Molecular Characterization of the Mitochondrial Presequence Translocase

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I hereby declare that this thesis has been written independently and with no other sources and aids than quoted.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

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Göttingen, 08. October 2017
- to Wibke -

- to my parents -

- to Maja, Fritz and Karl -
List of Publications

Parts of this thesis have been previously published in the following article:

*: these authors contributed equally

The following article is not part of this thesis:

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Acronyms

SI units and SI-derived units were used in this thesis without further declaration.

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<th>Definition</th>
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<td>AAC</td>
<td>ADT/ATP carrier</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BAM</td>
<td>bacterial β-barrel assembly machinery</td>
</tr>
<tr>
<td>CL</td>
<td>cardiolipin</td>
</tr>
<tr>
<td>Cox4</td>
<td>presequence of the cytochrome c oxidase subunit 4</td>
</tr>
<tr>
<td>DDM</td>
<td>n-Dodecyl β-D-maltoside</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERMES</td>
<td>ER-mitochondria encounter structure</td>
</tr>
<tr>
<td>IM</td>
<td>inner membrane</td>
</tr>
<tr>
<td>IMS</td>
<td>intermembrane space</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>lysogeny broth</td>
</tr>
<tr>
<td>LUV</td>
<td>large unilamellar vesicles</td>
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<tr>
<td>Mdm</td>
<td>mitochondrial distribution and morphology protein</td>
</tr>
<tr>
<td>MEGA-9</td>
<td>acyl-N-methylglucamine 9</td>
</tr>
<tr>
<td>MIA</td>
<td>mitochondrial intermembrane space assembly</td>
</tr>
<tr>
<td>MICOS</td>
<td>mitochondrial constact site and cristae organization sytem</td>
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Mim  mitochondrial import protein
NMR  nuclear magnetic resonance
OD   optical density
OM   outer membrane
Omp  outer membrane protein
PAM  presequence translocase-associated motor
PBD  presequence binding domain
PC   phosphatidylcholine
PE   phosphatidylethanolamine
PI   phosphatidylinositol
PiC  phosphate carrier
POTRA polypeptide-transport associated domain
PS   phosphatidylserin
PTFE polytetrafluoroethylene
rpm  rounds per minute
S. cerevisiae Saccharomyces cerevisiae
SAM  sorting and assembly machinery
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA  trichloroacetic acid
TEMED Tetramethylethylenediamine
TIM  translocase of the inner mitochondrial membrane
TOM  translocase of the outer membrane
VDAC voltage-dependent anion channel
WT   wild type
Chapter 1

Introduction

All cells are surrounded by biological membranes to shield and confine the cellular interior. In eukaryotic cells, biological membranes even separate functional compartments or organelles: confined environments that provide specific chemical conditions or harbor macromolecules and protein machineries to fulfill highly specialized tasks within the cells, like DNA replication and transcription, adenosin triphosphate (ATP) generation, lipid or protein biogenesis or fatty acid oxidation.

Biological membranes take on a very important role for the cell and its organelles. They limit diffusion of proteins, ions, toxic byproducts or protons, enable specific and directed transport of these solutes, harbor localized protein machineries and enable transient or permanent electrochemical gradients. As most functions of organelles are fulfilled by the organellar proteome, protein translocation over these membranes is of special importance for cell viability. While most protein importing organelles are of relatively simple constitution and maintain only few protein translocation systems, mitochondria and chloroplasts differ due to their unique, multi-membrane compartmenting structure (see
In yeast and mammals, protein import into mitochondria is arguably the most complex of all protein sorting mechanisms.

Figure 1.1: Sketch of a typical yeast cells with various organelles. Zoom into mitochondrion shows multi-membrane ultrastructure.

1.1 Protein Import into Mitochondria

Mitochondria are essential cellular organelles of all eukaryotic cells that play a pivotal role in many physiological processes. Their best known role is the involvement in oxidative phosphorylation, the most important ATP-generating metabolic pathway, making mitochondria vital for cell growth and viability. The organelle also harbors protein machineries involved in breaking down fatty acids via β-oxidation and play a significant role in biosynthesis of lipids. The mitochondrial iron-sulfur cluster assembly machinery not only matures Fe/S proteins within the organelle, but also contributes to biogenesis of cytosolic and
nuclear Fe/S proteins, making mitochondria essential even when respiration is not required, e.g. yeast is grown on fermentable media. Another critical involvement of mitochondria is in not cell growth but cell death, by setting the stage for early steps of the intrinsic apoptotic pathway: the release of cytochrome c\textsuperscript{8} and SMAC\textsuperscript{8}, triggering the caspase-9 cascade\textsuperscript{9}.

Historically, the mitochondrion originates from endosymbiotic \(\alpha\)-proteobacteria which left it with a double-membrane envelope and an own, albeit reduced, genome. After massive gene transfer to the nucleus\textsuperscript{88}, the mitochondrial genome nowadays only encodes for e.g. thirteen proteins in humans\textsuperscript{89} or eight proteins in the budding yeast \textit{Saccharomyces cerevisiae} (\textit{S. cerevisiae})\textsuperscript{10,11}, accounting for about 1% of the total mitochondrial proteome in general\textsuperscript{12}. These proteins mainly are components of respiratory chain complexes, e.g. subunits of the cytochrome c oxidase, of the ATP synthase and, in humans, of the nicotinamide adenine dinucleotide (NADH) dehydrogenase.

Similar to gram-negative bacteria or chloroplasts, which originate from endosymbiotic uptake of cyanobacteria, mitochondria possess two distinct membranes, dividing the organelle into four different subcompartments: The outer membrane (OM), the intermembrane space (IMS), the inner membrane (IM) and the matrix (see \textit{figure I.1}). Together, all four subcompartments are targets for import of over one thousand precursor proteins\textsuperscript{13,14}. In the last two decades, multiple pathways that work alone, in strictly consecutive or even in very complex cooperative manner have been revealed (see \textit{figure I.2}). At the hearts of these pathways protein-conducting channels have been discovered. Originally thought to be rare cases for translocation and insertion machineries, it is now accepted that such channels rather represent normality at least in mitochondria.
and were also discovered in other eukaryotic organelles\textsuperscript{18,19} and in bacteria\textsuperscript{20} to constitute protein translocases. A vital property of mitochondria, required for multiple functions, is the transmembrane potential across the inner membrane, created by an electrochemical gradient due to proton pumping from matrix to intermembrane space by respiratory chain complexes\textsuperscript{8}. The electrochemical gradient is used by protein translocation systems and by the ATP generating system of the inner membrane.
1.1. PROTEIN IMPORT INTO MITOCHONDRIA

The history of mitochondrial protein import research is a history of frequent revisions and of recurrent revolutions. Originally, mitochondrial protein import was believed to be performed by a single, streamlined pathway involving a translocase of the outer membrane (TOM) and a translocase of the inner membrane (TIM), importing unfolded proteins with an N-terminal presequence. Following this idea of a solitary pathway, the two translocases had to be responsible for translocation into all four subcompartmental of the mitochondrion: outer membrane and intermembrane space, inner membrane and matrix. While the presequence translocase of the inner membrane (TIM23) was indeed capable of both translocation into the matrix and lateral release into the inner membrane, the model of a solitary pathway had to be corrected shortly after. Studies regarding import of the inner membrane ADP/ATP carrier (AAC) family proteins revealed the essential involvement of Tim22, a protein organized in high molecular weight complexes. Not only did these Tim22-containing complexes show no association with TIM23, but import of AAC proteins was found to be fully independent of the presequence translocase. A new insertion pathway had been discovered: the carrier translocase of the inner membrane TIM22. For a long time afterwards, the trinity of protein translocases in mitochondria was mostly accepted and models were sought after how TOM itself could release membrane proteins into the outer membrane.

The triple-translocase model was challenged with the discovery of the sorting and assembly machinery (SAM) and the mitochondrial import protein Mim1 in the outer membrane. The small 13 kDa protein Mim1 inserts α-helical transmembrane proteins like the TOM receptor Tom20 into the OM independently of the TOM complex. It receives its substrates from the cytosol and can even accommodate multi-helical proteins. SAM on the other hand
is primarily inserting $\beta$-barrel proteins, like the TOM core component Tom40, and receives its substrates from the IMS side for which it cooperates with the TOM complex and soluble TIM-chaperones. These soluble chaperones and many other IMS-proteins contain characteristic -$\text{CX}_3\text{C}$- or -$\text{CX}_9\text{C}$- cysteine motifs and IMS sorting signals. In the IMS, they are recognized by the mitochondrial intermembrane space assembly (MIA), consisting of the disulfide relay system Mia40/Erv1. MIA catalyzes the formation of intramolecular disulfide bonds and facilitates oxidative folding. Membrane proteins encoded in the mitochondrial genome highlight the necessity of another protein insertion or translocation pathway in the inner membrane. Comparably early, the machinery for insertion of mitochondrially encoded proteins was identified in the OXA complex. Interestingly, the OXA machinery has been revealed to be much more than just the insertase for mitochondrially encoded substrates, as it is also involved in insertion of TIM23 substrates and even of TIM22 substrates.

1.2 Mitochondrial Protein Translocases

1.2.1 Translocase of the Outer Membrane (TOM)

The very first step for most mitochondrial import substrates after translation is the passage through the abundant mitochondrial entry gate, the TOM complex. In *S. cerevisiae*, the 450 to 500 kDa TOM complex consists of seven proteins: Tom40 is a 42 kDa $\beta$-barrel protein, forms the essential core of the complex and is present with two or three units per complex. Tom20 and Tom70 are the loosely associated receptors for presequence and carrier precursor proteins, respectively; Tom22 is the tightly associated central receptor,
handling preproteins both at the cytosolic exterior and in the intermembrane space. Tom22 is critical for complex formation, as deletion dissociates the TOM complex, heavily impairing mitochondrial biogenesis. Tom5/6/7 are small proteins and mainly linked to assembly and maintaining stability of the complex.

Translated on cytosolic ribosomes, preproteins destined for mitochondrial import are recognized by membrane bound receptors. Precursors with a positively charged presequence, ultimately enabling import by TIM23, are initially recognized in their secondary structure by the TOM receptor Tom20 and handed over to the general complex receptor Tom22. Carrier protein precursors with multiple internal targeting signals, on the other hand, are recognized by Tom70 before being handed over to Tom22. It has been reported that some carrier protein precursors are not translocated in a completely unfolded state but in a loop topology with the internal targeting signal leading ahead. After recognition by the central receptor, the respective preproteins are threaded through the pores formed by Tom40 and, after emerging from the channel, are again taken over by Tom22 on the IMS side. From there on, they are taken over and directed to other translocases by the respective targeting signal receptors. In the case of positively charged presequences, the release of preproteins from Tom22 is induced by Tim21, a subunit of the TIM23 complex, while inner membrane carrier preproteins as well as outer membrane β-barrel preproteins are recognized by small Tim chaperones in the IMS and further directed to TIM22 or SAM, respectively.

Initially, the protein conducting pore was found and characterized in isolated full TOM complexes after solubilization using the mild detergent digitonin. It
showed striking similarity to the peptide-sensitive channel (PSC) that was found ten years earlier\(^2\), indicating that these two channels are indeed identical. Later, the same pore characteristics were found in the smaller core complex isolated via a slightly harsher detergent n-Dodecyl \(\beta\)-D-maltoside (DDM), lacking Tom70 and Tom20 compared to the previously described intact complexes\(^3\). Furthermore, similar characteristics could be attributed to Tom40 isolated from *Neurospora crassa*\(^4\), Tom40 from *S. cerevisiae* and to both NcTom40 and ScTom40 heterologously expressed in *E. coli*\(^5\). Initially, there was some disagreement on the number of pores and conductance states (see Becker et al.\(^6\) for an experimental elucidation of the problem), though in the end a conclusive picture could be drawn: The TOM complex contains a water-filled translocation channel, the \(\beta\)-barrel protein Tom40 which forms a cation-selective pore \((P_+ : P_- \approx 10 : 1)\) with a main conductance \(G_{\text{main}} = 370\) to \(390\) pS and a subconductance gating state \(G_{\text{sub}} = 150\) pS (in 250 mM KCl). The pore diameter was assessed using different techniques like electron tomography, computational modeling or calculation from the conductivity of the channel. The independent experiments all led to a diameter of 2.1 to 2.6 nm for one pore unit, wide enough to import preproteins with two secondary structured stretches in parallel.

Surprisingly, the water-filled pore itself is capable of binding import substrates like the positively charged presequence of cytochrome c oxidase subunit 4 (Cox4) even without receptor units and, upon binding of substrates, exhibits a significant increase in channel activity. The full TOM complex shows similar behavior, though at a 40- to 60-fold increased sensitivity\(^7\). Recombinant Tom40 also exhibited a sensitivity to higher voltages regardless of sign that resulted in a reduced open probability, proving that a voltage sensor is also part of the channel protein and not exclusively located in other subunits of the TOM complex\(^8\).
High-resolution electrophysiology was further used together with detailed kinetic analysis\(^{22}\) to investigate substrate peptide interaction with isolated NcTom40. They revealed that pure peptide binding and full peptide translocation can be distinguished by temporal parameters of Tom40's substrate response\(^{23}\) and that the energy profile of peptide translocation through the channel can be extracted from temperature-dependent interactions\(^{24}\).

While the purified TOM complex was found to mostly constitute twin- or triple-pores\(^{22}\), the TOM core complex formed mainly twin-pores, no triple-pores, but a significant portion of single-pores. This distribution was recently confirmed and explained by the presence of two different populations of TOM complexes \textit{in vivo}, with a lack of receptors in the twin-pore conformation\(^{25}\). The single-pore appearance was also found for recombinantly expressed and reconstituted Tom40 from yeast\(^{24}\). Very recently, the structure of the TOM core complex was solved using electron microscopy\(^{25}\). The complex isolated with dodecyl-maltoside exhibits the twin-pore formation without Tom20 or Tom70, in line with the assumption that the milder detergent digitonin might be required to isolate the complex also in the triple-pore formation\(^{19,26,27}\). Bausewein et al.\(^{28}\) speculate that the triple-pore might represent a TOM-SAM supercomplex instead, though this assumption is not in agreement with previous model-free crosslink-data\(^{29}\).

### 1.2.2 Sorting and Assembly Machinery (SAM)

The second translocase in the outer membrane is constituted by SAM, also referred to as \textit{topogenesis of the outer mitochondrial membrane} \(\beta\)-\textit{barrel proteins} (TOB) complex. This complex is a relic of the endosymbiotic origin of mitochondria and shows homology to the bacterial \(\beta\)-barrel assembly machinery (BAM)
which inserts β-barrel proteins from the periplasm into the outer bacterial membrane. In yeast mitochondria, five different β-barrel proteins are known, all substrates of the SAM pathway: The outer membrane entry gate Tom40; the insertase of the SAM complex, Sam50, itself; the voltage-dependent anion channel (VDAC) which is related to Tom40; and the two mitochondrial distribution and morphology proteins Mdm10 and Mdm34. The SAM complex of S. cerevisiae consists of three proteins: the β-barrel protein Sam50, a member of the Omp85-family, the β-signal receptor Sam35, both essential, and the non-essential Sam37 which promotes release of the precursor proteins. Each protein is present with one unit in the 140 kDa core complex.

One of SAM’s substrates, the β-barrel protein Mdm10, is partially associated with the SAM complex. This association was initially thought to just originate from Mdm10’s nature as a SAM substrate, though the most abundant substrate VDAC was not co-isolated with SAM. Mdm10 was known to participate in mitochondrial fusion and fission and is, as well as Mdm34, a component of the ER-mitochondria encounter structure (ERMES). Mdm10 was also reported to play a crucial role in biogenesis of Tom40 and thus of the TOM complex itself, while biogenesis of VDAC was not impaired in *mdm10Δ* deletion strains of *S. cerevisiae*. Another temporary SAM constituent was found to be Tom5, one of the small TOM subunits, that plays a vital role in assembly of Tom40 at SAM but is not necessary for binding Tom40 in the first place, even indicating a two-step procession of β-barrel precursors at the SAM complex. In an earlier study of the same group, Mim1, which also interacts with TOM and SAM, was reported to be crucial for Tom40 biogenesis, though Mim1’s involvement was later linked only to Tom5 biogenesis, a common substrate of the Mim1-pathway. Surprisingly, a significant part of α-helical OM proteins is indeed not imported via Mim1 but in-
stead utilizes SAM proteins and even Mdm10 to be inserted\textsuperscript{[23][24]}. These substrates usually contain a transmembrane segment in their C-terminal half, like Tom22, or are tail-anchored at their extreme C-terminus.

SAM substrates are recognized by Sam35 by a $\beta$-signal which was first identified for \textit{Sc}Tom40. It resides in the last transmembrane $\beta$-strand of the protein\textsuperscript{[25]}, consistent with the targeting signal of prokaryotic $\beta$-barrels insert by BAM\textsuperscript{[26]}, and follows the semi-conserved sequence \textit{Po}-X-G-X-X-\textit{Hy}-X-\textit{Hy} (\textit{Po}: polar, \textit{G}: glycine, \textit{Hy}: hydrophobic). Based on these findings, a more complex, circular $\beta$-signal, containing the semi-conserved sequence, was identified that also contains all information for targeting the preprotein to mitochondria\textsuperscript{[2]}. The moment preproteins with such a $\beta$-signal enter the IMS through TOM, they are recognized by small Tim chaperones\textsuperscript{[28–31]}. They are six-bladed $\alpha$-propeller complexes formed either by the essential proteins Tim9 and Tim10 or by Tim8 and Tim13, as a trimer of dimers in triangular conformation\textsuperscript{[32][33]}. These chaperones are thought to shield the hydrophobic stretches of the preproteins from the aqueous IMS environment during shuttling from TOM to SAM or TIM22. After recognition at the SAM complex, the preproteins are handed over to the substrate receptor Sam35, where the preproteins are subsequently passed on to Sam50 and then released into the outer mitochondrial membrane, assisted by Sam37. It was originally assumed that the Tim chaperones take over emerging preproteins from TOM and then shuttle them to the independent SAM complex\textsuperscript{[34–36]} as a soluble chaperone-substrate complex. However, no soluble intermediates of $\beta$-barrel preproteins could be found in the IMS. Instead, a recent study reported the importance of the central TOM receptor Tom22 in not only import but oxidation and thus folding of $\beta$-barrel proteins at the SAM complex\textsuperscript{[37]}. Using intact complex
investigations, the authors of that study identified a transient 650 kDa TOM-SAM supercomplex that mediates the handover of preproteins from one complex to the other and is linked by Tom22 on the cytosolic side.

Mdm10 was not found to be part of this supercomplex, although its formation was studied using a Tom40-probe which should recruit Mdm10 to SAM at some point as seen before. One could speculate that Mdm10’s recruitment to SAM for Tom40 biogenesis might happen in a later stage, after supercomplex disassembly. Previous studies found that a core component of the mitochondrial contact site and cristae organizing system (MICOS), Mic60 (Fcjl/Mitofilin), binds both TOM and SAM complexes independently. While it might be tempting to speculate that simultaneous binding of both complexes by Mic60 represents an early stage of supercomplex formation, this seems unlikely as binding of SAM to Mic60 was shown to depend on an IMS-facing polypeptide-transport associated (POTRA) domain of Sam50, while supercomplex formation of SAM and TOM still happens after deletion of said POTRA and thus is independent of that POTRA domain.

The essential SAM component Sam50 has two noteworthy structural features: The β-barrel itself, predictably composed of 16 β-strands, and the N-terminal POTRA domain that is conserved in the Omp85-family, though with various number of copies per protein. The POTRA domain was initially proposed to function as a chaperone or as a receptor as it binds precursors, with partial deletion of the domain leading to growth defects. Only shortly thereafter though, an extensive study showed that deletion of the whole POTRA domain does not inhibit yeast growth or protein import and assembly by SAM at all, concluding that POTRA does not act as the main receptor domain. Instead, in a combined electrophysiological and biochemical approach the authors investigated the channel properties of the SAM complex and of Sam50 alone under...
various conditions. They found that both Sam50 and the full SAM complex exhibit very similar electrophysiological characteristics: The channel is mildly cation-selective ($P_+ : P_- \approx 4 : 1$) and is gating frequently between an open and a semi-open state with a conductance difference $\Delta G = 160 \text{ pS}$ (250 mM KCl). Upon addition of a shortened substrate, the $\beta$-signal of Tom40, Sam50 alone showed no alteration while the full SAM complex exhibits reduced gating frequency, remains mainly in the open state and even opens up to much higher conductance. Surprisingly, these increased conductance differences are multiples of the minimal conductance, i.e. the channel can open to 320 pS and rarely even to 640 pS, which might indicate that upon substrate binding the complex does not only undergo simple rearrangement but even that other, previously dormant units of Sam50 now contribute to channel formation.

