Protein import into yeast mitochondria

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Topological organization of Tim17 and Tim23 of the Tim complex of *Saccharomyces cerevisiae*

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Abstract

We have investigated the membrane topology of Tim17 and Tim23, two subunits of a complex for import of preproteins, which is located in the mitochondrial inner membrane from *Saccharomyces cerevisiae*. The amino acid sequences of Tim17 and Tim23 are similar, with exception of the N-terminal extension of Tim23 that is absent from in Tim17. In previous studies it was shown that the amino-terminus and the carboxy-terminus of Tim23, and the carboxy-terminus of Tim17 protrude into the intermembrane space (IMS), while hydrophilicity plots indicated that both proteins contain four membrane spanning domains. These data suggested a topology model where Tim17 and Tim23 contain two matrix exposed loops and one IMS exposed loop.

We have tested this model by determining the sensitivity to proteinase K of *c-myc* or HA epitope tagged proteins after their import and assembly into the mitochondrial inner membrane. Our results confirm that Tim23 spans the mitochondrial inner membrane four times and that the previously proposed topology model is correct [45].

Introduction

Most mitochondrial proteins are encoded by the nuclear genome and synthesized in the cytosol. They are often synthesized with an N-terminal targeting signal that directs these preproteins to the mitochondria. Mitochondria contain several multisubunit protein complexes for import of preproteins into the organelle. One of them is the *t*ranslocase of the mitochondrial *o*uter *m*embrane, the Tom complex, which is involved in the recognition of preproteins in the cytosol and their translocation across the outer membrane. The Tom complex consists of at least nine subunits, whose functions can be divided into proteins that have a receptor function and proteins that constitute the translocation channel, the general insertion pore (GIP) (see e.g. review [162]). Another complex is the Tim54-Tim22 complex (Tim, *t*ranslocase of the mitochondrial *i*nner *m*embrane) that, together with a number of components of the intermembrane space, constitutes a system for import and membrane insertion of members of the carrier family and other multipass inner membrane proteins into the inner membrane [101, 106-108, 186]. A third complex is the Tim17-Tim23 complex, which consists of Tim17, Tim23, Tim44 and mtHsp70 (mitochondrial Hsp70), and which is involved in the translocation across the inner membrane of imported proteins of the matrix space [45, 54, 127, 128, 154]. Current views suggest that the translocation channel of this import system is constituted of the integral membrane proteins Tim17 and Tim23, which cooperate with the ATP-dependent, matrix localized translocation motor, consisting of peripheral inner membrane protein Tim44 and mtHsp70.

Hydrophilicity plots predict four hydrophobic regions in Tim17 and Tim23, suggesting that both polypeptides traverse the inner membrane four times [45]. Previous protease accessibility experiments showed that the C-terminus of Tim17 and both the N- and C-terminus of Tim23 are exposed to the intermembrane space (IMS) side [7, 18, 41, 46, 75, 111, 160]. These results suggested a model for the topology of Tim17 and Tim23 in which their N- and C-termini protrude into the intermembrane space, while they span the inner membrane four times, resulting in proteins with two matrix localized loops and one IMS localized loop (Figure 2). No direct evidence has been obtained which provided further support for this topology model of Tim17 and Tim23. In this study we present data which indicate that the proposed anchoring of Tim23 in the inner membrane is correct.

Materials and Methods

Strains and media

Escherichia coli strain JF 1754 (*lac, gal, metB, leuB, hisB, hsdR*) was used for DNA manipulations. *E. coli* transformants were grown in YT medium (1.6% (w/v) bactotryptone, 1% (w/v) yeast extract and 0.5% (w/v) NaCl) containing 200 µg/ml ampicillin. *Saccharomyces cerevisiae* strain MB26 (*MATa ade2-101 trp1-289 ura3-52 his3-Δ200 tim17::LYS2 leu2-Δ1* + YCplac33::*TIM17*) [128] and *S. cerevisiae* strain MB29 (*MATa ade2-101 trp1-289 ura3-52 his3-Δ200 tim23::LYS2 leu2-Δ1* + YCplac33::*TIM23*) [45] were used for the transformation of plasmids harbouring tagged Tim genes. Transformation of yeast was performed according to Klebe *et al.* [104]. Double transformants were selected on minimal media containing 0.67% (w/v) yeast nitrogen base (Difco), 2% (w/v) glucose, 2% (w/v) agar supplemented with adenine, tryptophan and histidine (20 µg/ml). Plasmid shuffling was performed by growing double transformants overnight in YPD medium (2% (w/v) glucose, 1% (w/v) yeast extract, 2% (w/v) bactopeptone) and plating cells on solid minimal medium containing 1 g/l 5-fluoroorotic acid (5-FOA [14]), 0.67% (w/v) yeast nitrogen base (Difco) and 2% (w/v) glucose, supplemented with adenine, tryptophan, histidine and uracil (50 µg/ml).

