *A. thaliana* (*At.*) protein sequence (CAC81058.1) by using the ClustalW program.

A.TgNDH2-I gene

```
   1 ATGGCAGGGC AGTGGCTGCG GCTGCTGGCG GGGGCCTCTG TGCCTATGCT TAGCCTGCCG
  61 GCGCGCTGCG ACTCTCCAGC GTCTCCGTCG TCTCCTTCGT CTCCGAAAGA GCGCGTGTCT
 121 GCTCTGCTGG CTCAGCCCCC GCGTCCGTTC TCCGCCTTCT CCCCTGTCTC CACCTGGATG
 181 TCCACCAAGT GGACCAGCTT TCGCCTCCGA ACAGGCCTCC TCTCTCCCGC GGCGGTCGCC
 241 GCCTCTGCGG TCGCGTCTGC GTCTCCGGCT GCTCGCGAGG CGCCTGCGAG GAGACAGAAG
 301 GTGGTGGTTG TGGGGTCAGG CTGGGCAGCT GTCTCCTTCC TCGCGGACCT GGACATGACT
 361 CGCTACGAGC CTGTCGTCAT CTCTCCGCGC GACTACTTCA CCTTCACACC GCTGCTGCCG
 421 TCCGTCTGTG TGGGGACTTT GCCTGCGAGT GCATGCATGA CGGGTGTGAG GGAGTTGCTG
 481 GTTCGCGGCG GAGTGCCCTG CGGAAGTTTC TACGAAGGCC GCGTCGCGGA GATCTGCCCT
 541 ACAGAGAAGA AAGTTCGCTG TCAGTCGACT CACGGGAAGG CGCAGGACGC GCGCGAATGG
 601 GAGGAGAGCT ACGACTACCT CGTGGTCGCC GCGGGTGCCG ATGTGAACAC TTTTGGAGTC
 661 CCTGGAGTGA AGGAAAACGC GTTTTTCGTC AAGGAGTTGG AGGACGCAAG ACGCCTGCGA
 721 AGCGCGCTGT TCGACGTCAT CGAGACAGCT TCTGTCCCCG GCGTCTCCGA GGAAGAGAAG
 781 AAGAAACTTC TTCACTTCGT TGTCGTTGGA GCGGGTCCGA CAGGCGTGGA AGTCGCCGCA
 841 GAGATCGACG ACTTTTTCCA GGCCGAGGGC GCGACTCACT TCCCGCAGCT GCGGCCTCTC
 901 GTCCGGATCA CCGTGGTGGA GATGCTCCCG ACAGTCCTCG CAGCCTACAA CAACGACGTC
 961 CAGGCTTTCG CAAAACGCCT TCTGGAAGAG AATCCACGCG TCGACCTTTG CCTCCAGTCG
1021 CAGGTCGTCG GAGTCGGTCC AGACTCGGTC AAGGTTCGCA CAAAGCGCGC CGACGGCCAG
1081 GTGGAAGAAA AGGAGATGCC CTGCGGCCTC CTCGTGTGGG CGTCGGGGAT CAAGAGTCCA
1141 AAGGTCTGTC TCGACCTCGC CAGAAAGACC GCCGAGCTGC GCGAGGCTCA GCAGCAGTCG
1201 CCCGTAATCC TCGTCGACCA GCAAATGAAG GTGCGAGGCT GCGAAGGCGT CTACGCGCTC
1261 GGAGACTGCT GCAGACTCTC GCCGCCTCCT CTCGTCCAAC ATGCAGACAC CCTGTACGAA
1321 GCCGCCACGG CGAACGGAGC AGCCAgtacg tacagcacca gaagaagcaa gggagaagaa
1381 gaaaacaaga agagggagaa gagagagaga agaagagaga gagaagagaa gagagagaag
1441 aggagggaga taagaagaga gagaagagga gggagagaag agagagagaa gagaagagag
1501 agaagaagag agagagaaga ggagggagag aagagagaga gaagagaaga gagagaagaa
1561 gagagagaga agaggagaga gagaagagaa gagagagaag aagagagaga gaagaggaga
1621 gagagaagaa gagagagaga agagaagaga gagaagagga gggacaggaa gaccgagaag
1681 aagaaaagga agaagaacgc gaacgaagag caagggaaag acagcgcagg cagagagaac
1741 ggtgattgag atttgacctg ccagcgaatt gcgagctgcg cgcttcgtct ttcagGCACT
1801 GACTGGCTGG AGAGAGAGGC GCCGAAACTG AGCACTGTCT TCCCTCAACT GGCCAGCAGC
1861 AAATACGACT TTTCCCAGAA GCCGCGTCAA ACGCAAATGA CAAAGGAGCA GTTCGTGAAA
1921 CTTCTTGCGg tgagtggagc gcatttgctt cgttcctttc cttcactggg aagcggaatt
1981 ctcgttctct ttgagcaggc gctcgtgtgt aggttcctct cttctgactt ccccttctc
2041 cccgccgata gaggagcgtt gctgtagcgg gcgtcgtaga tcttccgcgc gcattgcatc
2101 ctcagtcctg ggtgcctgtc ttctctgcat ggtgtgtgtc tgcgagcttc cactcctcgg
```

```
2161 cgttcagagg agaccaagtg aggcaggcat ctggcagcgg tccgttgtct ttccgcctcg
2221 cgcttcttcg tctgtgcctc tcctgtcttc ccgcgtctat ccatctgctt cgcgtatgcg
2281 tttaagagct gctttcactc tctgtgtggc tgttcccccc ctcggggcgg cgcggtgtgg
2341 actcttcttc gctgcgggtg tctccgcccc cactgcgact cacaacgtct ttgcatctgc
2401 acggctgctc tgcagGACAT CGACGCGGCG TACCGCGCTC CGGCGCCGAC GGCGCAGAAC
2461 GCGAAGCAGG CAGGTCGCTA CTTGGCGCAG ACGTTCAACG CGTTTCCGAG TGTCGAGGAG
2521 AAGCGCCGAG CGCCTGCGTT TGTGAACCAG ACGCGCGGGG CGCTGGTCTA CCTCGGGCAT
2581 GGGCAAGCAG CTGCAGACAT CGAAGGATGG AGGACGTTCT TGGGCGGCGC GGCGACGCTG
2641 CTGCTGTGGA AGGCGGCGTA CCTCCAGATG CAGTTGACTT TGCACAACGC CGTCGCGTGC
2701 CTGGGCGGGT GGCTGCGAAC CAGTCTGGTC GGCAGGGCCG TCTGCCGAGA GCACCTCGAC
2761 GGAGAGACTG TGTACGGCGA CCGACGCAAG TGA
```

B.TgNDH2-I ORF(accession #: DQ211932)

```
   1 ATGGCAGGGC AGTGGCTGCG GCTGCTGGCG GGGGCCTCTG TGCCTATGCT TAGCCTGCCG
  61 GCGCGCTGCG ACTCTCCAGC GTCTCCGTCG TCTCCTTCGT CTCCGAAAGA GCGCGTGTCT
 121 GCTCTGCTGG CTCAGCCCCC GCGTCCGTTC TCCGCCTTCT CCCCTGTCTC CACCTGGATG
 181 TCCACCAAGT GGACCAGCTT TCGCCTCCGA ACAGGCCTCC TCTCTCCCGC GGCGGTCGCC
 241 GCCTCTGCGG TCGCGTCTGC GTCTCCGGCT GCTCGCGAGG CGCCTGCGAG GAGACAGAAG
 301 GTGGTGGTTG TGGGGTCAGG CTGGGCAGCT GTCTCCTTCC TCGCGGACCT GGACATGACT
 361 CGCTACGAGC CTGTCGTCAT CTCTCCGCGC GACTACTTCA CCTTCACACC GCTGCTGCCG
 421 TCCGTCTGTG TGGGGACTTT GCCTGCGAGT GCATGCATGA CGGGTGTGAG GGAGTTGCTG
 481 GTTCGCGGCG GAGTGCCCTG CGGAAGTTTC TACGAAGGCC GCGTCGCGGA GATCTGCCCT
 541 ACAGAGAAGA AAGTTCGCTG TCAGTCGACT CACGGGAAGG CGCAGGACGC GCGCGAATGG
 601 GAGGAGAGCT ACGACTACCT CGTGGTCGCC GCGGGTGCCG ATGTGAACAC TTTTGGAGTC
 661 CCTGGAGTGA AGGAAAACGC GTTTTTCGTC AAGGAGTTGG AGGACGCAAG ACGCCTGCGA
 721 AGCGCGCTGT TCGACGTCAT CGAGACAGCT TCTGTCCCCG GCGTCTCCGA GGAAGAGAAG
 781 AAGAAACTTC TTCACTTCGT TGTCGTTGGA GCGGGTCCGA CAGGCGTGGA AGTCGCCGCA
 841 GAGATCGACG ACTTTTTCCA GGCCGAGGGC GCGACTCACT TCCCGCAGCT GCGGCCTCTC
 901 GTCCGGATCA CCGTGGTGGA GATGCTCCCG ACAGTCCTCG CAGCCTACAA CAACGACGTC
 961 CAGGCTTTCG CAAAACGCCT TCTGGAAGAG AATCCACGCG TCGACCTTTG CCTCCAGTCG
1021 CAGGTCGTCG GAGTCGGTCC AGACTCGGTC AAGGTTCGCA CAAAGCGCGC CGACGGCCAG
1081 GTGGAAGAAA AGGAGATGCC CTGCGGCCTC CTCGTGTGGG CGTCGGGGAT CAAGAGTCCA
1141 AAGGTCTGTC TCGACCTCGC CAGAAAGACC GCCGAGCTGC GCGAGGCTCA GCAGCAGTCG
1201 CCCGTAATCC TCGTCGACCA GCAAATGAAG GTGCGAGGCT GCGAAGGCGT CTACGCGCTC
1261 GGAGACTGCT GCAGACTCTC GCCGCCTCCT CTCGTCCAAC ATGCAGACAC CCTGTACGAA
1321 GCCGCCACGG CGAACGGAGC AGCCAGCACT GACTGGCTGG AGAGAGAGGC GCCGAAACTG
1381 AGCACTGTCT TCCCTCAACT GGCCAGCAGC AAATACGACT TTTCCCAGAA GCCGCGTCAA
1441 ACGCAAATGA CAAAGGAGCA GTTCGTGAAA CTTCTTGCGG ACATCGACGC GGCGTACCGC
1501 GCTCCGGCGC CGACGGCGCA GAACGCGAAG CAGGCAGGTC GCTACTTGGC GCAGACGTTC
1561 AACGCGTTTC CGAGTGTCGA GGAGAAGCGC CGAGCGCCTG CGTTTGTGAA CCAGACGCGC
1621 GGGGCGCTGG TCTACCTCGG GCATGGGCAA GCAGCTGCAG ACATCGAAGG ATGGAGGACG
1681 TTCTTGGGCG GCGCGGCGAC GCTGCTGCTG TGGAAGGCGG CGTACCTCCA GATGCAGTTG
1741 ACTTTGCACA ACGCCGTCGC GTGCCTGGGC GGGTGGCTGC GAACCAGTCT GGTCGGCAGG
1801 GCCGTCTGCC GAGAGCACCT CGACGGAGAG ACTGTGTACG GCGACCGACG CAAGTGA
```