### 1.2.3 Carrier Translocase (TIM22)

The carrier translocase TIM22 is one of two inner membrane translocases that handle substrates previously imported by TOM. It inserts multi-spanning transmembrane proteins into the inner membrane in a strictly membrane potential dependent manner\cite{25,26,27}. Substrates for TIM22 are mainly metabolite carriers like the ADT/ATP carrier (AAC) or the phosphate carrier (PiC), each carrying three pairs of transmembrane $\alpha$-helices. TIM22 also imports protein translocases of the Tim17/22/23 homology family, with four $\alpha$-helices each. In *S. cerevisiae*, the 300 kDa TIM22 core complex is formed by the central translocation channel Tim22\cite{28}, the chaperone-receptor Tim54\cite{29}, Tim18\cite{30,31}/Sdh3\cite{32}, both required for complex assembly, and the peripheral inner membrane protein Tim12\cite{33,34} that interacts with the chaperones Tim9/10. Of the four integral
core components only Tim22 is essential for yeast growth, while deletion of the other proteins lead to more or less severe growth defects. Additional to the integral components, the peripheral membrane protein Tim12 was reported to be associated with the TIM22 complex via a large IMS-domain of Tim54, though it first forms a soluble complex with the chaperones in the IMS before becoming membrane-bound.

Similar to β-signal proteins, preproteins with multiple targeting signals are recognized by Tim9-Tim10 or Tim8-Tim13 chaperone complexes at the IMS side of the TOM complex. There, the Tim9-Tim10 chaperone complex promotes import of metabolite carrier proteins like SiC or AAC, while import of ScTim23 additionally requires the Tim8-Tim13 chaperone complex. After the chaperone-cargo complex is formed in the IMS, it is recognized by Tim12 and further directed to TIM22. Here, the soluble cargo-Tim9-Tim10-Tim12 complex is recruited to the membrane via interaction between Tim12 and Tim54, which leads to stable association of the peripheral membrane protein Tim12 with the TIM22 complex and the carrier preprotein is inserted by the central channel protein Tim22. Binding or tethering of the chaperone-cargo complex to TIM22 is independent of the mitochondrial membrane potential, while the initial insertion of α-helices into a pore of Tim22 requires a low polarization (ΔΨ > 60 mV) of the inner membrane. The final insertion and subsequent release into the membrane occurs only in the presence of both a targeting signal and a higher membrane potential (ΔΨ > 120 mV).

In an early study, Tim22 was identified as water-filled channel, the core of the TIM22 complex, and characterized electrophysiologically. Recombinantly expressed ScTim22 constitutes a cation selective channel with a maximum ob-
served conductance change of $G_{\text{main}} = (540 \pm 18)$ pS under symmetrical buffer conditions with 250 mM KCl. Interestingly, the channel gates in eight steps of $G_{\text{sub}} = (67 \pm 5)$ pS from open to closed and exhibits direct conductance transitions covering multiples of $G_{\text{main}}$ with frequencies much higher than expected for incidental simultaneous gating of independent pores. This indicates that the main conductance change $G_{\text{main}}$ indeed corresponds to gating of one functional subunit of a coupled channel. At asymmetric buffer conditions, Tim22 reveals a varying cation preference of $P_+ : P_- \approx 15 : 1$ in the smallest conductance state and $P_+ : P_- \approx 4 : 1$ in the fully open state. Isolated TIM22 complexes though exhibited a significantly reduced ion preference ($P_+ : P_- > 2.4 : 1$), indicating a possible reducing contribution by other complex constituents.

The channel is voltage-activated, i.e. at low membranes potential the channel is present in a closed conformation but starts to open at elevated voltages regardless of sign. Prolonged exposure to higher membrane potentials, on the other hand, again induces closure of Tim22, similar in principle to Tom40. While an open, substrate-susceptible TIM22 is a reasonable requirement for membrane potential dependent protein import, staying open would compromise the energized state of the inner membrane.

A follow-up study revealed the twin-pore nature of TIM22, using electron microscopy and electrophysiological investigations\[17\]. In electron micrographs, TIM22 appears as a twin-pore similar to the TOM core complex. Isolated TIM22 complexes fused to a planar lipid bilayer reveal a channel with identical pore conductances compared to recombinantly expressed Tim22, but appearing only in multiples of two. The possibility of two independent channels, e.g. an artifact of complex isolation or reconstitution, was refuted by the presence of direct conductance changes of $\Delta G_{\text{TIM22}} = 2 \cdot G_{\text{max, Tim22}}$, with a much higher fre-
quency than expected for independent pores. Interestingly, both native complex and recombinant protein are excitable using a combination of an uncharged targeting signal (internal signal peptide of the phosphate carrier) and a high transmembrane potential, leading to strongly increased gating activity of each channel. While this shows that the Tim22 protein contains both a targeting signal recognition mechanism and a voltage sensor, and does not require the full complex for this, the voltage threshold required for activity increase is much lower for the full complex ($\Delta \Psi_{\text{min,TIM}_{22}} \approx 70 \text{ mV}$) than for the recombinant protein ($\Delta \Psi_{\text{min,Tim}_{22}} \approx 140 \text{ mV}$).

1.2.4 Presequence Translocase (TIM23)

Designated mitochondrial matrix proteins and non-carrier inner membrane proteins are imported via the TIM23 pathway (see figure 1.3). These substrates typically bear positively charged, N-terminal presequences making up $\approx 70\%$ of the mitochondrial proteome. The presequences are cleaved after import by mitochondrial peptidases in the matrix or the IMS. Some substrates, like the cytochrome b$_2$, are even released back to the IMS after being processed by an IMS peptidase.

In *S. cerevisiae*, the TIM23 core complex consists of the channel-forming eponymous protein Tim23, its homolog Tim17 (both also homolog to Tim22), the main presequence receptor Tim50 and Mgr2. TIM23 exists in two different forms in the inner mitochondrial membrane to accommodate its dual function: Integral inner membrane proteins are inserted by the TIM23$^{\text{SORT}}$ complex, containing Tim2l as an additional integral component. For import of mitochondrial matrix proteins on the other hand, TIM23 recruits the presequence
Figure 1.3: Protein import through TIM23 requires dynamic switching of the complex between two different conformations: Preproteins with a presequence and hydrophobic sorting signals are inserted into the inner membrane via the Tim21-containing TIM23"SORT, assisted by electron transfer chain complexes III and IV. Preproteins with a presequence but without sorting signals are translocated into the matrix with the help of the PAM motor complex.
translocase-associated motor (PAM) on the matrix side. PAM consists of the chaperone heat-shock protein 70 (Hsp70), the membrane-anchored co-chaperone PamI8, the scaffold proteins PamI6 and Tim44, and the nucleotide exchange factor MgeI.

Upon emergence from TOM to the IMS, preproteins are bound by the central receptor Tom22. Tom22IMS was found to also interact with Tim23IMS and Tim21IMS, effectively linking the inner to the outer membrane. The preprotein is handed over to the TIM23 complex via the main receptor Tim50. The IMS domain of Tim50 contains two presequence-binding subdomains: Tim50core, which also interacts with TIM23IMS, and the essential Tim50PBD. Both Tim50IMS and Tim23IMS can bind presequences, though binding affinity was orders of magnitude lower for Tim23 in comparison, indicating that indeed binding to Tim50 is the first step in preprotein reception. During early steps of recognition and handover, protein domain interactions are competing in a complex manner. Tim50PBD is able to interact with both Tim50core and presequences with overlapping sites, presumably to enable preprotein handover between the two subdomains of Tim50. Further, Tim50IMS- and presequence-binding to Tim23IMS are mutually exclusive, i.e. IMS domains of Tim50 and Tim23 dissociate upon presequence handover to the channel protein. Now that the preprotein has reached Tim23IMS, it is inserted into the pore in an unknown manner and then electrophoretically threaded through the inner membrane.

If the preprotein contains a sorting signal, typically a hydrophobic stretch after the presequence, translocation through TIM23SORT proceeds until the hydrophilic presequences emerges on the matrix side. Here, most presequences are proteolytically processed by the mitochondrial processing peptidase (MPP).
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The protein is laterally released into the lipid bilayer through a yet unknown lateral gate. Mgr2[16,17] and Timl7[16,36] both interact with preproteins during lateral sorting of inner membrane proteins, with Mgr2 acting as the quality control of lateral protein release[19]. Interactions with preproteins and involvement in lateral release lead to the speculation that Mgr2 and Timl7 form or contribute to the lateral gate of TIM23[52,59,63]. Mgr2 and Timl21 are also critical for recruitment of respiratory chain complexes to TIM23SORT[15,43], which is speculated to ensure a sufficient membrane potential in close proximity to TIM23[33].

The mitochondrial membrane potential $\Delta\Psi$ is the sole energy source of membrane protein insertion by TIM23[17]. Dissipation of $\Delta\Psi$ leads to complete collapse of protein import for most TIM23 substrates, though interestingly, some preproteins lacking a classical cationic presequence are still imported by TIM23 at a basal rate[44]. Even more so, deletion of the only cationic amino acid in the targeting signal of such a preprotein, subunit $e$ of the F0F1-ATP synthase, leads to complete independence of its import from the membrane potential.

Designated matrix preproteins interact with Tim50 when they reach TIM23. Timl21 then dissociates from Tim50IMS and consequently from TIM23[23], which in turn recruits Paml7 to the complex[18]. After handover of the preprotein to Tim23, it is threaded through the channel in a $\Delta\Psi$-dependent manner. When it emerges on the matrix side, the PAM protein Tim44 can bind the presequence after its recruitment by Timl7 and Tim23[35]. Upon preprotein binding, Tim44 recruits the ATP-loaded main chaperone Hsp70[29], which in turn then binds to the emerging presequence[29,38]. ATP-hydrolysis by Hsp70 is stimulated by the J-proteins[39] Pam6/l8[31,41] that are recruited to TIM23 via interaction with Timl7IMS[39]. Conversion of ATP leads tightening of Hsp70 around the presequence[12] which pulls the preprotein a short distance into the matrix. Then, Hsp70$^{ADP}$ and the other
PAM proteins dissociate from the complex and fresh Hsp70<sup>ATP</sup> and co-chaperones bind to TIM23 and the presequence. This subsequent recharging of PAM maintains continuous presequence import<sup>31</sup> into the matrix.

### 1.2.5 Oxidase Assembly Machinery (OXA)

The oxidase assembly machinery (OXA) has a unique position among all mitochondrial protein translocation pathways. It has been identified as the only mitochondrial export machinery to handle mitochondrial encoded proteins<sup>15</sup> through a highly-conserved pathway. The OXA complex acts as an insertase of the inner membrane by directly binding mitochondrial ribosomes and inserting the nascent protein chain into the IM in a co-translational manner<sup>16</sup>. OXA is constituted by the membrane proteins Oxal, Mbal and Mdm38. Oxal is the core protein of the complex, contains five transmembrane $\alpha$-helices and spans the inner membrane in a N<sub>Out</sub>-C<sub>In</sub> topology. It is a member of the YidC/Alb3/Oxa<sub>1</sub> family<sup>14</sup> which is a small family of insertases with YidC and Alb3 being found in bacteria and chloroplasts, respectively. The individual function of each OXA protein appears not to be easily attributed as each component seems to participate in multiple processes in protein insertion and even beyond that. First of all, each of the three proteins was shown to bind mitochondrial ribosomes and they were speculated to act cooperatively with each other<sup>15</sup>-<sup>19</sup>. Early investigations of Mbal function showed that it can compensate for import defects in yeast strains with Oxal-mutants<sup>20</sup> and a very recent study bolstered the view that Mbal is not just a passive ribosome receptor but actively aligns the ribosome exit tunnel with the insertion complex<sup>31</sup>. Mdm38 on the other hand fulfills two major functions that are spatially distinct on the protein level: The transmembrane part acts as an $K_+/H_+$ antiporter<sup>22</sup>-<sup>24</sup> while the matrix exposed part has a ribosome binding domain with structural
similarity to 14-3-3 proteins. Both proteins also exhibit a regulatory function on translation of mRNAs and can compensate for each other in that, while loss of both proteins together leads to severe aberrant synthesis of respiratory chain proteins.

Substrates for the OXA pathway like ScCox2p can employ a cleavable targeting signal which is recognized by Oxal and processed by the inner membrane peptidase (IMP) for maturation and insertion of the substrate, while other substrates, e.g. the human HsCox2, do not have a cleavable leader sequence. OXA inserts not only mitochondrially but also some nuclear encoded inner membrane preproteins in a pathway referred to as conservative sorting. There, the precursor proteins make their way through TOM and TIM23 and are then not or only partially released to the IM by TIM23’s sorting mechanism. Instead, they are fully or partially translocated to the matrix where they are taken over by OXA to insert the remaining transmembrane segments. This pathway provides two different modes of action: chaperone-mediated release of transmembrane segments into the matrix by TIM23 and subsequent insertion by OXA (“conservative sorting”) or segment-wise insertion into the membrane directly by TIM23 (“stop-transfer”). Initially, both modes were thought to be conflicting models of protein insertion but were then proven to work in a complementary manner in during insertion of multispansing membrane proteins, as shown for the ATP binding cassette (ABC) transporter Mdl1. Another prominent substrate of this pathway is Oxal itself which is translated in the cytosol, imported via TOM and TIM and inserted via existing OXA complexes. Interestingly, Oxal was recently found to participate in biogenesis of another type of inner membrane proteins, the ATP/ADP carrier protein Aac2 which is actually inserted via TIM22. While the specific role of Oxal in this context is still unknown, the authors suggested a function in folding
newly inserted carrier proteins.

Recently, some light was shed on the molecular mechanism of protein insertion by OXA as an aqueous pore was identified in native OXA complexes. For this purpose, Oxa1 was isolated from three different sources, purified from *S. cerevisiae*, recombinantly expressed in *E. coli* and isolated as native OXA complexes, and subjected to planar lipid bilayer electrophysiology. This revealed a dynamic, cation-selective pore with a main conductance state $G_{\text{main}} = 530\, \text{pS}$, a minimal subconductance state $G_{\text{submin}} = 75\, \text{pS}$ and multiple conductance states in between, partially depending on the sign of the applied voltage. Oxa1 has a calculated diameter of $\approx 1.9\, \text{nm}$ and comes in minimal units of four pores. Incorporated channels reacted to αOxa1 antibodies by near-complete blockage, while incubation of Oxa1-channels with the presequence of the substrate ScCox2 led to a drastic increase in activity of the channel. Investigations further confirmed prior findings that Oxa1 is present as a homo-tetramer or, as two of four pores are functionally coupled, as a dimer of dimers. After the discovery tetrameric Oxa1 forming a water-filled pore in the inner mitochondrial membrane, crystal structures of the bacterial Oxa1-homolog YidC emerged. These structures show YidC in a monomeric state, a configuration that would not suggest channel activity. Instead the authors proposed that YidC forms a hydrophilic groove in the inner leaflet of the bilayer. This groove recruits the hydrophilic stretch of a substrate and transfers it to the extracellular space in an unknown manner whilst releasing the hydrophobic domain into the bilayer.

This obviously also challenged the idea of Oxa1 being a water-filled channel in an oligomeric state, as both proteins share a high degree of sequence similarity. While crystal structures provide a plethora of new and helpful information, also towards understanding the molecular mechanism of a protein, they naturally are
snapshots of a specific static state and often do not allow deeper insights into the protein dynamics. Even if the implications from the crystal structure were transferred from YidC to Oxal, one could speculate that the monomeric, crystallized form might represent an idle state whose oligomerization \textit{in vivo} needs to be induced to form the import-competent complex. It can also be speculated that two hydrophilic grooves, as found in YidC, together constitute one coupled double-pore, as found for Oxal complexes. It is tempting to imagine possible ways to merge electrophysiological data with crystal structures from putative, especially from non-\(\beta\)-barrel pores. While typical \(\beta\)-barrel channels often have stable shapes, fixed number of \(\beta\)-strands and were shown to form oligomers of pre-existing pores, but not assemble the pores by oligomerization\cite{65,65a}, the same cannot be said for \(\alpha\)-helical transmembrane proteins like Oxal or Tim23.

\section*{1.3 Molecular and Channel Properties of Tim23}

Tim23 has been subject of multiple molecular and electrophysiological investigations, trying to shed light on molecular mechanisms and the origin of channel properties. The protein consists of four \(\alpha\)-helical, C-terminal transmembrane segments\cite{65} and an unstructured, N-terminal domain facing the intermembrane space\cite{65}, which enables interaction with presequences\cite{65}. The IMS domain is able to homodimerize in the presence of a membrane potential, but dissociates upon binding of presequences\cite{65}, although the N-terminal IMS domain of Tim23 was found in an NMR study to exist purely as a monomer\cite{65}. Later, it was revealed that the dimerization requires a functional first transmembrane segment (TMS1)\cite{65}, possibly explaining the previous discrepancy. Interestingly, the third (TMS3) and fourth (TMS4) transmembrane segment were found dispensable for protein import\cite{65}, though it is unclear if they just do not take part in pore formation or
Tim 23 is the main channel-forming protein of the complex, was identified as a component of the inner membrane import machinery nearly 25 years ago and found to constitute the aqueous pore in the *multiple conductance channel* (MCC). It forms a triple pore when constituted alone and a twin pore in the full complex. Tim23 gates with a main conductance change $G_{\text{main}} = (450 \pm 11)$ pS and the subconductance change $G_{\text{sub},1} = (140 \pm 15)$ pS, with a single-pore diameter of 6.5 Å to 12 Å, which is wide enough to accommodate two parallel $\alpha$-helices without tertiary structure. Although the actual protein constitution of the pore within the TIM23 complex is unknown, both TimL7, containing four indispensable transmembrane $\alpha$-helices, and Mgr2, with two $\alpha$-helices, were found to mediate preprotein release into the bilayer and thus could be contributors towards pore constitution. According to basic calculations, eight to ten $\alpha$-helices could together form an aqueous channel with a size comparable to the Tim23 pore, without the need for Tim23-dimer formation.

The Tim23 channel is voltage-activated, but closes upon prolonged exposure to higher voltages. As with TIM22, this is in line with the need for an open channel to insert presequences while keeping the electrochemical barrier intact. The voltage-sensitivity is increased by the IMS domain of the receptor Tim50, drastically lowering the threshold for voltage-induced closing. Even without other complex constituents, recombinant Tim23 recognizes preproteins with its N-terminal IMS domain and reacts with fast gating (*flickering*) to site-specific presequence addition in electrophysiological studies. The voltage sensor of Tim23 is proposed to be formed by a leucine zipper motif in the IMS domain, re-
sponsible for voltage-regulated dimerization of the domain, though the voltage sensor can not be exclusively located in the N-terminal IMS domain, as a truncated, C-terminal version of Tim23 was found to also exhibit voltage-dependent channel activity. Similar to the full length protein, the truncated version also closed at high membrane potentials, indicating the presence of an membrane-based part of the voltage sensor.

The Tim23 channel is, compared to potassium channels, mildly cation-selective and prefers potassium ions over chloride ions with \( P_{K^+} : P_{Cl^-} \approx 16 : 1 \). Variations of the buffer-constituting cation revealed that the determining factor for ion permeability is dehydration energy, i.e. the likelihood of the ion to shed the hydration shell to move through the channel. An alternative determination factor is ion size, i.e. the ability to strongly bind to negative charges along the channel lumen. It was concluded that negatively charged amino acids in both IMS domain and channel lumen together constitute the ion filter of Tim23. Interestingly, the TIM23 complex shows slightly weaker cation selectivity \( P_{K^+} : P_{Cl^-} \approx 11 : 1 \) compared to recombinant Tim23, highlighting that net contribution of other complex subunits to the ion filter is small. The nonetheless present difference in selectivity could be attributed to Tim17 or Mgr2, putatively forming a part of the pore in fully assembled complexes.

Using a broad library of cysteine mutants of Tim23, interaction of Tim23 residues with preproteins during import was investigated via cross-link analysis. In line with the later finding that TMS3 and TMS4 are dispensable, the substrate was successfully cross-linked to the IMS domain and the first two helices of Tim23. As TMS2 showed a specifically high cross-link efficiency, it was further characterized using a sophisticated, environment-sensitive fluorophore.
CHAPTER 1. INTRODUCTION

labeling approach. The attached fluorophore changes its spectral properties depending on the polarity of its environment. It revealed that the TMS2 faces two different environments, the aqueous channel and the lipid or protein phase. The highly conserved, lumen-facing residues were further shown to switch to a more non-polar environment after preprotein incubation, indicating the TMS2 is in very close proximity to the substrate during translocation. In a follow-up study, the TMS2 of Tim23 was found to undergo structural rearrangements upon dissipation of membrane potential. In an energized membrane TMS2 forms a straight \( \alpha \)-helix, while upon depolarization the helix breaks into two smaller helices, presumably around residue I1162, at the IMS-facing end of the helix.\(^{74}\)

Taken together, multiple studies have been conducted to elucidate molecular characteristics and electrophysiological properties of Tim23. It was found to react to membrane potentials with increased gating and successive closing, enhanced by the voltage regulator, while membrane depolarization leads to helix-kinking and opening of the channel. The cation-selective Tim23 channel also closely interacts with positively-charged substrates via the channel lumen, and exhibits fast channel flickering upon incubation with presequences.
Chapter 2

Aims of this Thesis

Protein biogenesis of mitochondria is an essential requirement for not only mitochondrial fitness but also for cell viability. Despite its own genome and protein synthesis, the overwhelming majority of proteins is imported from the cytosol to one of the four distinct subcompartments of mitochondria, following multiple pathways. At the heart of these pathways lie protein-conducting, aqueous nanopores which cooperate in a complex orchestra.

