Protein import into yeast mitochondria
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Analysis of mutations affecting Tim17 and Tim44 function in mitochondrial protein import in *Saccharomyces cerevisiae*

Sandra van Wilpe
Ammy C. Maarse
Michiel Meijer
Analysis of mutations affecting Tim17 and Tim44 function

Abstract

A genetic screen previously resulted in the identification of a number of mutants disturbed in the import of cytosolically synthesized mitochondrial proteins of the yeast *Saccharomyces cerevisiae*. Complementation of these mutants by transformation with yeast genomic DNA libraries led to the identification of three genes encoding proteins that are involved in mitochondrial import of proteins of the matrix space. These proteins, Tim17, Tim23 and Tim44, constitute the protein translocase of the mitochondrial inner membrane (Tim). A rescreen with the same approach yielded many more *tim17* and *tim44* mutants and a few *tim23* mutants, but no other genes encoding translocator proteins were isolated. Thirteen *tim17* and nine *tim44* mutants were subjected to DNA sequence analysis to determine the nucleotide mutations and the resulting amino acid substitutions. Eleven out of thirteen *tim17* mutants contained an arginine to lysine substitution at position 105 (R105K), indicating an extreme bias towards this mutation. In a first attempt to characterize these newly isolated *tim17* mutants, we studied the effect of the mutations on the import efficiency or stability of Tim17 itself and its ability to assemble into the Tim complex, by measuring the steady-state level of mutant Tim17 protein in mitochondria by Western analysis and the stability of the Tim complex in blue-native gel electrophoresis. The steady-state level of the *tim17* R105K mutant protein was reduced to about 50% of the wild type level, which may be directly due to reduced import efficiency of this mutant protein into mitochondria. The amount of Tim complex in mitochondria of nine *tim17* R105K mutants varied from 0 to 100% of the wild type amount and correlated well with the variations in import efficiency of these mutant mitochondria. Variations may not be primarily caused by mutations in Tim17, which will be discussed.

Introduction

The first components of the translocase of the mitochondrial inner membrane (Tim) were identified several years ago with a biochemical approach [171] and a genetic screen [127]. The genetic selection procedure was based upon the mislocalization of a chimeric protein consisting of the presequence of the mitochondrial matrix protein SOD (manganese superoxide dismutase) and the coding sequence of *URA3*, a cytosolic enzyme with OMP decarboxylase activity. The chimeric protein is efficiently imported into the mitochondria of a wild type strain (carrying a deletion for *URA3*), resulting in a *ura*+ phenotype. After mutagenesis of the wild type strain carrying this construct, cells with mutations in proteins of the import pathway of the chimeric protein into the mitochondria were selected by means of their ura+ phenotype [127]. Further genetic analysis of these mutants led to the identification of three genes encoding proteins which appeared to have a role in mitochondrial protein import. These three proteins, Tim17, Tim23 and
Tim44, are components of the translocase which directs imported proteins of the matrix space across the inner membrane [45, 127, 128]. Tim17 and Tim23 are integral membrane proteins containing four predicted membrane spanning domains [45] and they are proposed to form the core of a protein translocation channel in the inner membrane. The interaction between Tim17 and Tim23 may be established by their hydrophobic domains. It is unknown if the translocation channel consists of additional components, what the structure of the channel is and by which mechanism the pore can open and close to allow the passage of preproteins. The hydrophilic N-terminus of Tim23 can form dimers that dissociate again upon the interaction with incoming preproteins [7]. This process is suggested to trigger opening of the translocation channel, thereby allowing further translocation of preproteins. Dimerization of Tim23 may therefore play a role in gating of the inner membrane translocation channel.

Tim44 is peripherally attached to the inner membrane and the bulk of the protein is localized in the matrix space [127]. Tim44 is transiently associated with a fraction of mtHsp70 and both proteins form a matrix localized translocation motor with the ability to pull proteins across the inner membrane towards the matrix space.

As mentioned, the N-terminus of Tim23 is involved in dimerization and its hydrophobic C-terminal domain is suggested to interact with Tim17 to form the translocation pore. Tim17 displays significant sequence similarity with the hydrophobic C-terminal domain of Tim23, but it lacks the hydrophilic N-terminal domain of this protein. The study of mutant Tim17 proteins and their effect on the assembly and activity of the Tim complex may provide useful clues to the role played by Tim17 in protein import.