C. TgNDH2-I Protein(accession #:ABA44355)

```
  1 MAGQWLRLLA GASVPMLSLP ARCDSPASPS SPSSPKERVS ALLAQPPRPF SAFSPVSTWM
 61 STKWTSFRLR TGLLSPAAVA ASAVASASPA AREAPARRQK VVVVGSGWAA VSFLADLDMT
121 RYEPVVISPR DYFTFTPLLP SVCVGTLPAS ACMTGVRELL VRGGVPCGSF YEGRVAEICP
181 TEKKVRCQST HGKAQDAREW EESYDYLVVA AGADVNTFGV PGVKENAFFV KELEDARRLR
241 SALFDVIETA SVPGVSEEEK KKLLHFVVVG AGPTGVEVAA EIDDFFQAEG ATHFPQLRPL
301 VRITVVEMLP TVLAAYNNDV QAFAKRLLEE NPRVDLCLQS QVVGVGPDSV KVRTKRADGQ
361 VEEKEMPCGL LVWASGIKSP KVCLDLARKT AELREAQQQS PVILVDQQMK VRGCEGVYAL
421 GDCCRLSPPP LVQHADTLYE AATANGAAST DWLEREAPKL STVFPQLASS KYDFSQKPRQ
481 TQMTKEQFVK LLADIDAAYR APAPTAQNAK QAGRYLAQTF NAFPSVEEKR RAPAFVNQTR
541 GALVYLGHGQ AAADIEGWRT FLGGAATLLL WKAAYLQMQL TLHNAVACLG GWLRTSLVGR
601 AVCREHLDGE TVYGDRRK.
```

A. TgNDH2-II GENE

```
   1 ATGGCGATGC TCTTCTCCAG CTCAGCAGCA GGCAGCCTGC CCTCGCGCAG AAGCCGATCC
  61 GTCGCTGCGA AGTTGGCGCC CTTCGCTTCT CCAATTTTCT CGTCTCCACT CTTGAAGCAA
 121 GGCGCAGATA CTGCCTTTGG TCTGCGCAGC CCCACACCTG GGCTGTCCGT ACACCCGAGA
 181 TTTGCTTCCA GAGGACCCTT GACACGGAGC GAGGAAGCCG GAAAGGCACC CTTCGGTTTC
 241 TCTACCGCGA GAGGCGAGCA GACGGCCACG GAGACAAATG CGCCGAGATT CGGGACGAGC
 301 CTTTGCTCTT CTTTTCCGTT GTCGGCGACT CAGAAAAGCC ACGGTTCGCG AGAGAAGGGC
 361 CTCACAAACG AACGGGCCTT CTCGACCTTC TCTTTTCAAG GCTTTATGAA AAATGTCAAG
 421 TCGAGAAACG CAAAGCCATA CACTGGACCC CCGCAGAAAG TCGTTGTCCT CGGAACCGGC
 481 TGGGCATCTG TCAATTTCTT CCGACACCTC GACCCAAACA Tgtacgggga atttgcttcc
 541 tgcttataaa cgatcaaaca ctgcatgcag tccaaaaaat gttcacagtt gtgtgtagtc
 601 gttgtatatg taactctaca tatacaccta tctttatata tatatatata tatatatata
 661 tatgtaccag tctatttttt ttatttttgc atgcgtatgc atgtacatgt gcaaatatat
 721 gtatacgaag gtgcatgtat gtgagatagg gtttctctat ataccgtagg ttggagtaat
 781 tcgatgcacc gctaggtagg tccatgtgcg tctccctcgt tacaggtgag gatttgctgt
 841 ctttcctaga taagaagaga gaaagacata actcggctga gaatgactag tcggccgcag
 901 cgctggaaac atgaaggacc tctcctctga cgaaaattcg ccactgccgg gtcttcccgg
 961 ttctccggct cacagtggga tgtgtgtcag ttggaaccgc atctgcttac aatatacttg
1021 ctcatcgacg ctgtgcatat gaggtgtaca tacactcgtt ctcactgccc gtcgattgcc
1081 gtcgaagaat agcgacggta atagctcagc ggctgcgctt tcgcgagttc gtgacctcta
1141 cggactctgc acaggtttcg ctgtcgcttt gtgttcgctt tcgcgacatc tacctctgca
1201 ctcaatggat ccagaattat ttctcttgaa agcttttctt cctctttatg catgcagCTA
1261 CGATGTCACT GTGATTTCGC CGCGCAACTA CTTCACTTTT ACGCCTCTTC TCCCCTCCGT
1321 CTGTGCCGGC ACCCTCTCGC CGCTCTCCTG CATTGAGgtc cgtcggagga tttccccgtc
1381 gtgagacaac gtctcggcgt cgtttctcga gcaaacagag tccttccatt tatagcgtca
1441 ttgcctccca gttctgtttc caaagtgtga cgtttcttag agtgtcctct gcatgcaaat
1501 gcgtcctcat acataattgt atgtattcga ttcacataag gatgtgcatg catatgtgtg
1561 tgtgtatcta tctacaagta gatgtgtata tatttttat atacgtatgc gtcatgtgca
1621 tacctgtgta ccattttaga ggtaaatgtg gacgtggtgc tgcagctttc gactttcctt
1681 tgggaaggcg ccagctcttc agacgagctc cttgctccca gcaagtgttg atttgtactg
1741 caccttctct cgagcctcaa acaggcgtgg atgtaccagt cgacgttgcc ttacggagaa
1801 aagcgctttc acctggatgt accgttacgg taacattcat cgattcgtat agatgtatgt
1861 gtgcctgtgt gcatctgtat ctgtctctct gcatctgtcc ccctcgatcg acgtctcagt
1921 ttctatgtcg gtctatcaag agatctccgt acctacctgt atttcgctgt ctctatgggc
1981 ggatctgttc gccgaccaat gccctgtcgc tccagatgta gtgtatatct gtgtatgtcg
2041 atgtacaact ttaaatgaag agtccggtgt ctttctgtcg ggtggtattc ctgtagCCTG
2101 TGCGCTCGTT GACCTACCGC AACGGCCGGA AGGTCGCTGA CTTCTACGAG GCTCACTGCA
2161 CAGATGTGGA CTTCAAGAAT CGCATCGTCG gtaggctttt ctttttctcg acttcaacgc
2221 gtgtttgcat cttctgtgcg tgctcattac acgacgagga agtgaagccc tgctaggtcc
2281 tcgctgggca ctttcacctg agatctgccg cctcaggtga agacgctgcc tcttcctccc
2341 cagttaccac cgcctcgtcg ctttcggcaa tgccttcgct ctatgcagac gcctaagctt
2401 atcttacatg catctaacca tatctataca ctcatataca taaatacata ggcaggcata
2461 tatacatgca tatacatata tatatatata tatgaaatct atatgaatga tatatatata
2521 tatatgtgaa tgtctatata aatatgcata tgcagctatg cgtgtatgtg tgcacgcatg
2581 tgtgtgcgtg tagatgcatc gtgaaagttt cgttcgtcga ctcggttttc ctgtcacgca
2641 cttttctgca tgcattttcg gttccaaaca gCCTGCGACA GCCGCCAGGG TGGACATTTC
2701 AAGGTGAAGT ACGACTACCT GGTCATTGCG GTTGGGTCGG AGTCGAACAC CTTCGGTATC
2761 AAGGACGTAG CTGCAAACGC GTTTTTTCTG AAGGAGGTGG AGCATGCAAT GGCCATCCGG
2821 AAGAAAGTGA TGAACAACTT CGAACTCGCA GCCCTGCCGC gtaagtcctt caatttctca
2881 ggtacctcga caaaagcaac caccacaggc acatttcaga tctgcctcta tatttctggt
2941 gttgaattaa acagaatgtc aaatccaagt gcagctgagt ttacatacat atttgtgcac
3001 ttgtaaacat ttgtgtagat gcacatgttg tagatattta tatgtatata catgtacgtt
3061 tgaatacctt gtatgtgaca tatttgtata tatctttata aaaacatctg catatgcatt
3121 tttgtgagtc tacgaattcg aatagcatgg aaatgcaatg tctcggagtt ctctgtatgg
3181 gctgctctcg agccctgttg gtgtcagcga gtgaatgcgt ttcccgactg acaagcagag
3241 ggcgttcgcg tctttgtgtt ctcgcgaagg attggtcctt ttattctcat ctgtggaggt
3301 ccaaggatga tcccggagga gggcagtgaa gaagagctac gataggaggc gacatgatca
3361 cactagcaag tcgggggctgc acatgtacaa ctgcttttgt cctttccctc agAAACATCT
3421 GAGAAAGAAC GCGACCGCCT TTTGCACTTT GTTGTCGTTG GCGGAGGCCC GACGGGTGTC
3481 GAAAGCGCAG CAGAGTTCGC CGATTTCATC AAAGAGGACA TGAGCAAGTA CTTCCCGCAG
3541 gtacgcgtgt gtttttgct ggctcgaaag aaaagccgct tcccttctag actgaacttc
3601 tgcagaaaaa gcggacacag ggtttccgga aaacgagacg aacctctgac gttgctagat
3661 gttccgagga ggacgcgact tggtctctcg tcacgctcgt gtctcgtcgc tttcgtagaa
3721 gaaagcgaga aggagcctgc atgcagtgaa cttctgcttc ttactgaggg cacatttcct
3781 ccacttattc acggttatta catatctgct tcacgatctt agcgcctgta cgaggaggcc
3841 ctcttcctag ctgagaaggg ggtgaggcag gctacactgt agtgatcgca gcagattgaa
3901 gagtcttgcc agcctttcct ggtcgatgga aacgacgaaa catctttcac gacgatggct
3961 tgaatcaaac gagcatctga gaagtctttt tcctcgcgcg ctatcaatac gactgcgaac
4021 agcgacccat acgtgacacc cagaaagcgt atataatgca gactcgacgt catcttgtac
```

```
4081 acctcacgtg cttgactcaa aaaagggttt ggtatatata tatatagata tagatatata
4141 tagatatata tagatatata tatatacact ttatatatat atatatatgt atatgtatat
4201 gtatatgtcg gcatctgaac tgggatgtgt ctgcatgcgc gtctgtgttt ctgtgcagCT
4261 GATTCCTCAC GTGAGCATTT CCTTGATCGA AGGCGGGAGC CGTTTGCTGG GGACGTACCC
4321 GCCAGACATT TCCGCTTTTG CGGAGAAAAC TCTGACGGAG GAGTTGCACG TCAAACTGTT
4381 GCTTCGAAGC ACCGTCGTCG GCGTGGACGC GACCTCTGTA CGTTATGTTT CCAACGAACC
4441 TGGCGCCTCA AAAGAGgtag gctgtcaccc tgtttgacgg ctgtcgaaaa cttcacgcgt
4501 ctttttttgg cagaggtttc tctagattcc gctacagctg gatccagctg agcagcagta
4561 ttgcggtgtc cacatcgaga tcactcagtt caatggtctc acgctccctt gtcttttcgg
4621 gcgcttgctg ttggttcctg aggtgacgcg gagctggtgt gtcgctttgc ggtgtcgacc
4681 gttcggctgt cgtctgcgaa cgtttgtatc acccattctt ttctttttcc ttctctctaa
4741 cgagctttcc gagtatcgcc ctggtccccg acaccccgcc ttcacactgt tcgccaatgc
4801 ccttcacacc gcatagtggc ttgtgctctg tggtctctcg cctctgtcgt ctctccttct
4861 tcgatcgctg ctccgtcgtc tcgttctctt ctctctttgt tcgctctgtc tgcttcggtc
4921 tctcgttatg ctctctctgt cttgttcagt ctctcggcat ccttccttcc cgcatgtttg
4981 tgtgcctccc ttgcctcaaa gccgtgaagg cgagccctgt cttcttcgtc tgtttttgaa
5041 gtcgtcggac catgcatccc gtgtgtttgc tctaataatt ctcctttcct tctcgaagac
5101 acccacattt agagctgcac attggccttc ttcagaggtg gagcgcgttc agggtccaca
5161 cgcgttcagg gtccacacgc ctgttctctg ttcttctctc cctgattctg ctttttttt
5221 atcaatctcg tttcctcgac tcttgggcgg cctgtagatc tactgtccgt gtagcatcat
5281 aactcgccat gtcggtgcct aggaatgcct gttgaagacc ttagtgtctg taggtccatc
5341 cgtgggtcca agtggatact aggttatata accagtgggc cgactcgtag aatagatgct
5401 cccgagaaac gatcaacagt cctgttgcag gtgattcctt ctagtacggt agtactagtc
5461 acactcgcat ctgagtagtc gtcgtcgcga ccttccccag agaacattaa ctcgttcagc
5521 cactgactgc tcccaggacg atggattgac tccagttgca ggcagaaatg tgacttttg
5581 tgtccagCCC AAGGAGTTGC TGCACGGATT TGTGTTATGG GCAAGCGGCG TGGGTGAGGT
5641 GCCCCTCGTG AAGAAGATTA TCGCAGAGAA TTTCCCAAAT GTCGAAGGAA AACCTCGTCT
5701 TCGAGGCCTT CCCGTCGACG CTCAGCTTCG CTTGCTGAAC CAGCCAAATG TGTACGCTTT
5761 AGGTGACTGC GCTGCCATTG CACCCCCACG GCTGGCGGAC GCTGCGCAAG AGCTGTTCTC
5821 GAAAGCGGGg taagtctgga aaagacctga ggaaaacagc gtctgcggag agaaggaacg
5881 ttaaagtagt ggcaacgagc ccttcgctat ttcagttcag tgatgaagtt gaaatatcca
5941 ctcaaaagcg aattcgcctt caaggtggag gcagtcgagg aaatgcctgc gctcaccggc
6001 accatgaaac gcccgagctt ttctcgtctt ggccttgtct tccgcagCGC TGCCGAACCG
6061 ACCCCCCAGT GGCTTGGCCG ACACGCACCG ACCCTAGCTC AACAGTTCCC TCAGCTGAGT
6121 CCTCTCAAGT TCAACTTCGC GAAACTTCAG TCCAACGAGC ATTTGCCTGC AGACCAGTTC
6181 AAGTCTTTCC TAGgtgcgtt tctcgcggat gtagaaaagc ctgtttcctc cttccagctg
6241 aactcagata cgccaaggat ttccatgggt gtgcccaagt acaaaggggt gcggcgcaaa
6301 ggcacccgag ccaggccttt ctggcggact gtcaccagtg ttccgcaagc ggttgggttc
6361 ttgtttgaat acgcgtcggt catgggtgtg cgaaaacaga atacttgagg tcttgctgca
6421 tggattggct gtccacctgt cgtttttctt gtagCCGAGA TCGACGCGGC TTACCGACCC
6481 CCTGCGCCGA CAGCTCAGAA CGCCCGTCAA GAAGGCATTT ACCTCGCGAA AGTGTTCAAC
6541 GAATGTCCCC ACCCAGAAGA AAAAGCCGAC GCGCCTGCTT TCCAGGAAAC GTGGAACGGC
6601 TCTCTCGCTT ATGTTGgtga gtaccaatgt atacgtgtgt acatagacct catagcgcgt
6661 atacatacat acgcgtacat gtatctgtat gagcgtccgt tctgcgcggc tgcaggtatg
6721 tctgaacgtc gtactggaga tctcctttgc atgtatcagg acacaagaaa cgcgcttgga
6781 ttcggacgtg tgttacatgt tctcttacat cattcgcatc acgaatttac cctcgcctgc
6841 atttcacaat atggcggctc atcagttttt tgtgtttatg gcttcttttc gtgtttttcc
6901 tgccccagGG AGTGGCCAGG CGGTGGCACA TTTGCCGTAC TTCAATATCA AGGGCGGTTT
6961 CCTCTCGCTC CCCTTCTGGA AGgtgagtac gctgtttgcg ggaatgtttc gctggagtcg
7021 gcagcttcac gacagtagcg ctgacgtctt catacctctt atatggtgga tcagctgaaa
7081 aggggcagtg actaccgtat gtagatataa atcttagatt ttcttccgac ttcgctgccg
7141 acaaatcgcg agcgtttctg caggcatccc ttggcctagc aagggtggag gattcccgca
7201 aagagacatg tgcatacaag gtgtacaaat atttcattca gtatggtaaa cgcgtgctcg
7261 tttccttttc cggggttcct gcccaaggga tgcgtgcgca gccacggaag ggaagtgggc
7321 gtctgcatca cagcctctgg atgatctaca tccgcttttg taccattctt ttgtatcttt
7381 tcgcctcgcg gtgaactgcg tggtggtcga gttcgagtag gggaaacttt ttacgtgcag
7441 cagatgttgt cctcgtgggc tgtcgataca cgtgtagctg tactcgtgaa gagagagaga
7501 caggtctcta cagttttcca tcgtgaaagg aaaggcgtga tgtgtgtctc cacgtgtctt
7561 tctcagGCCG TATATACGCA GATGCAAATC ACGTGGCGCA GTCGAACGAT ATGCCTCTTT
7621 GACTGGCTAA AGACGTTCTT TGCAGGCCGA GATGTTGGCC GGGATCACGA ATATTACAAC
7681 CACTGA
```