DNA manipulations

*Eco*RI restriction sites were introduced into the open reading frames of *TIM17* and *TIM23* for subsequent introduction of the sequence encoding a *c-myc* or HA epitope tag. The *Eco*RI restriction sites were generated in frame at positions of the coding sequences that were predicted to produce the IMS or matrix localized loops. A 1889 bp *Bam*HI-*Sal*I fragment carrying the *TIM17* gene, or a 1818 bp *Sph*I fragment carrying the *TIM23* gene were cloned into pSelect-1 (with a deleted *Eco*RI site) and site-directed mutagenesis using the Altered Sites *in vitro* mutagenesis system (Promega) was performed to introduce *Eco*RI restriction sites with the oligonucleotides listed in Table 1. Complementary *c-myc* or HA-oligonucleotides (Table 1) were annealed to form double stranded DNA encoding the *c-myc* epitope (EQKLISEEDLN) or the HA epitope (YPYDVPDYAL) flanked by the cohesive ends of *Eco*RI restriction sites. The double stranded fragments were phosphorylated with T4 polynucleotide kinase and cloned into the *Eco*RI sites of the modified *TIM17* and *TIM23*. Finally, the *Tim17-cmyc* and *Tim17-HA* mutants were subcloned as a *Bam*HI-*Sal*I fragment, the *Tim23-cmyc* and *Tim23-HA* mutants as a *Sph*I fragment into single copy vector YCplac111 and multi-copy vector YEplac181.

Isolation of mitochondria and gel electrophoresis

Mitochondria were isolated according to Glick *et al.* [65] from MB29 cells grown in rich glycerol medium (3% (v/v) glycerol, 1% (w/v) yeast extract, 2% (w/v) bactopeptone) and harvested at an

OD_{595nm} of 3. Protein concentrations were determined according to Bradford *et al.* [24]. Mitochondrial proteins were separated by SDS-PAGE [116], transferred to nitrocellulose membranes and subjected to immunodecoration with monoclonal antibodies against the *c-myc* tag and HA tag, and with polyclonal antibodies against AAC, $F_1\beta$ and Tim44.

no	*purpose*	*mutant*	*sequence*
1	*c-myc* coding strand		5'-aattcggaacaaaaactttatttctgaagaagatctgaatg-3'
2	*c-myc* non-coding strand		5'-aattcattcagatcttcttcagaaataagtttttgttccg-3'
3	HA coding strand		5'-aattcttacccatacgacgtcccagactacgctttg-3'
4	HA non-coding strand		5'-aattcaaagcgtagtctgggacgtcgtatgggtaag-3'.
5	*Eco*RI site, between aa 1 and 2	*Tim17-2*	5'-gtacacgggagcgttatgaattcgtcagccgatcattcg-3'
6	*Eco*RI site, between aa 44 and 45	*Tim17-5*	5'-gaaattcgccattagggaattctgagcgtggttcaggagc-3'
7	*Eco*RI site, between aa 83 and 84	*Tim17-6*	5'-gcgctgtgaaggccgttaggaattcaaagagagaggaccc-3'
8	*Eco*RI site, between aa 111 and 112	*Tim17-7*	5'-ggtggttggaggcatacgaattcaaggaacagttcgatcacc-3'
9	*Eco*RI site, between aa 130 and 131	*Tim23-4*	5'-gcccaatagtcccgggaattcaaaattgcaattgaac-3'
10	*Eco*RI site, between aa 170 and 171	*Tim23-5*	5'-caatagatgcactaaggaattcaggcaaacatgacac-3'
11	*Eco*RI site, between aa 192 and 193	*Tim23-6*	5'-ctttgttcaagtcttcgaattcaaaaggtttgaaacc-3'

Table 1. Sequence of oligonucleotides used in this study Oligonucleotides no. 1-4 were used to introduce the sequence of a *c-myc* or HA epitope tag into the coding sequences of *TIM17* and *TIM23*. Oligonucleotides no. 5-8 were used to introduce *Eco*RI restriction sites in the coding sequence of *TIM17*. Oligonucleotides no. 9-11 were used to introduce *Eco*RI restriction sites in the coding sequence of *TIM23*. Introduction of *Eco*RI restriction sites in the coding sequence of *TIM17* and *TIM23* results in the insertion of two amino acids (N and S) at the amino acid (aa) position indicated by the numbers in the 'purpose' column (startcodon is numbered '1').

