Fungal DAHP Synthases:

Evolution and Structure of Differently Regulated Isoenzymes

Dissertation
zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultäten
der Georg-August-Universität zu Göttingen

vorgelegt von
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aus Nürnberg

Göttingen 2001

D7

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Tag der mündlichen Prüfung: 29.01.2002

MUTATION

IT IS THE KEY TO OUR EVOLUTION.

IT HAS ENABLED US TO EVOLVE FROM A SINGLE CELL ORGANISM INTO THE DOMINANT SPECIES ON THE PLANET. THIS PROCESS IS SLOW AND NORMALLY TAKING THOUSANDS AND THOUSANDS OF YEARS.

BUT EVERY FEW HUNDRED MILLENNIA EVOLUTION LEAPS FORWARD.

PROFESSOR XAVIER

Mein Dank gilt an erster Stelle Herrn Prof. Dr. G.H. Braus, der durch sein Interesse, seine Anstrengungen und Diskussionen, sowie durch das angenehme Arbeitsklima das Entstehen dieser Arbeit maßgeblich gefördert hat.

Ebenso möchte ich mich bei Herrn Dr. H.U. Mösch und Herrn Dr. E. Kübler für die konstruktive Hilfe in schwierigen Phasen der Arbeit bedanken.

Weiter möchte ich mich bei der Labor Crew von 148 für das äußerst humorvolle und angenehme Arbeitsklima bedanken. Der Dank gilt natürlich auch dem Rest der Hefe, bzw. Aspergillus Gruppe.

Mein besonderer Dank gilt Frau Gabriele Heinrich, die durch ihre sorgfältige Arbeit nicht nur eine wertvolle Laborkollegin war, sondern auch durch ihr soziales Engagement wesentlich zum tollen Arbeitsklima beigetragen hat. Vielen Dank !

Zum Schluss möchte ich mich bei meiner Familie bedanken ohne deren Unterstützung diese Arbeit nicht möglich gewesen wäre.

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Summary

The first step of the biosynthesis of the aromatic amino acids is the condensation of erythrose-4-phosphate and phosphoenolpyruvate to 3-deoxy-D-arabino-heptulosonate 7-phosphate. This reaction is catalyzed by the DAHP synthase (EC 4.1.2.15). There is an isoenzymatic system with two or three enzymes, dependant on the organism, which are either unregulated or inhibited by the end products phenylalanine, tyrosine or tryptophan.

The yeast *Saccharomyces cerevisiae* carries two DAHP synthases, which are inhibitable by phenylalanine or tyrosine. Beside these two regulatory mechanisms *E. coli* carries a third isoenzyme which is inhibitable by tryptophan. The heterologous expression of this *E. coli* enzyme in yeast is stable. The gene product is able to rescue a *Δgcn4* yeast strain which is not able to activate its general control when inhibited by high amounts of tyrosine and phenylalanine. Random mutagenesis as well as site directed mutagenesis experiments showed that residues are involved in the regulation of the tryptophan-inhibitable DAHP synthase which are not important for regulation of the two other isoenzymes from *E. coli*.

In the filamentous growing fungus *Aspergillus nidulans* two DAHP synthases were identified and characterized. The two new isoenzymes are inhibitable by phenylalanine or tyrosine and carry a bivalent metal ion as cofactor. In contrast to the all known DAHP synthases both *A. nidulans* isoenzymes are not completely inhibitable by EDTA. Between these two DAHP synthases there a fine-tuning in regulation exists, which enables the fungus to control the carbon-flux into the shikimate pathway in a very sensitive way.

To understand the function of the DAHP synthase in a more detailed way the protein structure of the tyrosine-inhibitable isoenzyme of the yeast *Saccharomyces cerevisiae* was solved. It was found to be a TIM barrel fold with some extra parts. Various mutagenesis experiments revealed that these extra parts are crucial for the regulation of this isoenzyme. To exchange the regulation properties of both yeast isoenzymes it needs only one amino acid, glycine 226 (serine in the respectively isoenzyme).

Zusammenfassung

Der erste Schritt der aromatischen Aminosäurebiosynthese ist die Kondensation von Erythrose-4-Phosphat und Phosphoenolpyruvat zu 3-Desoxy-D-*arabino*-Heptulosonat 7-Phosphat. Diese Reaktion wird durch die DAHP Synthase (EC 4.1.2.15) katalysiert. Abhängig vom Organismus handelt es sich hierbei um ein Isoenzym-System von zwei bis drei Enzymen, die entweder unreguliert oder durch jeweils ein Endprodukt, Phenylalanin, Tyrosin oder Tryptophan negativ reguliert werden.

Die Hefe *Saccharomyces cerevisiae* besitzt zwei DAHP Synthasen, die durch Tyrosin oder Phenylalanin inhibierbar sind. Im Gegensatz dazu besitzt *E. coli* neben diesen beiden Regulationsarten noch ein drittes Isoenzym, welches Tryptophan-regulierbar ist. Dieses *E. coli* Enzym kann stabil heterolog in Hefe exprimiert werden. Das Genprodukt kann einen Hefestamm retten, der bei Inhibierung der eigenen DAHP Synthasen durch Tyrosin und Phenylalanin die allgemeine Kontrolle der aromatischen Biosynthese nicht mehr einschalten kann (Δgcn4). Zufallsmutagenesen und zielgerichtete Mutagense haben gezeigt, dass bei der Regulation der Tryptophan-inhibierbaren DAHP Synthase Aminosäurereste eine Rolle spielen, die bei den anderen beiden DAHP Synthasen nicht an der Inhibierung beteiligt sind.

Im filamentös wachsenden Pilz *Aspergillus nidulans* wurden zwei DAHP Synthasen identifiziert und charakterisiert. Die beiden neuen Isoenzyme werden durch Tyrosin oder Phenylalanin inhibiert und tragen ein zweiwertiges Metallatom als Kofaktor. Beide Enzyme lassen sich im Gegensatz zu den bisher bekannten DAHP Synthasen nicht vollständig durch EDTA inhibieren. Zwischen beiden Isoenzymen besteht ein Feinregulierungssystem, die es dem Pilz ermöglicht den Kohlenstofffluss in den Shikimat-Weg exakt zu kontrollieren.

Um die Funktionsweise der DAHP Synthase genauer verstehen zu können, wurde die Proteinstruktur der Tyrosin-inhibierbaren DAHP Synthase aus der Hefe *Saccharomyces cerevisiae* bestimmt. Hierbei handelt es sich um einen TIM barrel Fold mit einigen zusätzlichen Bestandteilen. Durch verschiedene Mutgenese-Techniken wurde festgestellt, dass diese zusätzlichen Teile der Struktur wesentlich an der Regulierung des Enzyms beteiligt sind. Um die Regulation zwischen Tyrosin und Phenylalanin auszutauschen bedarf es den Austausch einer einzigen Aminosäure, Glycin 226 (Serin im entsprechenden Isoenzym).

Abbreviations

APS	ammoniumpersulfate
bp	base pair
BSA	bovine serum albumin
DAHP	3-Deoxy-D-*arabino*-heptulosonate 7-phosphate
DAHPS	DAHP synthase
DTT	dithiothreitole
dNTPs	desoxy nucleotide triphosphates
EDTA	ethylene diamine tetraacetate
EtOH	ethanol
kDa	kilo Dalton
LB	Luria Bertani
LiOAc	lithium acetate
MCS	multi cloning site
MV	minimal vitamins
NaOAc	sodium acetate
OD	optical density
PAGE	polyacrylamide gelelectrophoresis
PEG	polyethylene glycole
PCR	polymerase chain reaction
PMSF	phenylmethanesulfonyleflouride
Rnase	ribonuclease
SC	synthetic complete
SDS	sodium dodecyl sulfate
TEMED	N,N,N',N' tetramethylethylenediamine
U	enzymatic Unit
wt	wild type
YEPD	yeast extract / peptone / dextrose

Chapter I

Introduction

1. Biosynthesis of Aromatic Amino Acids

Most microorganisms and plants are competent to synthesize *de novo* the three aromatic amino acids phenylalanine, tyrosine and tryptophan. Animals lack this capability and have to take up these compounds with their diet (Haslam 1974).

The aromatic amino acids are energetically the most costly amino acids for the living cell. Seventy-eight moles of ATP are required to synthesize one mole of tryptophan; the respective values for phenylalanine and tyrosine are 65 and 62 moles of ATP. On the average this is approximately twice the energy required for any other amino acid (Atkinson 1977). An outline of the biosynthetic pathway from carbohydrate through chorismate to the aromatic amino acids is given in Figure 1.

Shikimic acid was first described as a natural product from the plant *Illicium religiosum* by Eykmann in 1885, and it was from the Japanese name of this plant, shikimi-no-ki, that the name shikimic acid was derived (Haslam 1974). In the early 1950s, however, it was revealed that shikimic acid was an obligatory intermediate in the pathway from carbohydrate to the aromatic amino acids (Davis 1955, Sprinson 1961).

The seven enzyme-catalyzed reactions of the shikimate pathway from erythrose-4-phosphate and phosphoenolpyruvate to chorismic acid are common for all aromatic

amino acids. The series of reactions is invariable in all eukaryotic and prokaryotic organisms studied so far.

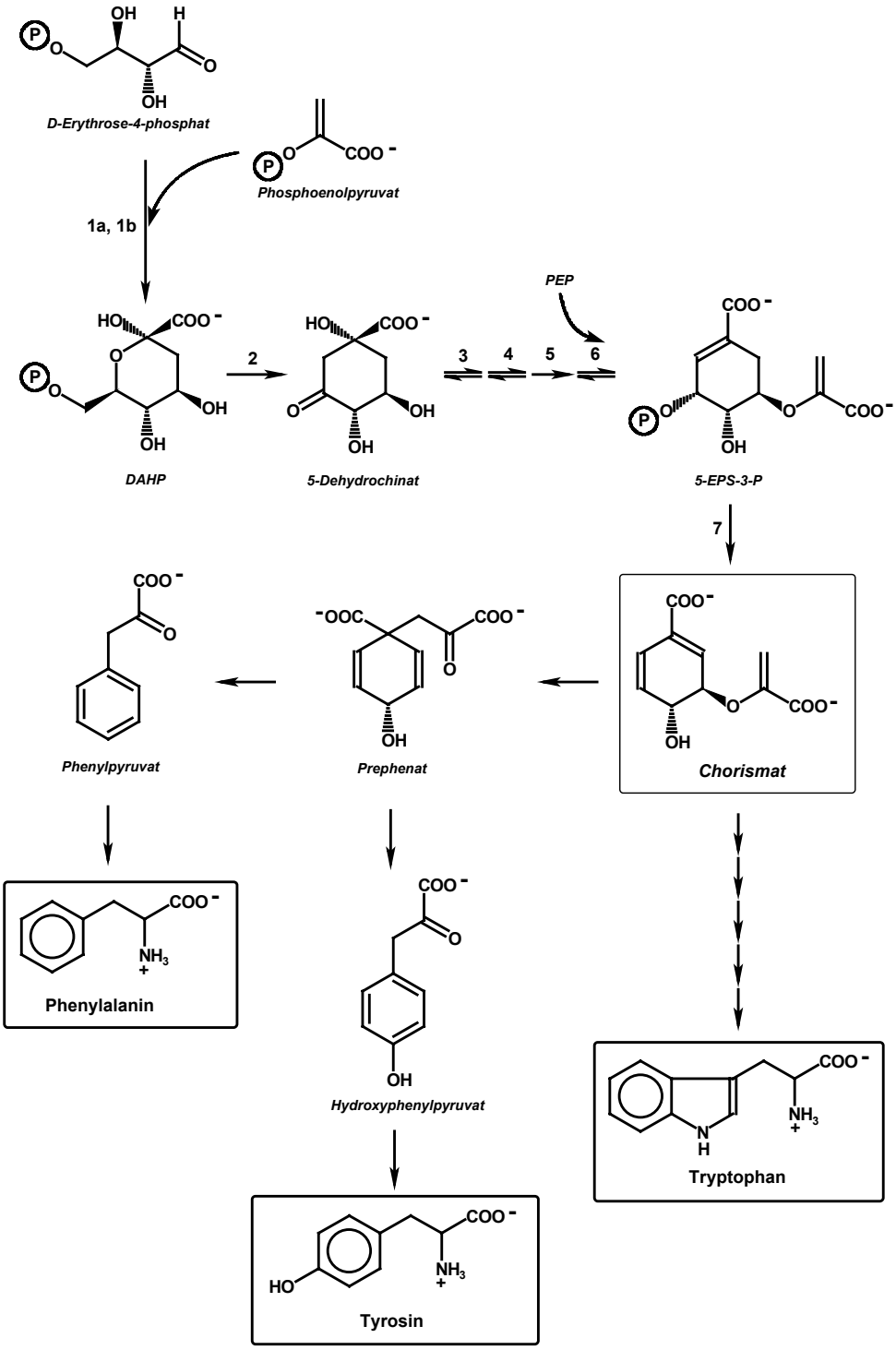

Fig. 1 Biosynthesis of aromatic amino acids (main pathway). 1a/1b DAHP Synthase (EC 4.1.2.15) **2** 3-Dehydroquinate Synthase (EC 4.6.1.3) **3** 3-Dehydroquinate-Dehydratase (EC 4.2.1.10) **4** Shikimate Dehydrogenase (EC 1.1.1.25) **5** Shikimate Kinase (EC 2.7.1.71) **6** 5-Enole-pyruvyleshikimate-3-phosphate Synthase (EC 2.5..1.19) **7** Chorismate Synthase (EC 4.6.1.4).

The absence of the shikimate pathway in animals is one of the metabolic differences between plants and animals. The aromatic amino acid pathway can therefore serve as a target for the action of herbicides. Glyphosate (N-phosphomethyl-glycine) is widely used as a non-selective herbicide, and beyond, is an inhibitor of microbial growth (LaRossa and Falco 1984). The finding that glyphosate is antagonized by one or more of the aromatic amino acids is true for many organisms including prokaryotes, algae and plants (Gresshoff 1979). Enzymological analysis has shown that glyphosate interferes with the enzyme EPSP synthase, catalyzing the 6th step of the shikimate pathway (Amrhein et al. 1980).

The specialized parts of the aromatic amino acids pathway proceed from chorismic acid either in five steps to tryptophan or in three steps to tyrosine and phenylalanine. Two separate routes to phenylalanine and tyrosine exist. Thus phenylalanine may be formed from arogenate or from phenylpyruvate, whereas tyrosine synthesis may proceed from either arogenate or 4-hydroxyphenylpyruvate (Herrmann and Somerville 1983). Tryptophan biosynthesis has become one of the best-studied examples of a biosynthetic pathway and has contributed to the understanding of topics such as control of flow through a pathway, enzyme structure, gene-enzyme relationships, molecular cloning and transcriptional and translational control in diverse prokaryotic and eukaryotic microorganisms (Crawford 1975, Yanofsky 1984, Hütter et al. 1986).

2. The Regulation of Biosynthesis of Aromatic Amino Acids

The biosynthesis of the aromatic amino acids is subject to a strict regulation. There are two ways of regulation. One being that the rate of enzyme synthesis can be modulated during transcription or translation and the other being the modulation of

the enzyme activity. Both mechanisms have been found in the amino acid biosynthetic pathways of various organisms.

2.1 Transcriptional Regulation and Attenuation in *E.coli*

The tryptophan biosynthetic genes in *E. coli* are arranged in a transcriptional unit, the trp-operon. These genes encode enzymes which catalyze the steps from chorismate to tryptophan. The compact arrangement allows for a contemporary regulation of transcription by repression and attenuation at the same time (Pittard 1996). The trp-operon is part of the trp-regulon, which is controlled by the trp-repressor.

The repressor-protein TrpR changes its conformation and can bind onto specific operator-sequences within the promoter when tryptophan is connected. However the binding areas for the RNA polymerase and the repressor-complex overlap, which means that the transcription initiation is inhibited by the repressor linkage on the operator.

An additional regulation mechanism of the trp-operon could be established in *E. coli* and relative bacteria and was named attenuation (Yanofsky 1984). The RNA polymerase must first overcome a 162 nucleotide long 5´untranslated area (leader region) before the transcription of the first structure gene of the trp-operon can start. Transcription can be terminated within the leader region under non-starvation conditions when sufficient amount of tryptophan is present. The first part of the leader consists of two tryptophan codons as well as the actual attenuation sequence. This is a GC-palindrome, which can form a hair-needle structure followed by a sequence with eight uridine residues. The mRNA can form two different secondary structure by building different base pairs. The structure which will be build depends on how long the ribosome needs to rest on the two tryptophan-codons. If the cell contains suffi-

cient amounts of loaded $tRNA^{Trp}$, which signalizes a high level of tryptophan in the cell, then a secondary structure in the mRNA called the termination loop is build. This leads to a disconnection of the RNA polymerase from the DNA strand. The transcription within the leader region is discontinued after the synthesis of a 141 nucleotide transcript. However if there is a high concentration of unloaded $tRNA^{Trp}$, then the ribosome has to pause at the two tryptophan codons before it can integrate the two amino acids into the polypeptide strand. This allows for an alternative secondary structure to be built which does not have a terminating function. Subsequently a poycistronic mRNA with the length of about 7000 nucleotides is synthesized. The alternative structure cannot be build when the tryptophan concentration is sufficient, because the required mRNA sequence is already protected within the ribosome at this point. However, the ribosome will pause when there is a shortage of tryptophan so the sequence will stay outside the ribosome for a longer period of time and will consequently hinder a termination loop from being build. This type of regulation is only possible in prokaryotes because transcription and translation are not separated into different components.

Attenuation modulates the transcription rate about ten times. The derepression increases the transcription rate about seventy times. Together these effects can increase the transcription rate about 700 times depending on the tryptophan concentration (Lewin 1994). Attenuation is not only used in tryptophan biosynthesis but also in the regulation of the transcription of biosynthetic genes for the amino acids such as histidine, phenylalanine, leucine, threonine and isoleucine.

2.2 The General Control of the Amino Acid Biosynthetic Pathway in Fungi

The biosynthesis of the aromatic amino acids in *S. cerevisiae* is controlled by the so-called general control (Hinnebusch 1988). Under amino acid starvation conditions the expression of at least 539 structure genes will be enhanced, which code for enzymes of 12 amino acid biosynthetic pathways (Natarajan et al. 2001). A lack of at least one of the concerned amino acids can cause the general control to be switched on (Wolfner et al. 1975, Niederberger et al. 1981).

The transcription activator Gcn4p in baker's yeast regulates the expression of nine of the 12 enzymes of the aromatic amino acid biosynthesis when there is a lack of amino acids (Braus 1991). Gcn4p binds onto a short UAS element with the consensus sequence 5'-TGACTC-3' and causes an increase in transcription of the concerned genes (Hinnebusch 1988).

Translation of the *GCN4*-mRNA will only take place on a very narrow level under optimal growth conditions. The reason for this is an unusual untranslated 5' region in the *GCN4*-mRNA which is situated 150-360 base-pairs in front of the *GCN4* start codon. Within this region there are four short open reading frames which code for only two to three amino acids (Mueller and Hinnebusch 1986). These open reading frames are also referred to as uORFs ("upstream open reading frames"). They inhibit the translation of the *GCN4*-mRNA when there is enough amino acid supply by interrupting the scanning process of the 43S-preinitiation complex. The 43S-complex binds onto the mRNA close to the CAP region which leads to the formation of an 80S ribosomal complex on the start codon of the first uORF. This 80S-complex will fall apart when reaching the stop codon of the same uORF.

A lack of amino acids is primarily detected by the Gcn2p which binds onto unloaded tRNAs (Lanker et al. 1992). The bondage leads to an activation of the kinase-domain

of Gcn2p. This interferes with the equilibrium between eIF2-GDP and eIF2-GTP by phosphorylizing the α-subunit of eIF2 and therefore decreases the rate in which the 43S-preinitiation complex is built. This means that the 40S ribosomal subunit reads over uORF2 up to uORF4. The next 43S-preinitiation-complex is built once the region between uORF4 and the AUG of the *GCN4* reading frame has been reached. The newly formed complex initiates the building of the 80S-ribosome which in turn leads to the translation of the *GCN4* ORF.

2.3 Regulation of Enzyme Activity of Aromatic Amino Acid Biosynthesis

Besides the control during transcription and translation there is a control which affects enzyme activity directly through feedback-regulation by endproducts of this biosynthetic pathway. The most important targets for this kind of regulation are the enzymes of the first branchpoint, where chorismate mutase competes with anthranilate synthase for the common substrate chorismate and DAHP synthase, which catalyzes the first step of the biosynthesis of aromatic amino acids.

2.3.1 Chorismate Mutase / Anthranilate Synthase Branchpoint

In the yeast *Saccharomyces cerevisiae*, the distribution of chorismate towards phenylalanine, tyrosine and tryptophan production is feedback regulated by end-products of the biosynthetic pathway. The reaction of chorismate to anthranilate, which is catalyzed by the heterodimeric anthranilate synthase, is regulated by allosteric feedback inhibition through tryptophan (Schürch et al. 1974, Miozzari et al. 1978, Graf et al. 1993). Tryptophan activates the chorismate mutase of yeast which is inhibited by one of its end-products, tyrosine (Schmidheini et al. 1989, Schmidheini

et al. 1990). The chorismate mutase of *Arabidopsis thaliana* is activated by tryptophan and feedback-inhibited by tyrosine and phenylalanine (Eberhard et al. 1993). *E. coli* possesses two chorismate mutases which are linked with prephenate dehydrogenase and prephenate dehydratase activities, respectively (Koch et al. 1971, Davidson et al. 1972, Dopheide et al. 1972). The chorismate mutase-prephenate dehydrogenase and chorismate mutase-dehydratase of *E. coli* are feedback-inhibited by tyrosine and phenylalanine, respectively, with most significant inhibition being exerted on the second activity. The activity of anthranilate synthase in *E. coli* is inhibited by tryptophan (Gibson and Gibson 1964, Ito and Crawford 1965).

2.3.2 DAHP Synthase Isoenzymes

Two isoenzymes have been identified in *Saccharomyces cerevisiae* which can be inhibited by two different endproducts of the aromatic amino acid biosynthesis. The *ARO3* encoded DAHP synthase is inhibited by phenylalanine and the *ARO4* isoenzyme is strongly inhibited by tyrosine. An isoenzyme which is inhibited by tryptophan is not present in baker's yeast (Teshiba et al. 1986, Paravicini et al. 1989a,b, Mewes et al. 1997). An auxotrophy for aromatic amino acids will only occur when the genes for both isoenzymes have been mutated (Künzler et al. 1992).

The amino acid sequence of Aro3p and Aro4p shows similarity of 75% of which 225 of the 370 amino acids are identical (Braus 1991). There is also a high similarity of 65% to 71% to the DAHP synthases from *E. coli* (Davies and Davidson 1982, Shultz et al. 1984, Zurawski et al. 1981).

The number of isoenzymes found in other organisms varies as well as the type of effectors which modulates enzyme activity. There are three isoenzymes in *E. coli* of which each one becomes inhibited by one aromatic amino acid (Brown and Doy

1966). The three isoenzymes AroF (tyr-inhibited), AroG (phe-inhibited) and AroH (trp-inhibited) contribute differently to the DAHPS total activity in *E. coli*. AroF contributes 20% of the activity, AroG almost 80% and AroH barely 1% to the total activity (Tribe et al. 1976). AroF and AroG are inhibited to more than 95% by their effectors tyrosine and phenylalanine, respectively (Herrmann 1983), whereas AroH becomes inhibited by tryptophan to maximum 56% to 70% (Pittard et al. 1969, Camakaris and Pittard 1974, Ray and Bauerle 1991). *Neurospora crassa* also possesses three DAHP synthases, which are individually inhibited by one of the aromatic amino acids (Nimmo and Coggins 1981). There is a similar situation in the fungi *Claviceps paspali*, *Schizosaccharomyces pombe* and *Candida albicans* as in *S. cerevisiae* (Lingens et al. 1967, Schweingruber and Wyssling 1974, Pereiera and Livi 1996). *Pseudomonas aeroginosa* contains two DAHP synthases (Whitaker et al. 1982). The first enzyme is inhibited strongly by tyrosine and only slightly by the intermediate phenylpyruvate. The other enzyme is inhibited strongly by tryptophan whereas chorismate is a weak inhibitor.

The evolution of the DAHP synthases has been thoroughly investigated in the prokaryotic Superfamily B to which the *E. coli* enzymes also belong (Fig. 1).

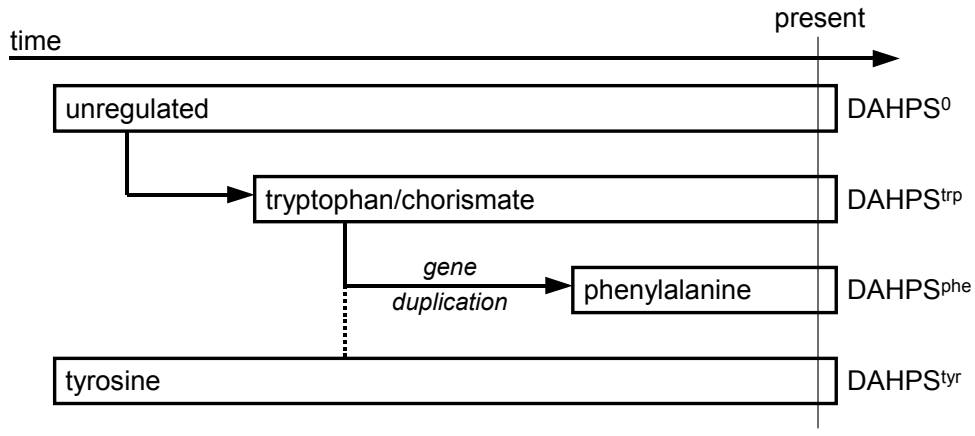

Fig. 1 Evolutionary development of the DAHP synthase isoenzymes of the prokaryotic Superfamily B. Each box represents a different regulated DAHP synthase which evolved during evolution. The respectively inhibitors of the enzymes are shown within the boxes. The dotted line indicates that the phenylalanine–inhibitable DAHP synthase could be evolved by gene duplication from the tryptophan/chorismate-inhibitable as well as from the tyrosine-inhibitable DAHP synthase.

An unregulated DAHP synthase ($DAHPS^0$) has been found in *Acinetobacter* species as well as in *Oceanospirillum minutulum*. These organisms also possess a DAHP synthase which can be inhibited by tyrosine. This state could probably be found in a common precursor of the Superfamily B. It is postulated that $DAHPS^0$ is the precursor of $DAHPS^{trp}$ (Ahmad et al. 1985). The newly developed $DAHPS^{trp}$ had supposedly the same sensitivity towards chorismate as to L-tryptophan. A weak chorismate sensitivity has stayed in the $DAHPS^{trp}$ of the rRNA group I Pseudomonades, a high sensitivity is to be found in the $DAHPS^{trp}$ of the small rRNA group V Pseudomonades. Enterobacteria have lost their entire $DAHPS^{trp}$ sensitivity towards chorismate (Ahmad 1981).