To isolate other genes encoding proteins involved in mitochondrial protein import, we performed a second screen using the same approach that yielded the first three Tim proteins. This second screen did not result in the identification of new proteins, but provided us with a number of additional Tim17, Tim23 and Tim44 mutants. Here we describe the determination of the mutations in thirteen tim17 and nine tim44 mutants by DNA sequence analysis. The mutations in the tim17 mutants show an extreme bias, since eleven out of thirteen mutants have an arginine to lysine (R105K) substitution at position 105. By Western analysis we determined the steady-state level of the mutant Tim17 proteins in mitochondria. This indicated that the amount of the tim17 R105K mutant is about 50% of the amount of wild type Tim17. The reduced steady-state level of this mutant protein may be directly caused by its inefficient import into mitochondria, since import studies indicated that import of this mutant Tim17 protein is about 30% of the import of wild type Tim17. We studied the effect of mutant Tim17 proteins on the stability of the Tim complex by blue-native gel electrophoresis. Mitochondria isolated from nine tim17 R105K mutants contained the Tim complex in amounts that varied from 0 to 100% and these variations correlated with the import efficiency of these mutant mitochondria. Mitochondria harbouring Tim17 with the R105K substitution can have wild type amounts of the
Tim complex, suggesting that variations in the amounts of Tim complex and import efficiency may rather be the results of secondary effects than directly caused by the Tim17 mutation.

Materials and Methods

Strains and media
Escherichia coli strain TG1 (traD36lacI9Δ(lacZ)HISproA1+5B+supEΔ(hsdM-mcrB)5(λ− me− McrB+) thiΔ(lacproAB)) was used for DNA manipulations. E. coli transformants were grown in YT medium (1% (w/v) yeast extract, 1% (w/v) bactotryptone and 0.5% (w/v) NaCl) containing 150 μg/ml ampicillin. Saccharomyces cerevisiae strain MB3 (MATα ade2-101 his3-Δ200 leu2-Δ1 lys2-801 ura3::LYS2) (derivative of YP102 (MATα ade2-101 his3-Δ200 leu2-Δ1 lys2-801 ura3-52); [127]) containing a test plasmid encoding a fusion protein consisting of the presequence of the mitochondrial matrix protein SOD and the coding sequence of URA3 was mutagenized by treatment with ethyl methanesulfonate (EMS) and mutants were selected by means of their ura+ phenotype [127]. Chromosomal DNA was isolated from yeast cells grown in YPD medium (2% (w/v) glucose, 2% (w/v) bactopeptone, 1% (w/v) yeast extract) [163]. Transformation of yeast was performed according to Klebe et al. [104]. Transformants of MB26 (MATα ade2-101 trp1-289 ura3-52 his3-Δ200 leu2-Δ1 tim17::LYS2 + YCplac33::TIM17) [128] were selected on minimal media supplemented with tryptophan, histidine and adenine (20 μg/ml). Plasmid shuffling was performed by growing transformants in YPD overnight at 28°C and plating a sample of the culture on 5-fluoroorotic acid (5-FOA; [14]) containing minimal medium plates (1 g/1 5-FOA, 2% (w/v) glucose,, 0.67% (w/v) YNB) supplemented with tryptophan, histidine, adenine and uracil (50 μg/ml).

Amplification of chromosomal DNA of tim17 and tim44 mutant strains by PCR
Chromosomal DNA was isolated from thirteen tim17 mutant strains and nine tim44 mutant strains according to standard procedures [163]. Amplification of chromosomal DNA of the tim17 mutant strains by PCR was performed with the primers 5’-attgaatacagaaatcttcggg-3’ and 5’-tttatgtaacaaaaaacatgcct-3’, which generated a 778 bp fragment harbouring the entire TIM17 gene, the adjacent 223 bp upstream flanking region and the adjacent 77 bp downstream flanking region. Amplification of the chromosomal DNA of the tim44 mutant strains by PCR was accomplished with the primers 5’-cagaaacgaaatatctgaacagacc-3’ and 5’-ggtcattgcgaacgagttg-3’, yielding a 2078 bp fragment bearing the entire TIM44 gene plus the adjacent 525 bp upstream flanking region and 257 bp downstream flanking region. All DNA amplifications were performed with Pfu DNA polymerase, with proofreading properties for high fidelity amplification.