One of the translocases of the inner membrane, TIM23, handles over 70% of all mitochondrial proteins and transports them either to the mitochondrial matrix or the inner membrane. While TIM23 requires a complex interplay of multiple subunits, switches between two different conformations to accommodate either type of substrate and might constitute the channel itself from two or three subunits, the basic properties of the water-filled pore originate from one subunit, the eponymous Tim23.

While its pore characteristics have been analyzed to some extent, studies on structural implications and molecular localization of specific channel mechanisms are scarce and suffer from the difficulties of handling hydrophobic
The aim of this study is to investigate the molecular origin of basic pore properties, interaction with regulators and substrates, and their implication for channel function. To this end, high-resolution single channel electrophysiology was employed to monitor channel behavior and interaction effects, combined with site-directed mutagenesis to study the impact of specific regions of the pore on its electrophysiological characteristics.
Chapter 3

Materials and Methods

3.1 Materials

Standard chemicals and laboratory consumables were purchased from Th. Geyer (Renningen, Germany), Carl Roth (Karlsruhe, Germany), Sarstedt (Nümbrecht, Germany), Sigma Aldrich (Taufkirchen, Germany). Oligonucleotides were purchased from Metabion (Planegg, Germany). Special chemicals and materials used in this study that were not purchased from Carl Roth are listed in Table 3.2.

Table 3.1: Lipids used in this study

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<th>Abbrev.</th>
<th>Name</th>
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<td>Avanti Polar Lipids</td>
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<tr>
<td>PE</td>
<td>L-α-phosphatidylethanolamine (Egg, Chicken)</td>
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<td>SIV-PC</td>
<td>L-α-Phosphatidylcholine (soybean, Type IV-S)</td>
<td>Sigma Aldrich</td>
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Table 3.2: Special consumables not purchased from Carl Roth

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<td>Proteinase inhibitor pills (EDTA-free)</td>
<td>Roche Applied Science, DE</td>
</tr>
<tr>
<td>PTFE-Film</td>
<td>GoodFellow GmbH, DE</td>
</tr>
<tr>
<td>Spectra/Por Standard RC Tubing 3.5 kDa</td>
<td>Spectrum Labs Inc, US</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>Sigma Aldrich, US</td>
</tr>
<tr>
<td>Wizard SV Miniprep DNA Purification Kit</td>
<td>Promega, DE</td>
</tr>
<tr>
<td>Wizard SV Gel and PCR Clean-Up Kit</td>
<td>Promega, DE</td>
</tr>
</tbody>
</table>

Table 3.3: Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Encoded protein</th>
<th>Vector</th>
<th>Marker</th>
<th>Origin</th>
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<tr>
<td>MM_87</td>
<td>sCTim23</td>
<td>pET10N</td>
<td>Amp</td>
<td>Truscott et al.</td>
</tr>
<tr>
<td>MM_103</td>
<td>sCTim23-NI50A</td>
<td>pET10N</td>
<td>Amp</td>
<td>this study</td>
</tr>
<tr>
<td>MM_104</td>
<td>sCTim23-GI53A</td>
<td>pET10N</td>
<td>Amp</td>
<td>this study</td>
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<tr>
<td>MM_105</td>
<td>sCTim23-YI59A</td>
<td>pET10N</td>
<td>Amp</td>
<td>this study</td>
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<tr>
<td>MM_117</td>
<td>sCTim23-AI52G</td>
<td>pET10N</td>
<td>Amp</td>
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<td>MM_118</td>
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<td>pET10N</td>
<td>Amp</td>
<td>this study</td>
</tr>
<tr>
<td>MM_190</td>
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<td>pET10N</td>
<td>Amp</td>
<td>this study</td>
</tr>
<tr>
<td>MM_191</td>
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<td>pET10N</td>
<td>Amp</td>
<td>this study</td>
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<tr>
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<td>Amp</td>
<td>this study</td>
</tr>
<tr>
<td>MM_196</td>
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<td>Amp</td>
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<tr>
<td>MM_197</td>
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<td>pET10N</td>
<td>Amp</td>
<td>this study</td>
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<td>MM_198</td>
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### Table 3.4: Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Encoded Mutation</th>
<th>Direction</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>MM13F</td>
<td>Tim23-N150A</td>
<td>Forward</td>
<td>AGAGGTCCCTTCTTAGTGCTAAT</td>
</tr>
<tr>
<td>MM13R</td>
<td>Tim23-N150A</td>
<td>Reverse</td>
<td>GAGAATCCCCCGCATTAGCAC</td>
</tr>
<tr>
<td>MM14F</td>
<td>Tim23-G153A</td>
<td>Forward</td>
<td>TCTCTAGTAAATCGGCGGATT</td>
</tr>
<tr>
<td>MM14R</td>
<td>Tim23-G153A</td>
<td>Reverse</td>
<td>GAGAATCCCCCGCATTAGCAC</td>
</tr>
<tr>
<td>MM15F</td>
<td>Tim23-Y159A</td>
<td>Forward</td>
<td>GCGTTAGCCGCAATATCATC</td>
</tr>
<tr>
<td>MM15R</td>
<td>Tim23-Y159A</td>
<td>Reverse</td>
<td>GCGTTAGCCGCAATATCATC</td>
</tr>
<tr>
<td>MM32F</td>
<td>Tim23-L155A</td>
<td>Forward</td>
<td>GCGTTAGCCGCAATATCATC</td>
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<tr>
<td>MM32R</td>
<td>Tim23-L155A</td>
<td>Reverse</td>
<td>GCGTTAGCCGCAATATCATC</td>
</tr>
<tr>
<td>MM33F</td>
<td>Tim23-A152G</td>
<td>Forward</td>
<td>CTTCTAGTAAATCGGCGGATT</td>
</tr>
<tr>
<td>MM33R</td>
<td>Tim23-A152G</td>
<td>Reverse</td>
<td>CTTCTAGTAAATCGGCGGATT</td>
</tr>
<tr>
<td>MM34F</td>
<td>Tim23-N163A</td>
<td>Forward</td>
<td>GCGTTAGCCGCAATATCATC</td>
</tr>
<tr>
<td>MM34R</td>
<td>Tim23-N163A</td>
<td>Reverse</td>
<td>GCGTTAGCCGCAATATCATC</td>
</tr>
<tr>
<td>MM36F</td>
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</tr>
<tr>
<td>MM36R</td>
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<td>MM109R</td>
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<td>MM110F</td>
<td>Tim23-K190A</td>
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</tr>
<tr>
<td>MM111R</td>
<td>Tim23-K190A</td>
<td>Reverse</td>
<td>CTTCTAGTAAATCGGCGGATT</td>
</tr>
<tr>
<td>MM12F</td>
<td>Tim23-G153L</td>
<td>Forward</td>
<td>CTTCTAGTAAATCGGCGGATT</td>
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<td>MM13R</td>
<td>Tim23-G153L</td>
<td>Reverse</td>
<td>CTTCTAGTAAATCGGCGGATT</td>
</tr>
<tr>
<td>MM19F</td>
<td>Tim23-A156L</td>
<td>Forward</td>
<td>GATGATATGTAGCTCAACGCAGCATTATCATC</td>
</tr>
<tr>
<td>MM20R</td>
<td>Tim23-A156L</td>
<td>Reverse</td>
<td>GATGATATGTAGCTCAACGCAGCATTATCATC</td>
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<tr>
<td>MM35F</td>
<td>Tim23-A156G</td>
<td>Forward</td>
<td>GATGATATGTAGCTCAACGCAGCATTATCATC</td>
</tr>
<tr>
<td>MM36R</td>
<td>Tim23-A156G</td>
<td>Reverse</td>
<td>GATGATATGTAGCTCAACGCAGCATTATCATC</td>
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### Table 3.5: Centrifuges and rotors used in this study

<table>
<thead>
<tr>
<th>Centrifuge</th>
<th>Rotor Type</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sorvall RC12BP</td>
<td>H-12000 BioProcessing</td>
<td>Thermo Scientific, US</td>
</tr>
<tr>
<td>Eppendorf 5810 R</td>
<td>A-4-62 swinging bucket</td>
<td>Eppendorf AG, DE</td>
</tr>
<tr>
<td>Sorvall RC6</td>
<td>F10S-6x500y mL</td>
<td>Thermo Scientific, US</td>
</tr>
<tr>
<td></td>
<td>JA-10</td>
<td>Beckman Coulter Inc., US</td>
</tr>
<tr>
<td></td>
<td>JA-20</td>
<td>Beckman Coulter Inc., US</td>
</tr>
<tr>
<td>Optima L-90K UC</td>
<td>Sw60 Ti swinging bucket</td>
<td>Beckman Coulter Inc., US</td>
</tr>
<tr>
<td>Eppendorf 5415 R</td>
<td>F45-24-11</td>
<td>Eppendorf AG, DE</td>
</tr>
<tr>
<td>Optima MAX-XP UC</td>
<td>TLA-55</td>
<td>Beckman Coulter Inc., US</td>
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### Table 3.6: Buffers used in this study

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Buffer composition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inclusion body purification buffers</strong></td>
<td></td>
</tr>
<tr>
<td>Lysis buffer</td>
<td>100 mM NaCl, 50 mM Tris-HCl, 10 µg mL⁻¹ Dnase I, 5 mM MgCl₂, 1 pill/50 mL complete protease inhibitor cocktail without EDTA pH 8.0</td>
</tr>
<tr>
<td>Triton X-100 buffer</td>
<td>100 mM NaCl, 1 mM EDTA, 10 mM DTT, 2% Triton X-100, 50 mM Tris-HCl, pH 8.0</td>
</tr>
<tr>
<td>TEN buffer</td>
<td>100 mM NaCl, 1 mM EDTA, 10 mM DTT, 50 mM Tris-HCl, pH 8.0</td>
</tr>
<tr>
<td>TN buffer</td>
<td>100 mM NaCl, 50 mM Tris-HCl, pH 8.0</td>
</tr>
<tr>
<td><strong>Chromatography buffers</strong></td>
<td></td>
</tr>
<tr>
<td>HisTrap buffer A</td>
<td>8 M urea, 150 mM NaCl, 10 mM Tris-HCl, 50 mM Imidazole, pH 8.0</td>
</tr>
<tr>
<td>HisTrap buffer B</td>
<td>8 M urea, 150 mM NaCl, 10 mM Tris-HCl, 500 mM Imidazole, pH 8.0</td>
</tr>
<tr>
<td>Size-exclusion buffer</td>
<td>8 M urea, 150 mM NaCl, 10 mM Tris-HCl, pH 8.0</td>
</tr>
<tr>
<td><strong>General experiment buffers</strong></td>
<td></td>
</tr>
<tr>
<td>Liposome buffer</td>
<td>150 mM NaCl, 20 mM MOPS-Tris, pH 7.0</td>
</tr>
<tr>
<td>Electrophysiology buffer 1</td>
<td>250 mM KCl, 20 mM MOPS-Tris, pH 7.0</td>
</tr>
<tr>
<td>Electrophysiology buffer 2</td>
<td>20 mM KCl, 20 mM MOPS-Tris, pH 7.0</td>
</tr>
<tr>
<td><strong>Other buffers</strong></td>
<td></td>
</tr>
<tr>
<td>Competent cell buffer 1</td>
<td>30 mM KAc, 10 mM CaCl₂, 50 mM MnCl₂, 100 mM RbCl, 15% glycerol, pH 5.8</td>
</tr>
<tr>
<td>Competent cell buffer 2</td>
<td>10 mM RbCl, 75 mM CaCl₂, 15% glycerol, 10 mM MOPS, pH 6.5</td>
</tr>
<tr>
<td>TAE buffer</td>
<td>20 mM Acetic acid, 40 mM Tris, 20 mM EDTA, pH 7.6</td>
</tr>
</tbody>
</table>
3.2 Methods - Molecular Biology

3.2.1 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was employed to perform site-directed mutagenesis. Primer pairs were designed to contain the desired base mutation in their center and purchased from Metabion (DE). For PCR, a 50 µL PCR reaction mix was set up, containing: 100 ng DNA template, 0.2 µM each forward and reverse primer, 3% DMSO, 0.2 µM dNTP mix, 1 U Phusion DNA polymerase and 1x Phusion HF buffer. The PCR mix was subjected to 20 cycles of Denaturing - Annealing - Elongation (see table XY). The methylated template DNA was digested with 1 µL DpnI and 5 µL of the digested PCR product was transformed into 50 µL *E. coli* XL1-Blue cells for plasmid amplification (see section 3.3.2).

3.2.2 DNA Sequencing

Plasmid DNA was sequenced, after amplification and isolation, by GATC Biotech AG (DE).

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
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<tr>
<td>Lid</td>
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</tr>
<tr>
<td>Initial Denaturing</td>
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<td>30 s</td>
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<tr>
<td>Denaturing</td>
<td>98 °C</td>
<td>50 s</td>
</tr>
<tr>
<td>Annealing</td>
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<tr>
<td>Elongation</td>
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<td>10 min</td>
</tr>
<tr>
<td>Final Elongation</td>
<td>68 °C</td>
<td>10 min</td>
</tr>
<tr>
<td>End</td>
<td>4 °C</td>
<td>20x</td>
</tr>
</tbody>
</table>

Table 3.7: Thermocycle setup for polymerase chain reactions
3.2.3 DNA Concentration Determination

DNA concentration was determined spectrophotometrically with a NanoDrop 2000 (Thermo Scientific, US) and its built-in Nucleic Acid method. The calculation uses on a modified version of the Lambert-Beer law \((\text{equation 3.1})\) which yields the concentration \(C_{\text{DNA}}\) by measuring the absorbance \(A\) at 260 nm, baseline-corrected by absorbance at 340 nm, with the path length \(d\) and an extinction coefficient \(\epsilon\).

\[
C_{\text{DNA}} = \frac{(A \cdot \epsilon)}{d}
\]

3.3 Methods - Cell Biology

3.3.1 E. coli Strains and Medium

In this study, three different \(E.\ coli\) strains were used \((\text{table 3.8})\). ScTim23 was expressed in BL21 (DE3) cells. Plasmid amplification for all plasmids was carried out in XLI-Blue cells.

All \(E.\ coli\) strains were grown on LB (lysogeny broth) with 10 g NaCl, 5 g yeast extract and 10 g tryptone per liter of culture. For LB-Agar solid medium, the LB was supplemented with 15 g L\(^{-1}\) Agar-Agar. Cells containing ScTim23 pET10N vector were grown in medium complemented with 100 mg L\(^{-1}\) Ampicillin.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>XL1-Blue</td>
<td>(\text{recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1})</td>
<td>Stratagene</td>
</tr>
<tr>
<td>BL2I (DE3)</td>
<td>(\text{F'}\ ompT h_{\beta} (r_{\beta}^{7} m_{\beta}^{6})\ gal dcm \lambda) (DE3)</td>
<td>Novagen</td>
</tr>
</tbody>
</table>

Table 3.8: \(Escherichia\ coli\) strains used in this study
3.3.2 Generation and Transformation of Chemically Competent *E. coli* Cells

*E. coli* cells were made chemically competent according to a previously published protocol. Briefly, a 1 L culture of *E. coli* BL21 (DE3), XLI-Blue or Rosetta (DE3) cells was grown without antibiotics to an optical density at 600 nm (OD) of 0.6 at 37 °C and cooled down on ice for 15 min. After centrifugation at 2700 g for 15 min at 4 °C, the cells were resuspended in 200 mL ice-cold competent cell buffer 1 and pelleted again at the same speed. After resuspension in 30 mL of the final competent cell buffer 2 the cells were aliquoted, frozen in liquid nitrogen and stored at -80 °C for further usage.

Transformation of competent cells was achieved by adding 100 ng plasmid DNA or 20 µL ligation PCR mix to 50 µL competent cells, thawed on ice, and incubation for minimum 5 minutes on ice. The mix was subjected to a heat-shock at 42 °C for 45 s and incubated on ice for minimum 2 minutes before 300 µL pre-warmed LB medium was added. The cells were recovered by shaking them for one hour at 37 °C and 450 rpm and subsequently plated on LB-Agar plates containing the appropriate antibiotics specified by the plasmid (100 µg mL⁻¹ Ampicillin for the pET10N-vector). The plates were left at 37 °C until bacterial colonies were visible.

For plasmid amplification after mutagenesis (see chapter section), XLI-Blue cells were transformed with the respective plasmid and plated on LB-Agar plates supplemented with 100 µg mL⁻¹ Ampicillin. Multiple colonies were picked and each grown in 10 mL LB medium with antibiotics for 12 – 24 hours. The cells were collected in 50 mL tubes by centrifugation (Centrifuge 5810 R, A-4-62 rotor, 4000 rpm or 3200 g, room temperature, 5 min). Cells were resuspended,
lysed and plasmids were isolated and purified, using *Wizard Plus SV Minipreps DNA Purification System* (Promega, DE) according to the manufacturers protocol.

### 3.3.3 Protein Expression and Inclusion Body Purification

Tim23 wild type and mutants containing an N-terminal His-Tag were expressed in *E. coli* BL21 (DE3) cells as described before\[^{16, 17}\]. To this end, the pET10N plasmid carrying Tim23 wild type or mutant expression gene was transformed into *E. coli* BL21 (DE3) cells which were plated on LB-Agar plates supplemented with Amp (100 µg/mL). After incubation for 24 h at 37 °C, single colonies were picked to inoculate a preculture of 10 mL LB (+Amp) for 6 h, which in turn was used to inoculate a 250 mL overnight culture of LB+Amp to an OD\(600\) (optical density at \(\lambda = 600 \text{ nm}\); OD\(600\) is proportional to cell density) of 0.05. The next morning, autoclaved LB medium in flasks (2 L medium per 5 L flask, typically 12 L total culture volume) was supplemented with Amp, inoculated with the overnight culture to an OD\(600\) of 0.05 and left in an incubator shaker until the culture reached an OD\(600\) of 0.6 - 0.8, which corresponds to the log or exponential growth phase, characterized by uninhibited cell growth i.e. doubling\[^{18}\]. Then, IPTG was added (1 mM) and expression was induced for 3 h at 37 °C before all cells were collected by centrifugation (Sorvall RC12BP with H-I2000 rotor, 4000 rpm or 5300 g, 18 °C, 20 min). The cell pellets were resuspended in 50 mL of cell resuspension buffer per 2 L of original culture and again collected via centrifugation (Sorvall RC6 with FI0S rotor, 5000 rpm or 4200 g, 20 min, 4 °C). The cell pellet was typically frozen at this step.

As Tim23 is a highly hydrophobic membrane protein, it must be isolated and purified from inclusion bodies. To this end, the pellet was resuspended in lysis
buffer (10 mL/g wet cells) and the cells were lysed by three passes at 1000 bar through a cooled EmulsiFlex-C3 (Avestin Inc., US). The cell lysate was supplemented with deoxycholic acid and lysozyme, stirred with a magnetic bar for 30 min at room temperature (to enable DNase activity) and centrifuged (Sorvall RC6 with F10S rotor, 5000 rpm or 4200 g, 60 min, 4 °C) to remove soluble proteins and bacterial cytoplasm. Next, membrane fractions and membrane-inserted proteins were removed by resuspension in 75 mL Triton X-100 buffer, stirring for 30 min at 4 °C and centrifugation (Sorvall RC6 with F10S rotor, 5000 rpm or 4200 g, 30 min, 4 °C). The inclusion body pellet was then further washed by resuspension in 75 mL TEN buffer, stirring for 1 h at 4 °C and centrifugation (as before). Ethylenediaminetetraacetic acid (EDTA) and dithiothreitol (DTT) were removed by resuspension in 75 mL TN buffer and centrifugation (as before), before the pellet was stored at -20 °C.

3.3.4 Affinity Chromatography

The protein was solubilized from inclusion bodies using high molar concentrations of urea, a chaotropic salt, and purified via His-tag affinity chromatography. Unlike protein tags, e.g. Glutathion-S-Transferase (GST), which require proper folding of the tag, peptide tags like polyhistidine tags enable affinity chromatography purification even for denatured proteins, though specificity is typically greater with GST-tag. The imidazole moiety of polyhistidine tags binds preferentially to complexed divalent metal ions, e.g. Ni2+-NTA or Co2+-NTA, with NTA being crosslinked to an agarose matrix. Proteins without polyhistidine tags do not bind at all or not tightly to the Ni2+-NTA and can be washed off even with low concentrations of imidazole, while the proteins of interest can be eluted from
the Ni²⁺-NTA by high concentrations of imidazole. This technique is typically used with either agarose beads as a matrix to enable purification in reaction tubes or with chromatography columns, either self-packed or commercially prepacked, to be used with chromatography setup, e.g. ÄKTA systems. In this study, affinity chromatography was used with prepacked HisTrapFF 1mL or 5mL columns (GE Healthcare, UK), operated on an ÄKTAPrime Plus system (GE Healthcare, UK).

