Protein import into yeast mitochondria
van Wilpe, S.

Citation for published version (APA):
van Wilpe, S. (2000). Protein import into yeast mitochondria

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
GENERAL DISCUSSION
Chapter 7
The first proteins with a function in mitochondrial protein import were discovered some ten years ago. All turned out to be components of a protein complex in the mitochondrial outer membrane, a complex now designated as the translocase of the mitochondrial outer membrane (Tom). The first members of a translocase of the mitochondrial inner membrane (Tim) were identified in 1992. This complex mediates the translocation across the inner membrane of those mitochondrial preproteins that are destined for the matrix- and the intermembrane spaces. A second Tim complex, which is specialized in the translocation and membrane insertion of polytopic membrane proteins, was discovered only three years ago.

Since their discovery, much effort has been put in the investigation of the Tom and Tim proteins to learn more about their exact function in protein translocation. Components of the Tom complex have been extensively studied, which resulted in a more detailed knowledge about their function. Tom20, Tom22, Tom37 and Tom70 function as receptor proteins on the mitochondrial surface and have different preferences for binding to preproteins with N-terminal or internal mitochondrial targeting signals. Tom40 forms the translocation channel of the outer membrane (general insertion pore, GIP). Tom5 represents a link between the receptor proteins and the GIP, by accepting preproteins from the receptors and mediating their insertion into the GIP. Tom6 promotes the association of receptor proteins with the translocation channel, whereas Tom7 promotes their dissociation. Together these two Tom proteins were proposed to modulate the dynamics of the Tom complex.

Tim44 and mtHsp70 are the most extensively studied components of the Tim complex. Together, these two proteins function as an ATP-dependent translocation motor, pulling preproteins into the mitochondrial matrix space. Tim17 and Tim23 are proposed to form a pore that functions as a translocation channel for preproteins. The structure of this channel is still unknown and it is unclear how it operates. The N-terminal domain of Tim23 was shown to be involved in Tim23 dimerization. Dimer formation is promoted by the presence of a membrane potential $\Delta \Psi$. Interaction with an incoming preprotein presumably causes the dimer to dissociate, thereby triggering the opening of the Tim channel. These data provided the first information about the operating mechanism of the inner membrane translocation pore.

Work described in this thesis has contributed to a better understanding of several aspects of protein import into yeast mitochondria. To obtain better knowledge about domains of Tim17 and Tim44 that are important for their function, we analyzed how mutations in these proteins affect their role in mitochondrial protein import. The membrane topology of Tim17 and Tim23 was determined to learn more about the structure of the inner membrane translocation pore. The outer membrane receptor protein Tom22, which was hitherto assumed to be essential for cell viability, was (via a mild genetic selection procedure) shown to be dispensable in yeast. Research described in this thesis indicates that Tom22 does not just function as a receptor and binding site for preproteins, but that it also regulates the interaction of the outer membrane
Chapter 7

receptor subcomplexes with the translocation channel. In addition, it was shown that Tom22 controls the opening of the outer membrane translocation channel to regulate the passage of preproteins. Import of heterologous and hybrid subunit VII or subunit VIII proteins of the \( bc_1 \) complex into yeast mitochondria showed that the currently known characteristics of mitochondrial targeting signals are not sufficient to correctly predict the presence and location of such a signal. Combining these results with those of a complementation analysis of subunit VII or VIII deletion strains in yeast with the same heterologous and hybrid proteins indicated that complementation analysis alone is not sufficient to determine the functionality of a mitochondrial protein.

In this chapter I will attempt to put results of the research described in this thesis into perspective in terms of the consequences for our knowledge (or lack of it) about protein translocation across the mitochondrial inner and outer membrane.