$DAHPS^{phe}$ is the youngest isoenzyme to ensue from the prokaryotic Superfamily B. The gene for the $DAHPS^{phe}$ probably originates from a duplication of the gene $DAHPS^{tyr}$ or $DAHPS^{trp}$ (Jensen and Byng 1981). $DAHPS^{trp}$ is most likely to be the precursor as there are more sequence and secondary structure similarities between $DAHPS^{trp}$ and $DAHPS^{phe}$ than between these isoenzymes and $DAHPS^{tyr}$ (Shultz et al. 1981).

3. α/β Protein Structures

The most frequent and most regular of the domain structures of soluble proteins are the α/β domains, which consist of a central parallel or mixed β sheet surrounded by α helices. All glycolytic enzymes are α/β structures as are many other enzymes as well as proteins that bind and transport metabolites. In α/β domains binding gaps are formed by loop regions. These regions do not contribute to structural stability but participate in binding and catalytic action.

3.1 TIM Barrel Fold – Structural Aspects

The most predominant α/β folding pattern is exemplified by the structure of triose phosphate isomerase (TIM) catalyzing the isomerization of dihydroxyacetonephosphate and glycerinealdehyd-3-phosphate in the glycolytic pathway. The $(\alpha/\beta)_8$ barrel structure of TIM is a folding motif that has been found to be the most common enzyme fold in the Protein Data Bank database of known protein structures (For examples see Fig. 2). It is present in many enzyme families, catalyzing completely

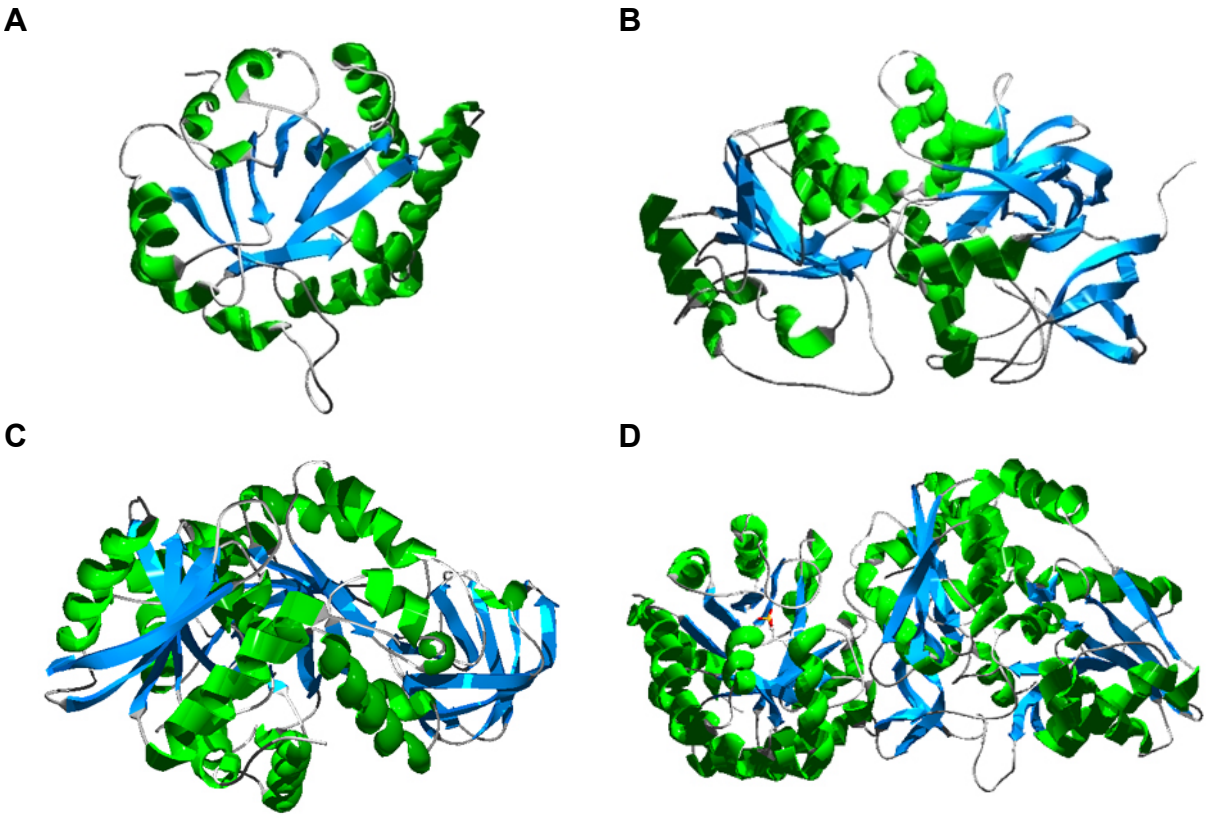

Fig. 2 $(\alpha/\beta)_8$ barrel structures. A Triose Phosphate Isomerase (Lolis et al. 1990) **B** Alcohol Dehydrogenase (Xie et al. 1997) **C** Pyruvate Kinase (Mattevi et al. 1995) **D** Tryptophan Synthase (Weyand and Schlichting 1999).

different reactions (Hegyi and Gerstein 1999). Eight parallel strands of β-sheet coil around sequentially to form a central β-barrel. Concentric with the barrel and linking the β-strands are the parallel segments of α-helices (Banner et al. 1975). Two key parameters of barrel structures are n (the number of strands in the barrel) and S (the

shear number). All (n=8, S=8) barrels are TIM barrels. For all TIM barrels n=8 and almost always S=8 (Nagano et al. 1999). The TIM barrel fold is spread over many biosynthetic pathways and because of this there much interest has been aroused in the structural, enzymological and evolutionary properties.

The typically TIM barrel domain has about 200 to 250 residues (Traut and Temple 2000). TIM barrel enzymes with only one domain such as hevamine (Terwisscha van Scheltinga 1996) can be small or very large, such as β-galactosidase (Juers et al. 1999).

The active sites of all TIM barrel enzymes are at the C-terminal ends of the β-strands. Therefore residues of the eight loops which follow the β-strands build the active site of these enzymes. These βα loops have variable length, but seems to be larger than the αβ loops, e.g. in TIM (Maes et al. 1998) and HisA and HisF (Lang et al. 2000). These αβ loops are considered to more important for the stability of the enzyme as they are located at the backside of the molecule (Urfer and Kirschner 1992).

3.2 Evolutionary aspects of the TIM barrel fold

Structure based sequence alignments reveal the presence of clusters of residues which are similar in physicochemically properties. They can be located in equivalent topological positions which therefore could directly stabilize and determine the common TIM barrel folding pattern (Selvaraj and Gromiha 1998). Circular permuted sequence variants of the TrpF TIM barrel enzyme have been shown to fold as the wild-type enzyme (Luger et al. 1989). It is uncertain whether all TIM barrel families have a common evolutionary origin. Copley and Bork suggested the model of a divergent evolution from a common ancestor for most of the TIM barrel enzyme families (Cop-

ley and Bork 2000). Due to recent directed evolution selection experiments it is clear that function can be altered by small sequence changes. One point mutation can be sufficient to change the HisA protein into a protein with TrpF function (Jurgens et al. 2000). Further it was shown that a directed evolution approach can change the TrpC protein in a protein with TrpF function (Altamirano 2000). In this study not only the substrate specifity but also the catalytic step was altered.

HisA and HisF share a sequence identity of approximately 25% (Altamirano 2000). In both enzymes there is an internal twofold repeat pattern in their sequence and fold (Altamirano 2000, Fani et al. 1994). This could lead to the assumption that both enzymes evolve from a common ancestor. This ancestor could have been the result of a gene duplication followed by gene fusion of a gene encoding a phosphate-binding half-barrel folding unit. Recent studies show that the N-terminal and C-terminal half-barrel domains of HisF have now been shown to be folding units. If HisF-N and HisF-C units are expressed separately they can be obtained as inactive homo-oligomers. They build fully active heterodimers when they are expressed jointly (Hocker et al. 2001).This has already been observed by Heinz et al. (1998).

Recent directed evolution experiments have shown that the substrate specifity and the catalytic specifity of TIM barrel enzymes can be changed by random mutagenesis. This indicates that in the future new TIM-barrel enzymes with completely novel catalytic activities could become available from structure-based, directed evolution experiments.

4. Aim of this Work

Isoenzyme systems are widespread in nature. The main purpose of such a system is to regulate a reaction in a very sensitive way. Two or more enzymes which can be differently regulated catalyze one reaction. This study tries to find some answers to questions like: Why does nature take a huge amount of energy into account to catalyze the same reaction with two or more different enzymes ?

In the first chapter we describe how the yeast *Saccharomyces cerevisiae* handles a third DAHP synthase from *Escherichia coli* which is tryptophan-regulated and naturally not present in the yeast. In the second chapter the regulative fine-tuning of the DAHP synthase system in *Aspergillus nidulans* is the topic. It is shown that both isoenzymes have their specific role in controlling the metabolic flux into the shikimate pathway. In the last chapter the structure of the tyrosine-regulated DAHP synthase of *Saccharomyces cerevisiae* is described. Further we prove that only one amino acid residue is crucial for specific regulation of each DAHP synthase from the yeast *Saccharomyces cerevisiae*.

5. References

Ahmad S, Rightmire B, and Jensen RA (1985) Evolution of the regulatory isoenzymes of 3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase present in the *Escherichia coli* genealogy. *J Bacteriol* **165**:146-154

Altamirano MM, Blackburn JM, Aguayo C, and Fersht AR (2000) Directed evolution of new catalytic activity using the alpha/beta-barrel scaffold. *Nature* **403**:617-622

Amrhein NB (1980) The site of the inhibition of the shikimate pathway by glyphosate. *Plant Physiol* **66**:830-834.

Atkinson DE (1977) Cellular energy metabolism and its regulation. Academic Press, New York

Banner DW, Bloomer AC, Petsko GA, Phillips DC, Pogson CI, Wilson IA, Corran PH, Furth AJ, Milman JD, Offord RE, Priddle JD, and Waley SG (1975) Structure of chicken muscle triose phosphate isomerase determined crystallographically at 2.5 angstrom resolution using amino acid sequence data. *Nature* **255**:609-614.

Braus GH (1991) Aromatic amino acid biosynthesis in the yeast *Saccharomyces cerevisiae*: a model system for the regulation of a eukaryotic biosynthetic pathway. *Microbiol Reviews* **55**:349-370

Brown KD and Doy CH (1966) Control of three isoenzymic 7-phospho-2-oxo-3-deoxy-D-*arabino*-heptonate-D-*erythrose*-4-phosphate lyases of *Escherichia coli* W and derived mutants by repressive and "inductive" effects of the aromatic amino acids. *Biochim Biophys Acta* **118**:157-172

Camakaris J and Pittard J (1974) Purification and properties of 3-deoxy-D-arabino-heptulosonic acid-7-phosphate synthetase (trp) from Escherichia coli. *J Bacteriol* **120**:590-597

Copley RR and Bork P (2000) Homology among $(\beta\alpha)_8$ barrels: implications for the evolution of metabolic pathways. *J Mol Biol* **303**:627-641

Crawford IP (1975) Gene rearrangements in the evolution of the tryptophan synthetic pathway. *Bacteriol Rev* **39**:87-120

Davidson BE, Blackburn EH, and Dopheide TAA (1972) Chorismate mutase-Prephenate dehydratase from *Escherichia coli* K-12. *J Biol Chem* **247**:4441-4446

Davies WD and Davidson BE (1982) The nucleotide sequence of *aroG*, the gene for 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase (phe) in *Escherichia coli* K12. *Nucl Acids Res* **10**:4045-4048

Davis BD (1955) Intermediates in amino acid biosynthesis. *Adv Enzymol* **16**:287-295

Dopheide TAA, Crewther P, and Davidson BE (1972) Chorismate mutase-Prephenate dehydratase from *Escherichia coli* K-12: *J Biol Chem* **247**:4447-4452

Eberhard J, Raesecke HR, Schmid J, and Amrhein N (1993) Cloning and expression in yeast of a higher plant chorismate mutase. *FEBS Lett* **334**:233-236

Fani R, Lio P, Chiarelli I, and Bazzicalupo M (1994) The evolution of the histidine biosynthetic genes in prokaryotes: a common ancestor for the hisA and hisF genes. J *Mol Evol* **38**:489-495

Gibson MI and Gibson F (1964) Preliminary studies on the isolation and metabolism of an intermediate in aromatic biosynthesis: chorismic acid. *Biochem J* **90**:248-256

Graf R, Mehmann B, and Braus GH (1993) Analysis of feedback-resistant anthranilate synthases from *Saccharomyces cerevisiae*. *J Bacteriol* **177**:1645-1648

Gresshoff PM (1979) Growth inhibition by glyphosate and reversal of its action by phenylalanine and tyrosine. *Aust J Plant Physiol* **6**:177-185

Haslam E (1993) Shikimic Acid. Wiley, Chichester New York, Brisbane

Hegyi H and Gerstein M (1999) The relationship between protein structure and function: a comprehensive survey with application to the yeast genome. *J Mol Biol* **288**:147-164.

Heinz DW, Essen LO, and Williams RL (1998) Structural and mechanistic comparison of prokaryotic and eukaryotic phosphoinositide-specific phospholipases C. *J Mol Biol* **275**:635-650

Herrmann KM (1983) *in* Herrmann KM and Somerville RL Amino Acids: Biosynthetic and genetic Regulation, pp. 301-322, Addison-Wesley Publishing Cp., Inc., Reading, MA

Hinnebusch AG (1988) Mechanisms of gene regulation in the general control of amino acid biosynthesis in *Saccharomyces cerevisiae*. *Microbiol Rev* **52**:248-273

Hocker B, Beismann-Driemeyer S, Hettwer S, Lustig A, and Sterner R (2001) Dissection of a (βα)8-barrel enzyme into two folded halves. *Nat Struct Biol* **8**:32-36

Hütter R, Niederberger P, and DeMoss JA (1986) Tryptophan biosynthetic genes in eukaryotic microorganisms. *Ann Rev Microbiol* **40**:55-77

Ito J and Crawford IP (1965) Regulation of the enzymes of the tryptophan pathway in Escherichia coli. *Genetics* **52**:1303-1316

Jensen RA and Byng GS (1981) The partitioning of biochemical pathways with isoenzyme systems. *Curr Top Biol Med Res* **5**:143-174

Juers DH, Huber RE, and Matthews BW (1999) Structural comparisons of TIM barrel proteins suggest functional and evolutionary relationships between beta-galactosidase and other glycohydrolases. *Protein Sci* **8**:122-136

Jürgens C, Strom A, Wegener D, Hettwer S, Wilmanns M, and Sterner R (2000) Directed evolution of a (βα)8-barrel enzyme to catalyze related reactions in two different metabolic pathways. *Proc Natl Acad Sci* USA **97**:9925-30.

Koch GLE, Shaw DC, and Gibson F (1971) The purification and characterization of chorismate mutase-prephenate dehydrogenase from *Escherichia coli* K-12. *Biochim Biophys Acta* **229**:795-804

Künzler M, Paravicini G, Egli CM, Irniger S, and Braus GH (1992) Cloning, primary structure and regulation of the *ARO4* gene, encoding the tyrosine-inhibited 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase from *Saccharomyces cerevisiae*. *Gene* **113**:67-74

Lang D, Thoma R, Henn-Sax M, Sterner R, and Wilmanns M (2000) Structural evidence for evolution of the beta/alpha barrel scaffold by gene duplication and fusion. *Science* **289**:1546-1550

Lanker S, Bushmann JL, Hinnebusch AG, Trachsel H, and Müller PP (1992) Autoregulation of the yeast lysyl-tRNA synthetase gene *GCD5/KRS1* by translational and transcriptional control mechanism. *Cell* **70**:647-657

LaRossa RA and Falco SC (1984) Amino acid biosynthetic enzymes as targets of herbicide action. *Trends in Biotech* **2**:158-161

Lewin B (1994) Genes V. Oxford University Press and Cell Press, Oxford, UK

Lingens F, Goebel W, and Uesseler H (1967) Regulation der Biosynthese aromatischer Aminosäuren in *Claviceps paspali*. *Eur J Biochem* **2**: 442-447

Lolis E, Alber T, Davenport RC, Rose D, Hartman FC, and Petsko GA (1990) Structure of yeast triosephosphate isomerase at 1.9-A resolution. *Biochemistry* **29**:6609-6618

Luger K, Hommel U, Herold M, Hofsteenge J, and Kirschner K (1989) Correct folding of circularly permuted variants of a beta alpha barrel enzyme in vivo. *Science* **243**:206-210

Maes D, Zeelen JP, Thanki N, Beaucamp N, Alvarez M, Thi MH, Backmann J, Martial JA, Wyns L, Jaenicke R, and Wierenga RK (1999) The crystal structure of trio-

sephosphate isomerase (TIM) from *Thermotoga maritima*: a comparative thermostability structural analysis of ten different TIM structures. *Proteins* **37**:441-453

Mattevi A, Valentini G, Rizzi M, Speranza ML, Bolognesi M, and Coda A (1995) Crystal structure of Escherichia coli pyruvate kinase type I: molecular basis of the allosteric transition. *Structure* **3**:729-741

Mewes HW, Albermann K, Bahr M, Frishman D, Gleissner A, Hani J, Heumann K, Kleine K, Maierl A, Oliver SG, Pfeiffer F, and Zollner A (1997) Overview of the yeast genome. *Nature* (6632 Suppl.) **387**:7-65

Miozzari G, Niederberger P, and Hütter R (1978) Tryptophan biosynthesis in *Saccharomyces cerevisiae*. Control of the flux of the pathway. *J Bacteriol* **134**:48-59

Müller PP and Hinnebusch AG (1986) Multiple upstream AUG codons mediate translational control of *GCN4*. *Cell* **45**:201-207

Nagano N, Hutchinson EG, and Thornton JM (1999) Barrel structures in proteins: automatic identification and classification including a sequence analysis of TIM barrels. *Protein Sci* **8**:2072-2084

Natarajan K, Meyer MR, Jackson BM, Slade D, Roberts C, Hinnebusch AG, and Marton MJ (2001) Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast. *Mol Cell Biol* **21**:4347-4368.

Niederberger P, Miozzari G, and Hütter R (1981) Biological role of the general control of amino acid biosynthesis in Saccharomyces cerevisiae. *Mol Cell Biol* **1**:584-593

Nimmo GA and Coggins RJ (1981) The purification and molecular properties of the tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase from *Neurospora crassa*. *Biochem J* **197**:427-436

Paravicini G, Mösch HU, Schmidheini T, and Braus GH (1989a) The general control activator GCN4 is responsible for a basal level of *ARO* 3 gene expression in Saccharomyces cerevisiae. *Mol Cell Biol* **9**:144-151

Paravicini G, Schmidheini T, and Braus GH (1989b) Purification and properties of the 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (phenylalanine-inhibitable) of *Saccharomyces cerevisiae*. *Eur J Biochem* **186**:361-366

Pereia SA and Livi GP (1996) Aromatic amino acid biosynthesis in Candida albicans: identification of the ARO4 gene encoding a second DAHP-Synthase. *Curr Genetics* **29**:441-445

Pittard J, Camakaris J, and Wallace B (1969) Inhibition of 3-deoxy-D-*arabino* heptulosonic acid-7-phosphate synthetase (trp) in *Escherichia coli*. *J Bacteriol* **97**:1242-1247

Pittard AJ (1996) Biosynthesis of the aromatic amino acids. *In: Escherichia coli* and *Salmonella*. F.C. Neidhardt, R. Curtiss, J.L. Ingraham, E.C.C. Lin, K.B. Low, B. Ma-

gasanik, W.S. Reznikoff, M. Riley, M. Schaechter and H.E. Umbarger, pp. 458-484. American Society for Microbiology, Washington, D.C

Ray JM, and Bauerle R (1991) Purification and properties of tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase from *Escherichia coli*. *J Bacteriol* **173**:1894-1901

Renault P, Godon JJ, Goupil N, Delorme C, Corthier G, and Ehrlich SD (1995) Metabolic operons in Lactococci. *Dev Biol Stand* 85:431-441

Schmidheini T, Sperisen P, Paravicini G, Hütter R, and Braus G (1989) A single point mutation results in a constitutively activated and feedback-resistant chorismate mutase of *Saccharomyces cerevisiae*. *J Bacteriol* **171**:1245-1253

Schmidheini T, Mösch HU, Evans JNS, and Braus G (1990) Yeast allosteric chorismate mutase is locked in the activated state by a single amino acid substitution. *Biochemistry* **29**:3660-3668

Schürch A, Miozzari J, and Hütter R (1974) Regulation of tryptophan biosynthesis in *Saccharomyces cerevisiae*: Mode of action of 5-methyl-tryptophan and 5-methyl-tryptophan-sensitive mutants. *J Bacteriol* **117**:1131-1140

Schweingruber ME and Wyssling HB (1974) Genes and isoenzymes controlling the first step in aromatic amino acid biosynthesis in *Schizosaccharomyces pombe*. *Biochim Biophys Acta* **350**: 319-327

Shultz J, Hermodson MA, and Hermann KM (1981) A comparison of the amino-terminal sequences of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase isoenzymes from *Escherichia coli*. *FEBS Lett* **131**:108-110

Shultz J, Hermodson MA, Garner CC, and Hermann KM (1984) The nucleotide sequence of the *aroF* gene of *Escherichia coli* and the amino acid sequence of the encoded protein, the tyrosine-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase. *J Biol Chem* **259**:9655-9661

Selvaraj S and Gromiha MM (1998) An analysis of the amino acid clustering pattern in (alpha/beta)8 barrel proteins. *J Protein Chem* **17**:407-415

Sprinson BD (1961) The biosynthesis of aromatic compounds from D-glucose. *Adv Carbohydrate Chem* **15**:235-270

Terwisscha van Scheltinga AC, Hennig M, and Dijkstra BW (1996) The 1.8 A resolution structure of hevamine, a plant chitinase/lysozyme, and analysis of the conserved sequence and structure motifs of glycosyl hydrolase family 18. *J Mol Biol* **262**:243-257

Teshiba S, Furter R, Niederberger P, Braus G, Paravicini G, and Hütter R (1986) Cloning of the *ARO3* gene of *Saccharomyces cerevisiae* and its regulation. *Mol Gen Genet* **205**:353-357

Traut TW and Temple BR (2000) The chemistry of the reaction determines the invariant amino acids during the evolution and divergence of orotidine5'-monophosphate decarboxylase. *J Biol Chem* **275**:28675-28681

Tribe DD, Camakaris H, and Pittard J (1976) Constitutive and repressible enzymes of the common pathway of aromatic biosynthesis in *E. coli* K-12: Regulation of enzyme synthesis at different growth rates. *J Bacteriol* **127**:1085-1097

Urfer R and Kirschner K (1992) The importance of surface loops for stabilizing an eightfold beta alpha barrel protein. *Protein Sci* **1**:31-45

Weyand M and Schlichting I (1999) Crystal structure of wild-type tryptophan synthase complexed with the natural substrate indole-3-glycerol phosphate. *Biochemistry* **38**:16469-16480

Whitaker RJ, Fiske MJ, and Jensen RA (1982) Pseudomonas aeroginosa possesses two novel regulatory isoenzymes of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase. *J Biol Chem* **257**:12789-12794

Wolfner MD, Yep F, Messenguey F, and Fink GR (1975) Integration of amino acid biosynthesis into the cell cycle of Saccharomyces cerevisiae. *J Mol Biol* **96**:273-290

Xie P, Parsons SH, Speckhard DC, Bosron WF, and Hurley TD (1997) X-ray structure of human class IV sigmasigma alcohol dehydrogenase. Structural basis for substrate specifity. *J Biol Chem* **272**: 18558-18563

Yanofsky C (1984) Comparison of regulatory and structural regions of genes of tryptophan metabolism. *Mol Biol Evol* **1**:143-161

Zurawski G, RP Gunsalus, KD Brown, and Yanofsky C (1981) Structure and regulation of *aroH*, the structural gene for the tryptophan-repressible 3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase of *Escherichia coli*. *J Mol Biol* **145**:47-73

Chapter II

Expression of a Tryptophan-Inhibitable DAHP Synthase in the Yeast *Saccharomyces cerevisiae*

Abstract

The tryptophan-inhibitable DAHP synthase (AroH) from *Escherichia coli* was heterologously expressed in the yeast *Saccharomyces cerevisiae*. The *aroH* open reading frame was fused with the yeast *ARO3*, *ARO4* and *MET25* promoter, respectively. The aroH protein was either expressed from genes integrated into the yeast genome or from low or high copy vectors. The enzyme activity originating from a single *aroH*-copy integrated into the yeast genome is sufficient to rescue an *aro3/aro4* deletion strain lacking any DAHP synthase activity. Activity of AroH was also able to rescue a Δ*gcn4* strain where *ARO3* and *ARO4* activity was suppressed by phenylalanine and tyrosine, respectively. The regulation of AroH has been investigated using an allelic *aroH*-library generated by random mutagenesis. This yielded five alleles that encode for mutant proteins which show significantly increased sensitivity towards tryptophan and 5-methyltryptophan. The positions of these amino acid exchanges indicate an alternative mode of regulation of DAHP synthases.

Introduction

Isoenzymes are wide-spread in nature. They catalyze the same biochemical reaction while they are differently regulated. The main target of isoenzymes systems are biochemical pathways with branch points such as the biosynthesis of the aromatic amino acids phenylalanine, tyrosine and tryptophan. Having such an isoenzymic system it is possible to perform a regulatory fine-tuning of the whole pathway. Not all of the enzymes have to be regulated with the same strength necessarily. Therefore most of the time the tribute to regulation of each isoenzyme is very different. To find out more about this topic we investigated the isoenzymic system of the yeast DAHP synthases which catalyze the first step of the aromatic amino acid biosynthesis.

The first step of the biosynthesis of aromatic amino acids, the stereospecific condensation of erythrose-4-phosphate and phosphoenolpyruvate, is catalyzed by the 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase. In *E. coli* three isoenzymic forms are known which can be distinguished by their different negatively regulation by the three aromatic amino acids phenylalanine, tyrosine or tryptophan (Brown and Doy 1966). The sum of specific activities which is differently distributed to the three DAHP synthases. AroF (tyrosine-inhibitable) with 20 % and AroG (phenylalanine-inhibitable) with 79 % deliver nearly all of the DAHP synthase activity for the cell. AroH (tryptophan-inhibitable) activity contribution is only 1 % (Tribe et al. 1976). Inhibition of AroF by tyrosine and AroG by phenylalanine is highly effective (>95 %) (Herrmann 1983). In contrast to this the grade of AroH inhibition is only 60 % (Pittard et al., 1969). In yeast *S. cerevisiae* there exist only two DAHP synthases which are inhibitable by phenylalanine (Aro3p) and tyrosine (Aro4p) (Paravicini et al. 1989a,b; Schnappauf et al. 1998). The biosynthesis of aromatic amino acids in *S. cerevisiae* is not only controlled on the enzymatic level, it is also part of a complex regulatory net-

work, which couples transcriptional derepression of at least 539 structural genes involved in multiple amino acid biosynthetic pathways (Natarajan et al. 2001). This cross-pathway regulation, known as the general amino acid control, also exists in other fungi like *Neurospora crassa* and *Aspergillus nidulans* (Carsiotis and Lacy 1965; Piotrowska 1980). It has been demonstrated, that the binding of the yeast Gcn4p to the promoter region of general control regulated genes increases initiation of transcription (Hope and Struhl 1985; Arndt and Fink 1986; Hill et al. 1986). The expression of nine aromatic amino acid biosynthetic genes is part of the general control network including *ARO3* and *ARO4* encoding the two DAHP isoenzymes (Braus 1991).