Prior to use, all chromatography buffers were sterile filtered through 0.22 µm filters and degassed. The pellet was resuspended in buffer containing 50 mM imidazole to reduce unspecific binding of contaminations. The resuspension was stirred for 60 min at room temperature and then centrifuged (Sorvall RC6 with JA-20 rotor, 12000 rpm or 17400 g, 20 min, room temperature) in a pre-warmed rotor. Urea is temperature sensitive at very high concentrations. Using the rotor right from the fridge would lead to precipitation of urea and thus of the protein of interest. Alternatively, instead of urea guanidine hydrochloride can be employed at 6 M concentrations and used at 4 °C as it is not temperature sensitive. The supernatant was filtered through 0.45 µm filters and loaded to the column with a flow rate of 0.5 mL/min to allow binding. The column was washed with 20 column volumes (CV) of HisTrap buffer A at 1 mL/min and the bound protein was eluted from the column with 4 CV of HisTrap buffer B, containing 500 mM imidazole, at 1 mL/min and fractionated in 1 mL steps.

3.3.5 Size-Exclusion Chromatography

Proteins pre-purified using affinity chromatography were further subjected to size-exclusion chromatography, where particles, like proteins, are not separated by binding affinity but by their size, more precisely their hydrodynamic
The size-exclusion matrix used in this study was made of crosslinked agarose with covalently bound dextran to form a porous matrix. While the smallest particles can enter the various nanometer-sized pores in the matrix, bigger particles pass by most pores and cavities. This leads to a much higher elution time for smaller particles that are trapped in the pores, whereas bigger particles just pass by the porous cavities. In this study, a HiLoad 16/600 Superdex 200 pg (GE Healthcare, UK) operated on an ÄKTAPrime Plus system (GE Healthcare, UK) and was used to separate Tim23 proteins from contaminations and aggregates, with a constant flow rate of 1 mL/min. Before the separation run, the column was equilibrated with 1 CV of ddH$_2$O and 1.5 CV of size-exclusion buffer. After injection, not exceeding a sample volume of 2 mL, the sample was eluted with 1 CV of sizeexclusion buffer and fractionated in 2 mL steps.

### 3.4 Methods - Protein Biochemistry

#### 3.4.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated and detected using discontinuous Tris-Glycine SDS-PAGE. Here, the negatively charged detergent SDS denatures proteins and binds to them approximately proportional to the protein mass with $\approx 2$ molecules of SDS per amino acid. Application of an electric field across the gel leads to migration of the, now negatively charged, proteins towards the anode. The polyacrylamide gel matrix acts as a sieve, with smaller molecules passing through the pores easier than bigger molecules, effectively separating smaller from bigger proteins.

The SDS polyacrylamide gels used in this study were cast with a stacking gel.
Table 3.9: SDS-PAGE gel matrix and buffer composition

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stacking gel</td>
<td>12.5% (30/0.8) acrylamide / bis-acrylamide, 0.1% SDS, 386 mM Tris/HCl pH 8.8, 0.1% APS, 0.08% TEMED</td>
</tr>
<tr>
<td>Running gel</td>
<td>5% (30/0.8) acrylamide / bis-acrylamide, 0.1% SDS, 126 mM Tris/HCl pH 6.8, 0.1% APS, 0.2% TEMED</td>
</tr>
<tr>
<td>Running buffer</td>
<td>0.1% SDS, 191 mM glycine, 25 mM Tris</td>
</tr>
<tr>
<td>Loading buffer</td>
<td>2% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.01% bromphenol blue, 60 mM Tris/HCl pH 6.8</td>
</tr>
</tbody>
</table>

above the actual running gel. The stacking gel had a low polyacrylamide concentration, and thus a higher porosity, and a pH of 6.8 and was used to focus the proteins to a single band after loading. With a higher polyacrylamide concentration, the porosity of the matrix becomes the limiting factor for protein migration, thus separating the proteins by size. For initial denaturing, the protein sample of interest was incubated with 1x Loading buffer (from 5x stock). To estimate molecular weight of protein bands, either Mark12 unstained marker (Novex) or PageRuler prestained marker (Thermo Scientific) were loaded on the gel as well.

### 3.4.2 Protein Visualization on SDS-PAGE

Proteins were visualized on gels after SDS-PAGE using Coomassie Brilliant Blue. For a lower detection sensitivity, the gel was immersed in Coomassie stainer, containing 25% ethanol, 10% acetic acid and 0.1% Coomassie Brilliant Blue R-250, heated in a microwave and incubated for 30 min on a shaker. Stainer was then discarded and replaced with destainer, same composition but without Coomassie Brilliant Blue, again heated and incubated for 30 min on a shaker. Destainer was replaced as needed for further destaining.

To reach a higher detection sensitivity or to visualize lipids on a gel, a colloidal
Coomassie stain was used, containing 0.12% Coomassie Brilliant Blue G-250, 10% ammonium sulfate, 10% phosphoric acid, and 20% methanol. The gel was immersed in the stain and incubated on a shaker for minimum one hour, typically over night, and then washed multiple times with ddH₂O. While normal Coomassie stain stains the whole gain and has to be thoroughly destained, background staining of colloidal Coomassie stain is minimal and can be washed with normal water.

### 3.4.3 TCA Precipitation

Protein samples with a too high volume or a too low concentration for SDS-PAGE had to be precipitated and redissolved in loading buffer. To this end, the sample were incubated with 15% trichloroacetic acid (TCA) for 30 min on ice and centrifuged (Centrifuge 5415 R, 13,200 rpm or 16,100 g, 4 °C, 30 min). The supernatant was carefully discarded, the pellet was washed with 1 mL ice-cold acetone and again centrifuged at the same conditions. Supernatant was again discarded and the samples were dried on air over night. SDS loading buffer was added to dried precipitation pellets. Due to the acidic precipitant, the redissolved pellet in loading buffer might turn yellow (due to pH-sensitive bromphenol blue) and was brought back to a higher pH by addition of 2 µL 1 M Tris.

### 3.5 Methods - Liposome Techniques

#### 3.5.1 Liposome Preparation

For liposome formation, PC, PE, PI, PS and CL (see [Table 3.1](#)) were first prepared in chloroform and then mixed in the desired molar ratios, i.e. either 70:15:15
CHAPTER 3. MATERIALS AND METHODS

PC:PE:CL or 45:20:15:5:15 PC:PE:PI:PS:CL, in glass test tubes. The lipid mixture in chloroform was dried under a nitrogen stream for 5 min followed by desiccation in vacuum for 2 h. The dried lipid film was fully resuspended with liposome buffer (150 mM NaCl, 20 mM MOPS/Tris, pH 7.0) for a final mass concentration of 10 mg mL\(^{-1}\). The lipid suspension was then subjected to at least seven freeze-thaw cycles, i.e. freezing in liquid nitrogen followed by thawing in cold water and vortexing for 3 min. To reach size-uniformity of the liposomes, the suspension was extruded 31 times through polycarbonate membranes with a pore size of 200 nm (Whatman). The assembled extruder setup was washed with 3x MeOh, 2x ddH\(_2\)O, 2x liposome buffer, 5 passes each.

3.5.2 Protein Incorporation into Liposomes

The Tim23 protein was incorporated into liposomes in a detergent mediated manner as described before\(^{[10]}\). For that, both extruded liposomes and Tim23 protein in Urea were incubated with 80 mM MEGA-9 (non-ionic detergent, critical micelle concentration \(cmc \approx 20\) mM, dialyzable) for 30 min, then mixed in 1:50 protein to lipid (w/w) ratio and incubated for another 45 min. The mixture was then dialyzed in membrane tubes with 3.5 kDa cutoff (Spectrum Labs) in 5 L liposome buffer to remove both Urea and MEGA-9, first for 2 h at room temperature followed by overnight dialysis at 4 °C.

3.5.3 Liposome Flotation Assay

To assess protein co-migration with liposomes, density gradient flotation was employed using nonionic Histodenz as we described before\(^{[12]}\). With this technique, membrane-unbound proteins can be separated from liposomes and membrane-bound proteins. While liposomes, both empty and with incorporated
protein, migrate to areas of lower density, unbound protein stays in the loading fraction (see figure 3.1). The base layer, 700 µL 46% Histodenz mixed with 100 µL proteoliposomes, was loaded in the bottom of a polycarbonate test tube and discreet layers of 20%, 10%, 5% and 0% in liposome buffer with 900 µL each were cast on top. The density gradient was centrifuged in a swinging bucket rotor (Optima L-90K UC with Sw60 Ti rotor, 55k rpm or 210,000 – 400,000 g, 4 °C, 1 h) and then fractionated in 500 µL steps from top to bottom. The fractions were TCA-precipitated and subjected to SDS-PAGE.

Figure 3.1: General principle of density gradient flotation as employed in this thesis. Liposomes and proteoliposomes migrate to low-density interfaces while unbound protein or unincorporated protein stays in the loading fraction.

3.5.4 Sodium Carbonate Extraction

To test if proteins are only attached or truly inserted into liposomes, proteoliposomes were diluted in 200 mM sodium carbonate (Na₂CO₃). This leads to detachment of peripherally attached proteins from the membrane due to distur-
bance of electrostatic interactions and unfolding of the protein. Proteoliposomes were used in a modified flotation assay with 900 µL 50% Histodenz mixed with 100 µl proteoliposomes as base layer, followed by 1 ml 20% Histodenz and 2.5 ml 0% Histodenz. After centrifugation, the liposomes were carefully extracted from the interface between 20% and 0% layers with a pipette. The flotated liposome samples were diluted in 10x 200 mM sodium carbonate, incubated on ice for 30 min and then centrifuged at 186,000 g for 45 min at 4 °C. Pellet and supernatant were separated, the supernatant was TCA-precipitated and both samples were subjected to SDS-PAGE.

3.6 Methods - Electrophysiology

3.6.1 General Principle of Electrophysiology

For electrophysiological considerations, reduced membrane systems and even whole cells can be brought down to an equivalent circuit diagram, that of a leaky capacitor. In a minimal system, as used in this thesis, the circuit consists of a series connection of resistor $R_{Electrolytes+Electrodes}$ representing the resistance of both the electrical setup (headstage and electrodes) and the electrolytes, with a parallel connection of $C_{membrane}$ and $R_{membrane}$, capacitance and resistance of the impermeable lipid bilayer, and $R_{channel}$, resistance (or inverse conductance $1/G$) of an incorporated ion channel (figure 3.2A). $R_{E1+E2}$ is small compared to $R_{channel}$ and can therefore be neglected in a series connection, while $R_{membrane}$ is big compared to $R_{channel}$ and can be neglected in a parallel connection, leaving a reduced circuit (figure 3.2B). Current flux through a capacitor depends on changes of applied potential, thus directly after setting a constant holding potential $U$, the capacitor $C_{membrane}$ is conducting for a few hundred milliseconds until exponen-
3.6. METHODS - ELECTROPHYSIOLOGY

tial decay reduces the respective current to near zero. Then, all current \( I \) is flowing through \( R_{\text{channel}} \), enabling direct calculation of the channel's conductance \( G \) via Ohm's first law:

\[
U = R \cdot I = \frac{I}{G} \iff G = \frac{I}{U}
\]

Figure 3.2: Complex (A) and reduced (B) circuit sketch of an ion channel in a biological membrane.

3.6.2 Reversal Potential and Ion Selectivity

In eukaryotic cells, the plasma membrane and many organellar membranes are nearly impermeable for ions and only allow ion passage through dedicated ion transporter. This restricted ion movement leads to an ion concentration gradient over the membrane. The concentration difference results in an electrochemical membrane potential which strives to equilibrate the ion concentration on both
sides of the membrane again. The potential that needs to be applied over the membrane to stop all ion flux through the membrane is termed *reversal potential*[^1]. If only one ion species, e.g. Na+, is transported over the membrane and contributes to the asymmetry, the resulting equilibrium potential can be calculated using the Nernst equation

$$U_{\text{rev}} = \frac{RT}{zF} \cdot \ln \frac{C_1}{C_2},$$  \hspace{1cm} 3.3

where $R$ is the universal gas constant ($R = 8.314 \text{ J K}^{-1} \text{ mol}^{-1}$), $T$ is temperature, $z$ is ion charge, $F$ is Faraday’s constant ($F = 96 485.332 \text{ C mol}^{-1}$) and $C_{1/2}$ is ion concentration on each side of the membrane.

If multiple anions and cations contribute to the electrochemical potential, it is not sufficient to only sum up the Nernst potentials (equation 3.3) for each ion as the ions may have different permeabilities through the membrane. At asymmetric salt concentrations, both anions and cations strive to equilibrate, and if the channel is not ion selective, all anions and cations migrate with the same rate, resulting in zero net current. Though, if the channel discriminates between ions, one type of ions has a higher permeability through the channel than the other, resulting in a higher ion flux for the preferred type which can be measured as a nonzero current at zero voltage. The reversal potential required to zero any current flux depends on the salt concentrations and the relative ion preference of the channel and can be calculated from the respective ion concentrations and their permeability, using the Goldman or Goldman-Hodgkin-Katz voltage equation[^2][^3]

$$U_{\text{rev}} = \frac{RT}{F} \cdot \ln \left( \frac{\sum P_C [C]_{\text{outer}} + \sum P_A [A]_{\text{inner}}}{\sum P_C [C]_{\text{inner}} + \sum P_A [A]_{\text{outer}}} \right),$$  \hspace{1cm} 3.4

where $[C] / [A]$ is the concentration of cations or anions, $P_{C/A}$ are the permeability
of cations or anions and *inner/outer* refers to the side of the membrane.

In this study, we use a potassium chloride buffer with known, different salt concentrations on trans and cis sides of the channel. We can solve the equation for the relative ion selectivity to

\[
P_{K^+} : P_{Cl^-} = \frac{[Cl^-]_{trans} \cdot \exp \left( \frac{u_{refK}}{RT} \right) \cdot [Cl^-]_{cis}}{\exp \left( \frac{u_{refK}}{RT} \right) \cdot [K^+]_{trans} - [K^+]_{cis}},
\]

where \( P_{K^+/Cl^-} \) are the permeability of potassium or chloride ions, \([K^+] / [Cl^-]\) are the respective ion concentrations, *cis/trans* correspond to the two sides of the bilayer, \( F, R, T \) are constants as declared before and \( U_{rev} \) is the measured reversal potential.

### 3.6.3 Conductance

As introduced in [section 3.6.1](#), ion flux through an open channel results in a recordable current \( I_{open} \) that depends on the applied voltage. After partial or full closure of a channel, the current is reduced to a \( I_{intermediate} \) or \( I_{closed} \). It has to be noted that \( I_{closed} \) can be non-zero, originating either from the membrane if it is not fully impermeable or from the channel if it allows a residual current even in its closed conformation.

Using Ohm’s law (equation 3.2), dividing recorded current by applied voltage, yields the channels conductance at that specific voltage. This is an indicator of how big the pore or multiple pores are. The diameter of a cylindrical pore can be calculated according to Hille [72] from the approximate length \( L \) of the cylinder, the resistivity \( \rho \) of the buffer and the recorded and calculated conductance \( G \) of the
channel. \( L \) can take on e.g. 0.5 nm for a very short constriction zone or 5 nm for a membrane-spanning cylinder. Resistivity \( \rho \) is approx. 50 \( \Omega \) cm for unrestricted electrophysiology buffer 1 (250 mM KCl, 20 mM MOPS-Tris, pH 7.0), whereas electrolytes exposed to strong electrical fields within a pore exhibit a much higher resistivity and a correction factor of 5 was determined for such a narrow channel.\(^{65}\)

The pore size can then be calculated:

\[
d = 2 \cdot G \cdot \rho \cdot \left( \frac{1}{4} + \sqrt{\frac{1}{16} + \frac{L}{G \cdot \rho \cdot \pi}} \right)
\]

Current recordings of a channel were also analyzed with regards to the occupation of open or closed states. To this end, the open probability \( P_{\text{open}} \) was calculated by dividing the mean current \( I_{\text{mean}} \), typically recorded over the course of a minute, by the maximum current \( I_{\text{max}} \) of a trace. Extracting the maximum current of a current recording can pose a difficulty if the channel is never fully open during the recording, possibly due to substrate or "stress" effects leading to closure.

### 3.6.4 Assembly of the Chamber

All electrophysiological experiments in this thesis were carried out using the planar lipid bilayer setup. A very detailed method review describing the vertical bilayer setup used in this thesis, with only minor alterations regarding the electrical setup (see section 3.6.3), was published recently.\(^{61}\)

Briefly, the bilayer chamber consists of two half-chambers with a circular aperture on each side. One aperture per half-chamber was closed with a glass plate and fixated with Parafilm and a tight PTFE ring. One half chamber was inserted with the glass front first into the metal cage. On the other side of the half chamber, Glisseal vacuum grease was applied to the flat front and a Polytetrafluoroethylene (PTFE) film with a needle-tip sized hole was glued to one half chamber. The hole
was created by carefully puncturing the PTFE film with a needle and trimmed with three pulses of a self-made spark gap. The other half chamber was greased without a PTFE film and inserted grease front first into the metal cage. There, the non-glass fronts of both half-chambers face each other and are sealed, upon contact, by the vacuum grease and the PTFE film after closing and carefully tightening the setup with a metal screw ring. Magnetic stir bars of 5 mm length are inserted in each half-chamber to allow buffer mixing with the magnetic stirrer underneath the metal cage.

Now, Type IV-S L-\(\alpha\)-Phosphatidylcholine (SIV-PC) in decane (redissolved after drying from chloroform in a dessicator, 3 mg lipid in 50 \(\mu\)L decane) is painted on the small hole in the PTFE film with a syringe and left for 20 min incubation time. The bilayer was created by first adding 3 mL of electrophysiology buffer I to each half-chamber and then repeatedly raising and lowering the buffer level over the lipid film to remove decane and excess lipids layer-wise. A lipid bilayer can be distinguished from a non-bilayer film by eye as described in Bartsch et al. or by its electrical properties, i.e. capacitance of a bilayer is much smaller than that of a non-bilayer film.

3.6.5 Electrical Setup and Software

Ag/AgCl electrodes were created by soldering silver wire (diameter 1 mm) to gold connectors and immerse the wire of the electrodes in 12\% NaCLO for minimum 3 h; alternatively, the Ag-electrodes can be chlorinated by immersing the wire and the ground in 1 M KCl and applying a voltage of 5 V over the electrode for 10 min. To eliminate liquid junction potentials, the electrodes were inserted into glass tubes and embedded in a 2 M KCl-Agar bridge. Electric recordings were performed with Ag/AgCl electrodes connected to a CV-5-1GU headstage and fur-
ther to a Geneclamp 500B current amplifier (both Axon Instruments, now Molecular Devices, US), with the trans-electrode used as reference electrode and the cis-electrode grounded. Currents were digitized using a Digidata 1440A AD/DA converter and recorded with a PC using the software AxoScope 10.3 for constant holding potentials or Clampex 10.3 for voltage ramps (Molecular Devices, US).

3.6.6 Fusion of Proteoliposomes

Proteoliposomes were added close to the bilayer in the cis compartment of the chamber. A salt gradient over the membrane was established with high salt in cis and low salt in trans compartment, to enable osmotically-driven fusion of proteoliposomes with the bilayer (figure 3.3). If fusion rates are low, CaCl$_2$ can be added to cis compartment to 10 to 20 mM. After fusion, the buffer in each chamber was perfused with 20 chamber volumes (60 ml) of standard 250 mM KCl buffer to set exact salt concentrations.

Figure 3.3: Sketch of electrophysiological setup and of channel insertion by osmotically-driven fusion of proteoliposomes to the lipid bilayer.

3.6.7 Data Reconstruction with R

As manual analysis of electrophysiological recordings can be prone to unintended bias (e.g. how to assess electrical noise to identify the true starting and
ending levels of a gating event), a sophisticated data analysis tool was further developed in collaboration with Inder Tecuapetla-Gómez (Institute for Mathematical Stochastics, University of Göttingen, DE), based on an estimator of stepwise constant functions, SMUCE, implemented in the R-package stepR. In brief, the SMUCE reconstructs the underlying clean data by taking into account the estimated filter effect, removing white noise from the presumed pre-filtered data and finally fitting constant segments to the denoised data. The reconstruction allows to easily identify and analyze conductance changes, dwell times and the general dynamic behavior of ion channels. A major advantage of SMUCE compared to previous techniques of data reconstruction is that SMUCE does not require any a priori information about the channel characteristics, e.g. Markov model parameter, to perform at least on the same level as established methods. With the core algorithm from stepR, we created a full reconstruction routine with RStudio (RStudio Inc., US) that automates gating event detection and dwell time calculation (see section 6.1). Routine parameters that were used in this study are displayed in table 3.10. The reconstruction routine exports data analysis in two .txt-files, a dwell time table and a list of gating events, that were further analyzed with OriginPro 8.5 (OriginLab, US).

For analysis of the temporal resolution of Tim23 channels, the very recent estimator JULES was used within our routine instead, keeping the formatting of data export identical (see section 6.2). JULES, related to SMUCE, is capable of accurately reconstructing conductance changes, by deconvolution of the data to fully remove the filter effect, that appear smaller that they were before filtering, thus giving information about the "true" conductance levels of very short events (see Pein et al. for a detailed presentation). This routine demands higher computational power than SMUCE if used for large amounts of current-voltage traces.
and was therefore only used for dwell time analysis.