Analysis of mutations affecting Tim17 and Tim44 function in mitochondrial protein import in \textit{Saccharomyces cerevisiae}

Tim23 dimer formation and dissociation is proposed to regulate the closing and opening of the Tim channel, thereby regulating the passage of preproteins across the inner membrane translocation channel. Tim17 displays significant sequence similarity with Tim23 and both proteins are associated in the same inner membrane translocator complex. Tim17 and Tim23 are essential for the viability of yeast and Tim23 cannot compensate for the loss of Tim17 and vice versa. Tim17 lacks the hydrophilic N-terminal domain that is involved in Tim23 dimerization. Until now, no additional function, apart from its function as a constituent of the translocation channel, could be attributed to Tim17. Determination of the nucleotide mutations in \textit{timl7} mutants and study of the effects of the resulting amino acid substitutions on protein import should provide information about domains that are important for the function of Tim17 in protein translocation. Chapter 2 describes the determination of mutations in thirteen \textit{timl7} mutants and the effects of the amino acid mutations on the amount of Tim17 in mitochondria and on the integrity of the Tim23-Tim17 complex. Each mutant contained a single nucleotide mutation and in total three different mutations were found, which are all located in transmembrane domains of Tim17. One of the mutant Tim17 proteins was present in reduced amounts in mitochondria compared to the wild type level. This may be due to a reduced import efficiency, because the mutation localizes to a domain that is proposed to function as the Tim17 mitochondrial targeting signal. Whether the mutant phenotype is due to a reduced amount of Tim17 in mitochondria or directly caused by the mutation remains to be established. Tim17 and Tim23 are proposed to form the translocation pore of the inner membrane. Mutations in Tim17 reduce the stability of this Tim23-Tim17 complex. This suggests that the mutations are localized in those regions of Tim17 that are involved in establishing or maintaining an interaction with
Tim23. Mitochondria of nine mutants with the same Tim17 mutation contained amounts of the Tim23-Tim17 complex varying from 0 to 100% compared to the wild type level. These mutant mitochondria were still able to import proteins, indicating that a membrane potential across the inner membrane must be present, although it could not be detected with standard assays. This low membrane potential suggests that the tim17 mutants may harbour mutations in proteins that are involved in maintaining a membrane potential across the inner membrane. The reduced amounts of the Tim23-Tim17 complex may therefore not be directly linked to the mutations in Tim17, but may be due to the presence of additional mutations in other proteins.

**Evolutionary conservation of Tom and Tim proteins**

The protein import complexes of the mitochondrial outer and inner membrane are evolutionarily conserved. Tom and Tim orthologues have now been identified in a wide range of organisms, including *Arabidopsis thaliana*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Rattus norvegicus*, *Mus musculus*, *Kluyveromyces lactis*, *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae* and *Homo sapiens* [20, 66, 107, 201]. Of all orthologues of import components identified so far, Tim17 is the most highly conserved protein. Alignment of Tim17 orthologues shows that sequence conservation extends throughout the entire protein sequence, implying that it performs an important function in protein import, as part of the protein translocation channel of the inner membrane. This function remains to be established. Determination of the functionality of orthologous Tim17 proteins in the corresponding *S. cerevisiae* deletion strain will provide more information about function and the nature of their interaction with other components of the import machinery. Tim17 of *K. lactis* and *S. pombe* are the first orthologues shown to substitute Tim17 of *S. cerevisiae*. Comparison of the protein sequences of these three yeast species with those of other Tim17 orthologues reveals that conservation of some amino acids is restricted to the yeast orthologues. These amino acids may be important for the function of Tim17 and it would be interesting to determine whether mutagenesis of these amino acids results in loss of function of the protein in *S. cerevisiae*.

Orthologues of other Tim components have also been identified, albeit in smaller numbers than for Tim17. Surprisingly, the mammalian orthologue of Tim44 is, in contrast to the peripherally membrane bound *S. cerevisiae* Tim44, partly soluble in the matrix space [201]. Recently, Tim8 was identified as a component of the Tim complex which is involved in translocation and membrane insertion of polytopic membrane proteins into the inner membrane. Tim8 is not essential for cell viability and is the yeast homologue of the human DDP1 protein (deafness dystonia peptide 1) and both proteins are localized in the intermembrane space of mitochondria. Mutations in the DDP1 protein give rise to a mitochondrial disease, leading to human deafness dystonia (Mohr-Tranebjaerg syndrome). It has been suggested that this syndrome is thus caused by defects in the mitochondrial protein import machinery. Better
knowledge of the function of Tim8 in yeast may lead to better understanding of the functioning of its counterpart in man.