B. NDH2-II ORF(accession #: DQ228957)

```
   1 ATGGCGATGC TCTTCTCCAG CTCAGCAGCA GGCAGCCTGC CCTCGCGCAG AAGCCGATCC
  61 GTCGCTGCGA AGTTGGCGCC CTTCGCTTCT CCAATTTTCT CGTCTCCACT CTTGAAGCAA
 121 GGCTCAGATA CTGCCTTTGG TCTGCGCAGC CCCACACCTG GGCTGTCCGT ACACCCGAGA
 181 TTTGCTTCCA GAGGACCCTT GACACGGAGC GAGGAAGCCG GAAAGGCACC CTTCGGTTTC
 241 TCTACCGCGA GAGGCGAGCA GACGGCCACG GAGACAAATG CGCCGAGATT CGGGACGAGC
 301 CTTTGCTCTT CTTTTCCGTT GTCGGCGACT CAGAAAAGCC ACGGTTCGCG AGAGAAGGGC
 361 CTCACAAACG AACGGGCCTT CTCGACCTTC TCTTTTCAAG GCTTTATGAA AAATGTCAAG
```

```
 421 TCGAGAAACG CAAAGCCATA CACTGGACCC CCGCAGAAAG TCGTTGTCCT CGGAACCGGC
 481 TGGGCATCTG TCAATTTCTT CCGACACCTT GACCCAAACA TCTACGATGT CACTGTGATT
 541 TCGCCGCGCA ACTACTTCAC TTTTACGCCT CTTCTCCCCT CCGTCTGTGC CGGCACCCTC
 601 TCGCCGCTCT CCTGCATTGA GCCTGTGCGC TCGTTGACCT ACCGCAACGG CCGGAAGGTC
 661 GCTGACTTCT ACGAGGCTCA CTGCACAGAT GTGGACTTCA AGAATCGCAT CGTCGCCTGC
 721 GACAGCCGCC AGGGTGGACA TTTCAAGGTG AAGTACGACT ACCTGGTCAT TGCGGTTGGG
 781 TCGGAGTCGA ACACCTTCGG TATCAAGGAC GTAGCTGCAA ACGCGTTTTT TCTGAAGGAG
 841 GTGGAGCATG CAATGGCCAT CCGGAAGAAA GTGATGAACA ACTTCGAACT CGCAGCCCTG
 901 CCGCAAACAT CTGAGAAAGA ACGCGACCGC CTTTTGCACT TTGTTGTCGT TGGCGGAGGC
 961 CCGACGGGTG TCGAAAGCGC AGCGGAGTTC GCCGATTTCA TCAAAGAGGA CATGAGCAAG
1021 TACTTCCCGC AGCTGATTCC TCACGTGAGC ATTTCCTTGA TCGAAGGCGG GAGCCGTTTG
1081 CTGGGGACGT ACCCGCCAGA CATTTCCGCT TTTGCGGAGA AAACTCTGAC GGAGGAGTTG
1141 CACGTCAAAC TGTTGCTTCG AAGCACCGTC GTCGGCGTGG ACGCGACCTC TGTACGTTAT
1201 GTTTCCAACG AACCTGGCGC CTCAAAAGAG CCCAAGGAGT TGCTGCACGG ATTTGTGTTA
1261 TGGGCAAGCG GCGTGGGTGA GGTGCCCCTC GTGAAGAAGA TTATCGCAGA GAATTTCCCA
1321 AATGTCGAAG GAAAACCTCG TCTTCGAGGC CTTCCCGTCG ACGCTCAGCT TCGCTTGCTG
1381 AACCAGCCAA ATGTGTACGC TTTAGGTGAC TGCGCTGCCA TTGCACCCCC ACGGCTGGCG
1441 GACGCTGCGC AAGAGCTGTT CTCGAAAGCG GGCGCTGCCG AACCGACCCC CCAGTGGCTT
1501 GGCCGACACG CACCGACCCT AGCTCAACAG TTCCCTCAGC TGAGTCCTCT CAAGTTCAAC
1561 TTCGCGAAAC TTCAGTCCAA CGAGCATTTG CCTGCAGACC AGTTCGAGTC TTTCCTAGCC
1621 GAGATCGACG CGGCTTACCG ACCCCCTGCG CCGACAGCTC AAAACGCCCG TCAGGAAGGC
1681 ATTTACCTCG CGAAAGTGTT CAACGAATGT CCCCACCCAG AAGAAAAAGC CGACGCGCCT
1741 GCTTTCCAGG AAACGTGGAA CGGCTCTCTC GCTTATGTTG GGAGTGGCCA GGCGGTGGCA
1801 CATTTGCCGT ACTTCAATAT CAAGGGCGGT TTCCTCTCGC TCCCCTTCTG GAAGGCTGTA
1861 TATACGCAGA TGCAAATCAC GTGGCGCAGT CGAACGATAT GCCTCTTTGA CTGGCTAAAG
1921 ACGTTCTTTG CAGGCCGAGA TGTTGGCCGG GATCACGAAT ATTACAACCA CTGA
```

C. NDH2-II PROTEIN (accession #: ABB17192)

```
   1 MAMLFSSSAA GSLPSRRSRS VAAKLAPFAS PIFSSPLLKQ GSDTAFGLRS PTPGLSVHPR
  61 FASRGPLTRS EEAGKAPFGF STARGEQTAT ETNAPRFGTS LCSSFPLSAT QKSHGSREKG
 121 LTNERAFSTF SFQGFMKNVK SRNAKPYTGP PQKVVVLGTG WASVNFFRHL DPNIYDVTVI
 181 SPRNYFTFTP LLPSVCAGTL SPLSCIEPVR SLTYRNGRKV ADFYEAHCTD VDFKNRIVAC
 241 DSRQGGHFKV KYDYLVIAVG SESNTFGIKD VAANAFFLKE VEHAMAIRKK VMNNFELAAL
 301 PQTSEKERDR LLHFVVVGGG PTGVESAAEF ADFIKEDMSK YFPQLIPHVS ISLIEGGSRL
 361 LGTYPPDISA FAEKTLTEEL HVKLLLRSTV VGVDATSVRY VSNEPGASKE PKELLHGFVL
 421 WASGVGEVPL VKKIIAENFP NVEGKPRLRG LPVDAQLRLL NQPNVYALGD CAAIAPPRLA
 481 DAAQELFSKA GAAEPTPQWL GRHAPTLAQQ FPQLSPLKFN FAKLQSNEHL PADQFESFLA
 541 EIDAAYRPPA PTAQNARQEG IYLAKVFNEC PHPEEKADAP AFQETWNGSL AYVGSGQAVA
 601 HLPYFNIKGG FLSLPFWKAV YTQMQITWRS RTICLFDWLK TFFAGRDVGR DHEYYNH.
```