Alignment studies of amino acid sequences between the DAHP synthases from *E. coli* and *S. cerevisiae* show 65-71 % similarities (Davies and Davidson 1982; Shultz et al. 1984; Zurawski et al. 1981). To date only one DAHP structure is known, the structure of the phenylalanine-inhibitable DAHP synthase from *E. coli* (Shumilin 1999). Resent data about the crystal structure of the tyrosine-inhibitable isoenzyme from *S. cerevisiae* suggests that the overall structure of both DAHP synthases are nearly equal (Data not shown).

This is the report of the expression of a tryptophan-inhibitable DAHP isoenzyme in the yeast *S. cerevisiae,* a organism, which is normally not able to regulate the first step of the shikimate pathway by tryptophan *in vivo*. Regulation-mutants of this enzyme were isolated by random mutagenesis indicating that this tryptophan inhibitable enzyme is regulated alternatively.

Materials and Methods

Materials, media and growth conditions. All chemicals were supplied by FLUKA or Sigma-Aldrich Chemie GmbH (Steinheim, Deisenhofen, Germany). Minimal vitamins and synthetic complete medium for the cultivation of yeast was described earlier (Miozzari et al. 1978, Sherman et al. 1986). Transformation of *Saccharomyces cerevisiae* was carried out by the LiOAc method (Ito et al. 1983).

Strains and plasmids. For overexpression, purification and the allelic library *Saccharomyces cerevisiae* strain RH2424 (*MATa*, *can1-100, GAL, aro3*::*HIS3, aro4*::*LEU2, ura3-1*) was used. For *GCN4* experiments RH1408 (*MATa*, *ura3*-52, Δ*gcn4*)was used. The plasmids used for this work are shown in Table 1.

Plasmid	Description	Reference
pCHA3	*E. coli aroH*-ORF under the control of the *tac*-promotor in the phagemid-vector pttS9	AKOWSKI und BAUERLE, 1997
pME1083	2.8 kb genomc *S. cerevisiae GCN4 Sal*I/*Eco*RI-fragment in pYCP50	HINNEBUSCH, 1985
pME1873	1.0 kb *aroH*-Fragment under the control of the yeast *ARO3* promoter in pRS416	this work
pME1875	1.0 kb *aroH*-Fragment under the control of the yeast *ARO4* promoter in pRS416	this work
pME1877	1.0 kb *aroH*-Fragment under the control of the yeast *MET25* promoter in pRS416*MET25*	this work
pME1878	1.0 kb *aroH*-Fragment under the control of the yeast *MET25* promoter in pRS426*MET25*	this work
pME1879	Like pME1878, but point mutation in the *aroH*-orf in codon 149: GGC_{Gly} - CCC_{Pro}	
pME1880	Like pME1878, but point mutation in the *aroH*-orf in codon 179: TCC_{Ser} – GCC_{Ala}	
pME1881	Like pME1878, but point mutation in the *aroH*-orf in codon 218: ACC_{Thr} – GCC_{Ala}	
pME1883	pRS416*MET25* carrying an *aroH* allele from the random mutagenesis experiments, Exchange in codon 333: ACC_{Thr} - AGC_{Ser}	this work
pME1884	pRS416*MET25* carrying an *aroH* allele from the random mutagenesis experiments, Exchange in codon 253: CAC_{His} - TAC_{Tyr}	this work
pME1885	pRS416*MET25* carrying an *aroH* allele from the random mutagenesis experiments, Exchange in codon 225: AAC_{Asn} - GAC_{Asp}	this work

pME1886	pRS416*MET25* carrying an *aroH* allele from the random mutagenesis experiments, Exchange in codon 124: CTG_{Leu} - CGG_{Arg}	this work
pME1887	pRS416*MET25* carrying an *aroH* allele from the random mutagenesis experiments, Exchange in codon 332: GAT_{Asp} - AAT_{Asn}	this work

Table 1 Plasmids used in this study.

Western Blot analysis. DAHP synthase was immunologically detected with a polyclonal rabbit antibody against the tyrosine-inhibitable DAHP synthase of *S. cerevisiae* and a second antibody with horseradish peroxidase activity was detected using the ECL-method (Tesfaigzi et al. 1994).

Mutagenesis experiments with *aroH.* The allelic *aroH*-library was constructed by PCR method in the presence of 20 µM Mn^{2+} ions. This ion concentration affect the accuracy of the Taq DNA-polymerase leading to random errors during the amplification process (Leung et al. 1989). Several Mn^{2+} ion concentrations were tested. To produce single mutations 20 µM was used as final concentration. All random mutated *aroH* alleles were cloned into pRS416 downstream of the *MET25* promoter. This promoter can be down-regulated to 10 % in the presence of 2 mM methionine. After transformation of the allelic library into RH2424 (*MATa*, *can1-100, GAL, aro3*::*HIS3, aro4*::*LEU2, ura3-1*) mutants were selected on synthetic complete medium without aromatic amino acids to screen only for functionally intact DAHP synthases. The resulting mutant colonies were transferred to minimal vitamins medium supplemented with 5 mM tryptophan to reduce DAHP synthase activity and 2 mM methionine to reduce expression of the mutated alleles to screen for alleles encoding tryptophan resistant AroH mutant enzymes. Only those strains were selected that show normal growth on minimal vitamins medium and more reduced growth on tryptophan supplemented medium than a strain carrying the wildtype *aroH* gene. A second selection approach was made to verify this effect, a dilution experiment. Starting off with 10^8

cells/ml dilution experiments were carried out. A drop of 10 µl was brought on minimal vitamins medium, MV medium containing 5mM tryptophan and MV medium containing 5 mM 5-methyltryptophan. Only strains which showed normal growth on MV medium and higher sensitivity towards the supplemented effectors were selected for further experiments. From all resulting *aroH* alleles the DNA sequence was determined (Rosenblum et al. 1997).

Site-directed mutations were introduced using oligo-nucleotide mutagenesis. The resulting alleles were cloned into pME1513 and transformed into yeast strain RH2424 for further analysis.

Enzyme assays. The DAHP synthase activities were determined with the stop assay described by Takahashi and Chan (1971) with the modifications described by Schnappauf et al. (1998). Enzyme activities are specified in International Units (1 U = appearance of 1 µmol product per minute). Specific enzyme activities are given as mU (mg protein $^{-1}$).

Results

The bacterial tryptophan-inhibitable DAHP synthase is able to maintain the flux into the shikimate pathway in *S. cerevisiae*. S. cerevisiae contains a phenylalanine- and a tyrosine-inhibitable DAHP synthase. Therefore *S. cerevisiae* has no instrument to regulate the first step of the shikimate pathway with tryptophan *in vivo*. We were interested how the expression of a tryptophan-inhibitable DAHP synthase from *E. coli* affects growth in *S. cerevisiae* lacking both genes for the corresponding yeast isoenzymes. To investigate this artificial regulatory change within the shikimate

pathway a 1.0 kb *aroH* fragment was brought in yeast under the control of the *ARO3*, *ARO4* or the *MET25* yeast promoters. The constructs were expressed in yeast RH2424 (*MATa*, *can1-100, GAL, aro3*::*HIS3, aro4*::*LEU2, ura3-1*) with different copy numbers. They were integrated and brought into yeast on low- or high copy number plasmids. Western blot hybridization experiments revealed that the tryptophan inhibitable DAHP synthase from *E. coli* was expressed and stayed stable in yeast (Fig. 1).

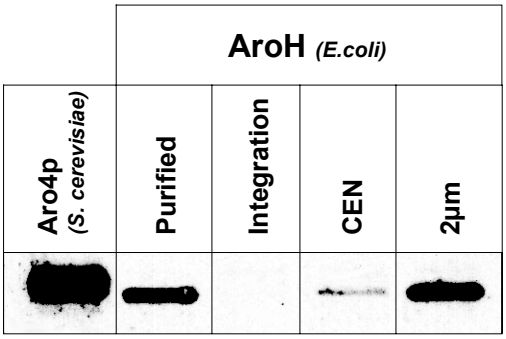

Fig. 1 Western blot hybridization experiments show cross reaction of an Aro4p raised polyclonal antibody and AroH protein. The lanes show Aro4p (10ng) from *S. cerevisiae* as control, purified AroH protein (100 ng) and crude extracts (each 2µg) of strains RH2424 carrying a genomic integration, cen-plasmid with *aroH* and a 2µm-plasmid carrying *aroH*.

Growth tests additionally showed that even the expression of the integrated copy of the *aroH* gene (cross reaction of the gene product with Aro4p not visible in the Western blot hybridization experiment) resulted in DAHP synthase activity which was sufficient to rescue the double deletion strain RH2424 (Fig. 2). The growth of strains carrying *aroH* constructs on low- or high copy number plasmids was comparable and also the usage of different promoters did not impair growth intensity (data not shown for strains carrying the high copy number plasmids).

Fig. 2 *aroH* expression in yeast. Growth of *S. cerevisiae* strains carrying an *aroH* integration or harboring low copy plasmids which carry *aroH*. The *aro3 aro4* deficient strain RH2424 which is not able to grow on this medium (data not shown) was transformed with *CEN*-plasmids, which express the *aroH* gene under the control of the *ARO3*- (pME1873), *ARO4*- (pME1875) and *MET25*-promoter (pME1877). Additionally a copy of the *aroH* gene was integrated into the yeast genome (*ARO3* locus) under the control of the *ARO3* promoter. To guarantee the growth of this integration strain, uracile and histidine was supplemented to the MV medium.

The tryptophan-inhibitable DAHP synthase from *E. coli* rescues a *GCN4*-deficient *S. cerevisiae* strain in the presence of phenylalanine and tyrosine. *S. cerevisiae* strain RH1408 (*Mat a*, *ura3-52*, Δ*gcn4*) lacks the transcription factor Gcn4p and is therefore not able to activate the general amino acid control under amino acid starvation conditions. It cannot grow on medium which is supplemented with 5 mM tyrosine and 5 mM phenylalanine. These amino acids inhibit the first step of the shikimate pathway and therefore a *gcn4* strain cannot grow due to tryptophan starvation (Fig. 3).

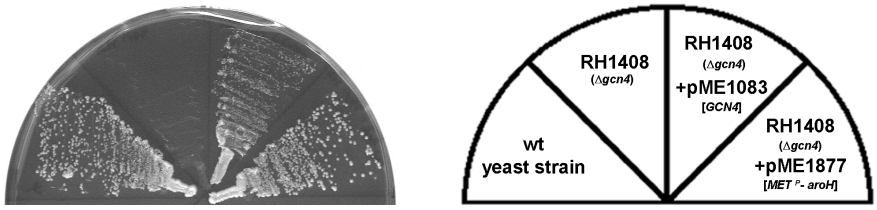

Fig. 3 *aroH* expression in yeast. Growth of the Δ*gcn4* yeast strain RH1408 (*ARO3*, *ARO4*) on MV medium supplemented with 5 mM phenylalanine and 5mM tyrosine was enabled by expression of *GCN4* or *aroH*.

A plasmid carrying a copy of the *GCN4* gene is able to rescue this strain by increasing the expression of all genes which are under the control of the general amino acid control, including *ARO3* and *ARO4*. We tested whether the expression of the trypto-

phan-inhibitable DAHP synthase from *E. coli* could be a second possibility to rescue this strain. We found that both, the plasmid carrying the *GCN4* gene as well as the expression of the *E. coli* enzyme enables the flux into the shikimate pathway again. This means that the yeast *S. cerevisiae* uses the additional DAHP synthase at the first step of the shikimate pathway to overcome the tryptophan starvation death.

Residues that are part of regulation are mainly located on the outside of the tryptophan-regulated DAHP synthase from *E. coli*. To find out more about the regulation of the tryptophan-inhibitable DAHP synthase from *E. coli* a random mutagenesis experiment was performed to identify residues which are involved into regulation. Therefore an allelic library was constructed by PCR in the presence of manganese to introduce single random mutations into the *aroH* gene of *E. coli*. The allelic library was transformed into *S. cerevisiae* strain RH2424 (*MATa*, *can1-100, GAL, aro3*::*HIS3, aro4*::*LEU2, ura3-1*) behind the methionine inhibitable *MET25* promoter. To select for tryptophan hypersensitive clones the selection was adjusted on the enzymatic level with 5 mM tryptophan and additionally on the transcription level by addition of 2 mM methionine. This amount of methionine decreased the expression of the mutated alleles to 10 %. As the wt-enzyme is inhibitable only up to 60 % the selection should result in alleles encoding enzymes, which are more sensitive towards tryptophan than the original *E. coli* enzyme. After the transformation process 1300 clones were selected, proved by retransformation and replica plated on minimal vitamin medium. For further experiments only clones which showed normal growth on MV medium but were more sensitive towards tryptophan or 5-methyltryptophan compared to the heterologous *E. coli* derived wildtype enzyme were selected. By confirming this effect with dilution tests on MV media with 5 mM tryptophan or 1 mM 5-

methyltryptophan only 5 clones remained which showed still hyper-sensitivity towards the tested effectors (Fig. 4).

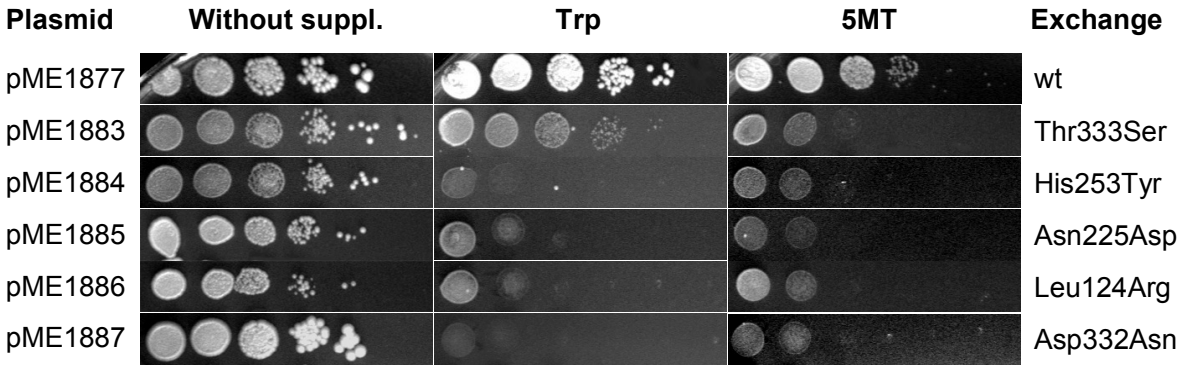

Fig. 4 Dilution experiments of the strain RH2424 (*MAT*a, *can1*-100, *GAL*, *aro3*::*HIS3*, *aro4*::*LEU2*, *ura3*-1) expressing the tryptophan hyper-sensitive mutants found by random mutagenesis. On MV medium there is nearly no difference in growth. By adding tryptophan or methyltryptophan to the medium the strains expressing the hypersensitive *aroH* mutants show strongly reduced growth compared to a strain expressing a wt *aroH* allele.

Sequencing revealed the positions of the mutations on the *aroH* gene and the respectively amino acid exchanges in the enzyme. Amino acid exchanges Leu124Arg, Asn225Asp, His253Tyr, Asp332Asn and Thr333Ser were found to be involved in tryptophan regulation (Fig. 5). Non of these residues were found to be involved in regulation of the tyrosine- or the phenylalanine-inhibitable DAHP synthase of *E. coli*. The projection of these residues on a modeled structure of the tryptophan-inhibitable DAHP synthase from *E. coli* showed that these residues are distributed all over the protein structure. Shumilin et al. (1999) discovered a certain hot spot which is important for regulation in the phenylalanine-sensitive isoenzyme. Most of their regulation mutants are located in this hot spot. Such a hot spot does not seem to exist for the tryptophan-regulated isoenzyme. Thus the tryptophan-regulated DAHP synthase seems to function by an alternative mode of feedback-inhibition.

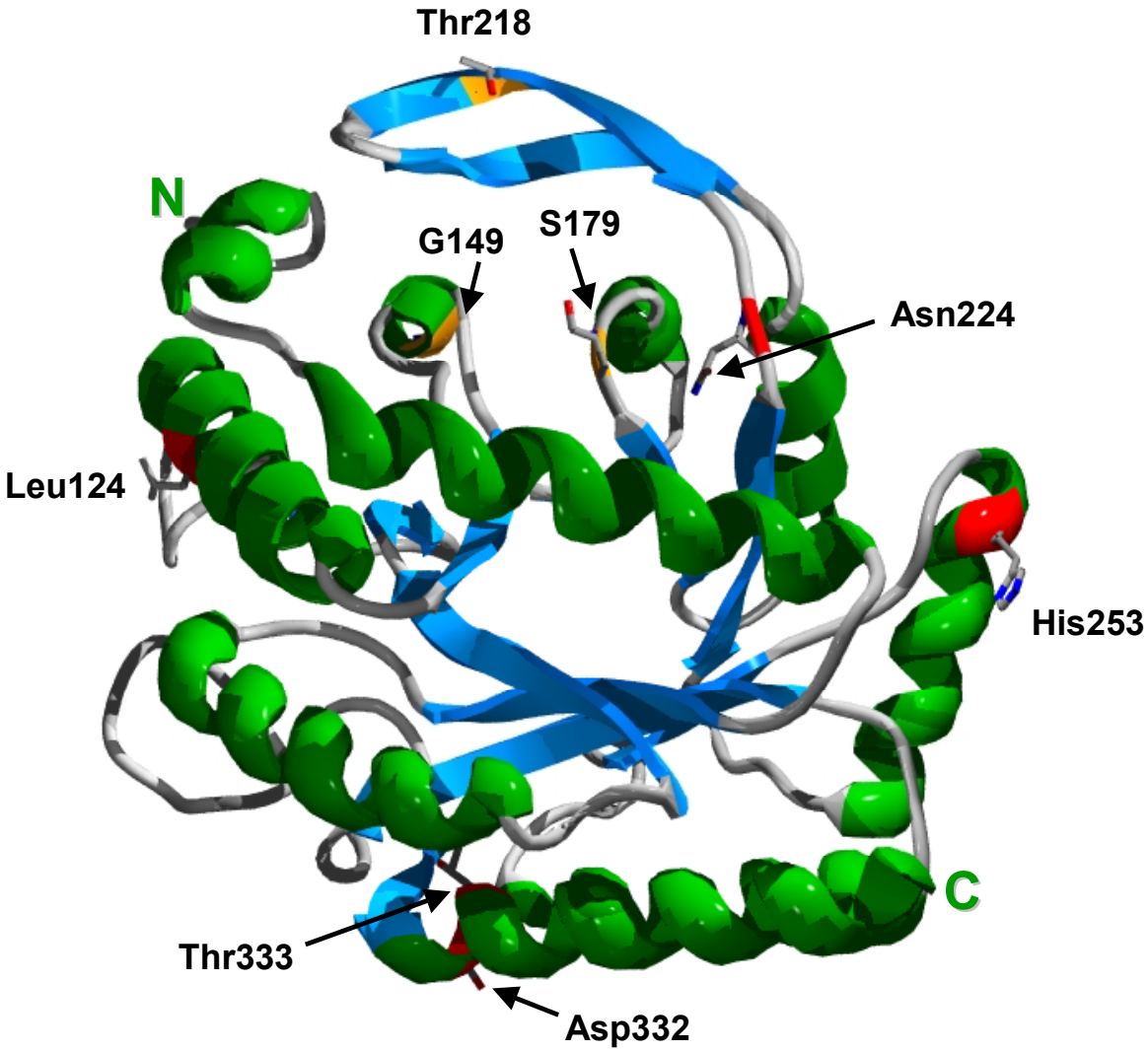

Fig. 5 Modeled overall structure of the tryptophan-inhibitable DAHP synthase from *E. coli*. The various amino acids which were exchanged by site-directed mutagenesis (orange) or found by random mutagenesis (red) are shown in this 3D structure. The residues which have an effect on tryptophan regulation are distributed on the outside on the protein molecule (Leu124, Asn 224, His253, Asp332 and Thr333).

Residues which are important for regulation of the phenylalanine- and tyrosine-inhibitable DAHP synthases do have a different function in the tryptophan-sensitive DAHP synthase from *E. coli*. Beside the random mutagenesis approach a site-directed approach was done to find out more about the regulation of the tryptophan-regulated DAHP synthase from *E. coli*. For this study residues which are already known to be involved in regulation of the two other DAHP isoenzymes were exchanged in the tryptophan-inhibitable enzyme.

Residue serine 180 is crucial for regulation of the phenylalanine-inhibitable DAHP synthase from *E. coli* (Ger et al. 1994). It is also essential for regulation in the tyrosine-inhibitable DAHP synthase from *S. cerevisiae* (data not shown). In the tryptophan-regulated *E .coli* enzymes it is the Ser 179 residue (Fig. 5). AroH DAHP synthase with a Ser179Ala exchange showed only slight reduction in regulation by tryptophan (Fig. 6).

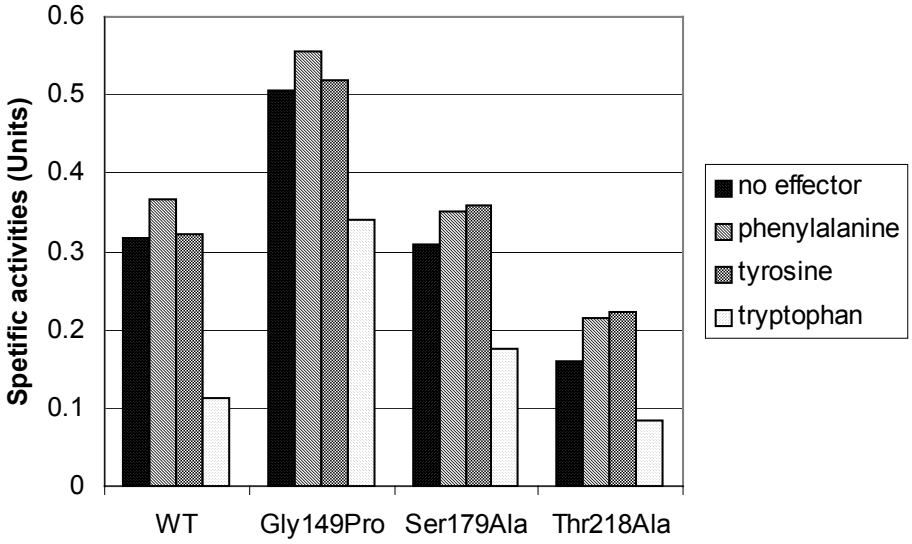

Fig. 6 Specific activities measured from crude extracts of RH2424 expressing the various constructed mutants of *aroH*.

Threonine 236 seems to play an important role in the regulation of the tyrosine-inhibitable DAHP synthase from *S. cerevisiae* (data not shown). This residue is threonine 218 in the tryptophan-regulated *E. coli* enzyme (Fig. 5). A Thr218Ala exchange did not show a significant change of the regulation pattern. Shumilin et al. (1999) proposed that glycine 149 could be essential for tryptophan regulation as in a sequence alignment this residue is a proline in all other not tryptophan-regulated DAHP synthases (Fig. 5). Glycine could provide the needed space for the steric side chain of tryptophan. Indeed exchange Gly149Pro showed a significant regulation decrease. The DAHP synthase is still regulated by tryptophan but the activity was raised to 63 % in presence of 1mM tryptophan compared to 32 % of the wt-enzyme

under these conditions. This suggests that glycine 149 is involved in inhibition of the tryptophan-regulated DAHP synthase and could therefore provide the space for the bulky sidechain as proposed in Shumilin et al. (1999).

To sum up all three mutant enzymes are still regulated by tryptophan. Serine 179 and threonine 218 seem not to be part of the regulation process. Looking at the crude extract measurements only glycine 149 could be involved in regulation of the tryptophan-inhibitable DAHP synthase from *E. coli*.

Discussion

The amino acid sequence of Aro3p and Aro4p from *S. cerevisiae* share 76 % similarity. These enzymes also show sequence similarities up to 73 % to the *E. coli* DAHP isoenzymes. The similarities of the other enzymes of the shikimate pathway between different organisms are significantly lower (Braus 1991). A high identity degree between isoenzymes is not very unusual. Remarkable is the high degree of identity between isoenzymes of two not very closely related microorganisms like *E. coli* and *S. cerevisiae*. This suggests that all microbial DAHP-synthases could be evolved from a common ancestor. Ahmad et al. (1985) suggested that in the superfamily B prokaryotes the phenylalanine-inhibitable DAHP synthase is closer related to the tryptophan-inhibitable isoenzyme than to the tyrosine-inhibitable DAHP synthase (Shultz et al. 1981). The results from the site-directed mutants suggest that the phenylalanine- and the tyrosine-inhibitable DAHP synthases are closer related. Two residues which are known to be involved in the regulation of the phe- and tyr-regulated isoenzymes are not involved in trp-regulation. Thus the regulation mechanism of the trp-inhibitable DAHP synthase could be different. Additionally the amino acid exchanges which

were obtained by the random mutagenesis and led to regulation mutants were distributed on the outside of the enzyme. Random mutagenesis approaches with the phe- and tyr-regulated DAHP enzymes of *E. coli* and *S. cerevisiae* led to a regulation hot spot at the inside of the enzyme (Shumilin 1999)

It was shown that the expression of the tryptophan-inhibitable DAHP synthase from *E. coli* can rescue a double deletion *S. cerevisiae* strain. Even the gene product of a integrated copy of the *aroH* gene in the yeast genome provides enough enzymatic activity to maintain the flux into the shikimate pathway. The question why *S. cerevisiae* does not carry a third DAHP synthase which is tryptophan-regulated could be answered with the presence of the general control of the amino acids which is absent in *E. coli*. In *S. cerevisiae* both DAHP synthases genes are regulated by the general control. The *ARO7* gene which codes for the last common shikimate pathway enzyme chorismate mutase is not under the general control (Schmidheini et al. 1990). This enzyme is negatively regulated by tyrosine and activated by tryptophan directing the flux towards tryptophan or tyrosine and phenylalanine, respectively. Gcn4p protects the cell from shutting down the shikimate pathway by inhibiting the two DAHP synthase isoenzymes in the presence of high tyrosine and phenylalanine concentrations. A third, tryptophan-regulated DAHP synthase in *S. cerevisiae* is therefore unnecessary. The existence of this enzyme in *E.coli* is important. It could be an emergency system in the presence of high aromatic amino acid concentrations. Without a general control system like in *S. cerevisiae* the flux into the shikimate pathway would stop in the presence of high concentrations of phenylalanine and tyrosine. Experiments with a yeast strain which has a *GCN4*-deletion showed that in *S. cerevisiae* a tryptophan-inhibitable DAHP synthase could also act as an emergency system. In the presence of high phenylalanine and tyrosine concentrations this yeast strain was not able to grow anymore. Growth was possible in the presence of *GCN4* introduced on

a plasmid, or the expression of the tryptophan-inhibitable DAHP synthase from *E. coli*. These experiments show that in the presence of a general control system of amino acids it is not necessary to carry a third DAHP synthase which is tryptophan-inhibitable. According to this fact it would be intriguing to find out why *Neurospora crassa* which possesses a general control system of amino acids still carries a third tryptophan-regulated DAHP synthase.