DNA sequence analysis of tim17 and tim44 mutant genes
Mutations in the tim17 and tim44 mutant alleles were determined by DNA sequence analysis of PCR fragments harbouring the TIM17 or TIM44 mutant genes as templates. For tim17 mutants, DNA sequence analysis was performed with the same primers as those used for the amplification of the TIM17 containing fragments. For tim44 mutants, DNA sequence analysis was established with the same primers to amplify the fragments harbouring TIM44 and the primers 5’-agtgggataaggggcaag-3’, 5’-atttgtcggacacctcgc-3’ and 5’-tcttgtcctcatacagcc-3’. DNA sequence analysis was performed by Eurogentec (Seraing/Belgium).

DNA manipulations
Cloning of tim17 mutant genes in YCplac111: DNA fragments containing the tim17 mutant alleles tim17-12-66 (G to R at position 34; G34R) or tim17-5-39 (G62D) were obtained by PCR and were digested with NcoI, yielding 194 bp DNA fragments with NcoI cohesive ends. Single copy vector YCplac111::TIM17(BstEII-HindIII)ΔNcoI contains the TIM17 gene which lacks this internal 194 bp NcoI fragment. The 194 bp NcoI fragment of each mutant was cloned into the unique NcoI site of TIM17 of
YCplac111::TIM17(BstEII-HindIII)\DeltaNcoI to obtain YCplac111::tim17-12-66 and YCplac111::tim17-5-39. Correct cloning and the presence of the mutations in these clones was verified by DNA sequence analysis. A DNA fragment containing the TIM17 gene of tim17-5-24 (R105K) was obtained by PCR on mutant chromosomal DNA as template with primers 5'-gcgaattcggtaacccggcattcttg-3' and 5'-gggtcgacattggtgctatc-3', yielding a 2401 bp fragment. This PCR product was cloned as an EcoRI-Sall fragment into YCplac111 digested with EcoRI and Sall to obtain YCplac111::tim17-5-24. The sequence of the cloned fragment and its correct cloning was checked by DNA sequence analysis.

Isolation of mitochondria and polyacrylamide gel electrophoresis of mitochondrial proteins
Mitochondria were isolated as described by Glick et al. [65]. Mitochondrial proteins (50 μg per lane) were separated by SDS-PAGE [116], transferred to a nitrocellulose membrane and immunodecorated with polyclonal Tim17 antibodies.

Blue-native gel electrophoresis
Mitochondria were lysed in 50 μl ice-cold digitonin buffer (1% (w/v) digitonin, 20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10% (v/v) glycerol, 1 mM PMSF) prior to the addition of sample buffer (5% (w/v) Coomassie brilliant blue G-250, 100 mM Bis-Tris, pH 7.0, 500 mM 6-aminocaproic acid). Mitochondrial protein complexes (100 μg of mitochondrial proteins per lane) were separated by blue-native electrophoresis on a 6-20% polyacrylamide gradient gel at 10°C [48]. Proteins were then transferred to a nitrocellulose membrane and subjected to immunodecoration with polyclonal Tim23 antiserum.

<table>
<thead>
<tr>
<th>tim17</th>
<th>mutation</th>
<th>substitution</th>
<th>tim44</th>
<th>mutation</th>
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<td>R105K</td>
<td>44-1-11</td>
<td>G515A</td>
<td>G172D</td>
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<td>44-2-9</td>
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Table 1. Nucleotide changes and amino acid substitutions in tim17 and tim44 mutants. Table listing the isolation numbers of the tim17 and tim44 mutants, their nucleotide changes and amino acid substitutions. Mutants are listed according to their isolation number. Numbers in the 'mutation' columns indicate the position of the nucleotide change (counting from A at the initiator ATG), numbers in the 'substitution' columns indicate the position of the amino acid substitution (counting from the initiator methionine), respectively. Amino acids are given in one-letter code.
Analysis of mutations affecting Tim17 and Tim44 function

Miscellaneous
Primers were obtained from Pharmacia or Eurogentec. *Pfu* DNA polymerase was from Stratagene and restriction and other enzymes were purchased from Gibco.