Protease accessibility experiments

Isolated mitochondria were resuspended in SEM buffer (250 mM saccharose, 1 mM EDTA, 10 mM MOPS pH 7.4) and converted to mitoplasts (mitochondria with a ruptured outer membrane) by hypotonic swelling after addition of 9 volumes EM buffer (1 mM EDTA, 10 mM MOPS pH 7.4) for 10 min. at 0°C. Mitochondria and mitoplasts were reisolated by centrifugation and resuspended in SEM buffer in the absence or presence of 100 µg/ml proteinase K and 1% (w/v) Triton X-100 for 30 min. at 0°C. The proteinase K reaction was stopped by adding PMSF to a final concentration of 5 mM and mitochondria and mitoplasts were again reisolated by centrifugation. Proteins from samples treated with Triton X-100 were collected by TCA-precipitation (final concentration 10% (w/v) TCA).

DNA sequence analysis

Proper insertion of the *Eco*RI restriction site, the *c-myc* and HA epitope tags into the open reading frames of *TIM17* and *TIM23* was verified by DNA sequence analysis according to Sanger *et al.* [164] to determine the orientation, in frame cloning and insertion of just one single restriction site/epitope tag.

Miscellaneous

Monoclonal anti-*c-myc* antibodies were purchased from Cambridge Research Biochemicals. Anti-HA monoclonal antibodies (12CA5) were obtained from Boehringer. Restriction and other enzymes were from Biolabs, Gibco or Boehringer and oligonucleotides were from Pharmacia. The HRP color

development and the ECL detection kits were purchased from Bio-Rad and Amersham Pharmacia Biotech, respectively.

Results

Topology model for Tim17 and Tim23

Multiple sequence alignment of the protein sequences of Tim17 and Tim23 shows that the proteins share similarity over their entire sequence, with the exception of the N-terminal region of Tim23 which is not present in Tim17 (Figure 1a).