References

Ahmad S, Rightmire B, and Jensen RA (1985) Evolution of the regulatory Isoenzymes of 3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase present in the *Escherichia coli* genealogy. *J Bacteriol* **165**:146-154

Akowski JP and Bauerle R (1997) Steady-state kinetics and inhibitor binding of 3-deoxy-D-arabino-heptulosonate 7-phosphate (Tryptophan sensitive) synthase from *Escherichia coli*. *Biochemistry* **36**:15817-15822

Arndt K and Fink GR (1986) GCN4 protein, a positive transcription factor in yeast, binds general control promoters at all 5´TGACTC3´ sequences. *Proc Natl Acad Sci* USA **83**:8516-8520

Braus GH (1991) Aromatic amino acid biosynthesis in the yeast *Saccharomyces cerevisiae*: a model system for the regulation of a eukaryotic biosynthetic pathway. *Microbiol Reviews* **55**:349-370

Brown KD and Doy CH (1966) Control of three isoenzymic 7-phospho-2-oxo-3-deoxy-D-*arabino*-heptonate-D-*erythrose*-4-phosphate lyases of *Escherichia coli* W and derived mutants by repressive and 'inductive' effects of the aromatic amino acids. *Biochim Biophys Acta* **118**:157-172

Carsiotis M and Lancy AM (1965) Increased activity of tryptophan biosynthetic enzymes in histidine mutants of *Neurospora crassa*. *J Bacteriol* **89**:1472-1477

Davies WD and Davidson BE (1982) The nucleotide sequence of *aroG*, the gene for 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase (phe) in *Escherichia coli* K12. *Nucl Acids Res* **10**:4045-4048

Ger YM, Chen SL, Chiang HJ, and Shiuan D (1994) A single Ser-180 mutation desensitizes feedback inhibition of the Phenylalanine-sensitive 3-deoxy- D-arabino-heptulosonate 7-phosphate (DAHP) synthetase in *Escherichia coli. J Biochem* **116**:986-990

Herrmann KM (1983) *in* Herrmann KM and Somerville RL Amino Acids: Biosynthetic and genetic Regulation, pp. 301-322, Addison-Wesley Publishing Cp., Inc., Reading, MA

Hill DE, Hope IA, MackeJP, and Struhl K (1986) Saturation mutagenesis of the yeast *HIS3* regulatory site: Requirements for transcriptional induction and for binding by GCN4 activator protein. *Science* **234**:451-457

Hinnebusch AG (1985) A hierarchy of trans-acting factors modulates translation of an activator of amino acid biosynthetic genes in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **5**:2349-2360

Hope IA and Struhl K (1985) GCN4 protein, synthesized *in vitro*, binds to *HIS3* regulatory sequences: Implications for the general control of amino acid biosynthetic genes in yeast. *Cell* **43**:177-188

Ito H, Fukuda Y, Murata K, and Kimura A (1983) Transformation of intact yeast cells treated with alkali cations. *J Bacteriol* **153**:163-168

Leung DW, Chen E, and Goeddel DV (1989) A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction. *Technique* 1:11-15

Miozzari G, Niederberger P, and Hütter R (1978) Tryptophan biosynthesis in *Saccharomyces cerevisiae*: control of the flux through the pathway. *J Bacteriol* **134**:48-59

Natarajan K, Meyer MR, Jackson BM, Slade D, Roberts C, Hinnebusch AG, and Marton MJ (2001) Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast. *Mol Cell Biol* **21**:4347-4368.

Paravicini G, Mösch HU, Schmidheini T, and Braus GH (1989a) The general control activator GCN4 is responsible for a basal level of *ARO* 3 gene expression in Saccharomyces cerevisiae. *Mol Cell Biol* **9**:144-151

Paravicini G, Schmidheini T and Braus GH (1989b) Purification and properties of the 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (phenylalanine-inhibitable) of *Saccharomyces cerevisiae*. *Eur J Biochem* **186**:361-366

Piotrowska M (1980) Cross-pathway regulation of ornithine carbamyoltransferase synthesis in *Aspergillus nidulans*. *J Gen Microbiol* **116**:335-339

Pittard J, Camakaris J, and Wallace B (1969) Inhibition of 3-deoxy-D-*arabino* heptulosonic acid-7-phosphate synthetase (trp) in *Escherichia coli*. *J Bacteriol* **97**:1242-1247

Pittard AJ (1996) Biosynthesis of the aromatic amino acids. *In: Escherichia coli* and *Salmonella*. F.C. Neidhardt, R. Curtiss, J.L. Ingraham, E.C.C. Lin, K.B. Low, B. Magasanik, W.S. Reznikoff, M. Riley, M. Schaechter and H.E. Umbarger, pp. 458-484. American Society for Microbiology, Washington, D.C

Rosenblum BB, Lee LG, Spurgeon SL, Khan SH, Menchen SM, Heiner CR, and Chen SM (1997) New dye-labeled terminators for improved DNA sequencing patterns. *Nucl Acids Res* **25**:4500-4504

Schmidheini T, Mösch HU, Graf R, and Braus GH (1990) A GCN4 protein recognition element in the *ARO7* promoter of *Saccharomyces cerevisiae* is not used in vivo. *Mol Gen Genet* **224**:57-64

Schnappauf G, Hartmann M, Künzler M, and Braus GH (1998) The two 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase isoenzymes from *Saccharomyces cerevisiae* show different kinetic modes of inhibition. *Arch Microbiol* **169**:517-524

Sherman F Fink GR, and Hicks J (1986) Methods in yeast genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Shultz J, Hermodson MA, and Hermann KM (1981) A comparison of the amino-terminal sequences of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase isoenzymes from *Escherichia coli*. *FEBS Lett* **131**:108-110

Shultz J, Hermodson MA, Garner CC, and Hermann KM (1984) The nucleotide sequence of the *aroF* gene of *Escherichia coli* and the amino acid sequence of the encoded protein, the tyrosine-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase. *J Biol Chem* **259**:9655-9661

Shumilin IA, Kretsinger RH, and Bauerle RH (1999) Crystal structure of phenylalanine-regulated 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase from Escherichia coli. *Structure* **7**:865-875

Takahashi M and Chan WWC (1971) Separation and properties of isozymes of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthetase from *Saccharomyces cerevisiae*. *Can J Biochem* **49**:1015-1025

Tesfaigzi J, Smith-Harrison W, and Carlson DM (1994) A simple method for reusing western blots on PVDF membranes. *BioTechniques* 17:268-269

Tribe DD, Camakaris H, and Pittard J (1976) Constitutive and repressible enzymes of the common pathway of aromatic biosynthesis in *E. coli* K-12: Regulation of enzyme synthesis at different growth rates. *J Bacteriol* **127**:1085-1097

Zurawski G, RP Gunsalus, KD Brown, and Yanofski C (1981) Structure and regulation of *aroH*, the structural gene for the tryptophan-repressible 3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase of *Escherichia coli*. *J Mol Biol* **145**:47-73

Chapter III

Regulative Fine-Tuning of the Two Novel DAHP Isoenzymes aroFp and aroGp of the Filamentous Fungus *Aspergillus nidulans*

Abstract

Two novel genes *aroF* and *aroG* from the filamentous fungus *Aspergillus nidulans* were isolated, and the regulative fine-tuning between the encoded, differently regulated 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthases was analyzed. A wide range of DAHP synthase isoenzymes of various organisms are known, but only a few have been characterized further. DAHP synthases (EC 4.1.2.15) catalyze the first committed step of the shikimate pathway, which is a putative target for anti-weed drugs. The reaction is the condensation of erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP) to yield DAHP. The two purified DAHP synthases showed different affinities to the substrates, being 175 µM for PEP and 341 µM for E4P for the aroFp and weaker affinities of 239 µM (PEP) and 475 µM (E4P) for the aroGp isoenzyme. The enzymes are differently regulated by tyrosine (aroFp) and phenylalanine (aroGp). The calculated k_{cat} values are 7.0 s^{-1} for the tyrosine-inhibitable and 5.5 s^{-1} for the phenylalanine inhibitable enzyme (aroGp). Tyrosine is a competitive inhibitor according to PEP in the aroFp DAHP synthase. Phenylalanine is a competitive inhibitor according to E4P in the isoenzyme aroGp. Both enzymes are inhibited by the chelating agent EDTA, which indicates a metal ion as cofactor.

Introduction

The enzyme 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHP synthase) catalyzes the first step of the aromatic amino acid biosynthetic pathway, form the condensation of phosphoenolpyruvate and erythrose-4-phosphate to DAHP and inorganic phosphate (Haslam 1993).

According to their regulatory properties, various isoforms and numbers of DAHP synthase isoforms exist in microorganisms and plants (Byng et al. 1982; Byng and Jensen 1983). The only known DAHP synthases from filamentous fungi are the three DAHP synthase isoforms of *Neurospora crassa*. They are each regulated by one of the following three aromatic amino acids: phenylalanine, tyrosine and tryptophan (Hoffman et al. 1972; Nimmo and Coggins 1981a; Nimmo and Coggins 1981b). Only one of the three isoforms, the tryptophan-inhibitable protein, has been analyzed further. Although all DAHP synthases catalyze the same reaction, they differ in size and modes of competivity. On average there is a size spectrum of 350 amino acids for *Escherichia coli* enzymes, 370 amino acids for the yeast *Saccharomyces cerevisiae* enzymes, and 540 amino acids for the plant DAHP synthases. Catalytic analyses have given insight on the mode of action of specific inhibitors. In yeast, tyrosine acts as a competitive inhibitor according to phosphoenolpyruvate and as a non-competitive inhibitor according to erythrose-4-phosphate. Phenylalanine acts competitive towards erythrose-4-phosphate and non-competitive towards phosphoenolpyruvate (Schnappauf et al. 1998; Paravicini et al. 1989). Three isoforms exist in *E. coli*, each regulated by one of the three aromatic amino acids. The competivity of tyrosine towards the substrates is comparable with yeast whereas phenylalanine acts competitive towards PEP and E4P (Schoner and Herrmann 1976; Staub and Denes

1969). The activity of all characterized DAHP synthases depends on bivalent metal ions. Various bivalent metal ions are able to restore enzyme activity either partially or fully after complexing the native metal with the chelating agent EDTA (Stephens and Bauerle, 1991).

The DAHP synthases accelerate the input reaction of the shikimate pathway, which means their activities have to be strictly regulated. In filamentous fungi, a regulatory network acts on gene expression, the so-called cross-pathway control (Paluh et al., 1988). The final effector of this system is a transcriptional activator protein that binds to conserved sequence elements in the promoter regions of target genes. Under conditions of amino acid starvation, the transcription of these target genes is induced to counteract the starvation. The cross-pathway control network regulates all characterized genes coding for DAHP synthases of fungal origin.

To date, no DAHP-synthase-encoding gene of the filamentous fungus *Aspergillus nidulans* has been identified. Here we present a detailed characterization of the *aroF* and *aroG* gene products, two proteins that regulate the flux into the shikimate pathway in *A. nidulans.*

Materials and Methods

Strains, Media, Libraries, Growth Conditions. All chemicals were supplied by FLUKA or Sigma-Aldrich Chemie GmbH (Steinheim, Germany). *Saccharomyces cerevisiae* strain RH2424 (*MATa*, *can1-100, GAL, aro3*::*HIS3, aro4*::*LEU2, ura3-1*) was used as recipient to clone the *aroF* and *aroG* genes by complementation of the auxotrophy, employing an inducible *A. nidulans* cDNA expression library (Hoffmann et al. 2000). The genomic regions were determined using the chromosome-specific

recombinant cosmid-library of *A. nidulans* constructed by Brody *et al.* (1991). Transformation of *S. cerevisiae* was carried out by the LiOAc method (Ito et al. 1983). Minimal vitamins medium for the cultivation of yeast was described earlier (Miozzari et al. 1978).

RNA Preparation and Analysis. Total RNA was prepared from vegetatively growing *A. nidulans* cultures using TRIzol™ reagent (Life Technologies, Rockville, MD) following the suppliers' instructions. Transcript levels were analyzed by Northern hybridization (Alwine et al. 1977) using a Bio-Imaging Analyzer (Fuji Photo Film, Tokyo, Japan).

Purification of *A. nidulans* DAHP synthases. For overexpression, a derivative of plasmid p426MET25 (Mumberg et al. 1994) was used in *S. cerevisiae* strain RH2424. The plasmid-carrying yeast cells were grown at 30°C in 10-l rotatory fermentors under aeration. Cells were harvested in mid-exponential phase at an OD_{546} of 3-4. For a typical purification procedure, 15 g of cells (wet wt.) were used.

The DAHP synthases from *A. nidulans* were purified according to the protocol of Schnappauf *et al.* (1998) with the following modifications: the anion-exchange chromatography was carried out with 10 mM potassium phosphate (pH 7.6) buffer instead of 10 mM Tris-HCl buffer. DAHP synthases were detected by SDS-polyacrylamide gel electrophoresis (Laemmli 1970) and enzyme assays. Protein solutions were concentrated in stirred cells with PM-10 ultra filtration membranes from Millipore (Eschborn, Germany). Protein concentrations were performed using the Bradford (1976) assay.

Western Blot analysis. DAHP synthases of *A. nidulans* were immunologically detected with a polyclonal rabbit antibody against the tyrosine-inhibitable DAHP synthase of *S. cerevisiae* and a second antibody with horseradish peroxidase activity was detected using the ECL-method (Tesfaigzi et al. 1994).

Enzyme assays. The DAHP synthase activities were determined with the stop assay described by Takahashi and Chan (1971) with the modifications described by Schnappauf et al. (1998). Enzyme activities are specified in International Units (1 U = appearance of 1 µmol product per minute). Specific enzyme activities are given as mU (mg protein $^{-1}$).

Determination of kinetic parameters. Initial velocity data were determined by varying the concentration of one substrate at various fixed concentrations of the second substrate (Bisswanger, 1979). Double-reciprocal plots of the initial velocity against concentrations of the varied substrate showed a set of intersecting lines. A plot of the interceptions of these lines with the ordinate against the reciprocal of the concentrations of the fixed substrate gave a straight line. The Michaelis constants were determined from the value of these lines on the abscissa, corresponding to the reciprocal of the absolute value. The reciprocals of the apparent maximal velocities $1/V_{max}$ are on the ordinate. Rate constants *k* were determined from these values, and the known enzyme concentration e_o was determined according to $k = V_{max}/e_o$. Inhibitory constants were determined by varying the concentrations of a single substrate under various fixed concentrations of the inhibitor. The second substrate was kept at a constant concentration. The data were transformed to double-reciprocal plots. The K_i value was determined from secondary plots of the reciprocal intersections of the lines with the ordinate (noncompetitive inhibition) or the slopes of the lines (competitive

inhibition) against inhibitor concentration. The intersection with the abscissa provided the value for K_i.

Results

Isolation of the *aroF* and *aroG* genes of *Aspergillus nidulans*. The genes *aroF* and *aroG* of *Aspergillus nidulans* encode a phenylalanine- and a tyrosine- inhibitable DAHP synthase, respectively. Both genes were isolated by functional complementation of the auxotrophic *Saccharomyces cerevisiae* strain (RH2424) in which the *ARO3* and *ARO4* genes encoding the two DAHP synthases have been deleted. This yeast strain is devoid of endogenous DAHP synthase activity and therefore not able to grow on minimal medium without supplementation of the three aromatic amino acids phenylalanine, tyrosine and tryptophan. S. cerevisiae strain RH2424 was transformed with an *A. nidulans* cDNA expression library under the control of the yeast *GAL1* promoter (Hoffmann et al. 2000). Transformants were selected on medium without aromatic amino acids containing 2 % galactose as sole carbon source to induce the *GAL1* promoter and as consequence expression of putative DAHP synthase encoding cDNAs. Sixty clones whose growth phenotype was rescued by the expression of a plasmid-encoded *A. nidulans* cDNA were isolated. Restriction digest analysis of the recovered plasmids revealed that two different cDNAs were able to complement the yeast auxotrophy. One cDNA of each group was subcloned into pME1513 for overexpression and DAHP synthase activity measurements in strain RH2424. Specific enzyme tests from crude extracts revealed that the two cDNAs encode DAHP synthases, one inhibited by tyrosine and the other by phenylalanine. No inhibition by tryptophan was detected. Even careful rescreening of the

cDNA library never revealed any gene encoding a tryptophan-dependent DAHP synthase. This suggests that no tryptophan-inhibitable DAHP synthase exists, however it cannot be completely excluded that a third gene exists which is not significantly expressed under hyphal growth condition. In accordance to the putative homologous genes of *E. coli*, the genes of *A. nidulans* were named *aroF* and *aroG*, respectively. Both cDNAs were analyzed by DNA sequencing. *aroF* has an open reading frame of 1089 bp encoding 362 codons with the capacity to express a polypeptide with a deduced molecular mass of 39.3 kDa. The open reading frame of *aroG* is 1125 bp long, encoding 374 codons resulting in a protein with a deduced molecular mass of 40.5 kDa. The deduced amino acid sequences show significant

ClustalW [%]		*A. nidulans*		*S. cerevisiae*		*E.coli*		
		aroFp	**aroGp**	**Aro3p**	**Aro4p**	**AroF**	**AroG**	**AroH**
A. nidulans	**aroFp**	**100**	**74.3**	**74.0**	**75.9**	**68.9**	**68.0**	**65.2**
	aroGp	**74.3**	**100**	**77.9**	**73.7**	**66.9**	**69.0**	**62.3**
S. cerevisiae	**Aro3p**	**73.4**	**77.7**	**100**	**76.4**	**69.8**	**70.4**	**62.8**
	Aro4p	**75.9**	**73.7**	**76.4**	**100**	**66.5**	**69.7**	**64.2**
E. coli	**AroF**	**68.9**	**66.3**	**69.8**	**66.5**	**100**	**70.8**	**66.5**
	AroG	**68.3**	**69.0**	**70.4**	**69.3**	**69.8**	**100**	**74.9**
	AroH	**65.2**	**62.3**	**62.8**	**64.2**	**66.5**	**74.9**	**100**

Table 1 Protein sequence alignment of the DAHP synthases from *A. nidulans*, *S. cerevisiae* and *E. coli*. The data shows the percentages of the sum of sequence identity and the rate of high similarity. The calculated values are based on the alignment method ClustalW (Weight matrix: BLOSUM; Window size: 5; Gap penalty: 3) and were carried out at the IBCP, Lyon (France); *http://pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page= /NPSA/npsa_clustalw.html*.

stretches of amino acid residues identical to those of known DAHP synthases, suggesting homologies between the encoding genes. The sum of identical and similar residues to the *Saccharomyces cerevisiae* and *Escherichia coli* enzymes vary be-

tween 62.3 and 77.7 % (Table 1). The genomic loci of the two genes were isolated from a chromosome-wise ordered cosmid library by colony hybridization of the cDNAs and shown to be located on chromosome V (*aroF*) and on chromosome VIII (*aroG*) (Fig. 1).

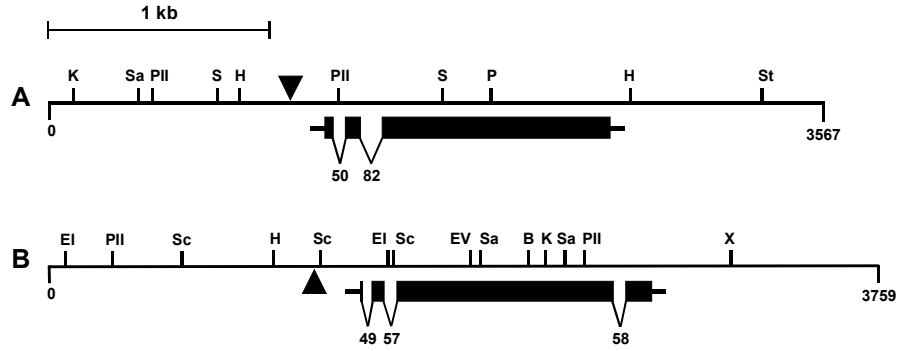

Fig. 1 Structure of the *aroF* (A) and *aroG* (B) genes of *A. nidulans*. The chromosomal loci of the two genes are shown. The GeneBank accession numbers for these sequences are AF283008 for the *aroF* gene and AF283009 for the *aroG* gene. The black boxes show the open reading frames, which are interrupted by introns, two introns in *aroF* and three *aroG*. ▼ indicate the putative CpcAp/Gcn4p binding sites. B, *Bam*HI; EI, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; P, *Pvu*I; PII, *Pvu*II; S, *Sph*I; Sa, *Sal*I; Sc, *Sac*I; St, *Stu*I; X, *Xba*I. Numbers indicate base pairs.

In a Southern hybridization experiment with the full-length cDNAs as probes, there was no detectable cross hybridization between the two genes (Fig. 2).

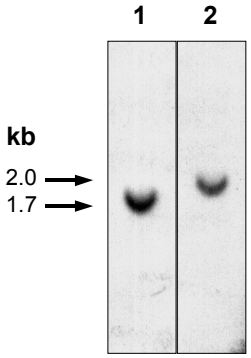

Fig. 2 Southern hybridization analysis of genomic DNA prepared from *A. nidulans* strain FGSC A234 (*yA2*, *pabaA1*, *veA1*). The Southern hybridization was carried out as described in Liu et al. (1992). The genomic DNA was digested with *Hind*III (1) or *Hind*III/*Xba*I (2), separated on an agarose gel, blotted onto nitrocellulose and probed with ^{32}P-labelled full-length *aroF* cDNA (1) and *aroG* cDNA (2).

No additional band was visible which could have been evidence for a third DAHP synthase of *A. nidulans*. The *aroF* open reading frame contains two introns, with the

length of 50 and 82 bp; three introns interrupt the *aroG* coding sequence with the length of 49, 56 and 57 bp.

Structural similarities between the DAHP synthases of *S. cerevisiae*, *E. coli* and *A. nidulans*. The phenylalanine-inhibitable *E. coli* enzyme was the first DAHP synthase whose crystal structure has been determined (Shumilin et al. 1999) and thus can be used for structure studies of other DAHP synthases. A deduced amino acid sequence alignment between the *aroF*- and *aroG*-encoded DAHP synthases of *A. nidulans,* the Aro3p and Aro4p enzymes of *S. cerevisiae,* and the phenylalanine-inhibitable DAHP synthase of *E. coli* reveals a high degree of identity between these enzymes (Fig. 3). Furthermore, it is shown that the crucial residues involved in metal- and PEP-binding in the *E. coli* enzyme are highly conserved in both *A. nidulans* enzymes. This suggests that the enzymes from all three organisms may have similar three-dimensional structures. Using the purified yeast Aro4 protein, we had previously raised polyclonal antibodies against the *S. cerevisiae* enzyme. By western blot analysis, we could show that this antibody also specifically recognizes the yeast Aro3 protein and both *A. nidulans* DAHP synthases aroFp and aroGp, when overexpressed in yeast (Fig. 4). This corroborates the results of the theoretical alignment studies that there are strong structural similarities between the DAHP synthases of these organisms.

The *aroF* and *aroG* genes are transcriptionally regulated by amino acid starvation. Putative CpcAp/Gcn4p binding sites were found in the *aroF* and *aroG* promoter regions (Dutton et al. 1997). The upstream positions of these sequence elements are –161 bp (5´-TTGAGTCTG-3´) and –220 bp (5´-GGATGACTCC-3´), respectively, relative to the start codon. These CpcAp/Gcn4p binding sites suggest

```
                  10        20        30        40        50        60
                   |         |         |         |         |         |
aroG_Ecoli   ---------------MNYQNDDLRIKEIKELLPPVALLEKFPATENAANTVAHARKAIHK
Aro3p_Scer   ------MFIKNDHAGDRKRLEDWRIKGYDPLTPPDLLQHEFPISAKGEENIIKARDSVCD
Aro4p_Scer   MSESPMFAANGMPKVNQGAEEDVRILGYDPLASPALLQVQIPATPTSLETAKRGRREAID
aroF_Anid    ---------------MSGQNEDTRVLGYDPLLSPEFVQSEIPSNEHSIPTVRSGRNQAVE
aroG_Anid    ---MSSFFLPNPNLGDSNHLEDSRIRGYNPLTPPNLLQHEIALTEKARQTVLQGRAEAIA
identity                          D R      L  P                    R

                  70        80        90       100       110       120
                   |         |         |         |         |         |
aroG_Ecoli   ILKGNDD---RLLVVIGPCSIHDPVAAKEYATRLLALREELKDELEIVMRVYFEKPRTTV
Aro3p_Scer   ILNGKDD---RLVIVIGPCSLHDPKAAYDYADRLAKISEKLSKDLLIIMRAYLEKPRTTV
Aro4p_Scer   IITGKDD---RVLVIVGPCSIHDLEAAQEYALRLKKLSDELKGDLSIIMRAYLEKPRTTV
aroF_Anid    IIEQRDD---RLLVVVGPCSIHDPETALEYATRLKELAARLSSDLCVIMRAYLEKPRTTV
aroG_Anid    VAHGTDTDKRRLLVVIGPCSIHDPEMALEYCDRLLKLKEKYKDELLIVMRSYLEKPRTTV
identity          D    R     GPCS HD   A  Y  RL          L   MR Y EKPRTTV

                 130       140       150       160       170       180
                   |         |         |         |         |         |
aroG_Ecoli   GWKGLINDPHMDNSFQINDGLRIARKLLLDINDSGLPAAGEFLDMITPQYLADLMSWGAI
Aro3p_Scer   GWKGLINDPDMNNSFQINKGLRISREMFIKLVEK-LPIAGEMLDTISPQFLSDCFSLGAI
Aro4p_Scer   GWKGLINDPDVNNTFNINKGLQSARQLFVNLTNIGLPIGSEMLDTISPQYLADLVSFGAI
aroF_Anid    GWKGLINDPDIDESYQINKGLRVSRKLYADLTGMGMPIASEMLDTISPQYMADLISLGAI
aroG_Anid    GWKGLINDPDIDNSFNINKGLRISRQLFVDLTNKGMPIASEMLDTISPQFLADCLSVGAV
identity     GWKGLINDP       IN GL   R           P   E LD I PQ   D  S GA

                 190       200       210       220       230       240
                   |         |         |         |         |         |
aroG_Ecoli   GARTTESQVHRELASGLSCPVGFKNGTDGTIKVAIDAINAAGAPHCFLSVTKWGHSAIVN
Aro3p_Scer   GARTTESQLHRELASGLSFPIGFKNGTDGGLQVAIDAMRAAAHEHYFLSVTKPGVTAIVG
Aro4p_Scer   GARTTESQLHRELASGLSFPVGFKNGTDGTLNVAVDACQAAAHSHHFMGVTKHGVAAITT
aroF_Anid    GARTTESQLHRELASGLSFPIGYKNGTDGNLTVAIDAIGAAAHPHRFLGVTKQGLAAITK
aroG_Anid    GARTTESQVHRELASGLSFPVGFKNGTDGSLDVAVDAIGSVKHPHHFLSVTKPGVVAIVG
identity     GARTTESQ HRELASGLS P G KNGTDG   VA DA       H F  VTK G  AI

                 250       260       270       280       290       300
                   |         |         |         |         |         |
aroG_Ecoli   TSGNGDCHIILRGGKE-PNYSAKHVAEVKEGLNKAGLP----AQVMIDFSHANSSKQFKK
Aro3p_Scer   TEGNKDTFLILRGGKNGTNFDKESVQNTKKQLEKAGLTDDSQKRIMIDCSHGNSNKDFKN
Aro4p_Scer   TKGNEHCFVILRGGKKGTNYDAKSVAEAKAQLPAGSNG------LMIDYSHGNSNKDFRN
aroF_Anid    TAGNPHGFVIMRGGNKGTNYDRESIQGAREALRGKKQRE----VLMVDCSHGNSKKNHRN
aroG_Anid    TVGNPDCFVILRGGKKGPNYDAASITEAKEKLIAKGLAP----RLMVDCSHGNSQKDHKN
identity     T GN     I RGG    N           L            M D SH NS K

                 310       320       330       340       350       360
                   |         |         |         |         |         |
aroG_Ecoli   QMDVCADVCQQIAGGEKAIIGVMVESHLVEGNQSLESGE---PLAYGKSITDACIGWEDT
Aro3p_Scer   QPKVAKCIYDQLTEGENSLCGVMIESNINEGRQDIPKEGGREGLKYGCSVTDACIGWEST
Aro4p_Scer   QPKVNDVVCEQIANGENAITGVMIESNINEGNQGIPAEG-KAGLKYGVSITDACIGWETT
aroF_Anid    QPLVAKEVADQLREGQDAIIGVMIESNINEGNQKVPPEG-PSGLKKGVSITDACIDWETT
aroG_Anid    QPKVAAVLAEQIAAGETAIMGVMIESNINEGNQKVPPEG-KAGLKYGVSITDACINWEDT
identity     Q  V      Q   G     GVM ES   EG Q          L  G S TDACI WE T

                 370       380
                   |         |
aroG_Ecoli   DALLRQLANAVKARRG---------
Aro3p_Scer   EQVLELLAEGVRNRRKALKK-----
Aro4p_Scer   EDVLRKLAAAVRQRREVNKK-----
aroF_Anid    VTVLEDLADAVRARRAVKSKANGTA
aroG_Anid    ESTLETLAKAVATRREKLAPSS---
identity        L  LA  V  RR
```

Fig. 3 Sequence alignment of the *aroG*-encoded DAHP synthase (phenylalanine-inhibitable) of *E. coli* with the *A. nidulans* and *S. cerevisiae* DAHP synthases. The residues shaded in light gray indicate the metal- and PEP-binding sites of the phenylalanine-inhibitable DAHP synthase of *E. coli*, the crystal structure of which was recently published (Shumilin et al. 1999). The bottom row shows amino acids which are identical in all five sequences in this alignment.