A. TgSDH-Fp GENE

```
   1 ATGCATGCGT CGGCATCCCT CACCCAGGCG CTGCGGCCCT ACGCGCGTCG TCCTCTCTCC
  61 TCTGCTCTCT CTCGATCCTC GCTTTCTCCA GCTCTCGCAG CTGGCGTAGC TCGTGGACTC
 121 AATGCCGCGC AGCCGCAGGC GACAGGACCT TCTTTTGTGC AGCAGAAACG CTTCTTCACG
 181 AATGTAAAGA AACCTGgtac gtcgcctcct cgcagctctt ctctcgaacc tctttgttgc
 241 tctctcggtc ctccggagtc gttctctctt ctgtcttctc tctcctgcac ttccgagaac
 301 gaatgcagcg tcgcagttct cttgcgtctc tcgttggccc cctcgctggc tctcgcgttt
 361 cctctcctga cggctgttcc agagcacaga cggatgcagc ttttcgacta cctcgagtcc
 421 tgcagaggtc gtcatccgcg catcttgctg cagtaatttt tgctcttctt ttttcctttt
 481 gtcgctcaca ttaaccgttg tttcattgca ccgcgccgcc agccttcacc tctgtttcgc
 541 cttcggtgtt tttacagCCT ACCGAGTCAT CGACCATGAG TATGATGCAA TCGTCGTCGG
 601 AGCAGGCGGC GCTGGTCTCC GAGCCGCCTT CGGCCTCGCG TCTGCAGgtt cgttctccac
 661 aagaaaaaaa tgaatcgagg tttcttctca cttttcgctt ttcctccctt cctcattctt
 721 ttcttctctt cctcttctct tcgtcttctt ttccttctct tctcttcgtt ttcttctctt
 781 cctcttctct tcgtcttctt ttcttcctct tctcttcgtc ttcttctctt tctcttctct
 841 ccatatcgcc ttcttcgtgg tgttctgccc tcttgcgtcg ccgtcttgtt cttcccctca
 901 cgaaggttcc actgaatgcc gcaagtcact ttggatagaa gcgaggccag caactcggct
 961 cctgtcaagg aggttccgtc ctcgaaaaag gcgctggggg cgttcagctg tcactaaagc
1021 ttcgactggg agccgccagc gagtgggcgg tctgcgagga agtttgcgtc gtaggtctct
1081 tgacgcctaa gacgacgcag cggcaaggag gcagggcgcg cgcatgcagc ttgcttccgc
1141 gttttctgtt tctttagGAA TCAAGACGGC CTGCCTTTCG AAGCTCTTCC CGACGCGCTC
1201 ACACACTGTC GCTGCCCAGG GTGGCATCAA CGCAGCTCTC GGCAACATGA CGGAGgtgag
1261 ctttgagggc aagaatttca gggagaaagc ttcgtcgcct gggcgaaaga cctgcggatc
1321 gcagtgcggc acagtcgagg attcgcacga gtttttttcc gatttgtttt tttggttttt
1381 tctcgacagG ACGACTGGCG CTGGCATGCG TACGACACGG TCAAGGGGTC GGACTGGCTC
1441 GGTGACCAGg caagtttttc ttttctcttt tggttctctg tggagccgcg ttttcagctg
1501 ctcgtgctct tctctctttt cgtcgaatcc ggatctttct cagactccct cgatcgagac
```

```
1561 aatcagactt tcaaggtcgt acttttttgc gagtgcaggc actcttccgc tgcaggtcgc
1621 ttgaacgccg cggcctcgct tcttcgcctt ctctttccgt tcttcgcctg cgtctgcctc
1681 tctcctgccg ccagctgttt ttgcgtttcg agagtttcct tcttcgcctg ggccgtgcga
1741 cggcctcgcc atgttctctg cagtcgcttg tctcttttcg tctcagGACG CCATCCAACA
1801 TATGTGCCGA GAGGCTCCGA AAGTTGTTCG GGAGCTCGAG TCCTACGGTC TCCCGTTCTC
1861 CCGCACTGAA GACGGACGCA TCTACCAGgt gcgtttgttt tttcgagaag caggaaaggg
1921 aggcgaaagg gagaaacgag aacagaagaa agcaagagac ttctgggcag tcaagacagg
1981 caggccataa gcagcggcgc gcctctgggg aaggaacttg ggtctccaag acgatagggg
2041 agagcggtgc atacgagcac agatcggatg tggaaagaaa acaacgcgtt tcaagaagat
2101 caaggttgaa actcacacaa cgagggatcc agggcgagcg tttctgtttc agcggggatg
2161 gtaaacgtct gtctgtctca gatgttgctc caaaaaggac gcgctttctc tcgatcggat
2221 ggttcttctc gagataagaa tggcgtcgtt ttccttctct cgagagactg ctttccccgt
2281 tcttgagagt tttgttcctt tgtttctctg atccttcagC GTGCATTCGG AGGACAGTCT
2341 CTGAGGTTCG GTAAAGGCGG CCAGGCGTAT CGCTGCGCCG CTGCGGCAGA TCGGACCGgt
2401 gaggaaaagg acgcgtctgt cgatgtctcg gaggactccg tcgctcttgc cttcttccgc
2461 gtcccctgtg gaagcgcgga ggccatctct gcagtccttc agtgctttca gagacactgg
2521 aacgccgttt gcaggctgag cctggctgcg atcggaaaga gcgttttctc tgctgtttgc
2581 cagcagccca gcgacgcgcg cagacaccgc gtcaacacaa agtgaactga ccgcgtgaac
2641 gccttttttc gagaactcac gaacgccggt tttcctctct gcttccatcc acggacgaac
2701 cgtagatcca tacaggcagc gcctctctct gtcgaaggtc accgtgcatt cgttcagccg
2761 cttgcgtgta gagaggcgtc tgcttgcatg tttgtttctg tgtgtacttg catgcatatt
2821 tatatgtatg ttttttttgtt cgcgtctcac ctgcggtgtt tggattttcg gcgaaagggg
2881 cgagtctgtg cgttgtgtgt gtagGCCATG CGATGCTGCA CTCGTTGTAC GGACAGAGTC
2941 TGGCCCACGA CTGCAAGTTT TTCGTGGAAT ACTTTGCCCT GGATTTGATG ATGAGCGGAG
3001 AGAACGACGA ACGCAAATGT CACGGAGTTG TCGCGATGTG CATGGAGGAC GGGAGCATCC
3061 ACCGATTCGC GGCGAAGCAC ACGGTCCTCG CCACTGGAGG gtatgcagga aggaaaagaa
3121 ggaaaggaag gaaaagaagg aaaggaagac ggcaggagaa tagaggacag agcgaaaaac
3181 ggacaagcga atgcgggcgt gaggaaagag agcgagagaa gagcagcgcg ggagcggagg
3241 aggcgaagtg aagagagaag gagaactgga cacagagggg caaacatgga aggcgcttct
3301 cacatactct gtggagagcg atttgcagga gagaagcaga ttcgcgaatc aaaagagaag
3361 aaatcagctg gcgaggggaa aagaggagaa agaagaagga acaccaatac gtgaaggaga
3421 cgaagtaggg gaagaagaga gaagaagcag acgttttagt cacgactcag ttaaaaagaa
3481 agtcagaaga gacaccggca gatcgccgcg cgaagggagg catttagagg actcgaacag
3541 gcaaacgctt cttcgagttt ccctaatttc cagttctttc caatcttaaa cttctctctc
3601 tctctccagA TACGGCCGCG CCTACCAGTC TTGCACCTCC GCTCACACCT GCACTGGAGA
3661 CGGCGGCGGG ATGgtgaggc tgcgtctgcg acgctctgcg aagaaggggа gaagcgtctc
3721 tcagaaagtc gaccaacgcg cgcaaagtcg agcggagcgc gatgcaggcg tacagacgat
3781 tgacgtcatc gcatgcggct gcaggcgtca ccccagagat tcgcacataa gcgtcgagat
3841 atgcatgttt ttgcacctct ctgtgtcgtt ctgtgtgggt gggtggacgt aggcttttag
3901 atcaatccaa cgaaagatcc atgtactgct gtagatcctc aggcagattc agaatcagat
3961 acagacactg gatgcgtgaa gctgtacagg taaacagctt gacagataga aaaagtcgaa
4021 cacagacaaa tagacggatg actgcgcgta catggatact ggtagatata cgtagtcaaa
4081 tagatatcca gagatacaga gatagatgca gatgtagcca taaatattgg catttgtaga
4141 tagatgtaga tggagcagtt ttctcatttg tctggagttt cacagaacgt agcttgcgct
4201 gctgcctttt ttttcagGTC TCTCGCGCTG GATTGCCTCT TGAAGACTTG GAATTCGTCC
4261 AGTTCCACCC CACCGGAATT TTCCCCGCCG gtacgtggaa gcgtcgtgtc tctctggttt
4321 gttttctctg catgcatcta tccgtccgga ggttctcttg agccttcgaa gtctctttct
4381 tcttgcagaa tacaagcaat cgcgagtcct ctttcgtgct cactagaaaa ctgggaacag
4441 gcctgacagc tcgagcgaga gtgcatgtgt ttcctcctaa gtgcgggctt gcaggaatgt
4501 tcaactgaga ctgaatgttt gtagagtgcc agattgcatg cgtttttcgt ttgagactgc
4561 atgcgttttt cagGCTGTCT CATCACCGAG GGATGCCGCG GAGAGGGTGG CATTCTCCGC
4621 AATGGCCAAG GCGAAGCTTT CATGGCCCGg taggtcgcgc atccttcagt ttcttgtttt
4681 cttcctcctc tttgtggtct tgtctcagcg gaatacttaa cgcgttacct ctagggtact
4741 gaatctatgt ctttaatgtc tcaagtaccg actatccatt gtgtacagtt aaggctcctg
4801 gggtatccaa tccgatttct tacaggcaaa agttgtctga aaatgtttgt ttttataatt
4861 cctattccaa gaatttagat aggaatttat gtttaaaaaa tccgttttcg ctcctctttt
4921 ttcgtcgctc tcttccgtct cgttcccttc ttccattttt ctcgttttgc tcttgtgccc
4981 tgcctctgcg tttttcctcg gctggagacc ggtctgttga tcattcactt ttcatttttt
5041 tcgccagGTA CGCCCCCACG GCGAAGGATC TAGCAAGCCG CGATGTCGTG TCACGATCCA
5101 TGACCATCGA AATCCGAGAA Ggtgcgtcag ataaaaagtc gatcttctgt ctttcctctc
5161 gtctttcatc ccctttttctt ctttttttct tgctctcttt cttctctcgc tctttcttct
5221 ctcgctcttt cttctctcgc tctttcttct ctctttcctc ggcgctactc tccttttttc
5281 cccgctacct cccttcttcc ctttctcctt ctgtatctcc tttgtcgttc attttttttc
5341 ttttttttct ttttttttct tttttatcga tcttttagGC CGCGGCTGTG GGCCCAATCG
5401 TGACCACATG CATTTGGATT TGACGCACTT GCCGCCGGCG ACTCTCCACG AGCGTCTTCC
5461 TGGAATCACC GAAACCGCGA AAATTTTCGC AGgttcgttt caaaatgaga cacatctgta
5521 attcttctcg agctgcttct tcgagacatc tgtcgagatc atttcagcaa tgtttttttcg
5581 gtttgttttt ggactgaagc ctcacagtgt tctcttcacc gtccttcaca acggagagac
5641 accccaccgg agcgtcatgt tcacttttca tgttttacaa ggcgcaacct accttgttct
5701 ctagaaggat acttcgatcg tttccttcgc agaaaaacag agaggcaatt gcgagagaga
```

```
5761 gagcatttttt tccttatcgc agcagcggca tccactacac tggcttctct gagagattga
5821 aaaactgttc tctctgttct ctgtgtgcct ccttcttctc tccagcgagg tgtgtgcgcg
5881 gatgatgttt ctgttacgtt gcgtttcgtt ccacaagccg gcttttcagt tggttttcag
5941 ctctcttctg tttccactcg ttggcaatcg agaggacggc tctctgcgcg ctgtgggacc
6001 tgcagggcct cgagggccgc cacaagatca tgctttcgtc tccgttggtt agacctgttg
6061 gttttctcgt gagttgcctg ttttttcact tgtttgtttt ttctgtgtct ttctgcgttt
6121 ctcagGAGTC AACGCAGAAA AAGAACCGAT TCCAGTGCTG CCAACGGTCC ACTACAACAT
6181 GGGGGGCATC CCAACCAACT GGCGCGCGCA GGTGCTCACC ACCTCCCGCT CTAAAGCTGG
6241 CGgtaggcgt tttctttttc ttttttttca ctgcatttct ctttctcctt tcttcttctt
6301 ccgtacgtct ctctctaccg cgtaggtctt ttctgctccc ttcagtccgt gctctctctg
6361 tcttcttcgc ttcgtctgcc tttctctttt ccgtctcttc ttctctctca gggtggaggc
6421 gcctcctcgc ggcaatggcg tcgagttcta gcgcatgtcc tcagccaaag ttcagtttcg
6481 tctgcttctg tgggccagag agaaagagaa cgaggacagt ccgagccgcg cgactccgca
6541 gcggaggcgg cacaccaatc ttcgttgtgg agtttttctt ctgcagGCCC CGACAAGATC
6601 GTTCAAGGCC TCTACGCCGC AGGCGAGGCT GCATGCGCTT CCGTGCATGG CGCCAATCGC
6661 CTCGGTGCAA ATTCGCTTTT GGACCTTGTC GTTTTTGGAC GCGAAGCTGC GCGCACAATC
6721 GCTGACGAAT GCAAGAAAGg tcagtttgtg tacgcttcgt gagacgcgtt gtgggtctgc
6781 tggcctcagc ttggcatccg ttgttcttca gtgtttgtgc aggtttttca aagagaggaa
6841 actttcagtg aactgtgtct tcgctgtgcg agctgtgttc tcccgccttt gcggcgggtc
6901 ccgaaacgcg cgagtgtatg tacacctggg ctcgttctgc gagtggcctt gatgtggact
6961 ctgtgaggtt gtaagtgtgg atggctctcg tcggcggtgg ttgagttctt tgcgaaaaaa
7021 gaatggtttc cccgtcttgc tgctgcatct gcttgctggt ctgcagACGC CTCGGCGCCG
7081 TCGCTGCCTC CGAACGCGGg tgagaacgct tttctttgct tgcgccggac gaaaagggag
7141 aacggcgttt ttcgctgttc ctctgcgagg cgatctgcgt cgaaacgcgc tgccgcacag
7201 ctgcacactc cgacgtagac gcagaccagc gacaaaaaaa ggaggagcag acgaaaggag
7261 agacaagagc cgcgctgagg aaaaggagag agggcgccac gagaggcaga ggcaggtttt
7321 gagagaaaga ggggggaacg ggggaacggt tctgcggcag acgagcaaag caccgagacg
7381 aacagcgcgc gtttcctcgt gtgtgaacaa cgcgctgttc tcagGAGAGG AGACACTTGC
7441 CATGGTCGAT CGGCTGAGGC ACTCCAACGG ACCGTTGACG ACGGCGCAAA TCCGTCAGCG
7501 CATGCAGAAG gtagggatcg ggaggagttc tacttgtgtc tcacgagtct ttgaggcgtc
7561 gcttttcagt cggatttcgg gccttcgctg ctgcttgtcg gagcggagca gtctcttctt
7621 cgcttttgcc agctgagagg gaacatcgag aaaaaagatg cgttctgctc tttcgaatgc
7681 gcttctgtga acgatgccaa ggtagtccac tgcttggggt tccacaaggt acagcgtttt
7741 cagaaaccga atgcttgctc agtgcagctt cgggggggatc tgtctccaca tgcacccgtc
7801 ggttctagag ctaactcgcg aaccttcgcg ttttttcaac tgtttctcgt gccttcctgc
7861 tctctctctc tctcatgctg gcgcctgcgc gcgcggccac cctgtccctg cgaggaacgc
7921 tcgtgacaag ccgtcaaaac tgccctggga ggagacactg aggaacgcac actccgtgtg
7981 cggctctctc tttctcagAC CATGCAGGAC CATGCGGCTG TCTTCCGAAC GGGTGCCGTC
8041 CTGCGAGAAG GCGTGGAGAT GATGAAGGAC GTTGCCAAGA GCTTCGCTGA CGTCGGCGTA
8101 AAGGACCGAT CGCTCTCGTG gtgaggcgaa tgcgcaccgg agaaactctt aggtggcgtc
8161 agaggccggc gcggtgtctc gtccttcaca gggaattcat ggaggacctc agacgcttca
8221 accgccacag atgcatgctg tctcgtatcc ggactccgca ggggaagcga gaggccgtcg
8281 gggggatgaa gaggggaagc cgatggaaga gacagagggt ctgctttctt cctcagacgg
8341 gcgtctcgat ctccgtccac ggcctctgtg cgttgctgca ctctctgcct ttgcgcgccg
8401 gtgtcgttcg ccgtcttttt gcttccttgc agGAACACAG ATTTGATCGA GACCTTGGAG
8461 CTACAAAACC TCCTGACGCA AGCCGTCCAG ACGATCGTCG GTGCAGAGGC GCGCAAGGAA
8521 AGCCGCGGCG CGCATGCGCG CGAAGATTTC AAGgtacacc gttgagcgca tgtcctctcc
8581 ctcgtttccg ccctcaccca acgcactcga acgtgtatct tatcaaatgt ccatccatat
8641 ccatacataa attcatatac atatatatat gtatatatat atatatatat gtatatatat
8701 gtatatatat atatatatgt atatatatat atatgtatat atatgtatat gtatatatat
8761 atgtatatat atatgtatat atgtatatac tctcctcgtg ttgttggagg tgttccgcga
8821 ggggatctgt ccatctacga gtgggttttt gaaggccttt cctgcagcag gtttcccaag
8881 agtcctcgct tgtgtgtctg ttgcatgcac gggtatgtca agctgtcgcg cgggtgtttt
8941 ctcggagccc tgagacgaga gacgaggcgg ttttttttct gcgcttcacg gaacgcgagc
9001 gttctgtctc tcctctgcac actgcagGAG CGTGACGACA AGAACTGGAT GAAACACACG
9061 TTGTCTTGGC AAACGCACCG GGAAGTGGAG AAAGCAGAGg tacgttttga agcttttgca
9121 ccattcggtg gcgcctgcag cttcagttag tctcgtccgt tcgacacccc actctccgac
9181 tccgtttcgt ttcccctgcc tcgcgttttc cttctgcaaa agccgcagga agggaagctg
9241 tttgcctgct tgtcacataa aagttccgct aatctgattc gtagcaccac cggatatcag
9301 acgtgaaggg gattttcgct aacgtcgtaa atggtgctcc aagtgagtcg agttggaacg
9361 ccggcgactc gttggtcttc agcttgtgcc gcccgttgaa aaccaacaga cactggtctt
9421 tctcggagcg ggaagagcgg ctgcaggaaa ctcagtagaa ggccctggac ctcgaggtgg
9481 acagacgcgt ctgtcagagg agtgaatcgg aagagggcaa acacccagcc agcatgtcac
9541 tctggagagc gtttctctct ctctctggcg tactcttcgt cgttctctga cgcgacgcgt
9601 ttcgcgagtt ctggcgtgat ggcacgagga cgaacgtctg ttgcgggtcg gtcggatgct
9661 cagaaacgtt gcatgcgtcg tttcctcctt ctgcgactgc ctgcagTTCC CCCTGACGTA
9721 CCGCCAGGTG ATCTCGCAGC CTCTTGACAA CGAAATGGAG CACATTCCGC CGGCGAAGCG
9781 CGTGTACTGA
```