a

```
tim17  ..........  ..........  ..........  ..........  .....  0
tim23  MSWLFGDKTP  TDDANAAVGG  QDTTKPKELS  LKQSLGFEPN  INNII  45

tim17  ..........  ..........  ..........  ...M■ADH■R  DPC■I  12
tim23  SGPGGMHVDT  ARLHPLAGLD  KGVEYLDLEE  EQLS■LEG■Q  GLI■S  90

tim17  VILN■.....  F■GAFAM■AI  G■VVWHGIK■  FR■S■LGER■  ....S  48
tim23  RGWT■DLCYG  T■AVYLL■LG  I■GFSGMMQ■  LQ■I■PNSP■  KLQLN  135

tim17  GAMSA■KA■A  ■V■■G■F■VW  GGLFSTFDCA  VK■V■KRE■P  WNA■I  93
tim23  TVLNH■TK■G  ■F■■N■A■IL  ALSYNIINST  ID■L■GKH■T  AGS■G  180

tim17  ■■FF■■GALA  VRG■WRHTRN  ■■ITC■CLLG  ■IEG■GL.MF  QRYAA  137
tim23  ■■AL■■ALFK  SSK■LKPMGY  ■■AMV■AACA  ■WCS■KKRLL  EK*..  222

tim17  WQAKPMAPPL  PEAPSSQPLQ  A*  158
tim23  ..........  ..........  ..  222
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b

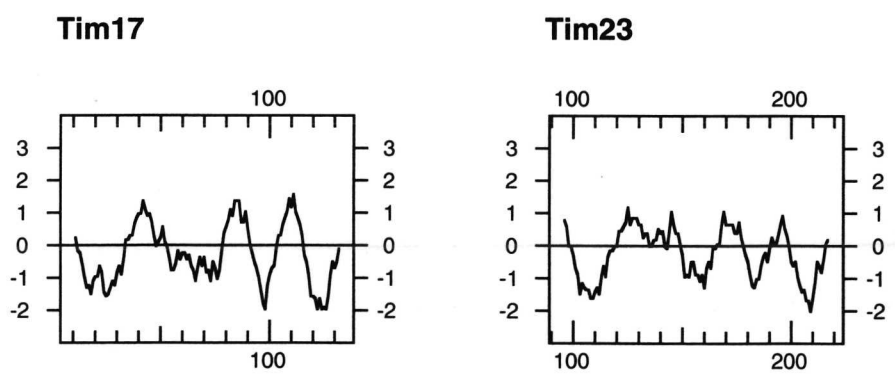

Figure 1. Multiple sequence alignment and hydrophilicity plots of the protein sequences of Tim17 and Tim23. **a,** Alignment of the amino acid sequences of Tim17 and Tim23 using the GCG PileUp program (gap weight 3.00; gap length weight 0.10) [56]. Conserved amino acids are indicated by a black box, similar amino acids are indicated by a grey box. Gaps are indicated (.). **b,** Hydrophilicity plots of Tim17 and Tim23 were created using the algorithm of Kyte and Doolittle [114] with a window size of 12. Negative values predict hydrophobic domains. Numbers correspond to the amino acid positions in the protein sequences.

Their hydrophilicity plots are also similar and display the presence of four potential hydrophobic domains that could function as transmembrane segments (Figure 1b), implying that both proteins may span the mitochondrial inner membrane four times.

The N- and C-terminus of Tim23 and the C-terminus of Tim17 are oriented towards the intermembrane space (IMS) side of the inner membrane [7, 41, 111, 160]. The finding that antibodies raised against the N-terminus of Tim23 can no longer detect Tim23 in mitoplasts treated with trypsin, also suggests that its N-terminal region protrudes into the intermembrane space [45]. The evidence for the localization of the N- and C-termini of Tim17 and Tim23, and the prediction of four hydrophobic domains in both proteins, suggested a topology model where the two proteins are embedded in the inner membrane as shown in Figure 2.

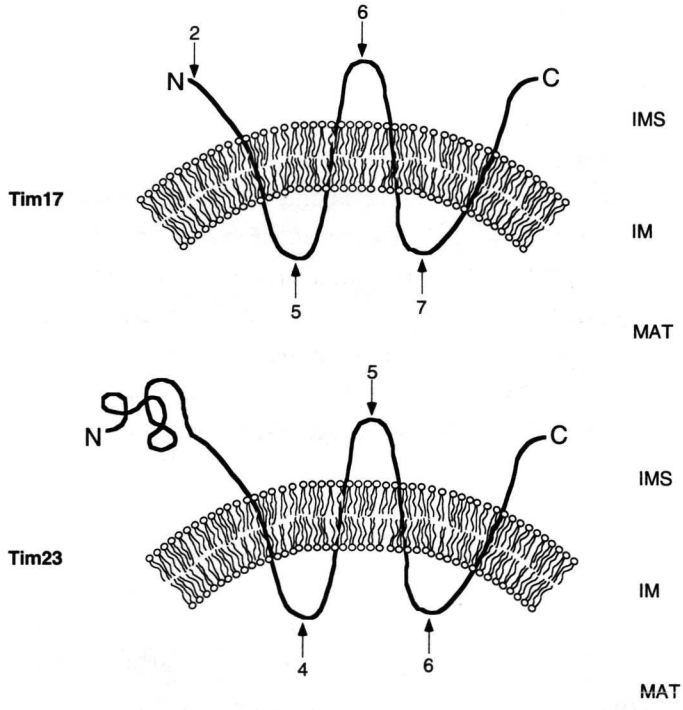

Figure 2. Model for the topology of Tim17 and Tim23 in the mitochondrial inner membrane. The N- and C-termini of both proteins protrude into the intermembrane space and both proteins span the mitochondrial inner membrane four times. The arrows indicate the positions were *c-myc* or HA epitope tags were inserted and the numbers refer to the corresponding *Tim17* and *Tim23* mutants listed in Table 1. IMS, intermembrane space; IM, inner membrane; MAT, matrix space.

To confirm this model, *c-myc* and HA epitope tags were introduced in Tim17 and Tim23 at positions indicated by the arrows in Figure 2. When introduction of these epitope tags does not interfere with the function of the proteins, we may assume that the mutant proteins are correctly

inserted into the inner membrane and that they have adopted their authentic topology. By determining the accessibility of the epitope tags of mutant proteins to externally added protease in mitoplasts, we have made an attempt to establish the membrane topology of Tim17 and Tim23.

Complementation test for functionality of epitope-tagged Tim17 and Tim23 proteins