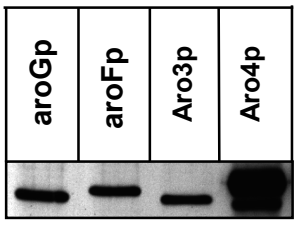

Fig. 4 Western blot analysis of the two DAHP synthases from *A. nidulans*. Crude extracts of yeast strain RH2424 harboring different 2µm plasmids were analyzed with a polyclonal rabbit antibody, raised against the Aro4p of *S. cerevisiae*, showing structural similarities of aroFp and aroGp to that enzyme. Each lane was loaded with 0.8 µg of protein.

that these genes are regulated by the cross-pathway control of amino acid biosynthesis by CpcA, the protein encoded by the *A. nidulans* homologue of the yeast *GCN4* gene. In fungi, amino acid starvation induces the expression of a genetic network including numerous amino acid biosynthesis genes. The central transcriptional activator Gcn4p of yeast, *cpcA* of *Aspergillus niger* (Wanke et al. 1997), and *cpc-1* of *N. crassa* (Paluh et al. 1988) have been characterized and bind to conserved target sequences within regulated promoters. As there are putative binding sites for these transcription factors in the promoter regions of *aroF* and *aroG*, the effect of amino acid starvation on the expression level of these genes was tested. *A. nidulans* strain FGCS A234 (*yA2*, *pabaA1*, *veA1*) was cultivated in liquid minimal medium for 20 h before the mycelium was transferred to fresh medium containing 3-amino-1,2,4-triazole (3AT). 3AT acts as false feedback inhibitor in the histidine biosynthesis pathway and therefore mimics amino acid starvation by depletion of the histidine pool (Hilton et al. 1965). Mycelium was harvested after different time points, and total RNA was prepared and subjected to Northern hybridization analysis using the *aroF* and *aroG* cDNAs as probes (Fig. 5).

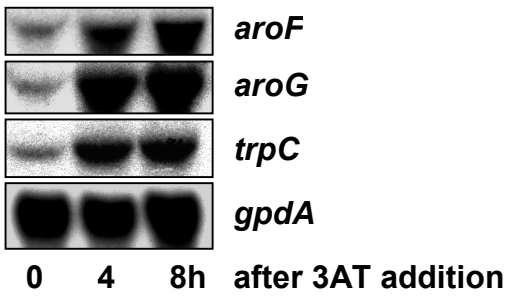

Fig. 5. Northern hybridization analysis of total RNAs prepared from *A. nidulans* strain FGSC A234 (*yA2*, *pabaA1*, *veA1*). The autoradiograph shows different time points after shifting to 3-amino-1,2,4-triazole (3AT)-containing medium. Each lane was loaded with 20 µg of total RNA. The RNA was labeled with probes specific for *aroF*, *aroG*, *trpC* or *gpdA*.

As controls, the expression of *gpdA* (Punt et al. 1988), a gene encoding a glycolytic enzyme (glyceraldehyde-3-phosphate dehydrogenase, EC 1.2.1.12) that is constitu-

tively expressed and *trpC*, a tryptophan biosynthesis gene reported to be induced upon amino acid starvation by the cross-pathway control (Eckert et al. 1999) were tested. Genes *aroF* and *aroG* were induced by a factor of 3.5 when 3AT was added to the culture. This induction was kinetically and quantitatively similar to the induction of the *trpC* gene (Yelton et al. 1983). This result suggests that *aroF* and *aroG* are targets of the cross-pathway control network, which is activated by the environmental signal amino acid starvation.

Feedback regulation of the *aroF*- and *aroG*-encoded DAHP synthases by tyrosine and phenylalanine, respectively. In order to characterize the enzymatic properties of aroFp and aroGp, the respective gene products were overexpressed in the yeast strain RH2424 under the control of the yeast *MET25* promoter, and the proteins were purified as described in Materials and Methods. The proteins were enriched 60-fold and purified to 95% homogeneity to analyze the enzyme activities and inhibition patterns of the two DAHP synthases of *A. nidulans* at various inhibitor concentrations (Fig. 6).

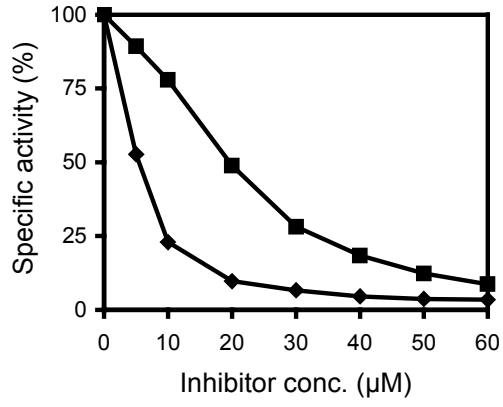

Fig. 6 Inhibition of the DAHP synthases from *A. nidulans* with their specific inhibitors tyrosine for aroF (■) and phenylalanine for aroG (◆). There were no cross inhibition effects detectable within the range shown. In addition, no inhibition by tryptophan was detected.

This inhibition study revealed a specifically different behavior of the isoenzymes towards increasing inhibitor concentrations. aroGp, which is inhibited by phenylalanine, is more sensitive to its inhibitor than the isoenzyme aroFp is to its inhibitor tyrosine. At an inhibitor concentration of 10 µM, the difference between the enzyme activities was 55 % in favor of the tyrosine-inhibited enzyme aroFp. This is a remarkable difference to the *S. cerevisiae* enzymes, where the sensitivities to the corresponding inhibitors are inverse (Schnappauf et al. 1998). No cross inhibition effects were detected. In addition, tryptophan at concentrations up to 60 µM had no effect on either enzymatic activity (not shown). This suggests that the regulation pattern of the DAHP synthase isoenzymes could be developed in evolution after separation between yeast and filamentous fungi.

Kinetic properties of the aroFp and aroGp. The kinetic parameters of the purified enzymes were measured. The reaction catalyzed by DAHP synthases is a two-substrate reaction. To obtain the steady-state kinetics, the concentration of one substrate was varied and the enzyme activity at different fixed concentrations of the other substrate was measured (Fig. 7A-D).

The two enzymes have a common intersection in the double-reciprocal plots (Lineweaver-Burk plots) of initial velocity plotted against the varying substrates. The kinetic parameters were obtained by secondary plots, where the reciprocal substrate concentrations were plotted against the intercepts of the y-axis (Table 2). Both K_m values (one for PEP and the other for E4P) of the aroFp enzyme are lower than that of the isoenzyme aroGp. This implies that the affinity of both substrates for aroFp is higher than for the aroGp protein. In addition, for both enzymes the affinity of PEP is higher than that of the other substrate E4P. With the apparent V_{max} values and the known enzyme concentration e_0, the turnover number k_{cat} of both enzymes was

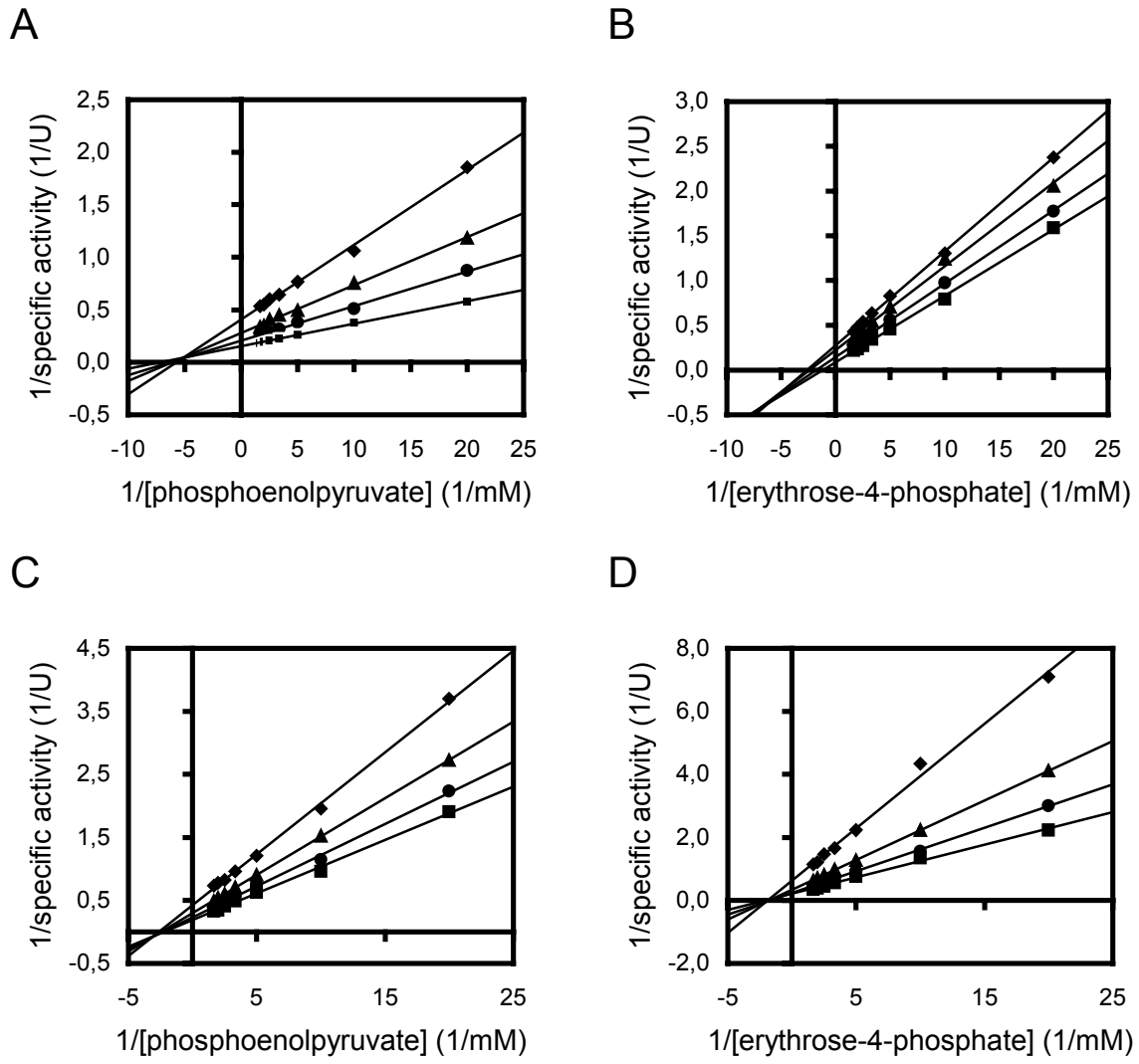

Fig. 7A-D Substrate saturations of the tyrosine- (A,B) and phenylalanine-inhibitable (C,D) DAHP synthases of *A. nidulans*. A,C Lineweaver-Burk plot with various phosphoenolpyruvate concentrations and fixed erythrose-4-phosphate concentrations of ◆ 0.1 mM, ▲ 0.2 mM, ● 0.3 mM and ■ 0.4 mM. **B,D** Lineweaver-Burk plot with various erythrose-4-phosphate concentrations and fixed phosphoenolpyruvate concentrations of ◆ 0.05 mM, ▲ 0.1 mM, ● 0.15 mM and ■ 0.2 mM.

	A. nidulans		*S. cerevisiae*	
	aroFp	**aroGp**	**Aro3p** [a]	**Aro4p** [b]
K_m (PEP) [µM]	**175**	**239**	**18**	**125**
K_m (E4P) [µM]	**341**	**475**	**130**	**500**
K_i [µM]	**8.4**	**1.2**	**10**	**0.9**
k_{cat} [s^{-1}]	**7.0**	**5.5**	**7.0**	**6.0**

Table 2 Kinetic parameters of the DAHP synthases from *A. nidulans* and *S. cerevisiae*. The K_i values refer to the specific inhibitors of each DAHP synthase. [a] Paravicini et al., 1989, [b] Schnappauf et al., 1998

calculated per monomer to 7.0 s^{-1} for the protein aroFp and 5.5 s^{-1} for the protein aroGp, which means that the tyrosine-inhibitable DAHP synthase is slightly more effective than the isoenzyme.

Inhibition studies of the DAHP synthases of *A. nidulans*. We had previously shown that the specific inhibitor tyrosine acts as a competitive inhibitor according to

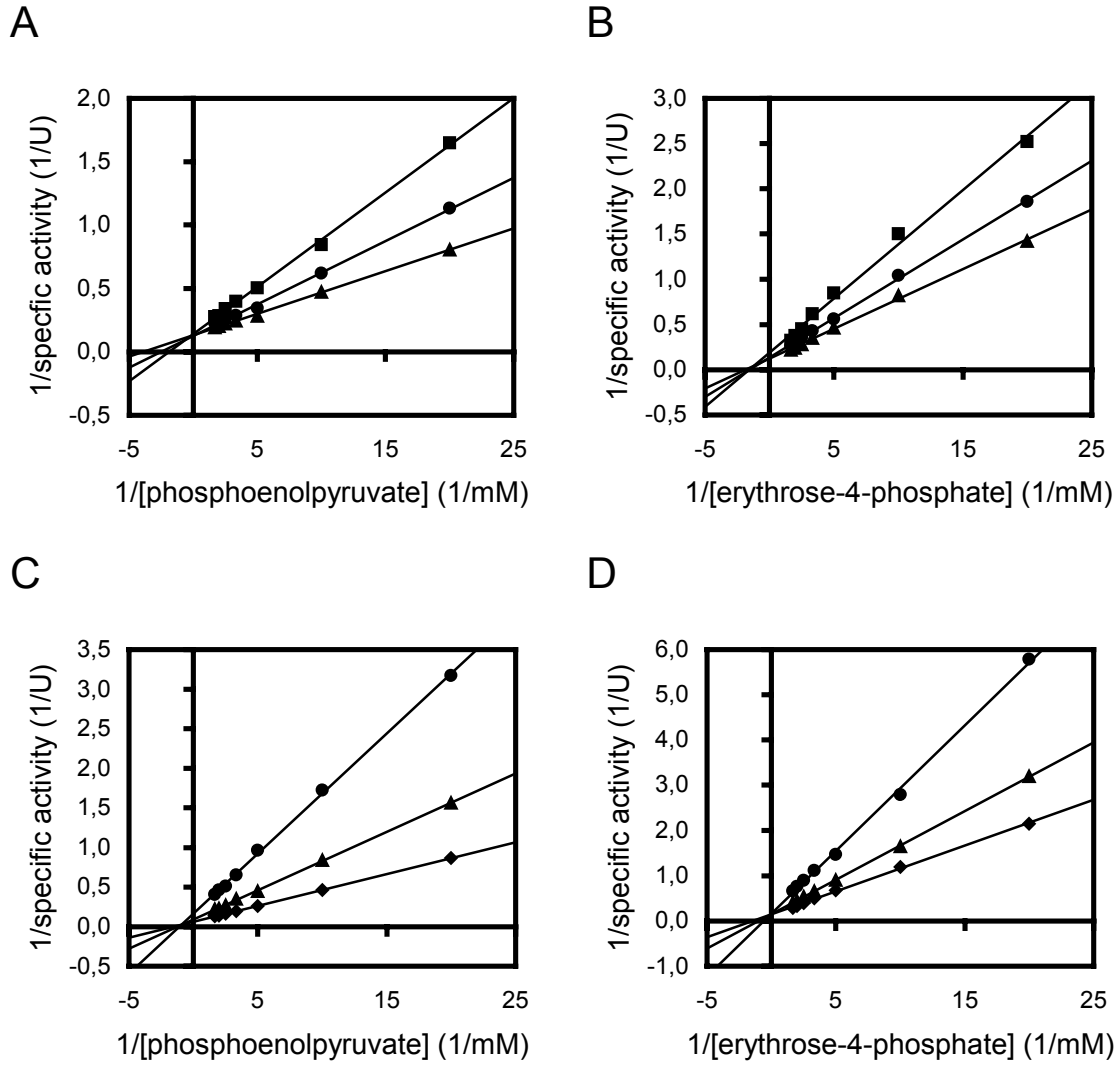

Fig. 8A-D Inhibition studies of the tyrosine- (A,B) and phenylalanine-inhibitable (C,D) DAHP synthases of *A. nidulans*. A,B Lineweaver-Burk plot with 0.5 mM substrate concentrations and fixed tyrosine concentrations of ▲ 5 µM, ● 10 µM and ■ 15 µM. **C,D** Lineweaver-Burk plot with 0,5 mM substrate concentrations and fixed phenylalanine concentrations of ◆ 1 µM, ▲ 2.5 µM and ● 5 µM.

phosphoenolpyruvate and that phenylalanine acts competitive according to erythrose-4-phosphate in yeast (Schnappauf et al. 1998). To verify the competivity

for the DAHP synthases of *A. nidulans*, enzyme tests with various concentrations of one substrate and a fixed concentration (0.5 mM) of the other were carried out. For inhibition, various fixed inhibitor concentrations of tyrosine (for aroFp) and phenylalanine (for aroGp), were used (Fig. 8A-D). In all Lineweaver-Burk plots, there is a common intersection of the lines representing different inhibitor concentrations. The intersection hits the ordinate at various phosphoenolpyruvate concentrations (tyrosine-inhibitable enzyme aroFp, Fig.8A) and at various concentrations of erythrose-4-phosphate (phenylalanine-inhibitable enzyme aroGp, Fig.8D). Thus, phenylalanine is a competitive inhibitor with respect to E4P for the aroGp isoenzyme, whereas tyrosine is a competitive inhibitor with respect to PEP for the aroFp DAHP synthase. Furthermore, phenylalanine and tyrosine are non-competitive inhibitors with respect to PEP and E4P, respectively. The K_i values are 8.4 µM for the tyrosine-inhibitable DAHP synthase and 1.2 µM for the phenylalanine-inhibitable isoenzyme. Therefore, phenylalanine has an inhibitory effect on the related enzyme, which is increased by one order of magnitude in comparison to the inhibitory effect of tyrosine on the corresponding enzyme.

Both DAHP synthases of *A. nidulans* are metalloproteins. For other DAHP synthases, it has been shown that enzyme activity decreases when the concentration of the metal-chelating agent EDTA increases, and that it is possible to destroy enzyme activity completely at high EDTA concentrations (Schnappauf et al. 1998). Therefore, it was of interest to determine whether the *A. nidulans* enzymes are also dependent on a bivalent metal; enzyme assays with increasing EDTA concentrations were carried out (Fig. 9A). At concentrations between 1 and 4 µM the enzymes lost 75 % of their activity. Higher concentrations up to 1.5 mM had no further significant effect on the enzyme activity.

If an enzyme is inhibitable by the chelating agent EDTA, whether this loss of activity is restorable by bivalent metals is of interest. Several bivalent metals of the first row of transition elements as well as Cadmium ions were tested. In the test system, the enzyme samples were incubated with 1.50 mM EDTA at room temperature. After 5 min incubation 1.75 mM of several Me^{2+} ions (one per test) were added and incubation was carried out for another 15 min at room temperature. After inhibition and reactivation of the proteins, enzymes were assayed to determine the rates of reactivation (Fig. 9B).

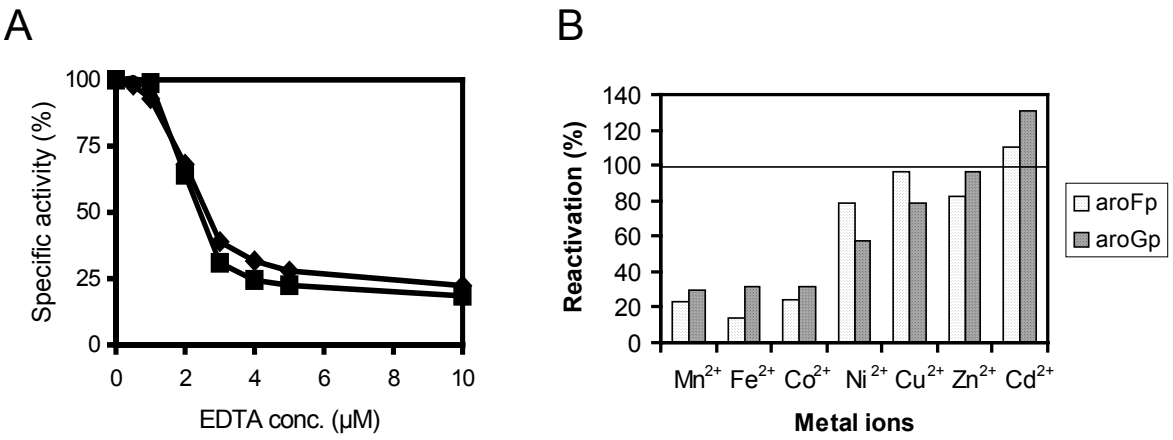

Fig. 9. Inhibition by EDTA. A Inhibition patterns of the DAHP synthases aroFp (■) and aroGp (□) from *A. nidulans* with varying EDTA concentrations. Between 1 and 3 µM EDTA there is 75 % loss of activity. Even with concentrations up to 1.5 mM it is not possible to increase this activity loss. **B** Reactivation of the DAHP synthase activities of *A. nidulans* with various bivalent metal ions after EDTA inactivation. The values reflect the activities in percentage compared to the EDTA- and metal-untreated enzyme.

Enzyme activities of both enzymes were restorable up to at least 80% by bivalent copper and zinc ions. Cadmium ions activated both *A. nidulans* DAHP synthases. Manganese, iron, and cobalt ions restored enzyme activities only up to 30%.

The pH dependence of DAHP synthases activity. The two DAHP synthase isoenzymes from *A. nidulans* show different pH optima. Enzyme activity starts at a pH of about 5.0 – 5.5, has an optimum between 6.5 and 7.5 and remain nearly stable to a

pH up to 8.0. The tyrosine-inhibitable aroFp had an activity optimum at a pH of 6.8; the phenylalanine-inhibitable isoenzyme had an optimum at a pH of 7.5.

Temperature effects on the *A. nidulans* DAHP synthases. The enzyme activity and stability of both DAHP synthases were tested at different temperatures. First, activities were measured at different temperatures with the enzyme assay described in Materials and Methods. In a second approach and to investigate the stability properties of both enzymes, the proteins were pre-incubated 5 min at different fixed temperatures between 25°C and 60°C. After incubation, the enzymes were assayed at 37°C to measure the remaining activities (Fig. 10A,B). Both enzymes are stable up to a temperature of about 40°C. Above this temperature the activity sharply decreased. At a temperature of about 55°C only 5 – 10 % of activity remained.

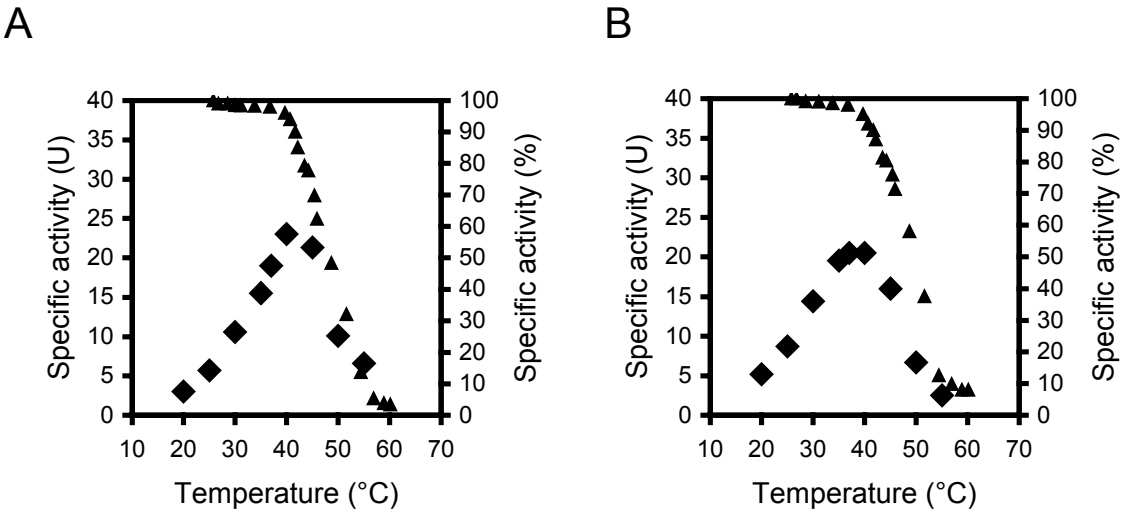

Fig. 10A,B. Temperature effect and heat inactivation of the aroF (A) and aroG (B) proteins of *A. nidulans*. The enzymatic assays were carried out with substrate concentrations of 0.5 mM of each substrate. The left y-axis shows the specific activity after 5 min reaction time (◆) given in units according to the test temperature. The right y-axis show the inactivation of the enzymes (▲) after incubating 5 min at a constant temperature. The values are given in percentage according to the enzyme treated at 37°C.