Results

DNA sequence analysis of the *tim17* and *tim44* mutants
A new screen for mitochondrial import mutants using the SOD-URA fusion protein mislocalization approach [127] resulted in a number of *tim17*, *tim23* and *tim44* mutants. For the analysis of the DNA sequence of thirteen *tim17* and nine *tim44* mutants, we generated DNA fragments harbouring the mutant genes by PCR. With chromosomal DNA isolated from the *tim17* mutants and the parent strain MB3 as a template and primers specific for the flanking regions of *TIM17*, a 778 bp PCR fragment harbouring the entire *TIM17* gene was obtained. This fragment contains a region of 223 bp which includes the sequence of the minimal promoter region of 146 bp upstream of the initiator ATG of the *TIM17* open reading frame, which is sufficient for expression of the *TIM17* gene [128]. PCR on chromosomal DNA isolated from nine *tim44* mutants with primers specific for the flanking regions of *TIM44* generated a 2078 bp fragment harbouring the entire *TIM44* gene and its upstream and downstream flanks (see Materials and Methods). DNA sequence analysis of the *tim17* and *tim44* mutants revealed that the mutants harboured only one mutation in each gene (Table 1). Of the thirteen *tim17* mutants whose DNA sequence was analyzed, eleven had the same G to A change at position 314, resulting in the amino acid substitution R to K. Two other *tim17* mutants carried a mutation at either position 100 or position 185, which resulted in the amino acid substitutions G to R and G to D, respectively. The mutations are indicated in the nucleotide sequence of *TIM17* and its deduced protein sequence shown in Figure 1.

The model of the topology of Tim17 in the inner membrane proposes that both termini of Tim17 are exposed to the intermembrane space and that the protein traverses the inner membrane four times. This would imply that the R105K mutation, in which the positively charged amino acid arginine at position 105 is substituted for another positively charged amino acid (lysine), is localized at the end of the predicted third hydrophobic domain. The G34R substitution is then located at the end of the predicted first hydrophobic domain and results in the substitution of the small uncharged amino acid glycine into the positively charged amino acid arginine. Finally, the G62D substitution results in the replacement of the neutral small amino acid glycine into the negatively charged glutamic acid, which is located at the beginning of the predicted second hydrophobic domain. All three mutations are thus localized in one of the transmembrane domains of Tim17, at positions which are close to the border of the matrix side of the inner membrane (Figure 2). Of particular surprise is the extreme bias towards the R105K class of
mutations, which is in agreement with our observation that four initially isolated \textit{tim\textsubscript{17}} mutants all carried this R to K substitution.

\begin{verbatim}
1  ATGTCAGCCGATATTGACGATGATCTATGTAAGCTACTAATT  48
1  M S A D H S R D P C P I V I L N  16
49  GATTTTCGTTGGCTTTTGCACCCTGGCTGCTTTGCTTTGCT  96
17  D F G A F A M Q A I G G Y V W  32
97  CAGGGAATTAAAGTTTATGAAATTCCACATTTAGGACGTTGCTCA  144
33  H G I K G F R N S P L G E R G S  48
145  GAGCCTATGAGCCTATGACCTCCTGACTGGCTATGGTGTAAT  192
49  G A M S A I K A R A P V L G G N  64
93  TTTGCTGGCTGGGGTTTTATTTTTTGCTCTGGCTGCTGGCG  240
65  P G V W G G L P S T F D C A V E  80
241  GCCGTTAAGAAGAGAGAAGATGCTATGACTGGCAGGGTTC  288
81  A V R K R E D P W H A I A G E  96
289  CTTCCACAGGCTGGCTTTTACGTTGAAGAGCTGGCTGTGCTGTC  336
97  F T G G A L A V R G G W R H T B  112
337  AACAGGCTATGAGCTACCTGATGGGCTTTGCTTGGGGCTTTGCTTGGCATTGGGAAGGGTGTG  384
113  N S S I T C A C L L G V I E G V  128
385  GAGCTATGTTTCAAGATATCGTCTGGCAGGCAGCAACATCGCTGCT  432
129  G L M F Q K Y A A W Q A K P H A  144
433  CCTGCGTTGCGCGAGAAGACATTTTCCTCAGGCTGAGCTGGATG  480
145  P P L P E A P S S Q P L Q A *  159
\end{verbatim}