Table 3.10: Reconstruction stepR-parameters

<table>
<thead>
<tr>
<th></th>
<th>SMUCE</th>
<th>JULES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum dwell time</td>
<td>$t_0 = 2\text{ ms}$</td>
<td>$t_0 = 0\text{ ms}$</td>
</tr>
<tr>
<td>Minimum conductance change</td>
<td>$c_0 = 40\text{ pS}$</td>
<td>$c_0 = 15\text{ pS}$</td>
</tr>
<tr>
<td>Fit segment length (points)</td>
<td>$LL = 15000$</td>
<td>$n_{\text{norm}} = 50000$</td>
</tr>
<tr>
<td>i.e. 0.3 s</td>
<td>resp. 1 s</td>
<td></td>
</tr>
</tbody>
</table>

3.6.8 Mean-Variance Analysis

A mean-variance analysis is a helpful tool to display and discover complex gating patterns of recorded current traces and is extensively described in Patlak. Briefly, pairs of mean and variance for windows with length $n$ are calculated and stored before moving the window forward step-by-step. Starting at the interval $[1, n]$ and storing $(M, V)_1$, next the window $[2, n + 1]$ and the pair $(M, V)_2$ is considered. Analyzing the whole data set with $N$ data points will yield $N$ pairs of mean and variance. The optimal choice of the window length $n$ depends on the kinetics, e.g. gating speed, of the channel. The faster the channel can change its conductance back and forth, the more transitions will be missed or biased using a long window length. Choosing a very short window length, on the other hand, might overvalue artifacts or noise.

Mean-variance analysis in this study was performed using a self-written R-script (see section 6.3) and a window length of 2 ms.
Chapter 4

Results

4.1 In-depth Characterization of the Tim23 Channel

4.1.1 Expression and Purification of Tim23

The expression plasmid for N-terminally His-tagged ScTim23 in a pET10N vector was originally created by Kaye Truscott. The plasmid was transformed into *E. coli* BL21 (DE3) competent cells and grown in liquid LB medium at 37 °C to an OD of 0.6 – 0.8 before inducing expression for 3 h at 37 °C with IPTG. Recombinantly expressed Tim23 is insoluble and aggregates in inclusion bodies at higher concentrations. These inclusion bodies were isolated from bacterial cell lysate by centrifugation, separated from soluble and membrane fractions via detergent, further washed and finally solubilized in 8 M urea used as a denaturing chaotropic agent. Chaotropic agents disrupt hydrogen bonds at very high concentrations, effectively leading to protein unfolding. The solubilized protein was purified by Ni\(^{2+}\)-NTA affinity chromatography utilizing the N-terminal deca-histidine tag.

To minimize the possibility of sample contamination by bacterial membrane
proteins, which often exhibit channel activity and would mask the signal of Tim23 in electrophysiological experiments, the protein was further purified using size-exclusion chromatography (figure 4.1D), despite its already high purity after Ni\(^{2+}\)-NTA affinity chromatography. SDS-PAGE analysis of size-exclusion fractions (figure 4.1C) showed that we could purify the protein to homogeneity. Note that the UV\(_{280\text{nm}}\)-absorption peaks at fractions 57 and 60 did not contain any protein and can be attributed to UV-absorbing oxidized DTT and imidazole, loaded onto the column together with the sample. Typical yield of Tim23 wild type protein expression after purification was \( \approx 10 \ \text{mg per liter of culture.} \)

While pore forming toxins or some beta barrel proteins are partly soluble in normal buffer and can insert into lipid bilayers on their own, this does mostly not hold true for more complex membrane proteins like Tim23. To embed this protein into lipid bilayers, it must be incorporated into liposomes first. To this end, large unilamellar vesicles (LUV) with a diameter of 200 nm were preformed from a lipid mixture of PC:PE:Pl:PS:CL with 45:20:15:5:15 (mol\%) before lipids and protein were, first separate then together, incubated with the mild dialyzable detergent MEGA-9. The mix was dialyzed in 5 L of liposome buffer to remove both urea and MEGA-9, forcing the protein to refold in the lipid bilayer of the liposomes. A density flotation assay was employed to investigate protein incorporation efficiency. The protein co-migrated upwards with the liposomes to a lower density (figure 4.2A), while unbound proteins would have stayed in the loading fraction (fraction 8 & 9). Co-migration of protein with liposomes can occur as integral or peripheral membrane protein. To distinguish between integration and attachment, co-migrated proteoliposomes were subjected to sodium carbonate extraction. Here, the sample is diluted in cold sodium carbonate (\( \text{Na}_2\text{CO}_3 \)) to unfold
4.1. IN-DEPTH CHARACTERIZATION OF THE TIM23 CHANNEL

Figure 4.1: Recombinantly expressed ScTim23 was purified to single band purity. (A) Expression and purification steps analyzed by SDS-PAGE. (B) UV$_{280\text{nm}}$-absorption profile of NiNTA-affinity chromatography. (C) Purification using size-exclusion chromatography monitored by SDS-PAGE. (D) UV$_{280\text{nm}}$-absorption profile of size-exclusion chromatography. Meike Wiegand assisted with SDS-PAGE analysis in a lab rotation under my supervision.

Figure 4.2: Tim23 was successfully incorporated into liposomes. (A) Histodenz density flotation assay analyzed by SDS-PAGE. (B) Ultracentrifuged proteoliposomes after treatment with 200 mM sodium carbonate. Meike Wiegand assisted with SDS-PAGE analysis in a lab rotation under my supervision.
and detach proteins only peripherally attached to a liposome. After ultracentrifuga-
tion, Tim23 was found in the pellet together with the liposomes (figure 4.2B) whereas peripherally attached proteins would stay in the supernatant, confirming that Tim23 is indeed incorporated into the liposomes as an integral membrane protein. After successful incorporation into liposomes, Tim23 wild type and mu-
tants were characterized electrophysiologically.

4.1.2 Electrophysiological Characterization of Tim23 Wild Type

The first task in the characterization of molecular mechanisms of Tim23 was to establish a detailed baseline of the channel behavior of recombinant Tim23 wild type. In addition to investigate whether the wild type channel behaves any different from the published channel features\cite{57,60,71}, it was required to exceed the level of detail present in these previous studies to classify putative incremental mutant effects.

Gating Analysis

The general gating behavior was investigated by recording the current flow through the channel at different voltages. It must be noted that recombinant Tim23 is a lightly rectifying channel, i.e. the conductance depends on the sign of the applied holding potential, and that it typically inserted with the same ori-
entation: Channel conductance is higher at positive than at negative voltages, when the cathode is emerged in the trans compartment of the cell (figure 4.3). The trans side corresponds to the IMS side of the channel as tested by addition of presequences or voltage regulator Tim50\cite{67,71} to either side. In this configura-
tion, the native electrochemical potential of the inner mitochondrial membrane corresponds to a holding potential of 150 to 180 mV. To assess the general gat-
Figure 4.3: Voltage ramp of a Tim23 wild type channel at symmetric buffer conditions.

ing behavior, constant holding potentials ranging from $-60 \text{ mV}$ to $140 \text{ mV}$ were applied to the bilayer incorporated channel in 20 mV steps for 60 s each. Exceeding this holding potential range was found to lead to irreversible closing of one or more pores. The respective channel orientation was determined after each fusion by recording a voltage ramp between $-60$ and $60 \text{ mV}$ and assessing the rectification.

The recorded current traces (see figure 4.4A as example) confirmed that current through Tim23 undergoes changes in three isometric steps (figure 4.4C), indicating the triple pore structure of recombinantly expressed ScTim23 as published earlier\textsuperscript{62}. All characterized channels contained three or multiples of three pores. Further, all current changes $\Delta I$ were extracted from the recordings using the reconstruction algorithm based on stepR\textsuperscript{24} (see section 3.6.7) and divided by the applied voltage $U$ to obtain conductance changes $\Delta G$. They were displayed in a histogram (figure 4.4B) and further modeled with multiple Gauss fits to ex-
Figure 4.4: Electrophysiological experiments show that Tim23 constitutes a voltage-sensitive triple-pore. (A) Current-recordings at different constant holding potentials. (B) Mean-variance analysis of a single Tim23 trace of 60 s at 120 mV with a window of 2 ms. (C) Gating event analysis of over 2000 gating events from 3 independent experiments.
tract the numeric values for the main conductance state $G_{\text{main}}$ and the primary and secondary subconductance states $G_{\text{sub1}}$ and $G_{\text{sub2}}$. The main conductance state $G_{\text{main}} = (461 \pm 31) \text{ pS}$ (Truscott et al.\textsuperscript{[53]}: $G_{\text{main}} = (450 \pm 11) \text{ pS}$) corresponds to one full closing or opening of a single pore of three, resulting in a total conductance of $G_{\text{total}} \approx 3 \cdot G_{\text{main}}$. Gating from open to closed or vice-versa appeared to be independent between the pores, i.e. the state of one pore did not obviously affect the state of another pore. The primary subconductance state $G_{\text{sub1}} = (172 \pm 30) \text{ pS}$ (Truscott et al.\textsuperscript{[53]}: $G_{\text{sub1}} = (140 \pm 15) \text{ pS}$) and the secondary subconductance state $G_{\text{sub2}} = (58 \pm 13) \text{ pS}$ are semi-open states a single pore can transition into from the fully open state, but not from the closed state (figure 4.4C).

Voltage-Dependency and Regulation

Increasing the applied holding potential above 100 mV, the channel gradually exhibits a lower open probability $P_{\text{open}} = I_{\text{mean}}/I_{\text{max}}$ (figure 4.5). The voltage sensitivity was further increased by addition of the soluble IMS-domain of ScTim50 to the trans side, which lead to a reduction in open probability above 40 mV (figure 4.5, in red), as published before\textsuperscript{[70]}.

Ion Selectivity

The presequence translocation channel Tim23 was found to be cation selective in electrophysiological experiments\textsuperscript{[63]}, very similar to the other mitochondrial translocases\textsuperscript{[25, 38, 43, 45, 463, 407, 476, 477, 478, 479]}. For this study, a reversal potential of $(47.2 \pm 0.4) \text{ mV}$ in the presence of a 12.5-fold KCl gradient (250 mm:20 mm) was determined experimentally for incorporated Tim23 wild type (figure 4.6). The corresponding ion selectivity of $P_{K^+} : P_{Cl^-} = 13.3 : 1$ was calculated using the GHK-
CHAPTER 4. RESULTS

Figure 4.5: Current-voltage ramps (left) and open probabilities (right) of bilayer incorporated Tim23 wild type before (black) and after (red) addition of 700 nM Tim50\textsuperscript{IMS} to the IMS-side of the channel. (n=3, mean±SD)

equation (equation 3.4), corresponding well to the published reversal potential of (49.60 ± 3.06) mV\textsuperscript{[10]}. 

Presequence Activation and Temporal Resolution

Tim23 reacts to substrate addition with fast gating\textsuperscript{[11,12]}. After adding the presequence peptide Cox4 to 300 nM or 500 nM to the IMS side of incorporated Tim23 channels and recording the current traces at $U = 120$ mV, a concentration-dependent increase in gating activity together with premature closing of the channel could be observed figure 4.7. After extracting the reconstructed data from three independent current traces for Tim23 with 0 nM and 500 nM Cox4 using JULES (see section 6.2), the gating events were analyzed in a histogram. The absolute gating frequency (figure 4.8A) confirmed the increase in overall gating event count (relative total increase by factor 18.9 for 500 nM Cox4). Normalization of the histograms showed that the main conductance state around 500 pS was significantly increased, while primary (around 180 pS) and secondary (around 75 pS)
4.1. \textit{In-depth Characterization of the TIM23 Channel}

Figure 4.6: Voltage ramp of a Tim23 wild type channel at symmetric buffer conditions. Subconductance states were not increased with the same rate.\[figure 4.8C\]
The temporal dimension of Tim23 gating could yield important insight into the channel dynamics. The dwell times, i.e. residence times of the channel at specific conductances, before and after addition of the Cox4 presequence peptide were analyzed. Tim23 not activated with Cox4 showed a primarily open channel that was able to close for very short times. For calculation of the regular minimal dwell time per condition, the lowest 1% was excluded. This results in a regular
minimal dwell time of unstimulated Tim23 of $3.2 \times 10^{-5}$ s (dashed black line in \figure{4.8C}). In the presence of 500 nM Cox4, gating frequency increased but the open conductance levels decreased slightly in the dwell time analysis, in line with the slight shift of the main conductance state peak in the gating event histogram (\figure{4.8A}). Despite the significant increase in gating events, the regular minimal dwell time of Cox4-stimulated Tim23 shifted to $5.6 \times 10^{-5}$ s (dashed red line in \figure{4.8C}), though the majority of short dwell times (below $5 \times 10^{-4}$ s) seems to peak not at constant values but proportional to the overall conductance of the occupied state (dash-dotted blue line in \figure{4.8C}). Even with a less strict view on regular minimal dwell time, short dwell times nearly exclusively occur for partially closed channels for unstimulated and even more so for stimulated Tim23. Data points of Tim23 and Tim23 plus Cox4, above $G = 1 \text{nS}$ and in the peak area of short dwell times (grey box in \figure{4.8C}), were extracted and analyzed in a dwell time histogram (\figure{4.9A}) and fitted with a logarithmic normal distribution with peak center $x_c = (62 \pm 1) \mu$s for unmanipulated Tim23 and $x_c = (184 \pm 2) \mu$s for Tim23 plus Cox4 (mean $\pm$ SD).
Figure 4.8: Cox4 induced increased gating activity in incorporated Tim23 channels, with no (black) or 500 nM (red) Cox4 added to the IMS side of Tim23. (A) Histogram of gating events per seconds per conductance change $\Delta G$. (B) Histogram of normalized gating frequency. (C) Dwell times at different conductances. Regular minimal dwell time indicated with black dashed line for no Cox4 and red dashed line or blue dash-dotted line for 500 nM Cox4. Grey box marks data used for figure 4.9A. Three independent experiments per condition.
Figure 4.9: Dwell time histogram of the peak area marked in grey in figure 4.8C for Tim23 before (A) and after (B) addition of Cox4. Fit of logarithmic normal distribution in red.
4.2 Tim50\textsubscript{core} Regulates Tim23 and Hands Over Preproteins

4.2.1 Voltage-Regulation by Tim50 Subdomains

To elucidate on the matter how molecular functions of the Tim50\textsubscript{IMS} domain are distributed between its subdomains, we investigated the interaction between these subdomains and Tim23 with electrophysiological techniques. All three Tim50 constructs, Tim50\textsuperscript{IMS} (aa 132-476), Tim50\textsuperscript{PBD} (aa 395-476) and Tim50\textsuperscript{core} (aa 132-365), were purified and provided by Christian Schulz and Alexander Benjamin Schendzielorz (both AG Rehling, University Medical Center Göttingen), as described in Schulz et al.\textsuperscript{23}.

As with wild type Tim23 and Tim50\textsuperscript{IMS} (see section 4.1.2), bilayer-incorporated Tim23 channels were characterized with voltage ramps and three full sets of constant voltage traces (60 s per voltage, from $-60$ mV to 140 mV in 20 mV steps). Then, Tim50\textsuperscript{core} or Tim50\textsuperscript{PBD} were added to the IMS-side of incorporated Tim23 channels with a final concentration of 730 nM before the buffer in each half-chamber was stirred and left to rest for two minutes each. After resting, a voltage ramp and another full set of traces was recorded.

Voltage ramps and open probabilities showed that Tim50\textsuperscript{core} was able to induce voltage-dependent closure in Tim23 channels (figure 4.10A), while Tim50\textsuperscript{PBD} did not induce any significant change in voltage ramps or open probability (figure 4.10B). Comparison with voltage-regulation by Tim50\textsuperscript{IMS} (figure 4.5) shows that no significant difference between voltage regulation by full IMS- and shorter core domain.
4.2. TIM50\textsubscript{CORE} REGULATES TIM23 AND HANDS OVER PREPROTEINS

Figure 4.10: Voltage regulation of Tim23 by Tim50 subdomains investigated via voltage ramps and open probabilities. (A) Voltage ramps (left) and open probability (right) of Tim23 before (black) and after (red) addition of Tim50\textsubscript{core} to the channels IMS side. (B) Voltage ramps (left) and open probability (right) of Tim23 before (black) and after (red) addition of Tim50\textsubscript{PBD} to the channels IMS side. (n=3; mean±SD)

4.2.2 Presequence Handover to Tim23 by Tim50

After it was confirmed that the regulatory function of Tim50 does not require the presequence binding domain PBD, we attempted to reopen Tim23 channels that were previously closed by Tim50 subdomains by addition of Tim23 substrates. In situ additions of the presequence peptide Cox4 to bilayer-incorporated Tim23 did not lead to any reactivation of Tim50-closed channels in previous studies\textsuperscript{24}. Instead, in that study Tim23 channels were preincubated with Tim50 and Cox4 prior to bilayer fusion, leading to channel-reactivation. Such a preincubation approach does not offer studying the unmanipulated system to detect minor differences, hence we tested the full preprotein b\textsubscript{2}(I67)\textsubscript{Δ}-DHFR instead of a short presequence peptide to reopen the channel in situ.

700 nM of the preprotein added to bilayer-incorporated Tim23 lead to a com-
combination of early closure and increased gating activity (figure 4.IIA). When first 730 nM Tim50\textsubscript{IMS} were added to the IMS side of Tim23 to induce closure before the preprotein was added to the same side, the channel was able to reopen partially (figure 4.IIB) in a manner observed for Tim23 + b\textsubscript{2}(l67)\textsubscript{A}-DHFR. Repeating the experiment with Tim50\textsubscript{core} instead of Tim50\textsubscript{IMS} again induced closure of Tim23 as observed before. Interestingly, the preprotein was again able to induce partial reopening of Tim23 (figure 4.IIC) even when Tim50 lacks its PBD. Analysis of current recordings at a constant holding potential of 120 mV confirmed that the preprotein was reactivating Tim23 to a similar extend after Tim50\textsubscript{IMS}- or Tim50\textsubscript{core}-induced channel closure.
4.2. TIM50core regulates TIM23 and hands over preproteins

Figure 4.11: Voltage ramps of Tim23 reactivation by $b_2(167)_\Delta$-DHFR after Tim50-induced closure. (A) Voltage ramp of Tim23 channel activation by $b_2(167)_\Delta$-DHFR. (B)+(C) Voltage ramps of Tim23 channel activation by $b_2(167)_\Delta$-DHFR after induced closing with Tim50ims (B) or Tim50core (C).

Figure 4.12: Tim23 traces with Tim50-induced closure at constant holding potentials. (A) Current traces of Tim23 at 120 mV without Tim50 (top) or closed by 730 nM Tim50ims (mid) or Tim50core. (B) Current traces of channels shown in (A), but after addition of 700 nM $b_2(167)_\Delta$-DHFR.
CHAPTER 4. RESULTS

4.3 Conserved TMS2 Residues Constitute Ion Filter

4.3.1 Design and Expression of Tim23 Mutants

To uncover molecular mechanisms that control electrophysiological properties of Tim23 from *S. cerevisiae*, single amino acid residues were mutated in and next to the second transmembrane segment (TMS2) that was shown to closely interact with presequence peptides in transit. Selection of most of these residues was based on a previous identification of amino acids within the second alpha helix (residues 145 – 166) that face an aqueous environment. As these pore-lining residues were also highly conserved among many different species, we speculated that these residues could constitute specific electrophysiological properties, e.g. voltage sensor, gating hinge or ion filter. The choice of amino acid residues and their distribution on a helical wheel with one side facing the pore interior is depicted in Figure 4.14. The residues were mutated to achieve a presumable loss-of-function by substitution with alanine (A), glycine (G). Additionally, GI53L was selected as a mutation as it was shown earlier that it constitutes a lethal phenotype when expressed in baker’s yeast. As AI56 was shown to shift from a polar to a non-polar environment in the presence of substrate, the residue was also mutated to more hydrophobic leucin (L) and phenylalanine (F) to shift it from the polar channel lumen even in the rested state, which could impact channel constitution, presequence handling or channel gating. In addition to the TMS2 mutants, two highly conserved, charged residues flanking the third transmembrane segment (TMS3) were mutated. The negatively charged aspartic acid at position 174 at the matrix side and the positively charged lysine at position 190 at the IMS side of TMS3 (residues 175 – 189) were substituted with neutral alanine. These mutations were introduced as a starting point to investigate whether
Figure 4.13: Sequence alignment of Tim23 from various species (Homo sapiens, Rattus norvegicus, Saccharomyces cerevisiae, Danio rerio, Neurospora crassa) calculated using ClustalW. Presumed transmembrane segments marked. Consensus: * identity; : high similarity; . low similarity
charges not inside but flanking the mostly neutral transmembrane helices could constitute or contribute to the channels ion filter.

All mutations were introduced to the wild type ScTim23 gene on a pET10N vector for recombinant expression in *E. coli* by site-directed mutagenesis. After PCR, transformation into *E. coli* XL-Blue competent cells, plasmid purification and sequencing, the isolated DNA was transformed into BL21 (DE3) competent cells, expressed and purified as described in section 4.1. Mariam Barbot (AG Meinecke, University Medical Center Göttingen) designed half of the mutants, performed the respective site-directed mutagenesis and expressed two mutants used in this study. Lennart Versemann (AG Meinecke, University Medical Center Göttingen) assisted with expression and incorporation of several mutants in a lab rotation and his bachelor thesis, both under my supervision.