Discussion

Two novel genes, *aroF* and *aroG* , encoding DAHP synthases of the filamentous fungus *A. nidulans* were isolated and the gene products were characterized. In contrast to the filamentous fungus *N. crassa,* no gene for a tryptophan inhibitable DAHP synthase could be recovered from a cDNA library, which was constructed from normally growing hyphae. Both genes are part of the cross-pathway control of *A. nidulans*. The activation by a transcriptional activator similar to Gcn4p of yeast with a factor of 3.5 for both genes is significant but weak compared to the reported genes so far (Paluh et al., 1988). This is similar in the yeast *S. cerevisiae,* where both enzymes are regulated by the general control. This means that the first step of the aromatic amino acid biosynthesis is a important step and has to be regulated precisely in eukaryotes.

As the DAHP synthases are Me^{2+} enzymes, it was not surprising that EDTA had an inhibitory effect on both *A. nidulans* isoenzymes. More surprising was the fact that even an EDTA concentration up to 1.5 mM did not completely abolish enzyme activity. In contrast, the *S. cerevisiae* enzymes are already inactive under these conditions. This suggests that complete removal of the metal ion from the enzyme is not possible under the conditions tested or that in contrast to the known DAHP synthases, the *A. nidulans* enzyme activities are not completely dependent on a bivalent metal. Reactivation was possible by copper and zinc ions, which indicates that these two metals could be cofactors of the native *A. nidulans* DAHP synthases *in vivo*. Cadmium even had an activating effect on the EDTA-inhibited enzymes of *A. nidulans,* but the biological implication of that is questionable. In general, the metal binding sites of DAHP synthases seem to be variable to bind more than one kind of metal ion as there are reports of different metal ions which can reactivate activity in differ-

ent microorganisms (Schnappauf et al. 1998; Paravicini et al. 1989; Stephens and Bauerle 1991).

Although DAHP synthases share common properties concerning their catalytic behavior and presumably their overall fold they show distinct differences according to regulation. They differ mostly in efficiency and inhibitor affinity. DAHP synthases of *A. nidulans* or *S. cerevisiae* with average k_{cat} values of 6 s^{-1} are less efficient than *E. coli* DAHP synthases with k_{cat} values of 21 s^{-1} (tryptophan-inhibitable) and 121 s^{-1} (tyrosine-inhibitable). In contrast to *S. cerevisiae* enzymes, the tyrosine-inhibitable isoenzyme of *A. nidulans* is more efficient. In addition the affinity of both substrates is higher to the tyrosine-regulated enzyme in *A. nidulans*. This is different than in yeast where the phenylalanine-regulated isoenzyme has a higher affinity to both substrates. Therefore, we conclude that the common ancestor of the DAHP synthases isoenzymes had a different regulation pattern.

Both characterized *A. nidulans* DAHP synthases are inhibited by an end product of the biosynthetic pathway. In the filamentous fungus *A. nidulans,* the phenylalanine-regulated enzyme is strongly inhibited with a K_i of 1.2 µM, whereas in *S. cerevisiae* phenylalanine inhibits the corresponding isoenzyme with a ten times lower magnitude of strength (K_i of 10 µM). The tyrosine-inhibitable isoenzyme of *A. nidulans* is regulated with the same magnitude of concentration (K_i of 8.4 µM). This is ten times weaker than the regulation of the tyrosine-isoenzyme in the yeast *S. cerevisiae* (K_i value of 0.9 µM). This means that in contrast to yeast, *A. nidulans* could regulate the flux into the shikimate pathway mainly by phenylalanine (Fell, 1992). Compared to the *E. coli* DAHP synthases, where the K_i values are much higher (8-fold) (Schoner and Herrmann 1976; Simpson and Davidson 1976) fungal DAHP synthases seem to be more carefully regulated on the enzymatic level.

Only two differently regulated isoenzymes were found in the filamentous fungus A. nidulans, in contrast to N. crassa, where three isoforms are present. Compared to the yeast S. cerevisiae, phenylalanine seems to play the more important role in regulation of the flux into the shikimate pathway in A. nidulans. This suggests that the evolution of the fine-tuning of the DAHP synthase enzyme regulation has developed after the taxonomic splitting of unicellular yeasts and filamentous fungi. It will be intriguing to determine whether phenylalanine or tyrosine is the more important pillar of regulation in *N. crassa*.

References

Alwine JC, Kemp DJ, and Stark GR (1977) Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. *Proc Natl Acad Sci* U.S.A. **74**:5350-5354

Bisswanger H (1979) Theorie und Methoden der Enzymkinetik. Verlag Chemie, Weinheim Deerfield Beach Basel

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **73**:248-254

Brody H, Griffith J, Cuticchia AJ, Arnold J, and Timberlake WE (1991) Chromosome-specific recombinant DNA libraries from the fungus *Aspergillus nidulans*. *Nucleic Acids Res* **19**:3105-3109

Byng GS, Kane JF, and Jensen RA (1982) Diversity in the routing and regulation of complex biochemical pathways as indicators of microbial relatedness. *Crit. Rev. Microbiol* **9**:227-252

Byng GS and Jensen RA (1983) Impact of isozymes upon partitioning of carbon flow and regulation of aromatic biosynthesis in prokaryotes. *Curr Top Biol Med Res* **8**:115-140

Dutton JR, Johns S, and Miller BL (1997) StuAp is a sequence-specific transcription factor that regulates developmental complexity in *Aspergillus nidulans*. *EMBO J* **16**:5710-5721

Eckert SE, Hoffmann B, Wanke C, and Braus GH (1999) Sexual development of *Aspergillus nidulans* in tryptophan auxotrophic strains. *Arch Microbiol* **172**:157-166

Fell DA (1992) Metabolic control analysis: a survey of its theoretical and experimental development. *Biochem J* **286**:313-370

Haslam E (1993) Shikimic Acid. Wiley, Chichester New York, Brisbane

Hilton JL, Kearney PC, and Ames BN (1965) Mode of action of the herbicide, 3-amino-1,2,4-triazole(amitrole): inhibition of an enzyme of histidine biosynthesis. *Arch Biochem Biophys* **112**:544-547

Hoffmann B, Wanke C, La Paglia SK, and Braus GH (2000) *c-Jun* and *RACK1* homologues regulate a control point for sexual development in *Aspergillus nidulans*. *Mol Microbiol* **37**:28-41

Hoffmann PJ, Doy CH, and Catcheside DEA (1972) The separation of three allosterically inhibitable 3-deoxy-D-arabino-heptulosonate 7-phosphate synthases from extracts of *Neurospora crassa* and the purification of the tyrosine inhibitable isoenzyme. *Biochim Biophys Acta* **268**:550-561

Ito H, Fukuda Y, Murata K, and Kimura A (1983) Transformation of intact yeast cells treated with alkali cations. *J Bacteriol* **153**:163-168

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680-685

Liu H, Krizek J, and Bretscher A (1992) Construction of a *GAL1*-regulated yeast cDNA expression library and its application to the identification of genes whose overexpression causes lethality in yeast. *Genetics* **132**:665-673

Miozzari G, Niederberger P, and Hütter R (1978) Tryptophan biosynthesis in *Saccharomyces cerevisiae*: control of the flux through the pathway. *J Bacteriol* **134**:48-59

Mumberg D, Müller R, and Funk M (1994) Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression. *Nucleic Acids Res* **22**:5767-5768

Nimmo G and Coggins RJ (1981a) The purification and molecular properties of the tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase from *Neurospora crassa*. *Biochem J* **197**:427-436

Nimmo G and Coggins RJ (1981b) Some kinetic properties of the tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase from *Neurospora crassa*. *Biochem J* **199**:657-665

Paravicini G, Schmidheini T, and Braus GH (1989) Purification and properties of the 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (phenylalanine-inhibitable) of *Saccharomyces cerevisiae*. *Eur J Biochem* **186**:361-366

Paluh JL, Orbach MJ, Legerton TL, and Yanofsky C (1988) The cross-pathway control gene of *Neurospora crassa*, *cpc-1*, encodes a protein similar to *GCN4* of yeast and the DNA-binding domain of the oncogene *v-jun*-encoded protein. *Proc Natl Acad Sci* U.S.A. **85**:3728-3732

Punt PJ, Dingemanse MA, Jacobs-Meijsing BJM, Pouwels PH, and van den Hondel CAMJJ (1988) Isolation and characterization of the glyceraldehyde-3-phosphate dehydrogenase gene of *Aspergillus nidulans*. *Gene* (Amst.) **69**:49-57

Schnappauf G, Hartmann M, and Braus GH (1998) The two 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase isoenzymes from *Saccharomyces cerevisiae* show different kinetic modes of inhibition. *Arch Microbiol* **169**:517-524

Schoner R and Herrmann KM (1976) 3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase. Purification, properties, and kinetics of the tyrosine-sensitive isoenzyme from *Escherichia coli*. *J Biol Chem* **251**:5440-5447

Shumilin IA, Kretsinger RH, and Bauerle RH (1999) Crystal structure of phenylalanine-regulated 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase from *Escherichia coli*. *Structure Fold Des* **7**:865-875

Simpson RJ, and Davidson BE (1976) Studies on 3-deoxy-D-arabinoheptulosonate-7-phosphate synthetase (phe) from *Escherichia coli* K12. 2. Kinetic properties. *Eur J Biochem* **70**:501-507

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98**:503-517

Staub M and Denes G (1969) Purification and properties of the 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (phenylalanine sensitive) of *Escherichia coli* K12. II. Inhibition of activity of the enzyme with phenylalanine and functional group-specific reagents. *Biochim Biophys Acta* **178**:599-608

Stephens MS and Bauerle R (1991) Analysis of the metal requirement of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase from *Escherichia coli*. *J Biol Chem* **266**:20810-20817

Takahashi M and Chan WWC (1971) Separation and properties of isozymes of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthetase from *Saccharomyces cerevisiae*. *Can J Biochem* **49**:1015-1025

Tesfaigzi J, Smith-Harrison W, and Carlson DM (1994) A simple method for reusing western blots on PVDF membranes. *BioTechniques* **17**:268-269

Wanke C, Eckert S, Albrecht G, van Hartingsveldt W, Punt PJ, van den Hondel CA, and Braus GH (1997) The *Aspergillus niger GCN4* homologue, *cpcA*, is transcriptionally regulated and encodes an unusual leucine zipper. *Mol Microbiol* **23**:23-33

Yelton MM, Hamer JE, deSouza ER, Mullaney EJ, and Timberlake WE (1983) Developmental regulation of the *Aspergillus nidulans trpC* gene. *Proc Natl Acad Sci* U.S.A. **80**:7576-7580

Chapter IV

Evolution of a Differently Regulated Isoenzyme by a Single Amino Acid Substitution

Abstract

The specific regulation of the tyrosine-inhibitable 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase from *Saccharomyces cerevisiae* (Aro4p) (EC 4.1.2.15) was compared with the phenylalanine-regulated isoenzyme (Aro3p). Chimera proteins built of Aro3p and Aro4p show different reactions towards the specific inhibitors. Random and site-directed mutagenesis experiments with Aro4p lead to tyrosine-resistant mutant enzymes. The Aro4p crystal structure was solved and revealed a cavity of the protein which is strongly involved in regulation. Part of the cavity in Aro4p is amino acid G226. This glycine residue is highly conserved within the tyrosine-inhibitable DAHP synthases. In the phenylalanine DAHP synthase isoenzymes this amino acid is a serine which is also highly conserved. The exchange of these amino acids in the *S. cerevisiae* DAHP synthases lead to a complete exchange of the regulation pattern of each enzyme suggesting that this amino acid residue is crucial for enzyme regulation. Therefore throughout evolution only one amino acid exchange was necessary to create two differently regulated DAHP synthase isoenzymes.

Introduction

Isoenzymes were at first regarded as interesting but rare occurrences. In the past 40 years the amount of information, however, has made likely that at least half of all enzymes exist as isoenzymes (Rider and Taylor, 1980). Amino acid sequences of isoenzymes are usually highly similar and allow studies on domains which have been conserved during the course of evolution. It is not possible to deduce from isoenzyme sequences the route of gene evolution exactly, as we are looking only at the end products of evolution, and it is impossible to know all the intermediate stages, even if many amino acid substitutions can be accounted for by only a single base pair change (Ryder and Taylor, 1980). It is therefore intriguing to study the evolved and essential differences between isoenzymes which make them unique and justify their existence. A model system for that are the DAHP synthase isoenzymes. DAHP synthases catalyze the first step of the biosynthesis of aromatic amino acids, the condensation of erythrose-4-phosphate and phosphoenolpyruvate (Haslam 1993). With regard to number and regulation pattern a high variability of DAHP synthase isoenzyme systems are distributed in microorganisms and plants (Byng et al. 1982; Byng and Jensen 1983). In *Escherichia coli* and *Neurospora crassa* there are three DAHP synthase isoenzymes, differently regulated by the three aromatic amino acids phenylalanine, tyrosine or tryptophan (Schoner and Herrmann 1976; Staub and Denes 1969, Hoffman et al. 1972; Nimmo and Coggins 1981a; Nimmo and Coggins 1981b). Only two isoenzymes are known in yeast *Saccharomyces cerevisiae*, the phenylalanine- (Aro3p) and tyrosine-regulated (Aro4p) DAHP synthase (Schnappauf et al. 1998; Paravicini et al. 1989). Although the DAHP synthases are differently regulated, they share high structural similarities throughout the known DAHP syn-

thase isoenzymes of microorganisms. The first reported crystal structure of a DAHP synthase isoenzyme was that of the phenylalanine-inhibitable DAHP synthase from *E. coli* (Shumilin et al. 1999). The monomer is a $(\alpha/\beta)_8$ barrel with several additional β strands and α helices. The active form was determined to be at least dimeric or tetrameric (Park et al., 1999). It was co-crystallized with PEP and a bivalent Pb^{2+} ion, which was added for crystallization. For yeast *S. cerevisiae* the first structural studies were reported by Schneider et al. (1999). As there are high structural similarities but different regulation patterns, it is intriguing to identify the crucial difference which makes regulation of each DAHP synthase within a microorganism unique.

Materials and Methods

Materials, media and growth conditions. All chemicals were supplied by FLUKA or Sigma-Aldrich Chemie GmbH (Steinheim, Deisenhofen, Germany). Minimal vitamins and synthetic complete medium for the cultivation of yeast was described earlier (Miozzari et al., 1978, Sherman et al., 1986). Transformation of *Saccharomyces cerevisiae* was carried out by the LiOAc method (Ito et al., 1983).

Strains and plasmids. *Saccharomyces cerevisiae* strain RH2424 (*MATa*, *can1-100, GAL, aro3*::*HIS3, aro4*::*LEU2, ura3-1*) was used for the chimera and mutagenesis experiments. Yeast strain RH1326 (*MATa*, *aro3-2*, *aro4-1*, *gcd2-1*, *leu2-2*) was used as recipient for overexpression of pME2015 encoding the protein Aro3p-S219G and pME2016 encoding the protein Aro4p-G226S. pME1513 was used to clone the alleles from the mutagenesis experiments. It is a derivate of *URA3*-carrying plasmid

Plasmids	Description	Source
pME1513	pRS426MET25 (Mumberg et al., 1994) with different MCS (*Sac*I pMet25 *Xba*I *Spe*I *Bam*HI *Sal*I *Sfi*I *Not*I *Xho*I T*CYC1 Kpn*I)	Krappmann et al., 2000
pME2017	pME825 (Graf et al, 1993) with *ARO4* promoter and terminator cloned into the MCS	This study
Chimera Protein Experiments		
pME1947	Fusion gene 3p-4p in pME1513, Gene product setup: Aro3p(1.181) / Aro4p(188.370)	This study
pME1951	Fusion gene 3p-(C-4p) in pME1513, Gene product setup:Aro3p(1.285) / Aro4p(287.370)	This study
pME1952	Fusion gene 3p-4p(C-3p) in pME1513, Gene product setup:Aro3p(1.181) / Aro4p(189.286) / Aro3p(286.370)	This study
pME1953	Fusion gene 4p-3p-(C-4p) in pME1513, Gene product setup:Aro4p(1.188) / Aro3p(182.285) / Aro4p(287.370)	This study
PCR Random Mutagenesis Experiments		
pME1976	Mutated 1.1 kb *ARO4* fragment in pME1513, Mutation of the corresponding gene product: S195P	This study
pME1977	Mutated 1.1 kb *ARO4* fragment in pME1513, Mutation of the corresponding gene product: R55G	This study
pME1978	Mutated 1.1 kb *ARO4* fragment in pME1513, Mutation of the corresponding gene product: D22G	This study
pME1980	Mutated 1.1 kb *ARO4* fragment in pME1513, Mutation of the corresponding gene product: T44I	This study
pME1982	Mutated 1.1 kb *ARO4* fragment in pME1513, Mutation of the corresponding gene product: E49G	This study
pME1983	Mutated 1.1 kb *ARO4* fragment in pME1513, Mutation of the corresponding gene product: F196S	This study
N-terminal Truncated Aro4p Enzymes		
pME1661	Truncated ARO4 allele in pME1513 without the first 20 codons	
pME1662	Truncated ARO4 allele in pME1513 without the first 32 codons	
Site-directed Mutagenesis Experiments		
pME1925	Mutated 1.1 kb *ARO4* fragment in pME1513, Mutation of the corresponding gene product: Y28F	This study
pME1931	Mutated 1.1 kb *ARO4* fragment in pME1513, Mutation of the corresponding gene product: G193K	This study
pME1932	Mutated 1.1 kb *ARO4* fragment in pME1513, Mutation of the corresponding gene product: G226A	This study
pME1933	Mutated 1.1 kb *ARO4* fragment in pME1513, Mutation of the corresponding gene product: G226T	This study
pME1935	Mutated 1.1 kb *ARO4* fragment in pME1513, Mutation of the corresponding gene product: T236R	This study
pME1989	Mutated 1.1 kb *ARO4* fragment in pME1513, Mutation of the corresponding gene product: Q166E	This study
pME2009	Mutated 1.1 kb *ARO4* fragment in pME1513, Mutation of the corresponding gene product: T162L	This study
pME2015	Mutated 1.1 kb *ARO3* fragment in pME1513, Mutation of the corresponding gene product: S219G	This study
pME2016	Mutated 1.1 kb *ARO4* fragment in pME1513, Mutation of the corresponding gene product: G226S	This study
pME2021	Mutated 1.1 kb *ARO4* fragment in pME1513, Mutation of the corresponding gene product: S195A	This study
pME2027	Mutated 1.1 kb *ARO4* fragment in pME1513, Mutation of the corresponding gene product: K229L	This study

Tab. 1 Plasmids used in this study.

p426MET25 (Mumberg et al. 1994). pME2017 is a derivate from pME825 (Graf et al., 1993) where the *ARO4* promoter and terminator was inserted into the multi cloning site.

Construction of chimera proteins. The chimera fusion genes were constructed by the overlapping PCR method. For chimeric gene 3p-4p (pME1947) the *ARO3*-part was amplified with the oligomers 5´-cgcggatccatgttcattaaaaacgatcacgc-3´ (flanking primer) and 5´-aggccaattctctgtgca gttgg-3´ (overlap primer) and the *ARO4*-part with the oligomers 5´-aactgcacagagaatt ggcct-3´ (overlap primer) and 5´-ggcctcgagttaaaaaaacatctatttctt gttaa-3´ (flanking primer). In a second PCR both resulting fragments and flanking primers were used to amplify the complete gene. For 3p-(C-4p) (pME1951) oligomers 5´-cgcggatccatgttcattaaaaacgatcacgc-3´ and 5´-tctgaaatccttattactgttgccgtg-3´ (*ARO3*) and 5´-ggcaacagtaataaggatttcagaaaccaacca-3´, 5´-ggcctcgagttaaaaaaacatctatttcttgtt aa-3´ (*ARO4*) were used. Oligomers 5´-cgcggatccatgttcattaaaaacgatcacgc-3´, 5´-aggccaattctctgtgcagttgg-3´, 5´-ggtaactccaataaagatttcaagaaccaa-3´ and 5´-ggcct cgagccctcatttacaggctattttt-3´ were used to construct the *ARO3*-parts of 3p-4p-(C-3p) (pME1952) whereas oligomers 5´-aactgcacagagaattggcct-3´ and 5´-cttgaaat ctttattggagttaccgcg-3´ were used to amplify the *ARO4*-part. For fusion gene 4p-3p-(C-4p) (pME1953) oligomers 5´-cgcggatccatgagtgaatctccaatgttcg-3´, 5´-ggatgct aattctctgtgcagttgaga-3´, 5´-ggcaacagtaataaggatttcagaaaccaacca-3´ and 5´-ggcctcgagttaaaaaaacatctatttcttgttaa-3´ were used to construct the *ARO4*-parts. The *ARO3*-part was amplified with the oligomers 5´-caactgcacagagaattagcatccggt-3´ and 5´-tctgaaatccttattactgttgccgtg-3´. Only within identical regions of the deduced protein sequence alignment the chimera genes were combined together in order to avoid misfolding of the resulting proteins. The basis for the construction of chimera proteins was a sequence alignment be-

tween the deduced Aro3p and Aro4p sequences on the protein level. The chimera genes were cloned into pME1513 behind the *MET25* promoter (Mumberg et al., 1994) for further analysis.

Mutagenesis experiments with Aro4p. The allelic *ARO4*-library was constructed by PCR method in the presence of 20 µM Mn^{2+} ions. This ion concentration affect the accuracy of the *Taq* DNA-polymerase leading to random errors during the amplification process (Leung et al., 1989). Several Mn^{2+} ion concentrations were tested. To produce single mutations 20 µM was used as final concentration. All random mutated *ARO4* alleles were cloned into pME1513 downstream of the *MET25* promoter. This promoter can be down-regulated to 10 % in the presence of 2 mM methionine. After transformation of the allelic library into RH2424 (*MATa*, *can1-100, GAL, aro3*::*HIS3, aro4*::*LEU2, ura3-1*) tyrosine-resistant mutants were selected in a final step on synthetic complete medium without aromatic amino acids to screen only for functionally intact DAHP synthases. The resulting mutant colonies were transferred to minimal vitamins medium supplemented with 5 mM tyrosine to reduce DAHP synthase activity and 2 mM methionine to reduce expression of the mutated alleles to screen for alleles encoding tyrosine resistant Aro4p mutant enzymes. A transformed yeast strain carrying a wt *ARO4* allele as control was not able to grow under this conditions. The plasmids pME1976-1978, 1980, 1982 and 1983 of the growing strains were isolated and retransformed to confirm the phenotype. From all resulting *ARO4* alleles the DNA sequence was determined (Rosenblum et al., 1997).

Site-directed mutations were introduced using oligo-nucleotide mutagenesis. The resulting alleles were cloned into pME1513 and transformed into yeast strain RH2424 for further analysis.

Enzyme assays. Enzyme activities are specified in International Units (1 U = appearance of 1 µmol product per minute). Specific enzyme activities are given as mU (mg protein $^{-1}$). The DAHP synthase activities were determined with the stop assay described by Takahashi and Chan (1971) with the following modifications: pH of the enzymatic reaction was adjusted to pH 6.8 instead of pH 6.5 and sodium periodate was dissolved in 0.25 instead of 0.125 M H_2SO_4. Assays were performed with substrate concentrations such that absorbance of the product was proportional to the amount of enzyme added and were measured on a Kontron Uvicon 922 photometer.

Overexpression, purification and kinetic analysis of A3-S219G and A4-G226S. Both alleles, A3-S219G and A4-G226S, were overexpressed in pME2017 and brought into RH1326 to result in a 55-fold overexpression compared to the respectively wt enzymes with only one genomic copy. The plasmid-carrying yeast cells were grown at 30°C in 10-l rotatory fermentors under aeration. Cells were harvested in mid-exponential phase at an OD_{546} of 3-4. For a typical purification procedure, 20 g of cells (wet wt.) were used.

The isoenzymes were purified according to the protocol of Schnappauf et al. (1998) with the following modifications: the anion-exchange chromatography was carried out with 10 mM potassium phosphate (pH 7.6) buffer instead of 10 mM Tris-HCl buffer. DAHP synthases were detected by SDS-polyacrylamide gel electrophoresis (Laemmli 1970) and enzyme assays. Protein solutions were concentrated in stirred cells with PM-10 ultra filtration membranes from Millipore (Eschborn, Germany). Protein concentrations were performed using the Bradford (1976) assay.

Initial velocity data were determined by varying the concentration of one substrate at various fixed concentrations of the second substrate (Bisswanger, 1979). Double-reciprocal plots of the initial velocity against concentrations of the varied substrate

showed a set of intersecting lines. A plot of the interceptions of these lines with the ordinate against the reciprocal of the concentrations of the fixed substrate gave a straight line. The Michaelis constants were determined from the value of these lines on the abscissa, corresponding to the reciprocal of the absolute value. The reciprocals of the apparent maximal velocities $1/V_{max}$ are on the ordinate. Rate constants k were determined from these values, and the known enzyme concentration e_o was determined according to $k = V_{max}/e_o$. Inhibitory constants were determined by varying the concentrations of a single substrate under various fixed concentrations of the inhibitor. The second substrate was kept at a constant concentration. The data were transformed to double-reciprocal plots. The K_i value was determined from secondary plots of the reciprocal intersections of the lines with the ordinate (noncompetitive inhibition) or the slopes of the lines (competitive inhibition) against inhibitor concentration. The intersection with the abscissa provided the value for K_i.

Crystallization. Crystals of DAHP synthase in complex with PEP were grown by the hanging drop vapor diffusion-method at 21 deg. C using a well solution of 14% PEG3K, 1mM DTT, buffered at pH 9.0 with 10 mM Tris-HC. Drops were made on siliconized cover-slips from 5 µl protein solution (15 mg/ml protein, 10mM Tris-HCl at pH 9.0, 1 mM DTT) and 5 µl well solution. Crystals reach a maximum size of 0.6 x 0.3 x 0.15 mm after three days and disintegrate in the following two days. The crystals belong to space group P1 with unit cell constants a=82.0 A, b=93.8 A, c=104.5 A, α=65.6, β=85.6, γ=75.4.

Data collection and processing. For data collection, a crystal was equilibrated with a solution of 16% PEG3000 buffered at pH 9.0 with 10 mM Tris-HCl and containing 30% Glycerol. Equilibration was done by increasing the glycerol concentration in

steps of 5% from 0 to 30%. The crystal was mounted in a fiber loop (Hampton Research, San Diego, CA, U.S.A.) and flash-cooled in a stream of gaseous nitrogen at 100 K (Oxford Cryosystems, Oxford, U.K.). Data were collected on Beamline X11 at EMBL c/o DESY (Hamburg, Germany) at a wavelength of 0.9076 Å and a temperature of 100 K. The data were integrated, scaled and merged with DENZO and SCALEPACK (Otwinowski, 1997). Processing statistics are summarized in Tab. 2.