In a first approach to determine the topology of Tim17 and Tim23, a *c-myc* epitope tag was inserted at the indicated positions in Figure 2. To test whether Tim17 and Tim23 with this tag can still function in mitochondria, a complementation assay was performed with a yeast strain which can be cured of either the wild type *TIM17* or the *TIM23* gene. The tester strains MB26 and MB29 contain respectively a disrupted *TIM17* or *TIM23* gene but are viable by virtue of the presence of single copy vector YCplac33 (*URA3* marker) containing the wild type *TIM17* (MB26)or *TIM23* (MB29) gene [45, 128]. MB26 and MB29 were transformed with YCplac111 harbouring Tim17 or Tim23 with a *c-myc* epitope insertion. As negative and positive controls, we also transformed the tester strains with YCplac111 and YCplac111 containing the wild type *TIM17* or *TIM23* gene. Instead of using YCplac111 as the plasmid vector we performed a similar set of transformations with the multi-copy vector YEplac181 as the plasmid encoding the tagged proteins. Double transformants were selected on minimal media, transferred to YPD, grown overnight and samples were finally plated on media containing 5-fluoroorotic acid (5-FOA). Cells that harbour YCplac33 (*URA3* marker) will synthesize the *URA3* gene product (orotidine-5'-phosphate decarboxylase [14]) and convert 5-FOA into 5-fluoro-uracil, which is toxic and kills the cells. However, for cells which contain the *LEU2*-marked plasmids YCplac111 or YEplac181 with a functional *c-myc* tagged protein, the YCplac33 plasmid is no longer essential. Cells which have lost the YCplac33 plasmid are in that case viable and able to grow on minimal medium plates containing 5-FOA (plasmid shuffling).

Table 2 shows that cells producing mutant proteins bearing a *c-myc* tag in a predicted IMS localized loop (*Tim17-6*, *Tim23-5*) or with a *c-myc* tag at the N-terminus (also IMS localized; *Tim17-2*) can grow in the absence of a wild type gene, indicating that these mutant proteins were functional. However, cells with mutant proteins with the *c-myc* tag in a predicted matrix localized loop (*Tim17-5*, *Tim17-7*, *Tim23-4*, *Tim23-6*) cannot grow on 5-FOA containing plates, indicating that these mutant proteins are not able to complement their corresponding deletion strains. Cells transformed with the multi-copy plasmid YEplac181 encoding these *c-myc* tagged proteins did not show growth on 5-FOA containing plates, indicating that even elevated expression of these proteins does not lead to complementation of the deletions strains. Cells transformed with YCplac111 or YEplac181 did not show any growth, indicating that these vectors cannot complement the deletion strains.

Inspection of the amino acid sequence of the *c-myc* epitope tag revealed four negatively charged amino acid residues, three glutamic acids and one aspartic acid. Mitochondrially and nuclearly encoded inner membrane proteins are biased in the distribution of positively charged amino acids within segments of the proteins facing both sides of the membrane. Positively charged residues are more frequently found in segments oriented towards the matrix space side than in segments facing the intermembrane space side ('positive-inside rule') [61].

mutant	YCplac111	YEplac181
	complementation	
Tim17-2-cmyc	+	+
Tim17-5-cmyc	-	-
Tim17-6-cmyc	+	+
Tim17-7-cmyc	-	-
Tim23-4-cmyc	-	-
Tim23-5-cmyc	+	+
Tim23-6-cmyc	-	-
TIM17	+	+
TIM23	+	+
YCplac111	-	
YEplac181		-