**Topology and function of Tim17 and Tim23**

As mentioned earlier, Tim17 and Tim23 are, with the exception of the N-terminal domain in Tim23, highly similar to each other. Hydrophilicity plots predict the presence of four potential membrane spanning domains in the protein sequences of Tim17 and Tim23. In line with this, both proteins were shown to behave as integral membrane proteins. Protease accessibility studies had already indicated that the N- and C-terminus of Tim23 and the C-terminus of Tim17 are exposed to the intermembrane space (IMS). This has led to a topology model for Tim17 and Tim23 in which both proteins span the inner membrane four times with their N- and C-termini protruding into the IMS. Protease accessibility experiments with epitope-tagged Tim17 and Tim23 showed that the proteins are embedded in the inner membrane exactly as predicted. Tim17 and Tim23 are proposed to form, perhaps in cooperation with yet unidentified Tim components, the core of a protein translocation channel of the inner membrane. As mentioned earlier, there are indications that Tim23 is not just a passive component of this translocation channel, but that it is also involved in gating of the pore. For Tim17, however, no other role could yet be assigned to the protein than as a structural component of the translocation pore.

**The role of Tom22 in protein import and the 'acid chain'**

Preproteins are imported into mitochondria by the consecutive action of chaperones in the cytosol, receptors on the mitochondrial surface, the Tom complex and the Tim proteins. Many proteins functioning in this mitochondrial preprotein import pathway contain clusters of negatively charged amino acid residues. The cytosolic chaperone Mft52, the import receptor protein Tom22, Tom20, Tom5 and Tom40 of the outer membrane translocation channel and the inner membrane protein Tim23 all contain patches of negative charge in their protein sequences. It has been proposed that these proteins are components of an 'acid chain' mechanism, in which the translocation of preproteins across the mitochondrial membranes is driven by the binding of the positively charged mitochondrial presequence to a series of strategically positioned acidic binding sites of increasing acidity. During the import process, the presequence is proposed to interact with a negatively charged region of one translocator protein and to be transferred to a domain with a higher negative charge present on a translocator protein further downstream in the import route.

The import receptor protein Tom22 contains a cytosolically exposed N-terminal domain with a net negative charge and is the only receptor protein with a negatively charged C-terminal domain protruding into the IMS. Tom22 is therefore thought to play a central role in the
translocation of preproteins and is proposed to be an important link of the 'acid chain'. Results either supporting or opposing the function of the negatively charged IMS localized domain of Tom22 as a trans binding site for preproteins and its role in the 'acid chain' were reported [17, 39, 110, 137, 143]. The proposed presequence binding domains of Tom22 on both sides of the outer membrane may explain why Tom22 was thought to be the only import receptor essential for the viability of yeast.

As presented in Chapter 5, a tom22Δ mutant is still able to import proteins into its mitochondria, albeit with a much lower efficiency compared to the corresponding wild type strain. This low level of import is sufficient to allow viability of mutant cells on fermentable carbon sources. Since earlier work showed that Tom22 was essential for cell viability [83, 122], the simplest explanation of these observations is that the tom22Δ mutant contains an extragenic suppressor mutation. Our results make this unlikely, since a back-cross of the mutant with the corresponding wild type strain indicated that viability of the tom22Δ strain could not be explained by the generation of extragenic suppressor mutations. Another explanation is that the function of Tom22 is of such great importance that mitochondria contain other proteins performing similar functions. However, no proteins with significant sequence similarity to Tom22 were found in Saccharomyces cerevisiae. The protein composition of tom22Δ mitochondria was comparable to that of wild type mitochondria, indicating that viability of the tom22Δ strain was not due to a higher level expression of proteins known to function in protein import. Whether a yet unidentified Tom component may perform a similar function as Tom22 and substitutes for this protein in tom22Δ mitochondria, remains to be investigated.