Structure solution and refinement. The structure was solved by molecular replacement using the program EPMR (Kissinger, 1999) with default parameters employing the dimer of chains A and B from *E. coli* DAHP synthase (Shumilin, 1999) after removal of all non-protein atoms as the search model. After correct placement of 4 dimers and rigid body refinement, the correlation coefficient and the R-factor for 22585 reflections between 15.0 and 4.0 Å were 60.8 and 42.8 percent, respectively. Structure solution employing a tetramer from the *E. coli* DAHP synthase structure was unsuccessful due to the different interdimer angle in *S. cerevisiae* DAHP synthase Aro4p. The interdimer angles were calculated at the angle between the vectors connecting the centers of mass of the respective molecules. Prior to refinement, 5% of the reflections were flagged in thin resolution shells for cross-validation (Brunger, 1992). Refinement was performed using CNS 1.0 (Brunger 1998) using standard command scripts employing the maximum likelihood target function (Pannu 1996) unless otherwise stated. After an initial round of torsion-angle refinement, one molecule was completely reconstructed using the sequence of Aro4p and then cloned to the other seven positions. Apart from the implicit use of non-crystallographic symmetry (NCS) at this stage, NCS constraints or restraints were not used in any later stage of the refinement. The resulting initial model was subjected to one round of high temperature simulated annealing to remove model bias. After coordinate and B-factor

minimization, R_{work} and R_{free} were 27.7 and 33.0%, respectively for all data between infinity and 1.9 Å. The 8 highest peaks in the corresponding F_{obs} - F_{calc} difference electron density map corresponded to the phosphate groups of eight bound PEP molecules. The PEP molecules were included in the model with their stereochemical parameters restrained to standard values as given in the CNS libraries where available and with values derived from searches in the Cambridge Structural Data Bank (Allen 1993) otherwise. Every round of refinement consisted of conjugate gradient minimization of atomic coordinates, individual B-factor refinement and automatic water picking from difference electron density maps. All manual rebuilding was done with XtalView (McRee 1999). During and after refinement the structure was analyzed using the programs PROCHECK (Laskowski 1993), WHAT IF (Vriend, 1990) and Ligplot (Wallace, 1995).

To validate the model in the regions around Gln166 and around the PEP binding site, simulated annealing omit maps (Hodel 1992) with at least 7.5% of all atoms removed from the model were calculated. The final model contains 8 molecules of DAHP synthase, corresponding to 2744 protein residues, 8 PEP molecules, and 1251 water molecules; the stereochemistry and the statistical agreement factors are good. Only two residue, His282 of molecules D and G have backbone dihedrals in the disallowed regions of the Ramachandran plot, for both these residues, the electron density is unambiguous. For all eight monomers, no electron density was found for residues Met1 to Asp22. The loop between α8 and β8 which was found to be disordered in the *E. coli* DAHP synthase Pb^{2+} complex is defined to varying degrees, ranging for the entire loop being ordered in molecule F to the entire loop being disordered in molecule B. The C-terminus is defined up to residue Asn368 in all molecules, the remaining two residues being visible to varying degrees.

Structure analysis and comparison. Based on an analysis of error-scaled difference distance matrixes as implemented in the program ESCET (Schneider, 2000), all eight molecules were found to be sufficiently similar to use all ordered atoms for least-squares superposition. All least-squares superposition were done using the program LSQKAB (Kabsch, 1976) from the CCP4 suite (Collaborative Computational Projekt, 1994) The 28 possible pair wise least-squares super positions (based on Cα atoms of residues 23 to 325 and 334 to 368) between all molecules gave rms displacements between 0.180 and 0.322 Å with a mean value of 0.243 Å. The initial 1-dimensional sequence alignment derived *via* ClustalW was manually adjusted to reflect the correspondence of amino acids between *E. coli* and the yeast Aro4p DAHP synthase (Fig. 1).

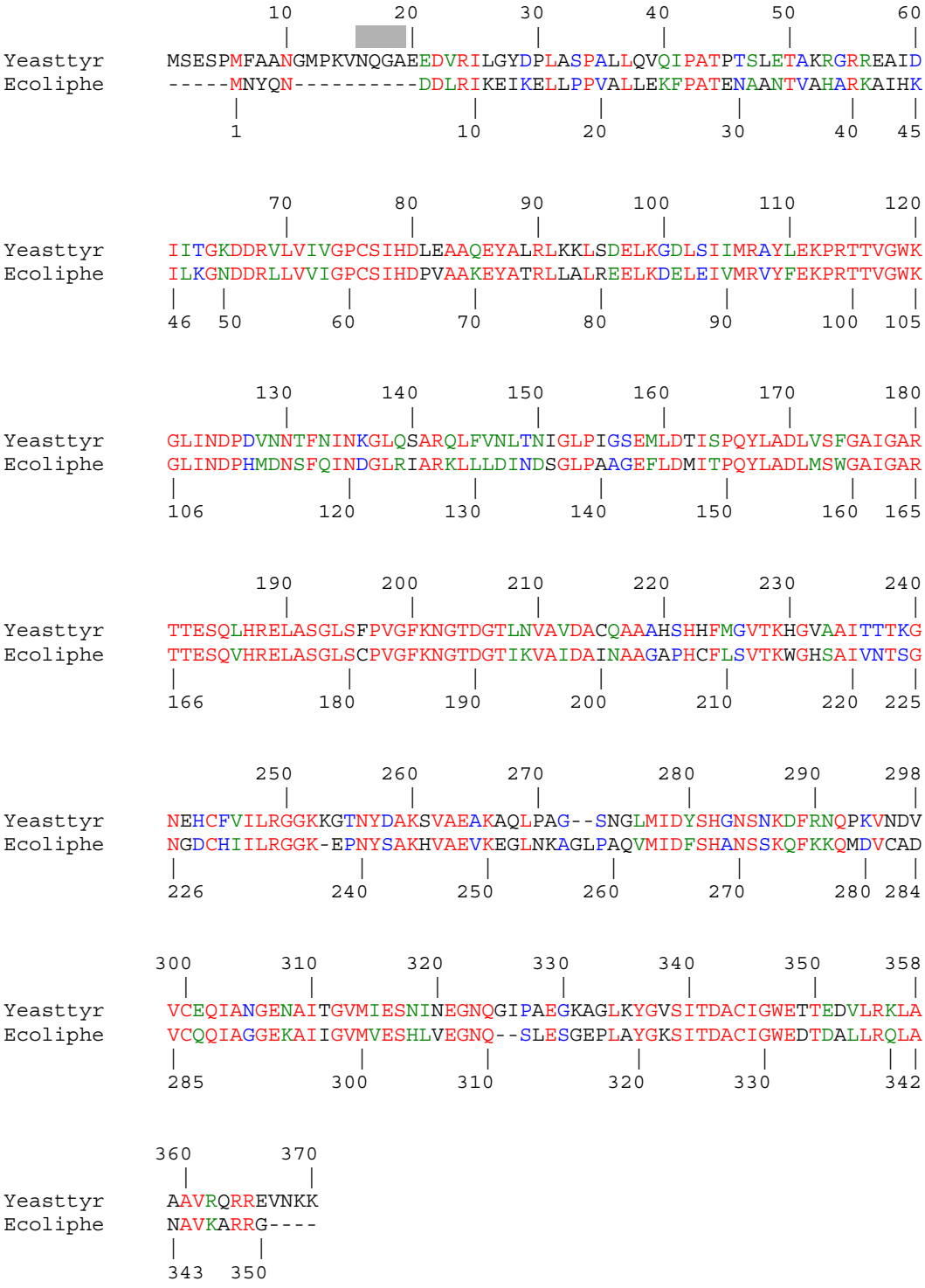

Fig. 1 Structure based alignment between the phenylalanine-inhibitable DAHP synthase from *E. coli* and the tyrosine-inhibitable isoenzyme from *S. cerevisiae*. Amino acids marked in red are identical, those are indicated in green are similar.

Results

The central regions of DAHP synthases are required for specific regulation. The two isoenzymes Aro3p and Aro4p from *S. cerevisiae* catalyze the same reaction of the shikimate pathway. Both proteins are built of 370 amino acids including 224 identical and 84 similar amino acids. 62 amino acids are completely different. The isoenzymes are differently regulated by either phenylalanine as feedback-inhibitor of Aro3p or tyrosine as feedback-inhibitor of Aro4p. We wanted to identify those of the 62 amino acid residues which have to be different to guarantee a specific distinctive regulatory pattern. As first approach, a set of chimera proteins of yeast Aro3p and Aro4p was constructed by fusing the corresponding parts of the encoding genes (Fig. 2 A, B). Chimera protein 3p-4p was a combination of the first part of Aro3p and the second part of Aro4p. The second chimera protein 3p-(C-4p) is predominantly Aro3p but carries the C-terminal end of Aro4p. Chimera proteins 3p-4p-(C-3p) and 4p-3p-(C-4p) carry the central region of Aro4p and Aro3p, respectively. The constructed fusion genes were expressed in *S. cerevisiae* strain RH24243 which does not possess any DAHP synthase activity. All fusion genes are driven by the *MET25* promoter (Mumberg et al., 1994). 3p-4p-(C-3p) chimera construct was not able to rescue a double deletion strain. All other chimera proteins where enzymatically assayed for their activity and specific inhibitor from crude extracts (Fig. 2C). The activities without any effector of the tested constructs where reduced to at least 15 % compared to wt Aro4p. These chimera proteins showed different regulative behavior. 3p-4p was not affected by phenylalanine and slightly inhibited by tyrosine. In contrast to this 3p-(C-4p) was strongly inhibited by phenylalanine and not affected by tyrosine showing a Aro3p-like behavior. 4p-3p-(C-4p) was unregulated neither by phenylalanine nor by tyrosine. Beside these chimera constructs 16 other possible chimeric combinations

were tested but all corresponding fusion genes were not able to rescue the double deletion strain RH2424.

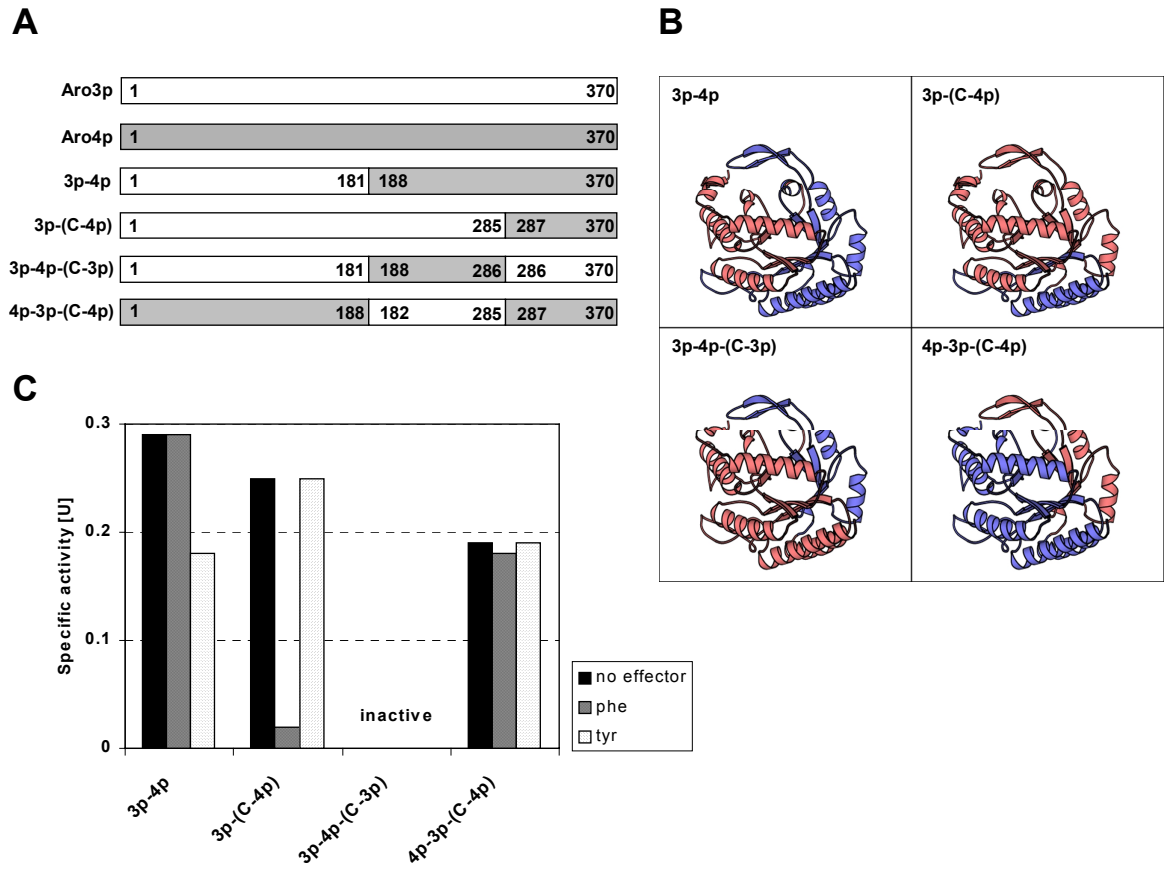

Fig. 2 Regulation of Aro3p/Aro4p chimeric DAHP synthase enzymes of the budding yeast *S. cerevisiae*. **A** Schematic structure of various chimeric fusion proteins of Aro3p and Aro4p. Parts of Aro4p and Aro3p are marked in gray and white respectively with the indicated amino acid positions as boundaries **B** Projection of the chimera proteins into the tertiary structure of Aro4p. Aro3p (red) and Aro4p (blue) derived parts are given in different colors. **C** Specific DAHP synthase activities from yeast crude extracts of the chimera proteins in the presence (1 mM phenylalanine: hatched; 1 mM tyrosine: white) or absence (black) of effector molecules.

These data suggest that the N-terminal part and the central part are involved in specific regulation of DAHP synthases by tyrosine and phenylalanine.

Single amino acid substitutions in the N-terminal region or the center of Aro4p abolish tyrosine feedback regulation. The chimera protein experiments suggested that at least two domains located in the N-terminus and in the center of the primary structure of the DAHP synthase protein are required for regulation. In a second ap-

proach we tested which single amino acid substitutions abolished tyrosine feedback regulation in the Aro4p DAHP synthase isoenzyme. Therefore we used a genetic screen starting with yeast strain RH2424 which lacks any DAHP synthase activity. A library of *ARO4* mutant alleles was constructed as described in Materials and Methods and was transformed into RH2424. Transformants were selected in presence of 5 mM tyrosine. Due to the tyrosine feedback-inhibition of the *ARO4* gene product, all transformants carrying the wildtype allele could not survive because they starved for the other two end-products phenylalanine and tryptophan. 20 transformants could grow on tyrosine suggesting a defect in regulation and were further analyzed. Plasmids were isolated and retransformed to confirm the effect. Sequencing of the isolated *ARO4* alleles revealed 15 alleles with single mutations and 5 with double mutations. Alleles with double mutations were not further analyzed. The resulting amino acid substitutions are all either located in the N-terminal or in the central part of the Aro4p confirming the data obtained from the chimeric enzymes. The N-terminal substitutions were D22G, T44I, E49G and R55G. The substitutions of the central part were S195P and F196S (Fig. 3). Mutations resulting in the amino acid substitutions D22G, R55G and S195P were found three times; substitutions T44I, E49G and F196S were found twice. These data confirm that the N-terminal end and the central part play a role in the regulation process of the tyrosine-inhibitable DAHP synthase.

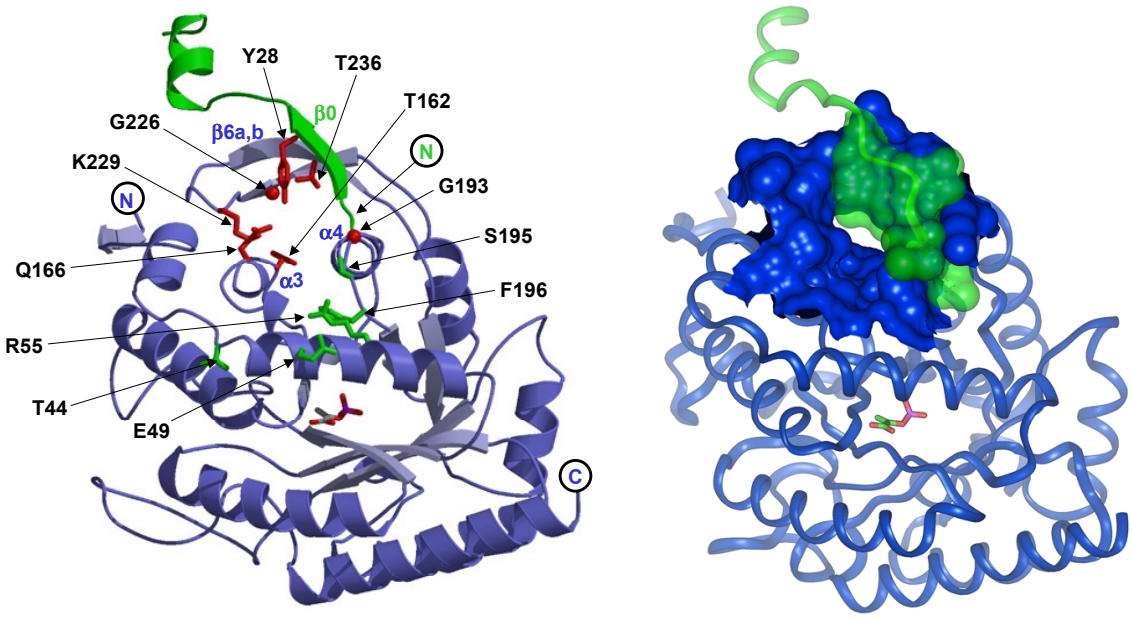

Fig. 3 (left) Location of amino acid substitutions in the monomeric structure of Aro4p. Amino acid substitutions changing the regulatory behavior of Aro4p and obtained by random mutagenesis are marked in green (T44; E49; R55; S195; F196). Red residues indicate amino acids substituted by targeted site-directed mutagenesis (Y28; T162; Q166; G193; G226; K229) deduced from the cavity tertiary structure shown and described in the right picture. The substitution Y28F is shown in the partly visible neighbor monomer (green). **(right)** Monomeric structure of the tyrosine-inhibitable DAHP synthase from *S. cerevisiae* indicating a cavity of the protein which is involved in regulation. The blue surface covers α3, α4, β6a and β6b from one monomer, the green surface covers β0 from the other monomer. The cavity essential for regulation is located in the center of the blue surface partly covered from the N-terminal β0-sheet from the green monomer (indicated transparently).

N-terminal truncation of 20 and 32 amino acid residues abolish tyrosine regulation and DAHP synthase enzyme function, respectively. The results of the chimera protein experiments as well as the genetic screen for alleles encoding unregulated enzymes suggested that the N-terminus and a central region are important for regulation. The N-terminal region involved was narrowed down by the random mutagenesis to the first 55 amino acid residues. To further elucidate the role of the N-terminus, *ARO4* alleles were constructed encoding truncated DAHP synthases. The *ARO4* derivate 4p-t20 encodes a protein which lacks the first 20 amino acids whereas *ARO4* derivate 4p-t32 coded for an Aro4 protein without the first 32 amino acids. Yeast strain RH2424 expressing the truncated mutant protein 4p-t32 showed no detectible DAHP synthase activity indicating that essential parts of the enzyme

function were deleted. Western experiments with an Aro4p raised polyclonal antibody showed cross hybridization indicating no folding deficiency (data not shown). The gene product of the deletion mutant 4p-t20 showed weak DAHP synthase activity but was not regulated by tyrosine anymore. The activity compared to wildtype Aro4p was reduced to 11 %.

The crystal structure of the eukaryotic tyrosine inhibitable Aro4p and the prokaryotic phenylalanine inhibitable aroGp show high similarities and distinct conformational differences. The crystal structure of the phenylalanine-inhibitable DAHP synthase from *E. coli* has been previously solved (Shumilin, et al., 1999) and represent the only fold of a DAHP synthase yet determined. To locate positions of all exchanges introduced into the yeast Aro4p resulting in a changed regulatory pattern the Aro4p crystal structure was determined. For molecular replacement the AB dimer of the DAHP synthase*Pb*PEP complex of the phenylalanine-regulated DAHP synthase from *E. coli* (Shumilin et al., 1999) was used as reference. Eight molecules of DAHP synthase were built into the obtained electron density maps. Eight PEP molecules were clearly visible in different electron density maps. The refinement resulted in a model with good overall stereochemistry and agreement factors (Tab. 2). The triclinic form of DAHP synthase*PEP complex from *S. cerevisiae* contains two tetramers (molecules ABCD and EFGH) in the asymmetric unit.

Resolution range [A]	40.0-1.9
Number of observations	423490
Number of unique reflections	210365
Rmerge (all/high) [%]	3.7 / 35.4
Completeness (all/high) [%]	96.3 / 89.9
Multiplicity (all/high)	2.0 / 1.7
I/sig(I) (all/high)	14.8 / 2.1
Number of reflections used in refinement	188463
Number of reflections in test set	9891
NUMBER OF ATOMS (<B> [A^2])	
Protein Atoms	20631 / 33.4
Ligand atoms	80 / 31.8
Water sites	1251 / 35.7
R [%]	20.8
R_{free} [%]	26.1
RMSD FROM IDEAL VALUES	
Bond length [A]	0.006
Angles [deg.]	1.4
Dihedral [A]	22.7
Impropers [deg.]	0.79
RAMACHANDRAN PLOT STATISTICS	
residues in most favored regions [%]	90.0
residues in allowed regions [%]	9.9
residues in disallowed regions [%]	0.1

Tab. 2 Data processing, refinement parameters and statistics of the x-ray analysis of the yeast Aro4p enzyme cocrystallized with phosphoenolpyruvate. Rmerge=3.7; highest resolution shell contains reflections between 1.97 and 1.90 Å. Statistics were generated by CNS and PROCHECK.

The angles between the dimers in the present structure (defined as the angle between two vector connecting the centers of mass of the monomers evolved)

(Aro4p(AB,CD)=74 deg; Aro4p(EF,GH)=74 deg) (Fig. 4, 5) are different from the corresponding angle in DAHP synthase from *E. coli* (AroG(AB,CD)=28 deg).

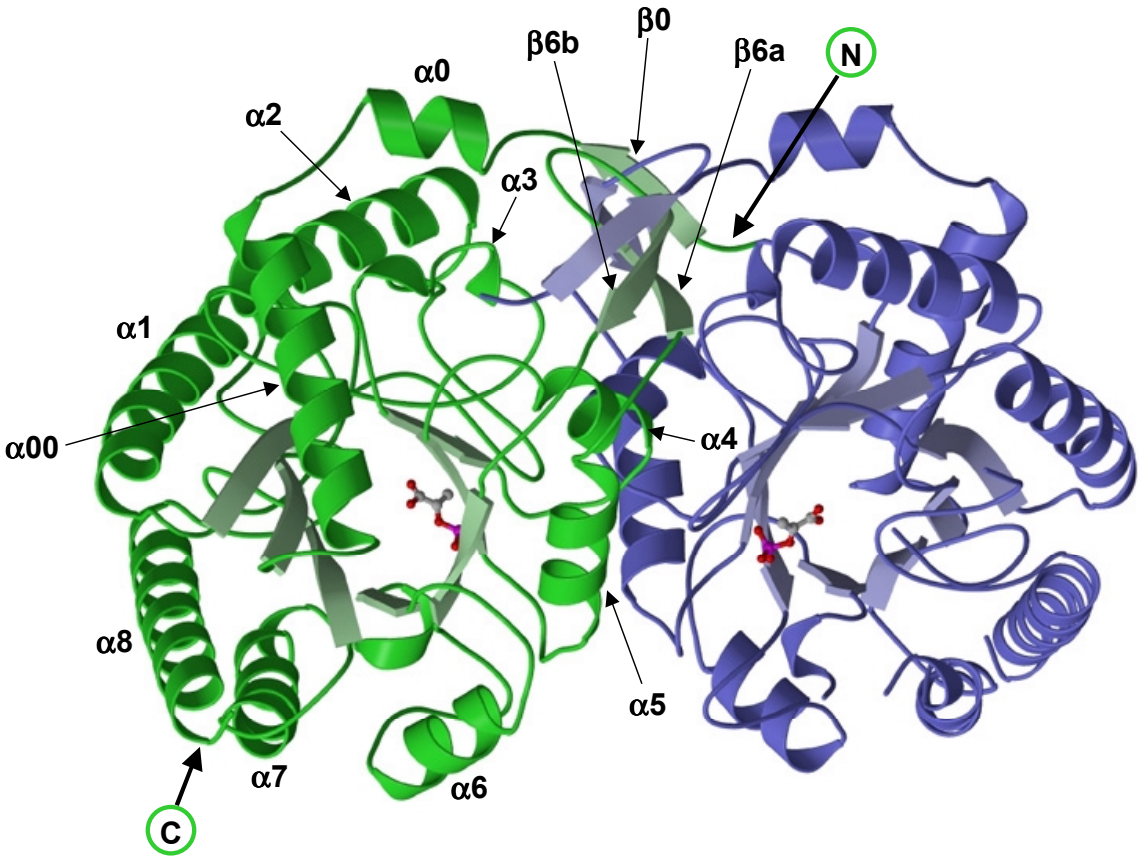

Fig. 4 Aro4p structure of *S. cerevisiae*. Tertiary structural fold of the tyrosine-inhibitable DAHP synthase from *S. cerevisiae* (Aro4p). The dimeric form is shown where two monomers are indicated in green and blue with one molecule phosphoenolpyruvate per monomer which was cocrystallized with the protein. α-helices, β-sheets, N-terminal and C-terminal ends are only shown for the green monomer.