B. TgSDH-Fp ORF(accession #: DQ228958)

```
   1 ATGCATGCGT CGGCATCCCT CACCCAGGCG CTGCGGCCCT ACGCGCGTCG TCCTCTCTCC
  61 TCTGCTCTCT CTCGATCCTC GCTTTCTCCA GCTCTCGCAG CTGGCGTAGC TCGTGGACTC
 121 AATGCCGCGC AGCCGCAGGC GACAGGACCT TCTTTTGTGC AGCAGAAACG CTTCTTCACG
 181 AATGTAAAGA AACCTGCCTA CCGAGTCATC GACCATGAGT ATGATGCAAT CGTCGTCGGA
 241 GCAGGCGGCG CTGGTCTCCG AGCCGCCTTC GGCCTCGCGT CTGCAGGAAT CAAGACGGCC
 301 TGCCTTTCGA AGCTCTTCCC GACGCGCTCA CACACTGTCG CTGCCCAGGG TGGCATCAAC
 361 GCAGCTCTCG GCAACATGAC GGAGGACGAC TGGCGCTGGC ATGCGTACGA CACGGTCAAG
 421 GGGTCGGACT GGCTCGGTGA CCAGGACGCC ATCCAACATA TGTGCAGAGA GGCTCCGAAG
 481 GTTGTTCGGG AGCTCGAGTC CTACGGTCTC CCGTTCTCCC GCACTGAAGA CGGACGCATC
 541 TACCAGCGTG CATTCGGAGG ACAGTCTCTG AGGTTCGGTA AAGGCGGCCA GGCGTATCGC
 601 TGCGCCGCTG CGGCAGATCG GACCGGCCAT GCGATGCTGC ACTCGTTGTA CGGACAGAGT
 661 CTGGCCCACG ACTGCAAGTT TTTCGTGGAA TACTTTGCCC TGGATTTGAT GATGAGCGGA
 721 GAGAACGACG AACGCAAATG TCACGGAGTT GTCGCGATGT GCATGGAGGA CGGGAGCATC
 781 CACCGATTCG CGGCGAAACA CACGGTCCTC GCCACTGGAG GATACGGCCG CGCCTACCAG
 841 TCTTGCACCT CCGCTCACAC CTGCACTGGA GACGGCGGCG GGATGGTCTC TCGCGCTGGA
 901 TTGCCTCTTG AAGACTTGGA ATTCGTCCAG TTCCACCCCA CCGGAATCTT CCCCGCCGGC
 961 TGTCTCATCA CCGAGGGATG CCGCGGAGAG GGTGGCATTC TCCGCAATGG CCAAGGCGAA
1021 GCTTTCATGG CCCGGTACGC CCCCACGGCG AAGGATCTAG CAAGCCGCGA TGTCGTGTCA
1081 CGATCCATGA CCATCGAAAT CCGAGAAGGC CGCGGCTGTG GGCCCAATCG CGACCACATG
1141 CATTTGGATT TGACGCACTT GCCGCCGGCG ACTCTCCACG AGCGTCTTCC TGGAATCACC
1201 GAAACCGCGA AAATTTTTGC AGGAGTCAAC GCAGAAAAAG AACCGATTCC AGTGCTGCCA
1261 ACGGTCCACT ACAACATGGG GGGCATCCCA ACCAACTGGC GCGCGCAGGT CCTCACCACC
1321 TCCCGCTCTA AAGCTGGCGG CCCCGACAAG ATCGTTCAAG GCCTCTACGC CGCAGGCGAG
1381 GCTGCATGCG CTTCCGTGCA TGGCGCCAAT CGCCTCGGTG CAAATTCGCT TTTGGACCTT
1441 GTCGTTTTTG GACGCGAAGC TGCGCGCACA ATCGCTGACG AATGCAAGAA AGACGCCTCG
1501 GCGCCGTCGC TGCCTCCGAA CGCGGGAGAG GAGACACTTG CCATGGTCGA TCGGCTGAGG
1561 CACTCCAACG GACCGTTGAC GACGGCGCAA ATCCGTCAGC GCATGCAGAA GACCATGCAG
1621 GACCATGCGG CTGTCTTCCG AACGGGTGCC GTCCTGCGAG AAGGCGTGGA GATGATGAAG
1681 GACGTTGCCA AGAGCTTCGC TGACGTCGGC GTAAAGGACC GATCGCTCTC GTGGAACACA
1741 GATTTGATCG AGACCTTGGA GCTACAAAAC CTCCTGACGC AAGCCGTCCA GACGATCGTC
1801 GGTGCAGAGG CGCGCAAGGA AAGCCGCGGC GCGCATGCGC GCGAAGATTT CAAGGAGCGT
1861 GACGACAAGA ACTGGATGAA ACACACGTTG TCTTGGCAAA CGCACCGGGA AGTGGAGAAA
1921 GCCGAGTTCC CCCTGACGTA CCGCCAGGTG ATCTCGCAGC CTCTTGACAA CGAAATGGAG
1981 CACATTCCGC CGGCGAAGCG CGTGTACTGA
```