The role of Tom22 in protein translocation is not restricted to that of preprotein receptor on the outer membrane surface. Different roles are attributed to the three domains of Tom22. The cytosolic domain specifically recognizes preproteins in the cytosol and functions as a docking point for the receptors Tom20 and Tom70. The IMS domain of Tom22 functions as a trans binding site for preproteins. The Tom22 membrane anchor coordinates the interaction of the receptor complexes with the general insertion pore (GIP) consisting of Tom40, Tom5, Tom6 and Tom7. Absence of this anchor (in tom22Δ cells) results in the dissociation of GIP complex into Tom40 dimers, which probably represent single translocation channels. Tom22 is proposed to tightly coordinate the opening of these channels. In wild type mitochondria, this channel is only open in the presence of precursor proteins, whereas in tom22Δ mitochondria it is already open in the absence of preproteins. Tom22 is therefore suggested to be a central organizer of the outer membrane translocase and it would be interesting to know how cells can survive in the absence of Tom22. As mentioned earlier, tom22Δ mitochondria did not contain elevated levels of proteins known to have a function in protein import. In addition, high copy expression of the known Tom proteins did not alleviate the mutant growth phenotype of the tom22Δ strain. Some of the questions that remain to be answered are whether other (yet unidentified) proteins can
substitute for the loss of Tom22 and which alternative mechanisms are applied by cells that lack Tom22 to still be able to import proteins into their mitochondria.

The mitochondrial targeting signal
Several computational programs have been designed to predict the presence and location of a mitochondrial targeting signal within a protein sequence. These programs rely on common features of N-terminally located targeting signals, including amphiphilicity and the ability to form an α-helix, the presence of predominantly positively charged amino acids and the absence of acidic amino acids. However, due to the lack of a consensus sequence, these programs sometimes fail to notice the presence of a targeting signal, or they do not correctly predict the location of such a signal. This is illustrated in Chapter 6, where computational programs have been used to predict the presence and location of a mitochondrial targeting signal in subunit VII and subunit VIII of the bc1 complex in *S. cerevisiae*. Subunit VII was predicted to contain an N-terminal targeting signal (amino acids 1-18), whereas subunit VIII was predicted to have an internal targeting signal (amino acids 34-51). Replacing the N-terminus of the subunit VIII orthologue from bovine heart, which was unable to direct the import of this orthologue into yeast mitochondria, with the N-terminal domain (amino acids 1-26) of *S. cerevisiae* subunit VIII, resulted in a hybrid protein that could be imported into yeast mitochondria in an *in vitro* system. This indicates that subunit VIII contains an N-terminal mitochondrial targeting signal which is capable of directing proteins to mitochondria, but which was not identified by the prediction programs. These results illustrate the complexity of mitochondrial targeting signals and the limitations of the prediction programs, and show that better definition of the features of mitochondrial targeting signals could improve the reliability of predictions made by these computational programs.

The *in vitro* import experiments with hybrid and heterologous subunit VII and subunit VIII proteins indicated that some orthologous proteins are not imported into yeast mitochondria, which may be due to lack of recognition of their targeting signal by the import apparatus of yeast. Exchanging the N-terminus of the subunit VIII orthologue from bovine heart with that from yeast subunit VIII gave rise to a protein that was (partially) functional in *S. cerevisiae*. Functionality of such a protein is determined by complementation analysis, a test which is commonly used to determine the functionality of orthologues of *S. cerevisiae* proteins. However, this analysis does not account for the fact that lack of complementation may be caused by inefficient import of the orthologous proteins into yeast mitochondria. Combining the results of the complementation analysis and the *in vitro* import experiments indeed showed that lack of functional complementation of subunit VIII from bovine heart was due to lack of import into yeast mitochondria. These results indicate that lack of functional complementation of a mitochondrial protein does not provide conclusive evidence about the functionality of this
protein in yeast. Furthermore, analysis of the import of heterologous proteins into isolated yeast mitochondria can provide additional information about the functionality of these proteins.
Chapter 7