### 4.3.2 Electrophysiological Screening of Tim23 mutants

Recombinantly expressed and incorporated mutants of Tim23 were screened for altered electrophysiological properties using the planar lipid bilayer technique. Mariam Barbot (AG Meinecke, University Medical Center Göttingen) contributed
partial electrophysiological characterization of one mutant to this study. Lennart Versemann (AG Meinecke, University Medical Center Göttingen) assisted with the initial screening of several mutants in a lab rotation and his bachelor thesis, both under my supervision, and contributed partial electrophysiological characterization of five mutants to this study.

**Gating Hinge**

The general gating behavior of TMS2 mutants was studied by applying constant voltages, in 20 mV-steps between $-60$ mV to 140 mV, to bilayer-incorporated channels and current was recorded for 60 seconds (see figure 4.4A for wild type). Conductance changes were extracted from current recordings ($\Delta G = \Delta I/U$) and plotted in a histogram. From these histograms, the main conductance peak representing the full closing of a single of three pores, and the primary subconductance peak corresponding to the biggest semi-stable state, were modeled with a multiple Gauss fit for each mutant (table 4.1) to extract the main conductance state $G_{main}$ and the primary subconductance state $G_{sub,1}$. Mutant $G_{main}$ and $G_{sub,1}$ do not significantly differ from the wild type levels where $G_{sub,1} = (461 \pm 31)$ pS and $G_{sub,1} = (172 \pm 30)$ pS.

**Voltage Sensor**

Recombinant Tim23 contains an internal voltage sensor, as evidenced by its strong voltage-dependent gating behavior even in the absence of the voltage regulator Tim50 [7]. While the IMS domain exhibits a $\Delta \Psi$-dependent dimerization capability, Tim23 reacts to membrane potentials even in a truncated form, lacking the IMS domain. This indicates that amino acid residues either in the transmembrane segments or in the loops between helices
contribute to the voltage sensor. To investigate if mutations in the conserved TMS2 residues alter the voltage sensitivity of recombinant Tim23, the open probability $P_{\text{open}}$ was calculated from the constant-voltage recordings. In general, no significant difference between wild type and mutant Tim23 was found in how the channels reacted to increased membrane potentials (figure 4.15).

Figure 4.15: (A)-(D) Open probability of Tim23 mutants, and wild type as reference, from three (wild type and N150A) or one full set of constant-voltage traces.

**Ion Filter**

It is unknown how the ion filter of the channel protein Tim23 is constituted. Previous studies show that the C-terminal transmembrane part of Tim23 still ex-
Figure 4.16: (A)-(E) Voltage ramps of Tim23 mutants, and wild type as reference, at asymmetric buffer conditions (250 mM:20 mM, 12.5-fold gradient).
hibits an ion preference, indicating that amino acid residues in the pore-forming transmembrane segments or the inter-helix loops facing IMS or matrix could constitute the ion filter. As with wild type Tim23, the ion selectivity of the mutant channels was assessed by recording a voltage ramp in the presence of a high salt gradient across the membrane, from 250 mM to 20 mM (figure 4.16). The reversal potentials of Tim23 were reduced for mutated residues 150, 153, 156, 159 (all highly conserved among species, see figure 4.13) and 160 (partially conserved among species). The cation preference $P_+ : P_-$ was calculated from the reversal potentials from three independent experiments each and was reduced by $\approx 30-50\%$ for most affected residues and over 65% for Tim23$^{N150A}$ (table 4.1). Remarkably, charge deletions at residues 174 and 190 flanking TMS3 did not lead to significant alteration of the ion selectivity. On the other hand, they exhibited a noteworthy reduction in fusion rates despite identical incorporation success as monitored by flotation and carbonate extration, indicating that the charges might be required for proper protein folding or complex assembly. Both mutants were not considered in further studies.
4.3. **CONSERVED TMS2 RESIDUES CONSTITUTE ION FILTER**

Table 4.1: Main conductance state $G_1$ (pS), subconductance state $G_2$ (pS), reversal potential $U_{rev}$ (mV) and the corresponding ion selectivity $P_\text{+} : P_\text{-}$ of all investigated Tim23 mutants. Conductance states modeled from histograms using multiple Gauss fits (Peak center ± SD). Reversal potential modeled from voltage ramps at asymmetric buffer conditions using linear regression (mean ± SD). Ion selectivity calculated from mean reversal potential 12.5-fold KCl gradient using GHK-Equation.

<table>
<thead>
<tr>
<th>Tim23 Mutant</th>
<th>$G_1$ (pS)</th>
<th>$G_2$ (pS)</th>
<th>$U_{rev}$ (mV)</th>
<th>$P_\text{+} : P_\text{-}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>461 ± 31</td>
<td>172 ± 30</td>
<td>47.2 ± 0.4</td>
<td>13.3 : 1</td>
</tr>
<tr>
<td>N150A</td>
<td>465 ± 55</td>
<td>181 ± 47</td>
<td>30.1 ± 0.7</td>
<td>4.4 : 1</td>
</tr>
<tr>
<td>A152G</td>
<td>442 ± 113</td>
<td>168 ± 62</td>
<td>47.6 ± 1.3</td>
<td>13.7 : 1</td>
</tr>
<tr>
<td>G153A</td>
<td>483 ± 50</td>
<td>122 ± 140</td>
<td>40.5 ± 1.8</td>
<td>8.1 : 1</td>
</tr>
<tr>
<td>G153L</td>
<td>498 ± 31</td>
<td>174 ± 42</td>
<td>44.0 ± 1.2</td>
<td>10.4 : 1</td>
</tr>
<tr>
<td>L155A</td>
<td>491 ± 83</td>
<td>209 ± 114</td>
<td>47.3 ± 1.0</td>
<td>13.4 : 1</td>
</tr>
<tr>
<td>A156G</td>
<td>497 ± 60</td>
<td>178 ± 59</td>
<td>39.7 ± 1.6</td>
<td>7.7 : 1</td>
</tr>
<tr>
<td>A156L</td>
<td>469 ± 29</td>
<td>165 ± 145</td>
<td>42.2 ± 1.7</td>
<td>9.1 : 1</td>
</tr>
<tr>
<td>Y159A</td>
<td>440 ± 121</td>
<td>198 ± 102</td>
<td>38.0 ± 1.4</td>
<td>6.9 : 1</td>
</tr>
<tr>
<td>N160A</td>
<td>469 ± 126</td>
<td>154 ± 113</td>
<td>42.0 ± 1.2</td>
<td>9.0 : 1</td>
</tr>
<tr>
<td>N163A</td>
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<td>192 ± 123</td>
<td>48.5 ± 1.9</td>
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</tr>
<tr>
<td>K190A</td>
<td></td>
<td></td>
<td>46.7 ± 2.3</td>
<td>12.7 : 1</td>
</tr>
</tbody>
</table>
4.3.3 Yeast Growth and Complex Integrity of Tim23 Mutants

In a close collaboration with Alexander Benjamin Schendzielorz (AG Rehling, University Medical Center Göttingen), we investigated if the observed alterations in electrophysiological properties, i.e. weakening of the ion filter, had implications for mitochondrial fitness in the yeast *S. cerevisiae*. To this end, the amino-acid substitutions in the second transmembrane segment were first introduced in the yeast plasmid expressing Tim23 wild type by site-directed mutagenesis, with the same primers as were used for mutagenesis of the pET10N-plasmid, and successful mutagenesis was monitored by plasmid sequencing. Half of the mutant plasmids were generated by me, the rest of the mutants and all further yeast handling and yeast experiments were performed by Alexander Benjamin Schendzielorz unless explicitly stated differently. All yeast experiments were performed as described in Denkert et al. [98].

*S. cerevisiae* cells with a chromosomal deletion of *TIM23* were complemented by a plasmid carrying both wild type *TIM23* and *URA3* genes. In normal medium, the cells survive by expressing Tim23 from the complementing plasmid. Yeast cells were transformed with a pRS413-plasmid containing *HIS3* gene as selective marker and either wild type or mutant *TIM23*. After transformation, yeast cells were plated on medium lacking histidine to select transformants that could grow on -His medium due to the *HIS3* gene. Transformants were further plated on medium containing 5-fluoroorotic acid (5-FOA), leading to *URA3*-induced loss of the *TIM23-URA3*-containing plasmid, to monitor Tim23 mutant ability to compensate for lack of wild type Tim23. Here, Tim23G153L exhibited a lethal phenotype as published before [99]. Complementing strains were grown on fermentable glucose and on non-fermentable glycerol at 18 °C, 30 °C or 37 °C. Four strains, ex-
pressing Tim23\textsuperscript{N150A}, Tim23\textsuperscript{L155A}, Tim23\textsuperscript{A156L} or Tim23\textsuperscript{Y159A} respectively, showed a significant growth defect on glycerol at 37 °C, while Tim23\textsuperscript{N150A} also lead to a mild growth defect on glucose at 37 °C (Table 4.2).

To investigate the origin of impaired growth, mitochondria were isolated from yeast cells expressing Tim23 mutants or wild type and mitochondrial protein levels of Tim23 were quantified from independent triplicates and corrected by levels of Por1, an outer mitochondrial membrane porin. The mutants Tim23\textsuperscript{L155A}, Tim23\textsuperscript{A156L}, Tim23\textsuperscript{Y159A} or Tim23\textsuperscript{N160A} have significantly reduced mitochondrial levels of the Tim23 protein (Figure 4.17A). In addition to reduced Tim23 levels, isolated TIM23 complexes from strains expressing Tim23\textsuperscript{A156L} and Tim23\textsuperscript{Y159A} show reduced levels of both Tim17 and Tim50, determined by co-immunoprecipitation using antibodies against Tim23 (Figure 4.17B). The wild type-like integrity of the inner membrane potential in Tim23\textsuperscript{N150A}-containing mitochondria was confirmed by measuring the dequenching of the \(\Delta\Psi\)-sensitive fluorophore DiSC\textsubscript{3}(5) (Figure 4.17C).

Table 4.2: *S. cerevisiae* strains expressing mutant forms of Tim23 were analyzed for growth on fermentable (glucose) and non-fermentable (glycerol) medium at different temperatures. Yeast handling was performed by Alexander Benjamin Schendzielorz.

<table>
<thead>
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<th>Glucose</th>
<th>Glycerol</th>
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<tr>
<td></td>
<td>30 °C</td>
<td>37 °C</td>
</tr>
<tr>
<td>WT</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N150A</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>A152G</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G153A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L155A</td>
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<td>+</td>
</tr>
<tr>
<td>N163A</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure 4.17: Protein levels, complex formation and membrane integrity were monitored for Tim23 mutant containing mitochondria. (A) Tim23 levels in mutant strain mitochondria were assessed by Western blot quantification normalized by Porr levels from four independent experiments (mean±SD). (B) TIM complex formation and interaction in mutant mitochondria was analyzed by co-immunoprecipitation with Tim23 antibodies. (C) Relative inner mitochondrial membrane potential was calculated from triplicates by dequenching of ΔΨ-sensitive fluorophore DiSC3(5) for wild type and Tim23N150A mitochondria. Figures were modified from Denkert et al. [19].
It can not be dissected if the growth defects observed for Tim23\(^{L155A}\), Tim23\(^{A156L}\) and Tim23\(^{Y159A}\) originate exclusively from reduced protein levels and compromised TIM23 complexes or if altered electrophysiological characteristics also reduce mitochondrial fitness, even in intact and proper complexes. Tim23\(^{N150A}\), on the other hand, showed a strong growth phenotype and the strongest reduction in ion selectivity, but was expressed and integrated into yeast mitochondria in wild type levels and maintained the full inner membrane potential.

To examine how Tim23\(^{N150A}\) could compromise mitochondrial fitness with wild type-like protein levels, complex integrity and membrane potential, we characterized protein import capabilities of mutant and wild type mitochondria. The matrix-destined substrates \(F_{1}\beta\) of the \(F_{1}F_{0}\)-ATP synthase, Cox4 of the cytochrome c oxidase and fusion protein \(b_{2}(I67)_{\Delta}\)-DHFR, and the inner membrane sorted fusion protein \(b_{2}(220)\)-DHFR, were translated with \(^{35}\)S-methionine in rabbit reticulocyte lysate and imported into isolated mitochondria. Tim23\(^{N150A}\)-containing mitochondria exhibit a reduced import capability for all substrates, as evidenced by import analysis after 10, 20 and 30 min at 30 °C (figure 4.18A). Import at 30 °C was quantified mid-time after 15 min in independent triplicates and indicated import reductions of Tim23\(^{N150A}\) by 30 to 40% of wild type levels. Note that import reduction of \(b_{2}(220)\)-DHFR at later time points becomes even more pronounced, compared to matrix targeted preproteins. Analysis of import kinetics of \(F_{1}\beta\) and Cox4 at non-permissive temperatures (figure 4.18C) revealed a stronger phenotype at later time points, i.e. a reduction to \(\approx 50\%\) of wild type levels after 30 min at 37 °C compared to 70 to 75% at 30 °C.
Figure 4.18: Import kinetics of various substrates were determined for wild type or Tim23<sup>N150A</sup>-containing mitochondria at (A) 30 °C or (B) 37 °C. (C) Import efficiency after 15 min at 30 °C was quantified from independent triplicates (n=3, mean±SD). Figures were modified from Denkert et al.^[98].
4.3. CONSERVED TMS2 RESIDUES CONSTITUTE ION FILTER

4.3.4 Presequence Titration of Cox4 to Tim23^{N150A} and Wild Type

After Tim23^{N150A} was identified as the only studied mutant with reduced ion selectivity, yeast growth defect and proper complex and membrane integrity, we showed that Tim23^{N150A} is importing significantly less preproteins in isolated mitochondria. Considering reduced protein import and the common assumption that cation preference of mitochondrial translocases might be directly connected to cationic presequences, we asked if Tim23^{N150A} exhibits an altered response to model presequences in vitro.

Before characterizing the presequence response of Tim23^{N150A}, the mutant was thoroughly characterized to avoid overlooking other alterations possibly responsible for impaired yeast growth or protein import, but neither detailed gating analysis (figures 4.19A to 4.19B) nor voltage-sensitivity with or without Tim50 (figure 4.19D) was altered compared to wild type. The only parameter that changed was the channel’s ion selectivity (figure 4.19C).

In section 4.1, it was shown that the most obvious and striking response of Tim23 to the presequences peptide Cox4 is a drastic increase in gating activity. When Cox4 was titrated step-wise to the IMS side of Tim23^{N150A} or wild type and the buffers on each side are first stirred and then left to calm for two minutes, the presequence induced increased gating frequency similar to Tim23 wild type (figures 4.20A to 4.20C), though it appeared that the relative increase is weaker for the mutant channel. For Tim23 wild type, a higher rate of partial closing at elevated voltages was observed for stimulated compared to unstimulated channels. It can not be easily distinguished, if this increased voltage-sensitivity originates from the presequence effect or from stress effects due to prolonged exposure to repeatedly applied holding potentials. When the effects of presequence peptides
Figure 4.19: General gating analysis of Tim23<sup>N150A</sup> (A) Constant-voltage recordings of Tim23<sup>N150A</sup> and different holding potentials. (B) Histogram of over 2000 gating events with main and subconductance states fitted. (C) Asymmetric voltage ramp for reversal potential analysis of Tim23<sup>N150A</sup>. (D) Current-voltage ramps (left) and open probabilities (right) of bilayer incorporated Tim23<sup>N150A</sup> before (black) and after (red) addition of 700 nM Tim50<sup>IMS</sup> to the IMS-side of the channel. (n=3, mean±SD). Figures were modified from Denkert et al. [95].
to gating frequency are to be analyzed in-depth, it is required that all pores of recombinant Tim23 remain open, and thus gating-capable, throughout the experimental process.

In an initial screening, the gating frequency of Tim23^{NI50A} at increasing Cox4 concentrations was analyzed at applied holding potentials of 80 mV, 100 mV and 120 mV. While the relative gating increase was roughly proportional to the Cox4 concentration for recordings at 80 mV, this does not hold true for more elevated holding potentials. Both at 100 mV and 120 mV, the channel started to close at higher Cox4 concentrations, leading to less open and gating-capable pores and thus underestimation of the gating frequency. Therefore, the quantification of gating activity of Tim23^{NI50A} and wild type were performed at 80 mV only. Here, both Tim23 channel variants showed an increase in activity, roughly proportional to the Cox4 presequence concentration. The relative increase in Tim23 wild type exceeded factor 50 above 500 nM of Cox4, while, for Tim23^{NI50A}, the increase reached a plateau at approximately factor 5. To exclude that this difference, by factor 10, between both channels originates from different gating activities of unstimulated channels, the absolute gating frequency was also analyzed, showing no significant difference between wild type and mutant Tim23.

Table 4.3: Open probability screening of stimulated Tim23^{NI50A} at different holding potentials.

<table>
<thead>
<tr>
<th>[Cox4]</th>
<th>Open probability</th>
</tr>
</thead>
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<tr>
<td>0 nM</td>
<td>0,998 0,991 0,984</td>
</tr>
<tr>
<td>300 nM</td>
<td>0,993 0,982 0,990</td>
</tr>
<tr>
<td>500 nM</td>
<td>0,99 0,982 0,957</td>
</tr>
<tr>
<td>700 nM</td>
<td>0,988 0,979 0,875</td>
</tr>
<tr>
<td>900 nM</td>
<td>0,97 0,825 0,805</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Holding potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80 100 120</td>
</tr>
</tbody>
</table>

Figure 4.20D.
CHAPTER 4. RESULTS

Figure 4.20: Gating frequency analysis of wild type and mutant Tim23 after stimulation with Cox4 presequence. (A)-(C) Constant-voltage recordings of Tim23 wild type (left) and Tim23<sup>N150A</sup> (right) with no (A), 500 nM (B) or 900 nM (C) Cox4. (D) Voltage dependency of gating frequency development for Tim23<sup>N150A</sup>. (E) Relative increase in gating frequency depending on Cox4 concentration for wild type (black) and mutant (red) Tim23 from independent triplicates (mean±SD). (F) Absolute gating frequency of unstimulated Tim23 variants (n=3, mean±SD). Figures were modified from Denkert et al. [9].
Chapter 5

Discussion

Tim23 is the eponymous pore-forming unit of the TIM23 translocon, the most complex protein translocation system of mitochondria. Alone, the subunit forms an aqueous pore with defined electrophysiological properties like voltage sensitivity, presequence activation or cation selectivity, that are also present to a similar extend in native TIM23 complexes. The molecular origin of Tim23’s channel properties is unknown and studies investigating molecular or structural characteristics of the channel-part were often hindered by the difficulty of handling hydrophobic \( \alpha \)-helical membrane proteins in vitro.

TIM23 utilizes the inner mitochondrial membrane potential \( \Delta \Psi \) as the energy source for cationic presequence translocation. The IMS domain of main receptor Tim50 hands over preproteins from the mitochondrial intermembrane space to the channel Tim23, but also acts as the voltage regulator, closing the channel at physiological holding potentials to avoid leakage.

In this study, I employed high-resolution single-channel electrophysiology with recombinant Tim23 to investigate channel characteristics in detail, explore origins and implications of these properties using loss-of-function mutations and
dissect interactions with the main receptor Tim50.

Both recombinant Tim23 and isolated TIM23 complexes interact with substrates of the TIM23 pathway bearing positively charged presequences and incubation of the channel with model peptides leads to stimulation and increased gating activity. Peptides with similar amino acid composition and identical charge but shuffled order of amino acids were not able to stimulate increased channel activity, indicating that secondary structure and charge alignment plays an important role in presequence recognition by Tim23.

5.1 Tim23 Kinetics Change upon Presequence Interaction

The presequence translocase TIM23 and the pore-forming subunit Tim23 were electrophysiologically characterized to some extend in the past. To enable reasonable analysis of Tim23 characteristics, e.g. between wild type and mutant channel or truncated forms of regulatory proteins, a detailed baseline of wild type properties had to be established in this thesis. While the general characteristics required for this thesis, like gating behavior, conductance states, ion preference or protein and peptide interactions, were consistent with published results, the aspect of temporal resolution of channel gating was not considered in previous studies.

Recently, studies on Tom40 substrate interaction showed that the process of substrate binding and translocation can be distinguished by analysis of kinetic gating parameters including dwell times of stimulated closing events in the submillisecond regime. A big difficulty in attempting to study channel kinetics at the resolution limit is that unified and consistent analysis and quantification parameters are hard to establish. In this study, sophisticated data reconstruction
tools capable of extracting channel properties like dwell times and conductance changes with unmatched consistency and reliability were employed and refined in collaboration. Using these computationally intensive routines, the time scale of Tim23 channel stimulation by presequence interaction was analyzed by extracting the dwell times, i.e. the time between two conductance changes, of short closing events. Unexpectedly, the minimal dwell time of these events increased after channel stimulation with presequence peptides which induced increased gating activity. Without substrate, regular short-timed events reached dwell times down to 32 µs. After addition of the Cox4 presequence, the regular minimal dwell time nearly doubled to 56 µs, although the majority of short events (below 500 µs) peaked at a much higher dwell time, around 180 µs.