C. TgSDH-Fp PROTEIN (accession #: ABB17193)

```
   1 MHASASLTQA LRPYARRPLS SALSRSSLSP ALAAGVARGL NAAQPQATGP SFVQQKRFFT
  61 NVKKPAYRVI DHEYDAIVVG AGGAGLRAAF GLASAGIKTA CLSKLFPTRS HTVAAQGGIN
 121 AALGNMTEDD WRWHAYDTVK GSDWLGDQDA IQHMCREAPK VVRELESYGL PFSRTEDGRI
 181 YQRAFGGQSL RFGKGGQAYR CAAAADRTGH AMLHSLYGQS LAHDCKFFVE YFALDLMMSG
 241 ENDERKCHGV VAMCMEDGSI HRFAAKHTVL ATGGYGRAYQ SCTSAHTCTG DGGGMVSRAG
 301 LPLEDLEFVQ FHPTGIFPAG CLITEGCRGE GGILRNGQGE AFMARYAPTA KDLASRDVVS
 361 RSMTIEIREG RGCGPNRDHM HLDLTHLPPA TLHERLPGIT ETAKIFAGVN AEKEPIPVLP
 421 TVHYNMGGIP TNWRAQVLTT SRSKAGGPDK IVQGLYAAGE AACASVHGAN RLGANSLLDL
 481 VVFGREAART IADECKKDAS APSLPPNAGE ETLAMVDRLR HSNGPLTTAQ IRQRMQKTMQ
 541 DHAAVFRTGA VLREGVEMMK DVAKSFADVG VKDRSLSWNT DLIETLELQN LLTQAVQTIV
 601 GAEARKESRG AHAREDFKER DDKNWMKHTL SWQTHREVEK AEFPLTYRQV ISQPLDNEME
 661 HIPPAKRVY.
```

A. TgCYTC1 GENE

```
   1 ATGGGAGGCG GCGGAGGCGG CGCGCTGAAC AAGCTGTTCC CTGGATACAA GGACAAGATC
  61 TGGATGAAAG TTCCAgttca ggtacgagcg atccaggctc tcccgtcaca cgaaatcgac
 121 tttgacgact cccctccacg cgctctgtgc gcggtcgagt ggaagacagg cgtctcagct
 181 cggtcttgtc tctctgctcc gcttcagata aaggagaggg aatcaacgcg gaagaaaggc
 241 aggtggaagg aagcaaaggg atgactcccc ggtgggggag aatgcagtcg gagactcaaa
 301 aaggagcaga aaggacaccc accaaggcga ccctctgttg gcgctcgcct cgcgacagag
 361 atgtctgtcc actggtttct gcacagggga aggtgaacgg gaggagagga agaagggaac
 421 tacagggggg aagtgacccg gaatgaggaa gcgggtgctg aggagaaagg gaggatgagc
 481 gagcggatca gaggcatgag aaagacacag gggtgactca gggcagagaa aggagccagc
 541 gagtcgtgcg tttactatcc gagaggtgac tgtgtgtcct gaagaaaggc atcgtgaacg
 601 aatgcagtgg aacccccctcg gcgcctgtta ccagacaggg cagacctcag gtgccgtctg
 661 cggcttcgga gcccggcgaa tctctcccac acgttctttt ctctctccct cccctctggc
 721 tttttcactc gcgccgcggt gtctcctcgc ggatcttctc tcgtgtctct ctcctcgttt
 781 cgcgggtgcg tccctcgtcg gctccagcgt ctgggccgca ctctgcacgt tgcacatttg
 841 ctttctgttt ttttctctct gtctctgcgc agTGGCGACA GCAGATGATT CAGCATTGGA
 901 ACAAGTCGTA CGAGAAGCAG GTGTACAGCG AGTCCGTCGC GTTGAACAGA ACTTTCCAGG
 961 CTCGCAACCA GCTGGTTCTG GATCGTCTGA AGCCGAGCGG AGCGTACCGT CTCCCCGCTG
1021 TGGACTACAA GAGACAACTC AGTCGAGGCA CGCTTGTCGA GGGAGCCGAC TTCTACCTCC
```

```
1081 CGACTGCTCA GGAGCAGCAA CGCCTGGCTC GCCACTTCGA GCCGTACTCC GAGCAAGAAC
1141 AAGAAGAAAG GAGAAAGTTT CGCTTCCAGA Ggtgagcagg gatctccagt tttctaaccg
1201 tccgttcttc gccgtgggaa aacgctcgaa agtgtaggcg cactgcaagg gcactggcgt
1261 acgttgggac agacaccaga ctgggagagc aagagacgca cttgcacgtg tgttctctta
1321 tgctctctcc ttgttcgcgg tcgagggttc cgtcaatctc tctctctctc tctggctgct
1381 tcgcctgaag aaacaggcgc tgttgatcga cgtctgtgtg ttctgcctgg tggaccgatc
1441 cggcttgggt acacgaaagc ttcactctcg ggtgtctaca taggcgtggg gacagtgtgt
1501 gcatgtgacg agcgctccgg gctcttcgtt tcccctatct ttctttttg ctgcggctcc
1561 tacgccaccg tgcctccagC ATTTCGGTGT ACTTGGCTGT CGCTCTCGGG GCCTCCTTCG
1621 TCCACGACTA CTTCTACCAG CGTCGACCAG TTGCGTGGTG TCTGGAGAAG GAACCCCCGC
1681 ATCCTCCGAG CTACCCTTTC TGGTTCAAAT CTCTCTTCCA CTCTCACGAC ATTCCCTCTG
1741 TCCGACGTGG CTATGAAGTC TACCGCAAGg tgaggggctt ctgcgcgtcc cgatctccaa
1801 aatgatctac ttctactact tgagacatcc ggctcgaaac gtatatgcta ccaagtcgac
1861 gcccgtctcg gtcactgatc cgccctatct atctgtatat atatatatat atatatgtcg
1921 atgtatatgc tggcatatac acacatacgt gtacgatcta aataggtagc accgatgtca
1981 aggagcatac gaaaaacgta tgcatgtata tatatatata tatatacatg tgtattgtgc
2041 acacggaatt cgttcaattt taacgtctgt ggcgggggtg tttgcctttg tcttcgtttc
2101 caactgtctg cgtgtctgtg gtgtgtatct cagttgtgaa tgtcactcgc tgtgaagtgt
2161 gcaagttgtg catgcgcttg tggggcccaa agtgtctggt tgacactgga caagaacgcg
2221 ccgttttgag tcttctcggc catttatgca gGTGTGTGCA ACATGCCACT CCATGGAGCA
2281 GCTGCATTTC CGCCATCTTG TCGGGGAGGT TCTTCCCGAG AAGCGCGTGA AGCAGATCGC
2341 TGCGGAGTAC GACGTGACCG ACGGTCCAAA TGATCAGGGC GAAATGTACA CGCGTCCTGG
2401 TATCCTCGGA GATGCTTTTC CCTCGCCGTA TCCGAACGAA GAAGgtaggt gcagctgctc
2461 aaatggtgaa aacagaggaa agacgctgct cgtgtcgcct gttgagtcaa ggggtcgtgc
2521 ttgcagacga tgaggctcac aatgtggact gcgtcagtgg cctcgcacgg tgtgcaaata
2581 cctcgttcgc aacgcgtcac tgtccactgt cggtcgctgt ttgtccacag CTGCGCGTTA
2641 CGCCAACGGA GGAGCCTATC CACCGGATCT TTCCCTCATC ACCGCGGCTC GCCATTTTGG
2701 TCCAGACTAT TTGATGGCTC TGCTTGGCGG CTACCGCGAC CCGCCTGAGG GTGTCGAGCT
2761 CCGACCTGGC CTGTACTGGA ATGTGTGGTT CCCCGGAAAC GCCATTGCCA TGCCTCCGCC
2821 CCTCATGGAC GAAATGATTG ACTACGAAGA TGGCACTCCC TGCAACATTT CTCAGATGgt
2881 aaggggaaaa cggggtcaag gtctgcagtg gtgggcagac gctctggtac agcgcgtcgg
2941 gcggtttcgc tttcccacca gaaaccgtca gtgtggcgtt ctgttagtga cgaggaaaga
3001 gaataccgtg aggtagacag tttcctacgg gtcgagaaaa tatgggtgca gggaacgatt
3061 cacgggcgtg gaagtcggtg cttctgcacg gacgcgtgag acaggattcg tgccactacc
3121 tggaacgcag ccagtgcgat gagcccctct gtgtgttttt gtgcgttgca gTCGAAGGAT
3181 GTTGTCAACT TTTTGACCTG GGCAACAGAG CCGACCGCGG ATGAGCGGAA ACTGTACGGG
3241 TTGAAGTGCG TGAGCGCCAT TGCGATCGGA ACTGTTTTGA TGACTCTGTG GTGGAGGTTC
3301 TACTGGGCCA TGTACGCGAC CCGCCGCATC GATTTCGGAA AGCTGAAGTA TTTGTGA
```