\textbf{Figure 1.} Nucleotide sequence of the wild type \textit{TIM17} gene and deduced amino acid sequence of the Tim17 protein. DNA sequence analysis of \textit{tim\textsubscript{17}} mutant alleles revealed nucleotide changes (italic font) resulting in single amino acid substitutions (bold font) indicated at the right of the sequence. Numbers indicate positions of nucleotides and amino acids relative to the A of the initiator ATG and the initiator methionine, respectively. Amino acids are depicted in the one-letter code. The predicted four hydrophobic domains in the amino acid sequence are underlined.

\textbf{Figure 2.} Schematic representation of the topology model of Tim17 in the mitochondrial inner membrane. The black circles indicate the positions of the G34R, G62D and R105K substitutions relative to the inner membrane. IMS, intermembrane space; IM, inner membrane; MAT, matrix space.
In contrast to Tim17, we found a broader scala of mutations in the tim44 mutants analyzed. Four of the mutations, present in eight of the nine mutants, are localized close to each other in the region comprising residues 151 to 173. The fifth mutation is located more to the C-terminal end of the protein, at the position of amino acid 299 (Figure 3).

Several domains have been distinguished in the Tim44 protein sequence and which may play a role in to the function of Tim44. These include domains with a putative ATP binding site, a highly charged region, a region with limited homology to DnaJ, a hydrophobic domain, an acidic region and a potential membrane anchor region (Figure 3). However, none of the amino acid changes found in these tim44 mutants are within one of these domains.

**Figure 3.** Schematic representation of the protein sequence of Tim44. Domains with proposed specific properties are represented by grey boxes and the numbers indicate positions relative to the initiator methionine. The black circles indicate the R151S, E167K, G172D, G173R and P299R substitutions in the mutants. N, N-terminus; C, C-terminus

**Plasmid shuffling to select cells with a mutant tim17 gene in a tim17Δ background**

The tim17 mutants that were identified with the genetic screen based on the mislocalization of the chimeric SOD-URA protein, were scored as mutants with a ura+ phenotype. To investigate whether the ura+ phenotype was indeed caused by the mutation in the TIM17 gene, strain MB26 (which carries a chromosomal deletion of TIM17, but remains viable by the presence of the single copy plasmid YCplac33 (URA3 marker) harbouring the wild type TIM17 gene) was transformed with YCplac111 harbouring one of the three tim17 mutant genes. As controls, MB26 was also transformed with YCplac111 or YCplac111 containing the wild type TIM17 gene. Double transformants were selected on minimal media, transferred to YPD, grown overnight and samples of the culture were finally plated on minimal medium containing 5-fluoroorotic acid (5-FOA). Cells that harbour YCplac33 (URA3 marker) will synthesize the URA3 gene product (orotidine-5'-phosphate decarboxylase) which converts 5-FOA into the toxic compound 5-fluoro-uracil [14]. However, for cells which contain a plasmid with a functional
mutant Tim17 protein, the YCplac33::TIM17 plasmid is no longer essential and cells which have lost the plasmid are in that case still viable and able to grow on minimal medium plates containing 5-FOA. Transformants containing YCplac111 with either of the three tim17 mutants did grow on 5-FOA containing plates as well as cells harbouring wild type TIM17. Thus they can functionally replace the wild type Tim17 protein. Control MB26 cells containing only the YCplac111 vector were not able to grow on 5-FOA containing plates (data not shown). With this plasmid shuffling approach we had thus selected cells that only harbour one of the tim17 mutant genes.

**Quantitation of steady-state level of mutant Tim17 in mitochondria**

Mitochondria from cells harbouring a mutant Tim17 protein were examined for their ability to import proteins. Because the mutation may have direct or indirect effects on the import or stability of Tim17, or its ability to assemble into the Tim complex, we determined the steady-state level of Tim17 in mitochondria isolated from cells expressing the mutant proteins. Mitochondria were isolated from MB3 expressing tim17-5-39, tim17-12-66, tim17-5-24 or the wild type TIM17.