If presequence incubation of Tim23 merely led to substrate binding to the IMS domain, randomized movement into and from the channel’s opening funnel, or presequences being trapped within the channel lumen, the increased gating frequency should follow a similar temporal distribution as unstimulated channels. The data instead shows that presequences block the channel for a rather defined and increased amount of time, suggesting that they traverse the channel from one end to the other. It is tempting to apply this new kind of data analysis to study channel-substrate interaction of presequences with various properties like charge or length.

### 5.2 Tim50core Regulates Tim23 and Hands Over Preproteins

The voltage regulator and main receptor Tim50 strongly interacts with Tim23 via their IMS domains. Tim50’s IMS domain is subdivided into two distinct domains, the very C-terminal presequence binding domain (PBD) and the
more N-terminal, globular core domain. Both subdomains bind and properly recognize presequences with similar affinity than the full IMS domain\textsuperscript{23}. PBD and core domain interact with each other\textsuperscript{24}, though only the core domain also interacts with Tim23\textsuperscript{IMS}. Still, the core domain can not compensate for PBD-function, as evidenced by the lethal phenotype of ΔPBD-strains\textsuperscript{24}. Further, Tim50\textsuperscript{IMS}-Tim23\textsuperscript{IMS} and Tim23\textsuperscript{IMS}-presequence interactions are mutually exclusive\textsuperscript{24,25,26}.

It is suggested that presequence translocation is first initiated by binding to the Tim50\textsuperscript{PBD} when Tim50\textsuperscript{core} is still bound to Tim23\textsuperscript{IMS}, effectively sealing the channel. Next, the presequence-loaded PBD interacts with the core domain and hands over the substrate due to a slightly higher affinity from core domain to presequence\textsuperscript{23}. Although the affinity of Tim23 towards substrates is orders of magnitudes lower\textsuperscript{23}, Tim23\textsuperscript{IMS} binds the presequence while dissociating from Tim50\textsuperscript{IMS}. Possibly due to force exertion of the transmembrane potential, the presequence is then threaded into the channel and partially translocated to the matrix before full translocation or membrane sorting is initiated by PAM resp. Tim21/Mgr2.

The question arises why the PBD of Tim50 is essential, i.e. which function of Tim50\textsuperscript{IMS} can not be compensated for by Tim50\textsuperscript{core}. The function of Tim50\textsuperscript{core} was addressed in this study by first investigating the regulatory effect of soluble Tim50\textsuperscript{IMS}, Tim50\textsuperscript{PBD} or Tim50\textsuperscript{core} on voltage-sensitivity of bilayer-incorporated Tim23 channels. Tim50\textsuperscript{PBD} did not induce any premature closing of the channel at elevated voltages, as expected by the lack of interaction between Tim50\textsuperscript{PBD} and Tim23\textsuperscript{IMS}. Tim50\textsuperscript{core}, on the other hand, increased the voltage-sensitivity of the channel to the same extend as Tim50\textsuperscript{IMS}. The data confirms that the voltage regulator property of Tim50 resides in the Tim23-interacting core domain.

Second, the ability of Tim50\textsuperscript{core} to initiate presequence handover to Tim23 without the PBD was investigated. In early studies, the presequence model pep-
The question whether the PBD is essential for initial presequence reception from the TOM complex was addressed in a previous study, where the presequence-triggered dissociation of Tim50 and Tim21 was investigated. Upon
binding of presequences to the main receptor, Tim50 dissociates from Tim21 which in turn leaves the TIM23 complex. Presequence reception was monitored indirectly via Tim50-Tim21 dissociation by cross-linking. The authors manipulated the mitochondria, e.g. by deleting the IMS domain of Tom22 which connects TOM to TIM23, or by gradually substituting Tim50\textsubscript{IMS} with Tim50\textsubscript{core}. Both manipulations only slightly reduced the level of dissociation, indicating that neither TIM23 link to TOM nor presence of Tim50\textsubscript{PBD} are required for initial presequence reception.

It appears that Tim50\textsubscript{PBD} has an essential function apart from voltage regulation, presequence handover or initial presequence recognition.

5.3 Tim23\textsuperscript{N150A} is Impaired in Presequence and Preprotein Translocation

Remarkably, all mitochondrial protein translocases exhibit an preference for cations in electrophysiological characterizations\textsuperscript{109} and many mitochondrial targeting signals contain positive net charges\textsuperscript{110}. The mitochondrial transmembrane potential $\Delta\Psi$ is required to import presequences and targeting signals via TIM23\textsubscript{N150A}, not only by inducing structural changes in the translocase\textsuperscript{111,112,113} but also by interacting with the positive charges themselves, pulling them through the channel\textsuperscript{113,114,115,116}. It was speculated that the cation selectivity of TIM23 is important for presequence recognition and translocation, though experimental evidence was missing.

One contribution to the ion selectivity of Tim23 was identified by chance when the N-terminal IMS-domain of the protein was cleaved of and the remaining C-terminal membrane part was found to still form a channel, though with a
Another effect on the ion filter was identified when a negative charge was introduced in the transmembrane segment 3 at position I86[167], which lead to significantly weakened ion selectivity and even reduced pre-sequence affinity. On the other hand, it is not surprising that introduction of arbitrary charges in the middle of a transmembrane $\alpha$-helix lead to alteration of channel properties and especially of a charge-specific process like ion selectivity. The authors of that study speculate[452,444] that the ion filter might be formed by charges in helix-flanking loops or by pore-facing charges within the channel lumen or a combination of both.

In this study, the origin of pore properties like the ion filter was addressed by introducing presumed loss-of-function mutations in uncharged amino acid residues of the second transmembrane segment (TMS2) of Tim23. These residues were identified as pore-lining and substrate-interacting in previous sophisticated fluorophore-mapping studies[13,74]. Additionally, for elucidation of the ion filter two highly conserved amino acid residues at positions 174 and 190, flanking the third transmembrane segment, were mutated to alanine to remove their respective charges. In an electrophysiological screening, the only property significantly altered was the ion selectivity of Tim23. Especially pore-facing TMS2-residues with very high sequence identity among species (figure 4.13) contributed to the strong cation preference.

It is remarkable that the charged residues flanking the TMS3 did not contribute to ion selectivity of the channel. This is in line with an experimental determination of Tim23’s pore size using the globular non-electrolyte PEG[161], where Tim23 was found to contain wide channel openings at channel entry and exit and a short and narrow constriction zone within the channel. This would indicate that helix-flanking residues are farer from the channel center and are less able to
manipulate ions by pure charge exposure.

Interestingly, uncharged amino acid residues from the matrix end (NI50) to the IMS side (NI60) of the pore contribute to the ion filter. These results imply that it is not the narrow constriction zone of Tim23 that sorts ions, as was proposed for large, ion selective β-barrel channels. It is tempting to speculate that the mode of ion filtering is shared at least with other large α-helical membrane channels, especially with homologs of the Tim17/22/23 family.

Most selectivity mutants also exhibited a growth defect when expressed in \textit{tim23Δ} yeast cells. Unfortunately, apart from Tim23\textsuperscript{NI50A}, the other ion filter mutants with a growth defect exhibited compromised mitochondrial steady state levels and consequently reduced TIM23 complex constitution, making it impossible to dissect contributions to their growth phenotype. Tim23\textsuperscript{G153A}, on the other hand, did not lead to impaired yeast growth despite showing a mediocre selectivity defect, indicating that a certain reduction in cation preference can be compensated for by the complex.

Tim23\textsuperscript{NI50A} though showed the strongest reduction in ion selectivity, a growth phenotype at 37 °C, wild type-like protein levels and complex integrity and no other deviation from wild type Tim23 regarding electrophysiological properties. Import assays for wild type and NI50A mutant with different TIM23 substrates revealed the reason for impaired cell growth, with Tim23\textsuperscript{NI50A}-containing mitochondria exhibiting significantly reduced import capabilities for both matrix and sorted proteins, even more pronounced at non-permissive temperatures.

Titrating the Cox4 presequence to bilayer-incorporated channels lead to a highly reduced gating activity induction for mutant channels, from a relative increase in gating frequency of \( \approx 50 \) for wild type, down to \( \approx 5 \) for mutant channels in the presence of over 500 nM Cox4 presequence. We conclude that the reduced
cation preference of Tim23 renders the channel insensitive towards presequences, which in turn explains the reduced import rates of mitochondria.

It was unknown whether the increase in gating activity came from presequence binding to the IMS domain or the channel entry, or if it corresponds to presequence translocation, for which a transmembrane potential is sufficient. The mutated residue N150 lies at the very C-terminal end of TMS2, basically forming the channel exit towards the matrix. The effect it still has, on not only on import rates in organello but also on gating activity in vitro, strongly suggests that the flickering of Tim23 channels upon presequence incubation represents an actual translocation rather than pure binding on the IMS side.
Chapter 6

Summary and Conclusion

In eukaryotic cells, mitochondria are organelles that play an essential role in multiple physiological processes like energy metabolism, fatty acid oxidation or lipid synthesis. Mitochondria have a double-membrane envelope, dividing it into four different subcompartments, which are all targets of complex protein import machineries within the organelle. The most complex translocase in the organelle is the presequence translocase TIM23, importing over 70% of all mitochondrial proteins into the inner membrane or the matrix.

The eponymous subunit Tim23 forms a dynamic, water-filled pore within the complex, with a cation preference conserved among mitochondrial translocases. Tim23 interacts with presequences of import substrates, leading to increased channel activity, and with the receptor and voltage regulator Tim50 which induces channel closing at elevated membrane potentials.

In this thesis, combining single channel electrophysiology and site-directed mutagenesis, multiple pore-lining amino acid residues of Tim23 were identified as constituents of the channel's cation filter. Unlike proposed before, the ion fil-
ter can not be constituted by a localized constriction zone within the channel, but possibly by providing an energetically favorable or unfavorable surface pathway for ions, spanning the whole channel lumen. Combining electrophysiology and yeast biochemistry, we showed that the cation preference is a key property in recognizing and especially translocating positively charged presequences \textit{in vitro} and preproteins \textit{in organello}. High-resolution analysis of electrophysiology data further indicate that the presequence-induced fast-gating state of Tim23 presumably corresponds to a translocating state with peptides in transit.

Further, we investigated the domain origin for critical functions of the main receptor Tim50. We could show that both voltage regulation of, and presequence handover to Tim23 is independent of the essential presequence binding domain PBD but is localized in the soluble core domain of Tim50.

In conclusion, we provided the first experimental evidence that the cation preference of a mitochondrial protein translocase is linked to its ability to transport substrates with a positively charged presequence. We also elucidated the submolecular localization of essential interactions between receptor and channel of TIM23.
Bibliography


BIBLIOGRAPHY


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Appendix - R scripts

6.1 R: Reconstruction of .abf Using SMUCE

```r
ls() # This is my default workspace from My Documents.
setwd(getwd()) # First change, if you haven’t already.
rm(list=ls()) # Delete the default workspace.

.getNamespace <- function(name) {Internal(getRegisteredNamespace(as.name(name)))
library( abf )
library( tools )
library( stats )
if( isNamespaceLoaded("stepR")) { detach("package:stepR", unload=TRUE) } else
 {}
library( stepR )

# # # # # # # # # #
# parameters to specify #
# record parameters
sampling<-5e4 #sampling frequency
cutoff<-5e3 #cutoff frequency of applied filter
# time window, when to apply fit
autotime<FALSE  #automatically specify time boundaries by checking voltage changes (timel=t(voltage)+ls, time2=t(voltage2)-ls)
time1<-6  #voltage is usually changed at t=5s, at timel=6s the signal should be mostly stable
time2<-65  #voltage change at t=65s
# size of each fit-segment
LL<-15000  #segment of 15k points work nicely, can also try 20k or 25k, but the fits take longer
# threshold for gating detection
c0<-15  #minimum conductance change in pS for detection of a gating event
t0<-0  #minimum dwell time,
# setting for displaying plots
PLOT<FALSE  #decide if a plot of every segment (with length LL) should be displayed
# setting for saving gating events and dwell times to txt-files
SAVE<TRUE
# # # # # # # # # #
```

119
Find. Indices. False. Positive <- function(v) {
  # this function returns a matrix containing the clusters of indices of false positive events
  v.aux <- c(v, v[length(v)])
  l <- length(v.aux)
  if (l > 0) {
    Matriz <- matrix(rep(0, (1 - l) * (1 - l)), nrow = (l - 1))
    temp <- c()
    for (i in 1:(l - 1)) {
      temp <- c(temp, v.aux[i])
      if (abs(v.aux[i] - v.aux[(i + 1)]) == 1) {
        temp <- c(temp, v.aux[(i + 1)])
      } else {
        temp <- unique(temp)
        Matriz[i, 1:length(temp)] <- temp[1:length(temp)];
        temp <- c()
      }
    }
    n.row <- sum(Matriz[, 1] != 0)
    M.aux <- matrix(rep(0, (1 - l) * n.row), nrow = n.row)
    ind.aux <- 1:(l - 1)
    for (j in 1:n.row) {
      k <- ind.aux[Matriz[, 1] != 0][j]
      M.aux[j, ] <- Matriz[k, ]
    }
    M.aux <- matrix()
  }
  ind. rep <- function(w) {
    # the parameter of this function, w, has to be the output of Find. Indices. False. Positive.
    temp <- matrix(rep(0, nrow(w) * 2), ncol = 2) # each row contains: the index of the first gating event in a seq. of consecutive gating events; length of seq. of gating events
    for (i in 1:nrow(w)) {
      temp[i, ] <- c(w[i, 1], length(w[i, w[i, ] > 0]))
    }
    # fit. idealization returns a list containing
    # 1) newFitValue, that is, after identifying false positive events, we removed them and replace their values accordingly.
    # 2) falsePositiveIndices, a vector containing the indices of all the false positive events.
    # 3) clustersIndices, a 2 x l matrix, first column contains the index of the first false positive event in a seq of consecutive false positive events,
    # second column contains the length of the seq of the corresponding consecutive false positive events.
    # 4) values, a vector containing ONLY the values of the idealization.
6.1. R: RECONSTRUCTION OF .ABF USING SMUCE

```r
fit.idealization <- function(fit, t1, t2) {  # fit is an object of the class fit (jsmurf or smuceR), t1 and t2 are thresholds
  n = length(fit$value)
  fit.leftEnd <- fit$leftEnd
  fit.rightEnd <- fit$rightEnd

  if(n>2) {
    ind1 <- 2:(n-1)
    fit.value <- fit$value[ind1]
    fit.value.fwd <- fit$value[3:n]
    fit.value.bwd <- fit$value[1:(n-2)]
    cril <- abs(fit.value - fit.value.fwd) >= t1
    cri2 <- abs(fit.value - fit.value.bwd) >= t1
    cri3 <- fit$rightEnd[ind1] - fit$leftEnd[ind1] < t2
    x <- ind1[cril & cri2 & cri3]  # indices of all false positive events
    fit.leftEnd
    gating <- Find.Indices.False.Positive(x)
    indices <- indices.replacement(gating)
    Y.aux <- fit$value

    for(i in 1:nrow(indices)) {  # here we update the vector fit$value, we remove the gating events and fill the gap accordingly
      indice <- indices[i, 1]
      length <- indices[i, 2]
      ifelse(length == 1, Y.aux[indice] <- fit$value[(indice-1)], Y.aux[indice:(indice+length-1)] <- rep(fit$value[indice-1], length))
    }

    for(i in (n-1):1) {  # delete subsequent identical levels
      if(is.finite(Y.aux[i])) {
        if((Y.aux[i] - Y.aux[i+1]) == 0) {
          Y.aux[i] <- Y.aux[i+1]
        }
        fit.leftEnd <- fit.leftEnd[-(i+1)]
        fit.rightEnd <- fit.rightEnd[-i]
      } else {
        } else {
          }
    }
    }
  }

  f = length(Y.aux)
  fit.value.new <- c()
  for(j in 1:f) {  # here we simply update the fitted function, cf. fit.aux<-fitted(fit) above
    l <- (fit.rightEnd[j] - fit.leftEnd[j]) + 1
    temp <- rep(Y.aux[j], l)
    fit.value.new <- c(fit.value.new, temp)
  }
} else {
```
x <- c(FALSE)
indices <- c()
fit.value.new <- c()

for (j in 1:f) {# here we simply update the fitted function, cf. fit.aux <-
  fitted(fit) above
  l <- (fit$rightEnd[j] - fit$leftEnd[j] + 1)
  temp <- rep(Y.aux[j], l)
  fit.value.new <- c(fit.value.new, temp)
}

List <- list("newFitValue" = fit.value.new, "falsePositiveIndices" = x,
            "clustersIndices" = indices, "value" = Y.aux, "leftEnd" = fit.leftEnd, 
            "rightEnd" = fit.rightEnd)
return(List)
}

### Gating Event Detection ###
# find transitions above tl, calculate the current changes and name them
# gatingEvents

GatingEventDetection <- function(idealized.fit, tl, V, LL, sampling, timel, t0)
{
  # idealized.fit is the outpt of fit.idealization, tl is the minimum
current change (in pA), V is the voltage applied in the trace, LL is the
length of the fit-segment, sampling is the sampling frequency, timel is
the starting time of the idealization within the real trace (like timel=6
s if voltage change occurred at 5s) and h is the turn number, i.e. which
fit-segment is currently processed

  nn <- length(idealized.fit$value)
if (nn>1){
  ind2 <- 1:(nn-1)
  ideal.value <- idealized.fit$value[ind2]
  ideal.value.fwd <- idealized.fit$value[2:nn]
  cri4 <- abs(ideal.value - ideal.value.fwd) >= tl

  gatingEvents <- abs(idealized.fit$value[c(FALSE, cri4)] - idealized.fit$value[c(cri4, FALSE)])
  conductance <- abs(gatingEvents/V*1000)
  gatingTime <- ((idealized.fit$leftEnd[c(FALSE, cri4)])/sampling) + timel
  Gating.temp <- cbind(gatingTime, idealized.fit$value[c(cri4, FALSE)], 
                       idealized.fit$value[c(FALSE, cri4)], gatingEvents, conductance, deparse.
level = 0)
  } else { Gating.temp <- c() }

  # tidy up, check 2nd threshold (minimum dwell time)
  Gating <- Gating.temp
  LG <- nrow(Gating)
if (LG>3){
  Gating.tc <- Gating
  for (i in LG:4){
    if (is.finite(as.numeric(Gating.tc[i])))
      diffG <- (as.numeric(Gating[i,1]) - as.numeric(Gating[(i-1),1]))*1000
  }
if (diffG < 0)
    Gating.t <- Gating.t[-i,]
LG.t <- nrow(Gating.t)
if (i < LG.t + 2)
    if (i > 4)
        diffG.bwd <- (as.numeric(Gating[i-1,1]) - as.numeric(Gating[(i-2),1]))
    else if (diffG.bwd > 0, Gating.t <- Gating.t[-(i-1),",","]
    } else if (i == 4, Gating.t <- Gating.t[-(i-1),])
Gating <- Gating.t
}

List <- list("Gating" = Gating)
return(List)

# # # # AFTER going through all trace-segments # # # #
# tidy up "ValuesDwell" and "Gating" to delete every entry below a set time
# minimum
#idealized.fit <- full.fit
#Gating$Gating
SmoothenIdeal <- function(idealized.fit, Gating, time1, time2, sampling, V, t1)
{
    GatingPoints.t <- (as.double(Gating[,1][1:nrow(Gating)])[1:time1] - time1)*sampling
    GatingPoints <- append(GatingPoints.t, length(idealized.fit$newFitValue), after = length(GatingPoints.t))
    GatingPoints <- append(GatingPoints, 0, after=0)

    for (i in 1:10)
        L <- length(GatingPoints) - 1
    Smooth.v <- c()
    if (L>1)
        for (i in 1:L)
            Smooth.v <- c(Smooth.v, as.double(summary(idealized.fit$newFitValue[
                GatingPoints[i+1]:(GatingPoints[i+1])][4])))
    }
    for (i in length(Smooth.v):2) { # i < L
        if ( (abs(Smooth.v[i]-Smooth.v[i-1]))<t1)
            GatingPoints <- GatingPoints[-i]
            GatingPoints.t <- GatingPoints.t[-(i-1)]
        } else {
    }