C. TgCYTC1 ORF(accession #:DQ228959)

```
   1 ATGGGAGGCG GCGGAGGCGG CGCGCTGAAC AAGCTGTTCC CTGGATACAA GGACAAGATC
  61 TGGATGAAAG TTCCATGGCG ACAGCAGATG ATTCAGCATT GGAACAAGTC GTACGAGAAG
 121 CAGGTGTACA GCGAGTCCGT CGCGTTGAAC AGAACTTTCC AGGCTCGCAA CCAGCTGGTT
 181 CTGGATCGTC TGAAGCCGAG CGGAGCGTAC CGTCTCCCCG CTGTGGACTA CAAGAGACAA
 241 CTCAGTCGAG GCACGCTTGT CGAGGGAGCC GACTTCTACC TCCCGACTGC TCAGGAGCAG
 301 CAACGCCTGG CTCGCCACTT CGAGCCGTAC TCCGAGCAAG AACAAGAAGA AAGGAGAAAG
 361 TTTCGCTTCC AGAGCATTTC GGTGTACTTG GCTGTCGCTC TCGGGGCCTC CTTCGTCCAC
 421 GACTACTTCT ACCAGCGTCG ACCAGTTGCG TGGTGTCTGG AGAAGGAACC CCCGCATCCT
 481 CCGAGCTACC CTTTCTGGTT CAAATCTCTC TTCCACTCTC ACGACATTCC CTCTGTCCGA
 541 CGTGGCTATG AAGTCTACCG CAAGGTGTGT GCAACATGCC ACTCCATGGA GCAGCTGCAT
 601 TTCCGCCATC TTGTCGGGGA GGTTCTTCCC GAGAAGCGCG TGAAGCAGAT CGCTGCGGAG
 661 TACGACGTGA CCGACGGTCC AAATGATCAG GGCGAAATGT ACACGCGTCC TGGTATCCTC
 721 GGAGATGCTT TTCCCTCGCC GTATCCGAAC GAAGAAGCTG CGCGTTACGC CAACGGAGGA
 781 GCCTATCCAC CGGATCTTTC CCTCATCACC GCGGCTCGCC ATTTTGGTCC AGACTATTTG
 841 ATGGCTCTGC TTGGCGGCTA CCGCGACCCG CCTGAGGGTG TCGAGCTCCG ACCTGGCCTG
 901 TACTGGAATG TGTGGTTCCC CGGAAACGCC ATTGCCATGC CTCCGCCCCT CATGGACGAA
 961 ATGATTGACT ACGAAGATGG CACTCCCTGC AACATTTCTC AGATGTCGAA GGACGTTGTC
1021 AACTTTTTGA CCTGGGCAAC AGAGCCGACC GCGGATGAGC GGAAACTGTA CGGGTTGAAG
1081 TGCGTGAGCG CCATTGCGAT CGGAACTGTT TTGATGACTC TGTGGTGGAG GTTCTACTGG
1141 GCCATGTACG CGACCCGCCG CATCGATTTC GGAAAGCTGA AGTATTTGTG A
```

C. TgCYTC1 PROTEIN(accession #: ABB17194)

```
   1 MGGGGGGALN KLFPGYKDKI WMKVPWRQQM IQHWNKSYEK QVYSESVALN RTFQARNQLV
  61 LDRLKPSGAY RLPAVDYKRQ LSRGTLVEGA DFYLPTAQEQ QRLARHFEPY SEQEQEERRK
 121 FRFQSISVYL AVALGASFVH DYFYQRRPVA WCLEKEPPHP PSYPFWFKSL FHSHDIPSVR
 181 RGYEVYRKVC ATCHSMEQLH FRHLVGEVLP EKRVKQIAAE YDVTDGPNDQ GEMYTRPGIL
 241 GDAFPSPYPN EEAARYANGG AYPPDLSLIT AARHFGPDYL MALLGGYRDP PEGVELRPGL
 301 YWNVWFPGNA IAMPPPLMDE MIDYEDGTPC NISQMSKDVV NFLTWATEPT ADERKLYGLK
 361 CVSAIAIGTV LMTLWWRFYW AMYATRRIDF GKLKYL.
```

A. TgATP SYNTHASE BETA GENE

```
   1 ATGGCGTCTC CCGCACTCCA AACTTGCTGG CGAAATCTTG CGCGCCTCTC CGGCGCGCAG
  61 GTTCGGCCGA GCCACTTTGG AGCCTTTTCT CTCGGCAGCC GCATGTCTCC TTTTTCTTCT
 121 CTCCTCGGCG CCCGCGCCTC TCCCATCGCC ACCGGCCGCG CCGGTCTCCG TTTTCTTTCT
 181 TCTGCGGCGC CGAATCCCGG AAAGAAACCA GCAAGCGCTG CCCCGCCAGC CGGAACGAAC
 241 CACGGCCGCA TCACCCAAGT CATCGGGGCC GTCGTGGACG TTCACTTCGA CGAACAACTC
 301 CCGCCAATTC TCAACAGCTT GGAAGTCCAG GGCCACACCA ACAGACTCGT CCTCGAGGTC
 361 GCGCAACACC TGGgtacgcg cgcgaagaaa ctcgcgttct gtctctggac tgtgccgttt
 421 ctgatctctc ccggcactct aatgcagaca aagaaaggcc actttgcgta atcgatgggg
 481 aagggggagt ctgaacggac tgcctcgcgc gaaaacgcct cttctgcaga catgcaggtc
 541 gatgtgtgac acagatagcc gggtcaactc caggtgtatg tacaccccgt gtaccgctgg
 601 aagcaggagt gactgcccga tattaatcga agagaatcca caaaagcgta tgactgctgc
 661 ggcgtgttcg cagttactgc gctcttcaca tgtggtgttg aatctgttcc ttctccgtgt
 721 tcaactgtac ttgctgagtt cgcagaacag agaagagtgt aacggctgaa tacacagtgg
 781 ggaggaaccg cacagcaccc ctcgcagaac gcgggaccgt ggcggtgtgt gtgtttctcg
 841 cctacatgct cgctgttcgt tttctttcgg aaactggagt gtccgaaaag gcgtttaagc
 901 gggctaacgt aggcctgaac ggtgtctctt aatctgatgt tcgatgctgc acaatggata
 961 ggggagaaat gcacaagtgc gtgggtgccg cgcagagcac ctggtcggct cgatctggaa
1021 ctcgggtgag gcttcgaaac gccaccaggg ctgttccgtc tgtgtataca gtggcgattg
1081 gattctgtac accacatcgt ctcgttttcc ctgagtgggc ggctgtttcg cagGCGAAAA
1141 CACGGTCAGG ACGATTGCGA TGGACGCGAC GGAGGGTCTC GTACGCGGAC AGAAAGTCGT
1201 CGACACAGGT GCTCCCATTC AGgtgaggcc gcgcggaagt cgtttccagt tcctcagctg
1261 aggaagaagc gaagaggaac gcacagcaga gtcttctttt ggtgtctcag cagctctgac
1321 gagcaactcc cttgtgcctt ttcccgatgc cccgacaggg cgtagacctg cctgccagct
1381 tccccggaag ctccaacgcg tccagaaggg aaggcaccgt tggcctcgag aggtgtacgc
1441 actcaccgcg gattattcga gtatctatgt gtggcgagtg ttgagacatg tggtgtcgtt
1501 tctgcactgt gggccgaaac agacgcagca gcgattccat ttttctcctc tgctctgttc
1561 gtccgccctc ctctgggctt tgcagGTGCC CGTCGGCGTT GAGACTCTGG GACGCATCAT
1621 GAACGTCATT GGAGAGCCTG TGGATGAGTG TGGACCGGTT CCTGCGAAGA AGACCTACTC
1681 GATTCACCGC GCAGCTCCAC TCTTTGCCGA CCAGTCGACG GAACCCGgtg cgtcttccta
1741 tgttgcatat ttgtcgagaa gggaagagtt tcgcgtgtcg cacgggagac gcagtgtctg
1801 caggtcggcg ggagttcttt tctgccgggg aggtgaacgc gccgcatcgc atgctttcag
1861 cgttgctccg tggcgcaggc acagatcgcg aggttgtggg gaaaaggggg gggaggggggg
1921 gctcgttttt ctcacttctc gcggcggcgc gttctgtctg cagcgtgagg tgtcgtgggt
1981 tgcagggtaa gcctttcgct gtttgcttcg ttgtttcagG CCTTCTGCAG ACCGGCATCA
2041 AGGTCGTGGA TCTCCTGGCG CCGTACGCGA AGGGAGGGAA AATCGGTCTC TTTGGCGGTG
2101 CAGGAGTCGG CAAGACTGTG TTGATTATGG AGTTGATCAA CAACGTCGCG AACAAGCACG
2161 GAGGTTTCTC TGTGTTTGCG GGTGTCGGCG AGAGGACGCG CGAGGGGAAC GACTTGTACC
2221 ACGAAATGAT GACGACTGGA GTGATTAAGC GGAAGAAGTT GGAAGATGGC AAGTTCGACT
2281 TCACCGGCTC CAAGGCTGCG CTGGTGTACG GACAGATGAA CGAGCCTCCC GGTGCGCGTG
2341 CGCGCGTCGC CCTGACTGCT CTGTCTGTCG CCGAGTACTT CCGTGACGAG CAAGGCCAAG
2401 ACGTGTTGCT TTTCATCGAC AACATCTACC GCTTCACTCA AGCTGGCTCT GAGGTGTCTG
2461 CGTTGCTCGG ACGCATTCCC AGCGCCGTCG GATACCAGCC GACGCTGGCG ACCGATCTCG
2521 GGCAGCTCCA GGAGCGAATC ACCACGACGA AGAAGGGATC GATTACCTCC GTCCAGGCCG
2581 TCTACGTACC TGCAGATGAC TTGACGGATC CTGCGCCCGC GACAACTTTT GCTCATCTCG
2641 ACGCCACCAC TGTGCTGTCT CGGCAGATCG CCGAGCTGGG GATTTATCCC GCCGTCGACC
2701 CGCTCGACTC GACGAGTCGC ATGCTGGCTC CGGAGATCGT CGGTCAGGAG CACTACGACA
2761 CGGCCCGCGC GACTCAGAAG CTGCTCCAGG ACTACAAGTC CCTGCAGGAT ATCATCGCCA
2821 TTCTGGGAAT GGACGAGCTG AGTGAGGAAG ACAAGCTGGT CGTCTCGCGC GCGCGCAAGA
2881 TCCAGAGATT CCTCTCCCAG CCGTTCACAG TCGCGGAAGT CTTCACGGGC AAACCCGGAC
2941 GCTTCGTCGA GCTGCCCGAA ACCATCAAGA GCGCGCAGAC GATCCTCCGA GGCGAGTGCG
3001 ACGACTTGCC CGAAATGGCT TTCTACATGT GCGGCGGCCT CGAGGAAGTG CGTTCGAAGG
3061 CCGTGAAGAT GGCGCAGGAA GCGGCGAGCG GAAAGTAG
```