![Figure 4](image_url)

**Figure 4.** Western blot analysis to determine the steady-state level of Tim17 in mitochondria isolated from strain MB3 expressing wild type or mutant Tim17 proteins. Mitochondrial proteins (50 μg per lane) were separated on a 15% SDS-PAGE and then blotted to nitrocellulose. Immunodecoration was performed with polyclonal Tim17 antibodies.

Mitochondrial proteins were separated by SDS-PAGE and subsequently blotted to nitrocellulose. Immunodecoration with a polyclonal Tim17 antiserum showed that the amount of Tim17 in tim17-5-39 and tim17-12-66 mitochondria is not significantly reduced in comparison to wild type mitochondria (Figure 4, lanes 2-3 vs lane 1), whereas the level of Tim17 in tim17-5-24 mitochondria is reduced to ± 50% of the wild type level (Figure 4, lane 4 vs lane 1). We thus conclude that the mutations in tim17-5-39 and tim17-12-66 do not affect the import or stability of Tim17 in mitochondria, whereas the mutation in tim17-5-24 does.
Figure 5. Assembly of the Tim complex in the presence of mutant Tim17. Mitochondria were isolated from strain MB3 expressing either a wild type (lanes 1, 6 and 10) or a mutant Tim17 (lanes 2-5, 7-9 and 11-13) protein. Mitochondrial protein complexes (100 μg per lane) were separated by blue-native gel electrophoresis with a 6-20% gradient gel. After electrophoresis, proteins were blotted to a nitrocellulose membrane and subjected to immunodecoration with polyclonal anti-Tim23 serum. ‘import efficiency’, efficiency of protein import into mitochondria with a mutant Tim17 protein; ‘+’, efficient import; ‘±’, less efficient import; ‘-’, inefficient import. The quantitation of the amount of 90 kDa complex is indicated in ‘%’ (relative to wild type, which was set at 100%).

Assembly of the Tim complex in the presence of mutant Tim17

In wild type mitochondria, Tim17 and Tim23 are assembled into a complex of about 90 kDa which is stable during blue-native gel electrophoresis [46]. To investigate whether the mutant Tim17 proteins can assemble correctly and are still able to form a stable 90 kDa complex, mitochondria were isolated from strain MB3 expressing the mutant Tim17 proteins. Protein complexes were separated by blue-native gel electrophoresis [165], blotted to a nitrocellulose membrane which was immunodecorated with polyclonal anti-Tim23 antibodies. Mitochondria of strains expressing a mutant Tim17 protein were prepared in three consecutive isolations (Figure 5, lanes 2-5, 7-9 and 11-13), each time simultaneously with the isolation of mitochondria from the corresponding wild type strain (Figure 5, lanes 1, 6 and 10). The amount of the 90 kDa complex in mitochondria harbouring the tim17-5-39 mutant protein is about 30% of the amount found in wild type mitochondria (Figure 5, lane 8 vs lane 6). Mitochondria of nine different strains, expressing the Tim17 protein with the R105K mutation, contained the 90 kDa complex in amounts that varied from 0 to 100% (Figure 5, lanes 2-5, 7, 9 and 11-13). We noticed that the amount of 90 kDa complex correlated with the efficiency of protein import into these mutant
mitochondria (experimental data not shown). Variations in the amount of 90 kDa protein complex and in the efficiency of protein import of mitochondria harbouring the same mutant Tim17 protein, may be explained by the presence of mutations in other proteins as a result of the EMS mutagenesis. Varying levels of the 90 kDa complex may therefore rather be a secondary effect of these additional mutations than primarily caused by the mutations in Tim17. This is supported by the observation that the amount of 90 kDa complex in mitochondria harbouring Tim17 with the R105K mutation can be equivalent to that in wild type mitochondria (Figure 5, lane 7).