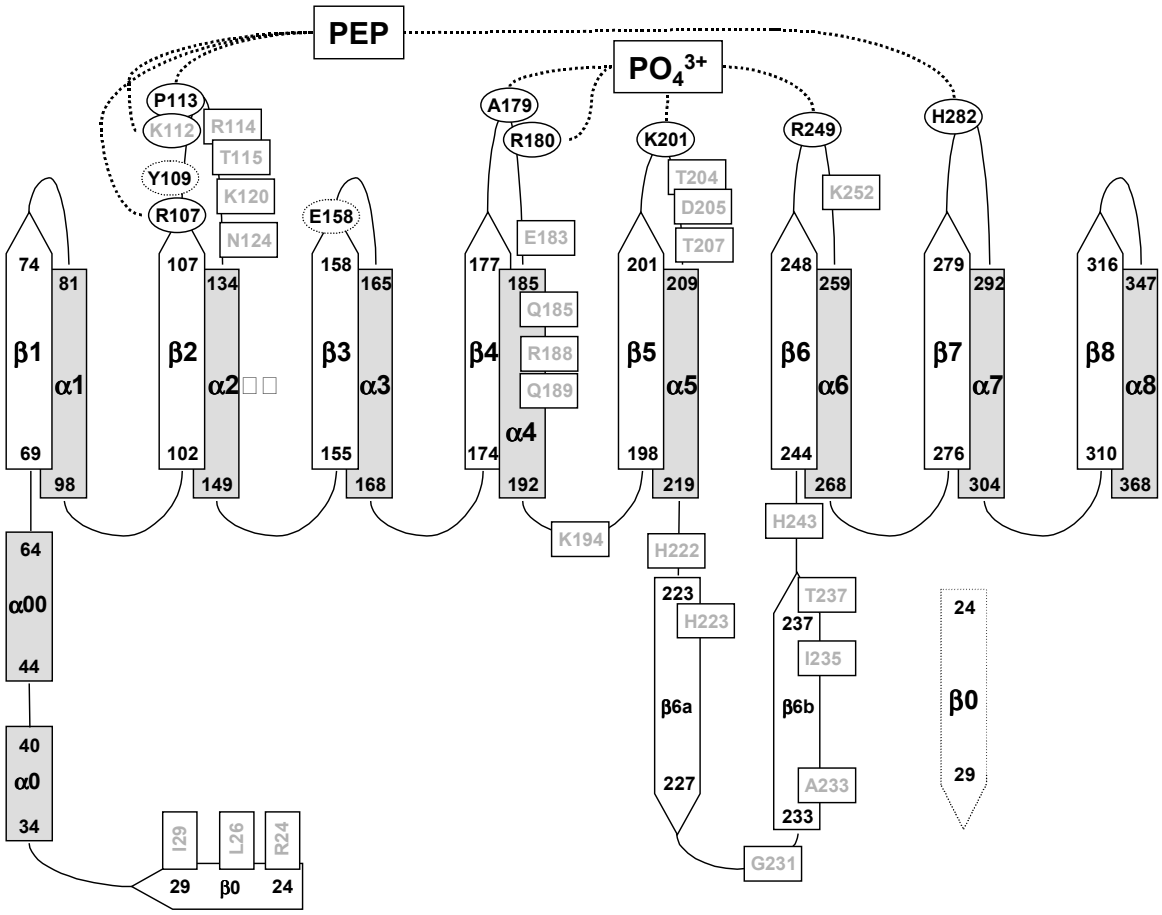

Fig. 5 Topology plot of the Aro4p overall fold. The typical $(\alpha/\beta)_8$ TIM barrel conformation is shown in the center, marked with numbers from one to eight with arrows and squares indicating β-sheets indicate α-helices, respectively. The dotted arrow indicates sheet β0 including amino acids at positions 24 to 29 from the neighbor monomer. The helices α0 and α00 and sheets β0, β6a and β indicate extra parts of the TIM barrel. The numbers in small circles and squares indicate amino acid positions. The substrate binding site and the dimer interface are marked in black and gray, respectively.

Parts of two monomers form a cavity within Aro4p which is involved in tyrosine regulation. The results from the various mutant Aro4p enzymes showed that the N-terminal 20 amino acids, single amino acid residues between position 21 and 55 as well as the central part from position 188 to position 286 of the deduced primary structure are involved in tyrosine regulation of Aro4p. The location of all these amino acids within the crystal structure was determined. The N-terminal end up to amino acid D22 is not visible in the crystal structure indicating a high degree of variability of that part of the protein. All amino acid residue changes resulting in unregulatable

DAHP synthase enzymes are surrounding a cavity which is defined by α3, α4, β6a and β6b of monomer A and β0 of monomer B (Fig. 3 right). To verify whether this cavity is part of the regulatory site further site-directed mutagenesis experiments of the *ARO4* allele were performed resulting in additional mutant enzymes. Single amino acid substitutions were introduced into Aro4p by changing the corresponding codons of the ARO4 open reading frame: Y28F located in β0, T162L, Q166E located in α3, G193K, S195A, G226A, G226S, G226T located in β6a, K229L and T236R located in β6b (Fig. 3 left). The resulting alleles were transformed into yeast strain RH2424 and were able to complement the double deletion of this strain even in the presence of 5 mM tyrosine. The activities of the constructed Aro4 mutant proteins were assayed for activity and regulative behavior from yeast crude extracts (Fig. 6).

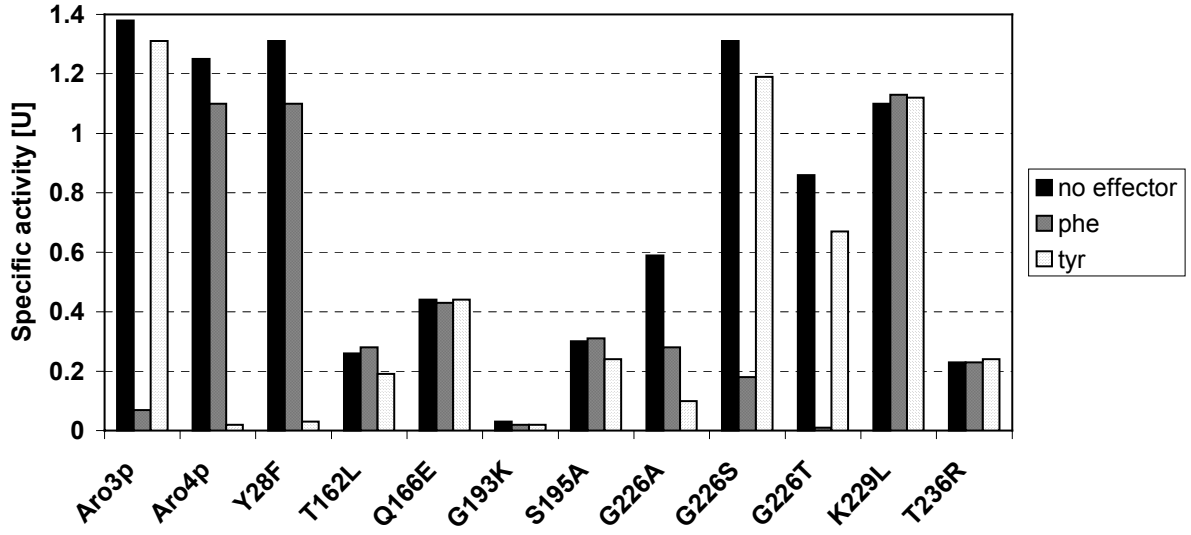

Fig. 6 Specific DAHP synthase activities of yeast Aro4 enzymes carrying various amino acid substitution introduced by site-directed mutagenesis and described in Figure 3. The activities were measured from yeast crude extracts without effector (black) or in the presence of 1 mM phenylalanine (grey) and 1 mM tyrosine (light gray), respectively.

The substitutions at codon positions 162, 193, 195 and at position 226 in β6a and position 236 in β6b had a strong effect on tyrosine inhibition which was reduced to 20 % in presence of 1 mM tyrosine (wt Aro4p: 98 %). Regulation was even diminished by substitutions at position Q166 in α3, K229 and at position T236 in β6b. Substitu-

tion at position 28 had no effect on tyrosine regulation. All substituted residues pointed towards this cavity (Fig. 3B, 7A). Further analysis of the eight molecules of the crystal unit cell showed high variability of the residues Q166 and K229 indicating lack of a potential binding partner (Fig. 7B). This data demonstrates an essential role of amino acid residues at positions 162, 166, 193, 195, 226, 229 and 236 in the vicinity of the cavity for tyrosine regulation of Aro4p.

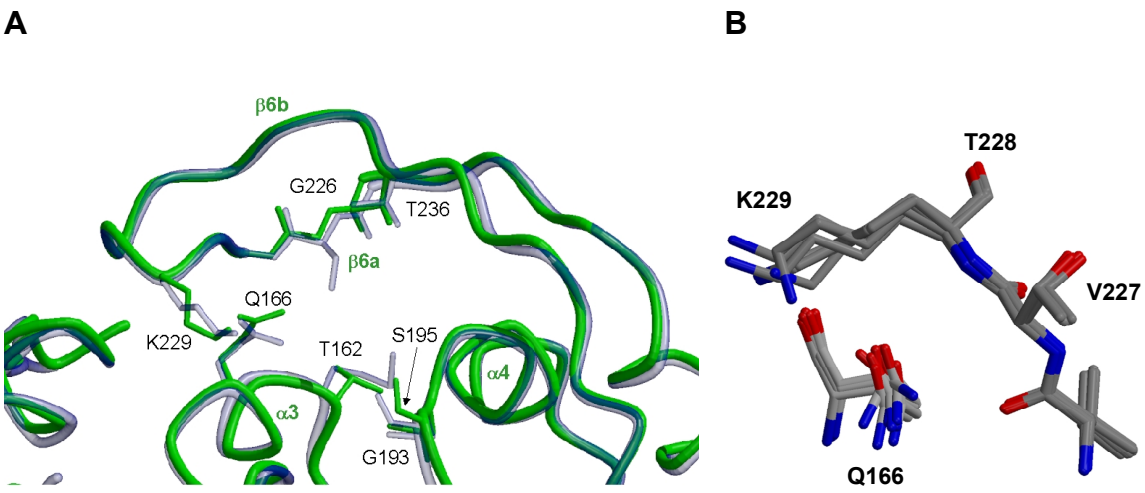

Fig. 7 A Comparison between phe- and tyr-regulated DAHP synthase isoenzymes. Superimposition of parts of the phenylalanine-regulated DAHP synthase aroG from *E. coli* (gray) and corresponding parts of the tyrosine-regulated isoenzyme Aro4p (green). Details of the Aro4p carrying amino acid substitutions which result in a changed regulatory behavior are shown. **B** Superimposition of the amino acids Q166, V227, T228 and K229 within the discovered eight molecules of the crystal structure unit cell. The figure shows a high degree of variability of amino acid residues K229 and Q166.

Evolution required a single amino acid substitution for switching the tyrosine-inhibitable Aro4p into a phenylalanine-inhibitable enzyme and vice versa. The development of differently regulated isoenzymes is an important step in the evolution of versatily adapted organisms. As the deduced primary sequences of both differently regulated isoenzymes Aro3p and Aro4p differ in 62 amino acids, it was intriguing to find out how many amino acid substitutions are necessary to switch from phenylalanine to tyrosine regulation and vice versa. Sequence alignments of primary se-

quences of known tyrosine-regulated DAHP synthases and phenylalanine-inhibitable DAHP synthases gave further insights (Fig. 8). A comparison of both alignments revealed a highly conserved serine in all phenylalanine-regulated enzymes and a highly conserved glycine in all tyrosine-inhibitable enzymes at position 226 of yeast Aro4p. All enzymes showed these residues at the identical relative positions.

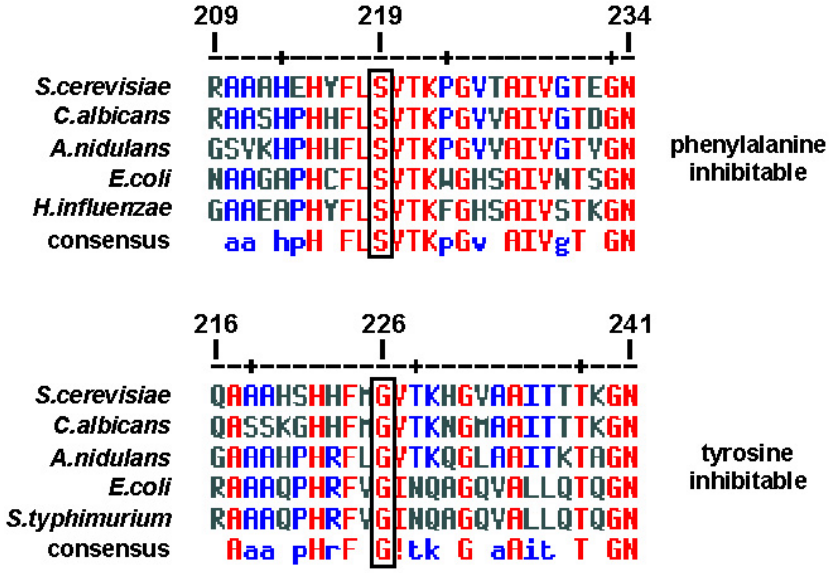

Fig. 8 Amino acid alignment of phenylalanine- and tyrosine-inhibitable DAHP synthases from various organisms. The indicated amino acids serine at position 219 and glycine at position 226 are highly conserved within phenylalanine- and tyrosine-inhibitable DAHP synthases respectively. They are located at the same position of the primary and tertiary structure. The numbers indicate the positions using the *S. cerevisiae* isoenzymes.

In the tertiary structure of yeast Aro4p the glycine at position 226 is located on top of the cavity built by both monomers which has been shown by the results above to be essential for regulation. A direct comparison of this cavity in the tyrosine-regulated DAHP synthase of *S. cerevisiae* with that of the phenylalanine-regulated DAHP synthase of *E. coli* revealed a major difference (Fig. 9). The cavity of the phenylalanine-regulated enzyme is significantly reduced. This reduction is caused by the side chain of the highly conserved serine in comparison of the single hydrogen

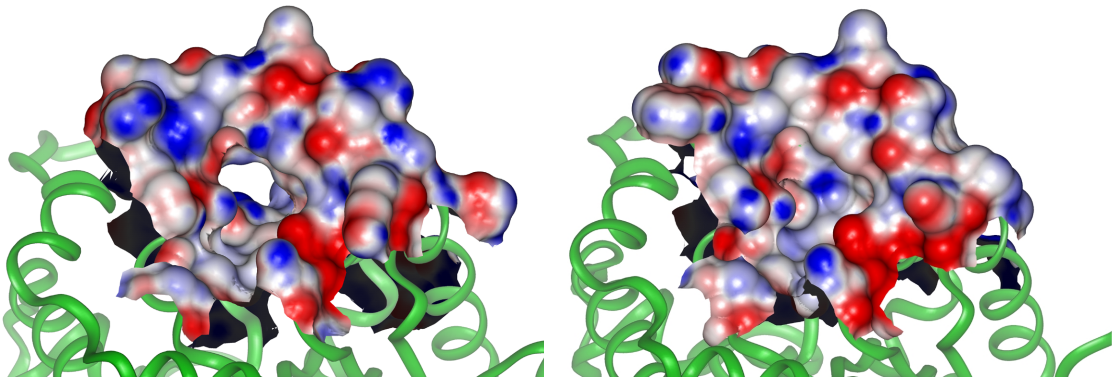

Fig. 9 Comparison of the cavity of the tyrosine-regulated DAHP synthase Aro4p (*S. cerevisiae*) on the left and the corresponding region of the phenylalanine-regulated DAHP synthase aroG (*E. coli*) on the right. The cavities are covered with a surface showing the electrostatic distribution of this area (red = negatively charged, blue = positively charged, white = neutral). The phenylalanine isoenzyme includes only a cavity with reduced space in comparison to the tyrosine-inhibitable DAHP synthase which is enlarged towards the back-side of the projection.

atom which is the side chain of glycine 226 in the tyrosine-regulated enzyme of *S. cerevisiae*. Therefore we wondered whether during the course of evolution a single amino acid exchange would be sufficient and responsible for distinct regulation specifity towards phenylalanine or tyrosine as effector of DAHP synthases. To test this hypothesis we exchanged the S219 codon in *ARO3* to G as well as the G226 codon of *ARO4* to S. The resulting alleles were expressed in yeast and the mutant enzymes Aro3p-S219G and Aro4p-G226S purified. The kinetically parameters of the enzyme in the presence or absence of phenylalanine or tyrosine as inhibitor were determined (Tab. 3) The mutant enzymes showed similar activities compared with their corresponding wildtype-enzymes.

	mutants		wt enzymes	
	A3-S219G	**A4-G226S**	**Aro3p [a]**	**Aro4p [b]**
K_m (PEP) [µM]	**27**	**122**	**18**	**125**
K_m (E4P) [µM]	**136**	**349**	**130**	**500**
K_i [µM]	**1**	**25**	**10**	**0.9**
k_{cat} [s^{-1}]	**8.8**	**7.9**	**7.0**	**6.0**

Tab. 3 Kinetic parameters of the DAHP synthase mutants A3-S219G and A4-G226S from *S. cerevisiae*. The K_i values refer to the specific inhibitors phenylalanine for the Aro4p-G226S and tyrosine for the Aro3p-S219G protein. [a] Paravicini et al., 1989, [b] Schnappauf et al., 1998.

The k_{cat} values are 8.8 for Aro3p-S219G and 7.9 for Aro4p-G226S. Surprisingly, the exchange of a single amino acid residue resulted in a completely exchanged regulation pattern for both enzymes. The formerly phenylalanine-regulated Aro3p enzyme was in its Aro3p-S219G version strongly regulated by tyrosine (K_i = 1.0 µM). Vice versa the tyrosine-regulated Aro4p became phenylalanine-inhibitable (K_i = 25 µM) when Aro4p-G226S was analyzed. Tyrosine regulation was abolished in the mutant enzyme. Both proteins kept their behavior towards the substrates PEP and E4P (K_m values).

In summary, these data suggest that although the development of a N-terminal and central regulatory domain was necessary as prerequisite of regulation, a single amino acid substitution in the central region of Aro4p was sufficient to produce a first Aro3p precursor. This residue is located at an important cavity required for DAHP synthase regulation. The additional 61 different amino acid residues which can be found in our days in both isoenzymes are not important for regulation and might therefore be secondary events after the primary evolutionary step which separated the regulatory behavior of both isoenzymes.

Discussion

Different approaches were carried out to analyze the regulation process of the Aro3p/Aro4p DAHP synthase isoenzyme system in the yeast *S. cerevisiae*. Data from chimera protein experiments suggest that the central part of the DAHP synthases is involved in regulation. As 3p-4p was only weakly regulated and 4p-3p-(C-4p) was not regulated, a cooperation of the central part with the N-terminal region seems to be important for the specifity of regulation process. This is supported by the effect of the N-terminal truncated mutant 4p-t20 which was not regulated anymore. A remarkable fact is that the extra β-sheets 6a and 6b, which are absent in a normal TIM barrel fold, are located in the central part (amino acid 222-243).

The PCR random mutagenesis experiments resulted in tyrosine resistant mutant enzymes. The mutations are located within the codons of the first 55 amino acids and in the central part. This also corroborates with the fact that some kind of communication between the central part of the DAHP synthases with the N-terminal region is necessary for specific regulation. The crystal structure revealed a cavity at the top of the enzyme. It is defined by α3, α4, β6a β6b of one monomer and β0 of the neighbor monomer. Site-directed mutagenesis experiments within the codons of this cavity produced an additional set of deregulated mutant enzymes. All substituted amino acid residues pointed directly into this cavity. Single substitutions at positions 166, 193, 229 and 236 lead to completely unregulated enzymes indicating that this cavity is involved in inhibition of the enzyme. Q166 and K229 are highly variable in all eight molecules of the unit cell. High variability of amino acid residues in a crystal structure suggests that a direct binding partner could be missing. It is therefore possible that both residues were not able to find a partner to form a H-bond to another amino acid

of Aro4p or that they could directly be involved in effector binding by waiting for the effector molecule.

The same cavity exists in the phenylalanine-regulated isoenzyme of *E. coli*. A comparison of both cavities revealed a decisive difference between both isoenzymes. The cavity of the phenylalanine-regulated *E. coli* enzyme is reduced in contrast to the cavity of the tyr-regulated yeast enzyme which is enlarged. Comparison of the sequence alignments showed which amino acid residue is responsible for this difference. The highly conserved and small glycine (tyrosine-regulated DAHP synthases) increases the space of the cavity. In contrast to this, the conserved serine (phenylalanine-regulated DAHP synthases) decreases the cavity. By comparing the two sets of sequence alignments, this is the only clear and conserved difference between the two differently regulated isoenzyme-groups. Exchange of this particular amino acid between the differently regulated isoenzymes formed the mutants A3-S219G and A4-G226S. Kinetical analysis revealed that the regulative behavior of these mutants has also been completely exchanged. A3-S219G was Aro4p-like tyrosine-regulated and A4-G226S was Aro3p-like phenylalanine-regulated. Therefore it was possible to switch the specific regulative behavior of each DAHP synthase isoenzyme from *S. cerevisiae* by exchanging one amino acid.

In enzyme evolution, there is strong evolutionary pressure against a change in enzyme structure (Rider and Taylor, 1980). It has been suggested that the structure of an 8-fold α/β barrel, first described for the enzyme triosephosphate isomerase (Phillips et al., 1978), seems to be particularly suited for the evolution of new functions: surface loop modifications in the regions between the α-helices and β-strands alter the properties of binding and catalysis without affecting the basic structure of the enzyme (Lindqvist and Brädén, 1985). Therefore it is not surprising that the known crystal structures of DAHP synthases isoenzymes are 8-fold α/β barrels. The few addi-

tional α-helices and β-strands which are part of the described cavity seem to reflect important molecules for the evolution of feedback inhibition of the enzymes. Overall structures of both crystal structures are nearly identical. Nevertheless, in *S. cerevisiae,* evolution resulted in two differently regulated DAHP synthase isoenzymes with 62 different amino acids. It was a surprising result of this study that 61 amino acid substitutions are irrelevant for the regulatory behavior, but the crucial difference in regulation can be pinpoints to a single glycine-serine exchange.

References

Allen FH and Kennard O (1993) 3D Search and Research Using the Cambridge Structural Database. *Chemical Design Automation News* **8**:31-37

Bisswanger H (1979) Theorie und Methoden der Enzymkinetik. Verlag Chemie, Weinheim Deerfield Beach Basel

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **73**:248-254

Brunger AT (1992) The Free R Value: A Novel Statistical Quantity for Assessing the Accuracy of Crystal Structures. *Nature* **355**:472-474

Brunger AT, Adams PD, Clore GM, DeLano WL, Gros P, Grosse-Kunstleve RW, Jiang JS, Kuszewski J, Nilges M, Pannu NS, Read RJ, Rice LM, Simonson T, and

Warren GL (1998) Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Cryst* **D54**:905-921

Byng GS, Kane JF, and Jensen RA (1982) Diversity in the routing and regulation of complex biochemical pathways as indicators of microbial relatedness. *Crit. Rev. Microbiol* **9**:227-252

Byng GS and Jensen RA (1983) Impact of isozymes upon partitioning of carbon flow and regulation of aromatic biosynthesis in prokaryotes. *Curr Top Biol Med Res* 8:115-140

Collaborative Computational Projekt, Number 4 (1994) The CCP4 Suite: Programs for Protein Crystallography. *Acta Cryst* **D50**:760-763

Graf R, Mehmann B, and Braus GH (1993) Analysis of feedback-resistant anthranilate synthases from Saccharomyces cerevisiae. *J Bacteriol* 175:1061-1068

Haslam E (1993) Shikimic Acid. Wiley, Chichester New York, Brisbane

Hodel A, Kim SH, and Brunger AT (1992) Model Bias in Macromolecular Crystal Structures. *Acta Cryst* **A48**:851-859

Hoffmann PJ, Doy CH, and Catcheside DEA (1972) The separation of three allosterically inhibitable 3-deoxy-D-arabino-heptulosonate 7-phosphate synthases from extracts of *Neurospora crassa* and the purification of the tyrosine inhibitable isoenzyme. *Biochim Biophys Acta* **268**:550-561

Ito H, Fukuda Y, Murata K, and Kimura A (1983) Transformation of intact yeast cells treated with alkali cations. *J Bacteriol* **153**:163-168

Kabsch W (1978) A discussion of the solution for the best rotation to relate two sets of vectors. *Acta Cryst* **A34**:827-828

Kissinger CR, Gehlhaar DK, and Fogel DB (1999) Rapid automated molecular replacement by evolutionary search. *Acta Cryst* **D55**:484-491

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685

Laskowski RA, MacArthur MW, Moss DS, and Thornton JM (1993) PROCHECK: a program to check the stereochemical quality of protein structures. *J Appl Cryst* **26**:283-291

Leung DW, Chen E, and Goeddel DV (1989) A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction. *Technique* **1**:11-15

Lindqvist Y and Brädén CI (1985) Structure of glycolate oxidase from spinach. *Proc Natl Acad Sci* USA **82**:6855-6859

McRee DE (1999) XtalView/Xfit – A Versatile Program for Manipulating Atomic Coordinates and Electron Density. *J Struc Biol* **125**:156-165

Mumberg D, Müller R, and Funk M (1994) Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression. *Nucleic Acids Res* **22**:5767-5768

Nimmo GA and Coggins RJ (1981a) The purification and molecular properties of the tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase from *Neurospora crassa*. *Biochem J* **197**:427-436

Nimmo GA and Coggins RJ (1981b) Some kinetic properties of the tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase from *Neurospora crassa*. *Biochem J* **199**:657-665

Otwinowski Z and Minor W (1997) Processing of X-ray Diffraction Data Collected in Oscillation Mode. *Methods in Enzymology* **276**:307-325

Pannu NS and Read RJ (1996) Improved structure refinement through maximum likelihood. *Acta Cryst* **A52**:659-668

Paravicini G, Schmidheini T, and Braus GH (1989) Purification and properties of the 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (phenylalanine-inhibitable) of *Saccharomyces cerevisiae*. *Eur J Biochem* **186**:361-366

Park OK and Bauerle R (1999) Metal-catalyzed oxidation of phenylalanine-sensitive 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase from *Escherichia coli*: Inac-

tivation and destabilization by oxidation of active-site cysteines. *J Bacteriol* **181**:1636-1642

Phillips DC, Sternberg MJE, Thornton JM, and Wilson IA (1978) An analysis of the structure of triosephosphate isomerase and its comparison with lactate dehydrogenase. *J Mol Biol* **119**:329-351

Rider CC and Taylor CB (1980) Isoenzymes. Chapman and Hall, New York

Rosenblum BB, Lee LG, Spurgeon SL, Khan SH, Menchen SM, Heiner CR, and Chen SM (1997) New dye-labeled terminators for improved DNA sequencing patterns. *Nucl Acids Res* **25**:4500-4504

Schnappauf G, Hartmann M, and Braus GH (1998) The two 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase isoenzymes from *Saccharomyces cerevisiae* show different kinetic modes of inhibition. *Arch Microbiol* **169**:517-524

Schneider TR, Hartmann M, and Braus GH (1999) Crystallization and preliminary X-ray analysis of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (tyrosine inhibitable) from *Saccharomyces cerevisiae*. *Acta Cryst* **D55**:1586-1588

Schneider TR (2000) Objective comparison of protein structures: error-scaled difference distance matrices. *Acta Cryst* **D56**:714-721

Schoner R and Herrmann KM (1976) 3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase. Purification, properties, and kinetics of the tyrosine-sensitive isoenzyme from *Escherichia coli*. *J Biol Chem* **251**:5440-5447

Sherman F Fink GR, and Hicks J (1986) Methods in yeast genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Shumilin IA, Kretsinger RH, and Bauerle RH (1999) Crystal structure of phenylalanine-regulated 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase from *Escherichia coli*. *Structure Fold Des* **7**:865-875

Staub M and Denes G (1969) Purification and properties of the 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (phenylalanine sensitive) of *Escherichia coli* K12. II. Inhibition of activity of the enzyme with phenylalanine and functional group-specific reagents. *Biochim Biophys Acta* **178**:599-608

Takahashi M and Chan WWC (1971) Separation and properties of isozymes of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthetase from *Saccharomyces cerevisiae*. *Can J Biochem* **49**:1015-1025

Vriend G (1990) WHAT IF: a macromolecular modeling and drug design program. *J Mol Graph* **8**:52-56

Wallace AC, Laskowski RA, and Thornton JM (1995) LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions. *Protein Eng* **8**:127-134

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