B. TgATP SYNTHASE BETA ORF(accession #: DQ228960)

```
   1 ATGGCGTCTC CCGCACTCCA AACTTGCTGG CGAAACCTTG CGCGCCTCTC CGGCGCGCAG
  61 GTTCGGCCGA GCCACTTCGG AGCCTTTTCT CTCGGCAGCC GCATGTCTCC TTTTTCTTCT
 121 CTCCTCGGCG CCCGCGCCTC TCCCATCGCC ACCGGCCGCG CCGGTCTCCG TTTTCTTTCT
 181 TCTGCGGCGC CGAATCCCGG AAAGAAACCA GCAAGCGCTG CCCCGCCAGC CGGAACGAAC
 241 CACGGCCGCA TCACCCAAGT CATCGGGGCC GTCGTGGACG TTCACTTCGA CGAACAACTC
 301 CCGCCAATTC TCAACAGCTT GGAAGTCCAG GGCCACACCA ACAGACTCGT CCTCGAGGTC
 361 GCGCAACACC TGGGCGAAAA CACGGTCAGG ACGATTGCGA TGGACGCGAC GGAGGGTCTC
 421 GTACGCGGAC AGAAAGTCGT CGACACAGGT GCTCCCATTC AGGTGCCCGT CGGCGTTGAG
 481 ACTCTGGGAC GCATCATGAA CGTCATTGGA GAGCCTGTGG ATGAGTGTGG ACCGGTTCCT
 541 GCGAAGAAGA CCTACTCGAT TCACCGCGCA GCTCCACTCT TTGCCGACCA GTCGACGGAA
```

```
 601 CCCGGCCTTC TGCAGACCGG CATCAAGGTC GTGGATCTCC TGGCGCCGTA CGCGAAGGGA
 661 GGGAAAATCG GTCTCTTTGG CGGTGCAGGA GTCGGCAAGA CTGTGTTGAT TATGGAGTTG
 721 ATCAACAACG TCGCGAACAA GCACGGAGGT TTCTCTGTGT TTGCGGGTGT CGGCGAGAGG
 781 ACGCGCGAGG GGAACGACTT GTACCACGAA ATGATGACGA CTGGAGTGAT TAAGCGGAAG
 841 AAGTTGGAAG ATGGCAAGTT CGACTTCACC GGCTCCAAGG CTGCGCTGGT GTACGGACAG
 901 ATGAACGAGC CTCCCGGTGC GCGTGCGCGC GTCGCCCTGA CTGCTCTGTC TGTCGCCGAG
 961 TACTTCCGTG ACGAGCAAGG CCAAGACGTG TTGCTTTTCA TCGACAACAT CTACCGCTTC
1021 ACTCAAGCTG GCTCCGAGGT GTCTGCGTTG CTCGGACGCA TTCCCAGCGC CGTCGGATAC
1081 CAGCCGACGC TGGCGACCGA TCTCGGGCAG CTCCAGGAGC GAATCACCAC AACGAAGAAG
1141 GGATCGATTA CCTCCGTCCA GGCCGTCTAC GTACCTGCAG ATGACTTGAC GGATCCTGCG
1201 CCCGCGACAA CTTTTGCCCA TCTCGACGCC ACCACTGTGC TGTCTCGGCA GATCGCCGAG
1261 CTGGGGATCT ATCCCGCCGT CGACCCGCTC GACTCGACGA GTCGCATGCT GGCTCCGGAG
1321 ATCGTCGGTC AGGAGCACTA CGACACGGCC CGCGCGACTC AGAAGCTGCT CCAGGACTAC
1381 AAGTCCCTGC AGGATATCAT CGCCATTCTG GGAATGGACG AGCTGAGTGA GGAAGACAAG
1441 CTGGTCGTCT CGCGCGCGCG CAAGATCCAG AGATTTCTCT CCCAGCCGTT CACAGTCGCG
1501 GAAGTCTTCA CGGGCAAACC CGGACGCTTC GTCGAGCTGC CCGAAACCAT CAAGAGCGCG
1561 CAGACGATCC TCCGAGGCGA GTGCGACGAC TTGCCCGAAA TGGCTTTCTA CATGTGCGGC
1621 GGCCTCGAGG AAGTGCGTTC GAAGGCCGTG AAGATGGCGC AGGAAGCGGC GAGCGGAAAG
1681 TAG
```

C. TgATP SYNTHASE PROTEIN(accession #: ABB17195)

```
   1 MASPALQTCW RNLARLSGAQ VRPSHFGAFS LGSRMSPFSS LLGARASPIA TGRAGLRFLS
  61 SAAPNPGKKP ASAAPPAGTN HGRITQVIGA VVDVHFDEQL PPILNSLEVQ GHTNRLVLEV
 121 AQHLGENTVR TIAMDATEGL VRGQKVVDTG APIQVPVGVE TLGRIMNVIG EPVDECGPVP
 181 AKKTYSIHRA APLFADQSTE PGLLQTGIKV VDLLAPYAKG GKIGLFGGAG VGKTVLIMEL
 241 INNVANKHGG FSVFAGVGER TREGNDLYHE MMTTGVIKRK KLEDGKFDFT GSKAALVYGQ
 301 MNEPPGARAR VALTALSVAE YFRDEQGQDV LLFIDNIYRF TQAGSEVSAL LGRIPSAVGY
 361 QPTLATDLGQ LQERITTTKK GSITSVQAVY VPADDLTDPA PATTFAHLDA TTVLSRQIAE
 421 LGIYPAVDPL DSTSRMLAPE IVGQEHYDTA RATQKLLQDY KSLQDIIAIL GMDELSEEDK
 481 LVVSRARKIQ RFLSQPFTVA EVFTGKPGRF VELPETIKSA QTILRGECDD LPEMAFYMCG
 541 GLEEVRSKAV KMAQEAASGK .
```

Figure 6.5: DNA and protein sequences of TgNDH2-I, TgNDH2-II, TgSDH-Fp, TgCYTC1, and TgATP-β. (A). Complete DNA sequence of the gene from ToxoDB with putative coding sequences (exons) in capital letters, and non coding sequences (introns) with small letters. **(B).** The open reading frame (ORF) which is confirmed sequencing cDNA from tachyzoites of RH strain, introns are spliced out. **(C).** The translated amino acid sequence of the open reading frame (ORF).

Department of Med. Microbiology
Faculty of Medicine,
Georg August University,
Kreuzbergring 57,
D-37075 Goettingen
Germany.
E-mail: asaleh@gwdg.de
ahmadsaida@yahoo.com

AHMAD M. SALEH

Curriculum Vitae

➢ PERSONAL INFORMATION

First Name: **Ahmad** **Family Name: Saleh** **Middle Names**: Mahmoud Hasan

Gender: Male **Marital Status**: Single **Age**: 32 years

Birth Date: 04/05/1974 **Birth Place**: Saida - Palestine **Nationality**: Palestinian

➢ EDUCATIONAL BACKGROUND

Degree	Subject	G.P.A	Note	Rank	Date	University /Country
Doctorate (PhD)	**Molecular Microbiology**		**Very Good**		**2006**	**Georg August University, Germany**
Master (M.Sc.)	**Biological Sciences**	**83.2%**	**Very Good**	**Second**	**2001**	**An-Najah N. University; Palestine**
Bachelor (B.Sc.)	**Biological Sciences**	**81.8%**	**Very Good**	**First**	**1997**	**Yarmouk University; Irbid-Jordan**
Tawjihi (H.S.S.C)	**Scientific Stream**	**88.4%**	**Very Good**		**1992**	**Attil Sec. School, Palestine.**

- **Nov. 2006**: PhD of molecular and biochemical microbiology; Georg August University of Goettingen, Germany.
- **June 2001**: M.Sc. of Biological Sciences; An-Najah National University, Nablus -Palestine.
- **July 1997**: B.Sc. of Biological Scinces; Yarmouk University, Irbid-Jordan.
- **July 1992**: High Secondary School Certificate (Tawjihi), Scientific stream; Attil Secondary Boys School, Palestine.

➢ SCHOLARSHIPS & AWARDS

- A full scholarship awarded by the "Deutscher Akademischer Austausch Dienst" –DAAD- (German Academic Exchange Service - Bonn) covering living and tuition expenses in Germany for 4 years (**2002-2006**).
- Annual honor awards from Yarmouk University (**1993-1997**).
- Patent for a new anti-malarial and anti-toxoplasmosis drug (HDQ) registered at Georg August University of Goettingen, Germany.

➢ WORK EXPERIENCE

. **1996-1997**: Research assistant in molecular microbiology research laboratory in Biology Detp.,Yarmouk University.

- **1997-2002**: Biology teacher for secondary classes at Al-Islamiya Secondary School in Nablus–Palestine.
- **2003-2006**: Scientific research on *T. gondii* energy metabolism in the laboratory of Prof. Dr. med. Uwe Gross.

➢ RESEARCH PROJECTS

- **B.Sc.-Research project-** Genotyping of *E. coli* by different molecular biological techniques including PCR, PFGE, and RFLP.
- **M.S. -Thesis-** Molecular Typing of Methicillin Resistant *Stahylococcus au*reus (MRSA) by Antibiogram, RS-PCR and AP-PCR.
- **PhD -Thesis-** Characterization of alternative NADH dehydrogenases in the respiratory chain of *Toxoplasma gondii* as a novel drug targets.

➢ TECHNICAL SKILLS

Computer Software: Windows XP, Microsoft Office (Word, Excell, Power Point, Access, …), Statistical analysis software (SPSS, PRISMA), Bioinformatics (PHYLIP, DNAstar, Clone manager, Enhance, multiple alignments software), and Internet.

Research : Cloning, DNA sequencing, recombinant protein expression and purification, immunization and antibody production; western, southern and northern blotting; transfection, culturing of *T .gondii* and *P. falciparum*, immunofluorescence staining and microscopy, anti-sense RNA, RT-PCR, quantitative gene expression analysis by light cycler (real time RT-PCR), enzymatic activity analysis, drug inhibition assays, and other related molecular biology techniques.

➢ WORKSHOPS & CONFERENCES

- Workshop of molecular biology at Bethlehem University organized by UNESCO, October 2000.
- International congress of the DGHM and VAAM, 25-28.09.2005 Goettingen, Germany. (Abstract +Poster)
- International conference of Candidosis (IFOCAN 2005), 23-25.09.2005 Goettingen, Germany.
- 2nd Short course of young parasitologists, 19-22 March 2006, Vienna, Austria. (Abstract + Oral Presentation)
- International congress of parasitology, 23-25 March 2006, Vienna, Austria. (Abstract + Poster)
- Cost Action 857 3rd annual workshop: Apicomplexan biology in post-genomic era,17-20 May 2006, Dresden, Germany (Abstract + Poster)

➢ PUBLICATIONS

- **Saleh A. et al**, Typing of methycillin resistant *Staphylococcus aureus* by ribosome spacer and arbitrarily primed PCR, Turk J Med Sci, 34 (2004) 5-9. (published)
- **Saleh A., Friesen J., Gross U., Bohne W.,** (2006) Growth inhibition of *Toxoplasma gondii* and *Palsmodium falciparum* by nanomolar oncentrations of HDQ (1-hydroxy-2-dodecyl-4(1H)quinolone): High affinity inhobitor of alternative (type II) NADH dehydrogenases. *Anti. Microb. Chem.* (In press, accepted)
- **Saleh A., Gross U., Bohne W.,** Identification of two isoforms of alternative (type II) NADH dehydrogenases in *Toxoplasma gondii*. *Mol. Biochem. Parasit.* (In press, submitted)
- **Fleige T*., Saleh A.*, Gross U., Bohne W.,** Localization and quantitative steady state mRNA level expression analysis of glycolysis, TCA cycle and respiratory chain components of *Toxoplasma gondii* (in preparation).

➢ LANGUAGES

- **Arabic** : native language
- **English** : Speaking , reading, writing and understanding very good. (TOEFL score: 550; 213 computer based test).
- **German** : 6 months of intensive courses and DSH certificate (very good).