Discussion

In this chapter we report the analysis of thirteen tim17 and nine tim44 newly obtained mutants which were isolated according to a previously described genetic screen [127]. This screen did not lead to the isolation of new Tim genes, suggesting that it is either saturated or that the use of the SOD-URA chimeric protein imposes restrictions to the type of mutant that can be detected. DNA sequence analysis of PCR fragments derived from these mutants revealed the presence of three different mutations in the tim17 mutants and five different mutations in the tim44 mutants. The amino acid substitutions in Tim17 are located in three of the four proposed hydrophobic domains and are all spatially localized at the border with the matrix side of the inner membrane. Eleven out of thirteen tim17 mutants expressed Tim17 with an R to K substitution at position 105, a mutation which had previously also been found in the initially isolated four tim17 mutants (M. Meijer and A.C. Maarse, unpublished results). The extreme bias towards the occurrence of this arginine to lysine substitution in Tim17 suggests that either the type of fusion protein used in the genetic screen preferentially selects for this mutation, or that the arginine residue at position 105 is of great importance for the function of Tim17. Multiple sequence alignment of the protein sequences of the Tim17 orthologues of nine different species indicates that this arginine residue is conserved in all examined species and further underscores the importance for its role in protein import (Chapter 3, Figure 2). The amount of Tim17 in mitochondria with the tim17-5-24 (R105K) mutant protein is reduced to about 50% of the wild type level. Since the Western blot was not immunodecorated with antibodies against other mitochondrial proteins, it is possible that variations in Tim17 protein are the result of differences in the amounts of mitochondrial protein loaded on gel. DNA sequence analysis of the minimal promoter region of the TIM17 gene excluded the presence of mutations in this region, indicating that this reduction is not caused by a low level of expression of Tim17. The reduced mitochondrial amount of Tim17 may be caused by inefficient import of the mutant protein into mitochondria or by increased proteolytic breakdown of the mutant Tim17 protein. Tim17 has no cleavable mitochondrial targeting signal [128] and the region of amino acid 103 to 112 was proposed to
function as a targeting sequence (IS17, import signal 17) [97]. Since the R105K mutation is localized in this sequence, we tested the possibility whether the import of the mutant Tim17 into mitochondria was reduced. Import studies with isolated wild type mitochondria showed that the tim17-5-24 (R105K) mutant protein is imported with an efficiency of only about 30% compared to the wild type protein (data not shown). The defective import of mitochondria with the tim17 R105K mutation is therefore possibly a direct consequence of the inefficient import of this protein into mitochondria.

The glycine residue at position 62 is also conserved in all known Tim17 orthologues, whereas the glycine residue at position 34 is not always present in other organisms. Neither of the mutations, G34R and G62D, is localized in IS17 and the amount of mutated tim17 in tim17-12-66 and tim17-5-39 in mitochondria was comparable to that of wild type mitochondria. The effect of both mutations on protein import is thus not clear.

One possibility is that mutations in Tim17 may cause a reduced association with Tim23 to form the 90 kDa complex. The amount of the 90 kDa complex in mitochondria isolated from cells expressing Tim17 with the G62D mutation is about 30% compared to the amount found in wild type mitochondria. The region of Tim17 where this glycine residue is located may therefore be involved in forming the interaction with Tim23. The amount of 90 kDa complex in mitochondria containing Tim17 with the R105K mutation, varied from 0 to 100% of the wild type level. The ability of mitochondria isolated from these mutants to import proteins indicated that their import efficiency correlated well with the amount of 90 kDa complex that was present. The ability of these mitochondria to import proteins, indicates that a membrane potential Δψ across the inner membrane must be present, since import of most mitochondrial proteins is dependent on it. However, a residual membrane potential was below the detection level of the assay used to detect Δψ (data not shown). This suggests that, in addition to the R105K mutation in Tim17, these strains may also harbour mutations in proteins that directly or indirectly have a function in maintaining the membrane potential across the inner membrane. Thus, it is more likely that the varying amounts of 90 kDa complex in these mutants is primarily caused by these additional mutations.

In contrast to the tim17 mutants, up to five different amino acid substitutions occur in the tim44 mutants, four of which are clustered at a location encompassing amino acid 151 to 173. None of these substitutions is localized in one of the regions of Tim44 that were proposed to have specific properties, such as those depicted in Figure 3. DNA sequence analysis of the initially isolated tim44 mutants has revealed the presence of two other amino acid substitutions, affecting the function of Tim44 (M. Meijer and A.C. Maarse, unpublished results). Amino acid mutations in Tim44 may for instance have an effect on the interaction of Tim44 with proteins such as Tim17, Tim23 or a recently identified protein of 14 kDa which was shown to be in close contact.
to Tim44 [139]. Future experiments are necessary to establish the role in protein import of the amino acids that were found to be mutated in the \textit{tim17} and \textit{tim44} mutants.

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