    L <- length(GatingPoints) - 1
    Smooth.l <- c()
    Smooth.r <- c()
APPENDIX - R SCRIPTS

```r
Smooth.value <- c()
for (i in 1:L) {
  Smooth.value <- c(Smooth.value, as.double(summary(idealized.fit$newFitValue[[
    GatingPoints[i+1]:(GatingPoints[i+1])]][4])) # mean value between
  # gating event "i" and "i+1"
  Smooth.l <- c(Smooth.l, GatingPoints[i+1]) # left End of each
  Smooth.r <- c(Smooth.r, GatingPoints[i+1]) # right End of each
}
L2 <- length(GatingPoints) - 1
if (length(Smooth.value) > 1) {
  Smooth.Gating <- cbind((GatingPoints.t/sampling) + timel, Smooth.value[1:(L2-1)]
    , Smooth.value[2:L2], abs(Smooth.value[1:(L2-1)] - Smooth.value[2:L2])
    , abs(abs(Smooth.value[1:(L2-1)] - Smooth.value[2:L2]) * 1000/V), deparse.level=0)
} else {
  Smooth.Gating <- cbind("-", "-", "-", "-", "-", deparse.level=0)
}

Smooth.DwellTime <- (Smooth.r - Smooth.l) / sampling
Smooth.DwellLevelC <- Smooth.value / V
Smooth.DwellLevelI <- Smooth.value
Smooth.Dwell <- c()

Smooth.Dwell <- cbind(Smooth.DwellTime, Smooth.DwellLevelI, Smooth.DwellLevelC,
  
  # Loading datasets
files <- list.files(getwd(), pattern = "\abf\$, all.files=TRUE, full.names=
  TRUE, ignore.case=TRUE, include.dirs=TRUE, recursive=TRUE)
files <- files[1:length(files)]
files
for (f in 1:LF) { # go through all files that are present in the main folder
  trace <- abfload(files[f])
  if (nrow(trace$tags) > 1) { # check if there has been a voltage applied
    Vc <- trace$tags[2,2]
    if (autotime) # if autotime==TRUE, set timel and time2 next to voltage changes
      timel <- trace$tags[2,1]+1
      if (nrow(trace$tags) > 2) { 
```

6.1. R: RECONSTRUCTION OF .ABF USING SMUCE

```r
time2 <- trace$tags[3,1] - 1
} else{ time2 <- 64 }
] else{ }

name <- basename(files[f])
dir <- dirname(files[f])
nameL <- nchar(basename(files[f]))
name <- substr(name,1,nameL-4) # reformat filename for data export later

lbound <- traces$s > time1;
ubound <- traces$s < time2;
traces <- traces[lbound & ubound] # skim the trace to

t2 <- t0*sampling/1000  # threshold for dwell time in points
t1 <- abs(V < c0/1000)  # threshold for gating events, scaled by voltage to account for smaller current changes

LcountR <- ceiling(length(traces)/LL) # round up number of runs
if(LcountR==1){
  LL <- length(traces)
} else{ }

full.fit <- c()
Gating <- c()
VD <- c()

for(h in 1:LcountR){
  LB <- (h-1)*LL + 1
  if(h*LL <= length(traces)){
    RB <- h*LL
  } else{ RB <- length(traces) }
  ion.chan <- traces[LB:RB]
}

# Setting the fit

# JSMURF for ion channel
dfil <- dfilter("bessel", list(pole=4, cutoff = cutoff / sampling)) # define filter
fit.ion.chan <- jsmurf(ion.chan, param=dfil, r=1e2, confband=FALSE) # reconstruction

fit.aux <- fitted(fit.ion.chan)
fit <- fit.ion.chan

ideal.ion.chan <- fit.idealization(fit.ion.chan, t1, t2) # idealization of reconstruction

full.fit$newFitValue <- c(full.fit$newFitValue, ideal.ion.chan$newFitValue)
full.fit$value <- c(full.fit$value, ideal.ion.chan$value)
full.fit$rightEnd <- c(full.fit$rightEnd, (ideal.ion.chan$rightEnd + LL*(h-1)))
full.fit$leftEnd <- c(full.fit$leftEnd, (ideal.ion.chan$leftEnd + LL*(h-1)))
```
APPENDIX - R SCRIPTS

Gatings<-c()
Smooth<-c()
Gatings<-GatingEventDetection( full.fit , t1 , V , LL , sampling , timel , t0 ) # extract gating events from idealization
Smooth<-SmoothenIdeal( full.fit , Gatings$Gating , timel , time2 , sampling , V , t1 ) # smoothen idealization data between detected gating events

# export matrices to txt, separator is tab for easy import. no names to avoid which messes up with importIntoEnv
header<-cbind( paste("Minimum Dwell Time:" , t0 , "ms ---") , paste("Minimum Gating","c0","pS ----") , "","","","",deparse.level=0)
headerVD<-cbind("Dwell Time (s)" , "Current (pA)" , "Conductance (nS)" , "","","",deparse.level=0)
headerGating<-cbind("Time (s)" , "From (pA)" , "To (pA)" , "Diff (pA)" , "Gating (pS)" ,
                      deparse.level=0)
Gating.final<-rbind( header , headerVD , Smooth$Gating , deparse.level=0)
VD<-rbind( header , headerVD , Smooth$DwellTime , deparse.level=0)

if(SAVE){
  # export two files
  write.table( Gating.final , file = paste( dir , "/", V , "mV_" , name , "-SMUCE-gating.txt" ,
                                            sep="\t" , row.names=FALSE , col.names=FALSE , sep="\\t" , quote=FALSE)
  write.table( VD , file = paste( dir , "/", V , "mV_" , name , "-SMUCE-dwell.txt" , sep="\t" ), row.
                      names=FALSE , col.names=FALSE , sep="\\t" , quote=FALSE)
} else{ }

if(PLOT){
  # plot the original trace, reconstruction, idealization and
  # smoothened fit
  par(mfrow=c(1,1))
y.range(ion.chan)
dy=y[y>2]-y[y<1]; y=y+y+.1*dy*(-1,1) # adjusting the y-axis
plot( ion.chan , type = "p" , pch = "." , ylim=y , cex.lab=1.5 , cex.axis=1.25 , xaxt="n",
yaxt="n" , ylab="pA" , xlab="time (ms)"
mtext( 'A' , side = 3 , adj = 0 , line = 1.2 , cex = 2 , font = 2)
axis(2 , las = 1)
axis(1 , at = seq(0, LL , by = LL / 3) , labels = c( timel+(h-1)*SL , timel+SL/3+(h-1)*SL ,
          timel+2*SL/3+(h-1)*SL , timel+SL+(h-1)*SL ))
lines(1:length( fit.auxl ) , fit.auxl , type = "l" , lwd=2.5 , lty=2 , col = "green3"
lines(1:length( full.fit$newFitValue ) , full.fit$newFitValue , type = "l" , lwd=5 , lty = 3 ,
          col = "blue3"
lines(1:length( Smooth$newFit ) , Smooth$newFit , type = "l" , lwd=3.5 , lty=4 , col = "red3"
legend("bottomright" , inset = .1 , legend = c( "jsmurf-fit" , "Final Idealization",
          "Smooth Fit" ) , lty = c( 2, 3 ) , col = c( "green3" , "blue3" , "red3")
}
6.2  R: Reconstruction of .abf Using JULES

```r
# This is my default workspace from My Documents.
default_workspace <- getwd()

# First change, if you haven’t already.
setwd(getwd())
if (default_workspace) {
  load("DependencyIonChannel-111.RData")
  rm(list=ls(), default_workspace)
}
ls()

library(tools)
library(gtools)
library(abf2)
if (isNamespaceLoaded("stepR")){
  pkg <- "package:stepR"
  detach(pkg, character.only=TRUE, unload=TRUE)
} else {
  library(stepR2)
  library(dbacf)
}

time.start <- as.double(proc.time() [3])

GatingEventDetection <- function(iidealized.fit, t1, V, sampling, t0){
  idealized.fit is the output of fit.idealization, t1 is the minimum
  conductance change (in pA), V is the voltage applied in the trace, LL is
  the length of the fit-segment, sampling is the sampling frequency, timel is
  the starting time of the idealization within the real trace (like
  timel=6s if voltage change occurred at 5s) and h is the turn number, i.e.
  which fit-segment is currently processed

  # idealized.fit <- postDeconv
  # t1 <- c0
  # V<-voltage
  # sampling<-sr
  # t0

  nn <- length(iidealized.fit$value)
  if (nn>1){
    ideal.value <- idealized.fit$value[1:(nn-1)]
    ideal.value.fwd <- idealized.fit$value[2:nn]
  }
}
```

### Use as test environment

# idealized.fit <- postDeconv
# t1 <- c0
# V<-voltage
# sampling<-sr
# t0

```
cri4 <- abs( ideal.value-ideal.value.fwd ) >= t1/1000 & ideal.value.fwd*
abs(V) >= -4 & ideal.value*abs(V) >= -4 # filter out events that go
far over zero (farer than 4pA)
for (i in 1:length(cri4)){
  if(is.na(cri4[i])){
    cri4[i] = FALSE
  }
}
gatingEvents <- abs( idealized.fit$value[c(FALSE, cri4)]*V - idealized.
fit$value[c(cri4, FALSE)]*V)
conductance <- gatingEvents/abs(V)*1000
gatingTime<- idealized.fit$leftEnd[c(FALSE, cri4)]
Gating.temp <- cbind(gatingTime, V*idealized.fit$value[c(cri4, FALSE)], V
*idealized.fit$value[c(FALSE, cri4)], gatingEvents, conductance, 
deparse.level = 0)

# tidy up, check 2nd threshold (minimum dwell time)
Gating<-Gating.temp
LG<-nrow(Gating)
if (LG>=3){
  Gating.t<-Gating
  for (i in LG:4){
    if(is.finite(as.numeric(Gating.t[i]))){
      diffG <- (as.numeric(Gating[i,1]) - as.numeric(Gating[(i-1),1]))*1000
      if (diffG<0){
        Gating.t<-Gating.t[-i,]
      }
      LG.t<-nrow(Gating.t)
      if(i<LG.t+2){
        if(i>4){
          diffG.bwd<-(as.numeric(Gating[i-1,1])-as.numeric(Gating[(i-2)
,1]))*1000
          ifelse (diffG.bwd>0, Gating.t<-Gating.t[-(i-1),],"")
        } else{ ifelse (i==4,Gating.t<-Gating.t[-(i-1),])
        }
      }
    }
  }
  Gating<-Gating.t
  DwellTime <- c(Gating[,1], tail(idealized.fit$rightEnd, n=1)) - c(idealized.fit$leftEnd[1], Gating[,1])
  DwellLevelI <- c(Gating[,2], tail(Gating[,3], n=1) )
  DwellLevelC <- DwellLevelI/V
  if (LG>0){
    DwellTime <- c(Gating[,1], tail(idealized.fit$rightEnd, n=1)) - c(idealized.fit$leftEnd[1], Gating[,1])
    DwellLevelI <- c(Gating[,2], tail(Gating[,3], n=1) )
    DwellLevelC <- DwellLevelI/V
  } else{
    DwellTime <- c(Gating[,1], tail(idealized.fit$rightEnd, n=1)) - c(idealized.fit$leftEnd[1], Gating[,1])
    DwellLevelI <- c(Gating[,2], tail(Gating[,3], n=1) )
    DwellLevelC <- DwellLevelI/V
  }
6.2. R: RECONSTRUCTION OF .ABF USING JULES

```r
avgC <- sum((idealized$fit$value * (idealized$fit$rightEnd - idealized$fit$leftEnd))/ (idealized$fit$rightEnd[nn] - idealized$fit$leftEnd[1]))
DwellLevelC <- avgC
DwellLevelII <- avgC * V

}
}
}

List <- list("Gating"=Gating , "DwellTime"=FullDwellTimes)
return(List)

#---------------------------------------------------------------------
# PARAMETERS
#---------------------------------------------------------------------
sr <- 5e4  # sampling rate
cf <- 5e3 / 5e4  # cutoff factor, filter/sampling

m <- 30L
n_norm <- 5e5  # number of data points per fit segment
alpha <- 0.05  # quality parameter of fit

#---------------------------------------------------------------------
# GatingEventDetection
#---------------------------------------------------------------------
c0 <- 15  # gating threshold in pS
t0 <- 0  # dwell time threshold in ms

SingleFile <- FALSE  # fit only one file?
FileNumber <- 13  # if yes, which?
FullTrace <- FALSE  # consider full trace betw. V changes?
ManualStart <- 6  # starting point if above is FALSE
ManualEnd <- 65  # ending point if above is FALSE
PLOT <- TRUE  # plot each 10s segment?
SAVE <- TRUE  # save Gating Events and Dwell Times?

filter <- dfilter(type = "besselButter", param = list(poleBessel = 4, cutoffBessel = 0.1, R = 500, C = 3300e-12), sr = 5e4, len = 30)
kern <- filter$kern

correlations <- filter$param$sacf

files <- list.files(getwd(), "\\.abf\$", all.files=TRUE, full.names=TRUE, ignore.case=TRUE, include.dirs=TRUE, recursive=TRUE)
files <- files[mixedorder(files)]
```
```r
files
if(SingleFile){LF<-1} else{LF <- length(files)}

for(f in 1:LF){ #reverse order
    if(SingleFile){f<-FileNumber} else {

        name <- basename(files[f])
        dir <- dirname(files[f])
        nameL <- nchar(basename(files[f]))
        name <- substr(name,1,nameL-4)

        abf <- abfload(files[f])

        if(FullTrace){
            start <- ceiling(abf$tags[2,1] + 0.3)*sr
            end <- floor(abf$tags[3,1] - 0.1)*sr
        } else {
            start <- ManualStart*sr
            end <- ManualEnd*sr
        }

        startTime <- start/sr
        endTime <- end/sr

        L <- ceiling((end-start)/n_norm)
        LastStep <- (end-start)/n_norm - floor((end-start)/n_norm)
        FullTime <- abf$s
        voltage <- abf$tags[2,2]
        current <- abf$traces[1,]
        FullData <- current / voltage
        rm(abf) #remove abf again, its huge!

        GatingFinal = list()

        for(i in 1:L){ #i<-1
            time.quantiles1 <- as.double(proc.time()[3])
            if(i==L & LastStep!>0){
                n <- (LastStep*n_norm)
                time <- FullTime[start + (i-1)*n_norm + 1:n]
                data <- FullData[start + (i-1)*n_norm + 1:n]

                stat <- t(readRDS(paste(getwd(), "/dyaLen/dyaLen", 2^ceiling(log2(n)), "
                rds", sep = "")))
                q <- critVal(stat = stat, n = n, family = "mDependentPS", alpha = alpha,
                    covariances = correlations, intervalSystem = "dyaLen", output = "
                    vector")
            } else {
                stat <- t(readRDS(paste(getwd(), "/dyaLen/dyaLen", 2^ceiling(log2(n_norm)
                ), ".rds", sep = "")))
            }
        }
    }
}
```
q <- critVal(stat = stat, n = n_norm, family = "mDependentPS", alpha = alpha, covariances = correlations, intervalSystem = "dyaLen", output = "vector")

time <- FullTime[start + (i-1)*n_norm + 1:n_norm]
data <- FullData[start + (i-1)*n_norm + 1:n_norm]
}
time.quantiles <- as.double(proc.time()[3]) - time.quantiles1

time.Firstfit1 <- as.double(proc.time()[3])
estSD <- stepR::sdrobnorm(data, lag = m + 1L)

fit <- stepFit(data, x = time, family = "mDependentPS", q = q, confband = TRUE,
covariances = estSD^2 * correlations, intervalSystem = "dyaLen")
FirstFit <- fit
postIncr <- postFilterIncremental(fit, t0 = m)
time.Firstfit <- as.double(proc.time()[3]) - time.Firstfit1

# threshold to filter small jumps
# increasing sequences work better than computing just one round
time.iteration1 <- as.double(proc.time()[3])
for (i in 1:(c0/1000/0.005 -1)) {
  postFpIterative <- postFilterFalsePositiveAbsoluteValue(postIncr, data, threshold = i*0.005)
}
postFpIterativeOld <- postFpIterative
postFpIterativeNew <- postFilterFalsePositiveAbsoluteValue(postFpIterativeOld, data, threshold = (c0/1000/0.005 -1)*0.005)
counter <- 0
while (length(postFpIterativeNew$value) != length(postFpIterativeOld$value)) {
  postFpIterativeOld <- postFpIterativeNew
  postFpIterativeNew <- postFilterFalsePositiveAbsoluteValue(
    postFpIterativeOld, data, threshold = (c0/1000/0.005 -1)*0.005)
  counter <- counter + 1
}
postFp <- postFpIterativeNew
time.iteration <- as.double(proc.time()[3]) - time.iteration1

thresholdShortSegment <- 10 + m * 2
shiftStart <- m
shiftEnd <- m
messages <- TRUE
postDeconv <- deconvolveLocally(fit = postFp, data = data, x = time, filter =
filter, covariances = filter$param$sacf * estSd^2, thresholdShortSegment = thresholdShortSegment,
shiftStart = shiftStart, shiftEnd = shiftEnd, messages = TRUE)
time.deconv <- as.double(proc.time()[3]) - time.deconv
# output similar than with jsmurf: postDeconv$leftEnd; postDeconv$value;
postDeconv$rightEnd
GatingFinal.t <- c()
GatingFinal.t <- GatingEventDetection(postDeconv, c0, voltage, sr, t0)
GatingFinal$Gating <- rbind(GatingFinal$Gating, GatingFinal.tsGating,
deparse.level = 0)
GatingFinal$DwellTime <- rbind(GatingFinal$DwellTime, GatingFinal.tsDwellTime,
deparse.level = 0)
if (PLOT){
plot(time, data, type='p')
lines(FirstFit, col='blue3')
lines(postFp, col='red3')
lines(postDeconv, col='orange')
} else {}  
}
  # end of the for-loop going through the whole data set of a
  single file

GatingFinal$leftEnd <- cbind(paste("Minimum Dwell Time:", t0, "ms ----"),
paste("Minimum Gating :", c0, "pS ----"), ",", ",", ",", deparse.level = 0)

headerVD <- cbind("Dwell Time (s)", "Current (pA)", "Conductance (nS)", ",", ",", deparse.level = 0)
headerGating <- cbind("Time (s)", "From (pA)", "To (pA)", "Diff (pA)", "Gating (pS)",
"","","",deparse.level = 0)
Gating.final <- rbind(header, headerGating, GatingFinal$Gating,
deparse.level = 0)
VD <- rbind(header, headerVD, GatingFinal$DwellTime, deparse.level = 0)

if (SAVE){
write.table(Gating.final, file=paste(dir,"/","JULES-G.txt",sep=""),
row.names=FALSE, col.nam = FALSE, sep="\t",quote=FALSE)
write.table(VD, file=paste(dir,"/","mV_",name,"_",startTime,"to",
endTime,"=JULES-DT.txt",sep=""),row.names=FALSE, col.names=FALSE,
sep="\t",quote=FALSE)
} else {}  
}
  # end of the for-loop going through all abf-files

time.full <- as.double(proc.time()[3]) - time.start
time.full
6.3 R: Mean-Variance Analysis from Dwelltime-List

```r
ls()   # This is my default workspace from My
       Documents.
setwd(getwd())   # First change, if you haven't already.
rm(list=ls())   # Delete the default workspace.
ls()           

.lib <- function(name) .Internal(getRegisteredNamespace(as.name(name)))
library(gtools)
library(stats)

##########
W.base <- 100   # window length for MV plot
freq.base <- 50000
fil <- 1   # reduce number of datapoints and window length by factor <fil>

files <- list.files(.lib, pattern = "*dwell*\.txt$", all.files=TRUE, full.names=TRUE, ignore.case=TRUE, include.dirs=TRUE, recursive=TRUE)
# files <- list.files(.lib, pattern = "*DT*\.txt$", all.files=TRUE, full.names=TRUE, ignore.case=TRUE, include.dirs=TRUE, recursive=TRUE)
files <- files[orderDescending(files)]
files
LF <- length(files)
MVtot <- c()

for (f in 1:LF){
data.orig <- read.table(files[f], header=FALSE, sep="\t", skip=2L, fill=TRUE,
   strip.white=TRUE, blank.lines.skip=TRUE)
data <- data.orig
data<-data[-5]
data<-data[-4]
data<-data[-2]
data

for (i in 1:nrow(data)){
   if (data[i,1] > W){
      data[i,1] <- W
   }
}
data
```
```r
fulldata <- c()
LL <- nrow(data[1])

for (i in 1:LL){
  L <- data[i,1]
  V <- data[i,2]
  fulldata <- c(fulldata, L)
  V
  fulldata.t <- c()
  n <- 1
  while (n < (L+1)){
    n.temp <- n
    fulldata.t[n.temp] <- V
    n <- n + 1
  }

  fulldata <- append(fulldata, fulldata.t)
}

Ltotal <- length(fulldata)
LW <- Ltotal - W
MV.M <- c()
MV.V <- c()

m<1
while (m <(LW+1) ){
  mean.t <- c()
  var.t <- c()
  window <- fulldata [m:(m+W-1)]
  mean.t <- mean(window)
  var.t <- var(window)

  MV.M[m] <- mean(window)
  MV.V[m] <- var(window)
  m <- m+1
}

name.t <- basename(files[f])
dir <- dirname(files[f])
nameL <- nchar(basename(files[f]))
name <- substr(name.t,1,nameL-4)

MV<--cbind(MV.M, MV.V, deparse.level = 0)
plot(MV[,1], MV[,2], type="l")
write.table(MV, file=paste(dir,"/",name,"_MV.txt", sep=""), row.names=FALSE, col.
  names=FALSE, sep="\\t", quote=FALSE)

MVtot <- rbind(MVtot, MV, deparse.level = 0)
plot(MVtot[,1], MVtot[,2], type="l")
```

./Rscripts/MVanalysis.R
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