Table 2. Complementation analysis after plasmid shuffling in strain MB26 or strain MB29 with plasmid encoded Tim17-*cmyc* or Tim23-*cmyc* tagged proteins. Tim17 or Tim23 plasmid shuffling strains MB26 and MB29 were transformed with single copy (YCplac111) or multi-copy (YEplac181) plasmids containing the genes encoding *Tim17-cmyc or Tim23-cmyc* tagged proteins. Double transformants were selected on minimal media, then grown overnight in YPD, and samples were subsequently plated on 5-FOA containing minimal media. Growth at 23°C was monitored for several days and compared to growth of cells transformed with control plasmids harbouring wild type *TIM17* or wild type *TIM23* (normal growth), or YCplac111 or YEplac181 (no growth). Growth and functional complementation are indicated by '+', no growth and lack of functional complementation are indicated by '-'.

Even though this bias is more pronounced for mitochondrially encoded proteins, it is also observed for nuclearly encoded proteins. Probably even more important is the finding that the latter protein group displays an extremely biased distribution of glutamic acid residues, which are found ten times more at the intermembrane space side than at the matrix space side of the inner membrane [61]. This suggests that introduction of the *c-myc* tag in the predicted matrix localized loops of both Tim proteins hampers the correct translocation of these loops across the inner membrane, resulting in a non-functional protein.

To circumvent this problem we replaced all *c-myc* epitopes (except in mutant *Tim17-7*) by an HA epitope tag which lacks glutamic acid residues [146]. Table 3 shows that proteins with an HA tag inserted in the predicted amino-terminal matrix localized loop of either Tim17 or Tim23, and in the carboxy-terminal matrix facing loop of Tim23, are all able to complement the corresponding null mutants (*Tim17-5*, *Tim23-4*, *Tim23-6*). Cells transformed with multi-copy

plasmids encoding these mutant proteins could, as expected, also complement the deletion strains, whereas cells transformed with YCplac111 or YEplac181 could not. The results of the complementation analysis of Tim23 proteins furnished with an HA tag in the *tim23* null strain MB29 are illustrated in Figure 3. The *c-myc* tag in mutant *Tim17-7* was not replaced by an HA tag, since we found that the insertion of two amino acids due to the introduction of the *Eco*RI restriction site at this position already yielded a non-functional protein (data not shown). Insertion of amino acids in the carboxy-terminal matrix-localized loop of Tim17 is apparently not compatible with functionality of this protein. For the other Tim17 and Tim23 tagged proteins, we believe that these results are in agreement with the positive inside rule.

mutant	YCplac111	YEplac181
	complementation	
Tim17-2-HA	+	+
Tim17-5-HA	+	+
Tim17-6-HA	+	+
Tim17-7-HA	nd	nd
Tim23-4-HA	+	+
Tim23-5-HA	+	+
Tim23-6-HA	+	+
TIM17	+	+
TIM23	+	+
YCplac111	-	
YEplac181		-

Table 3. Complementation analysis of plasmid encoded *Tim17* or *Tim23* HA tagged proteins after plasmid shuffling in strain MB26 or strain MB29. Tim17 or Tim23 plasmid shuffling strains MB26 and MB29 were transformed with single copy (YCplac111) and multi-copy (YEplac181) plasmids containing the genes encoding *Tim17-HA* or *Tim23-HA* tagged proteins. For further experimental details of the shuffling assay, see legend to Table 2. Growth and functional complementation are indicated by '+', no growth and lack of functional complementation are indicated by '-'; nd, not determined.

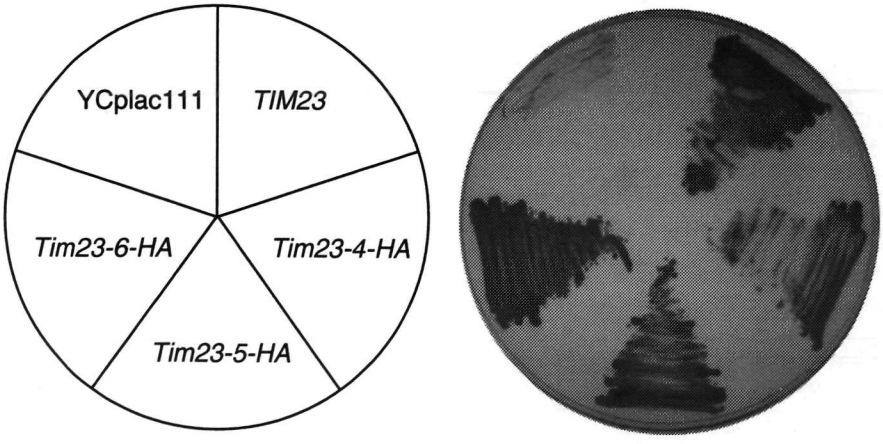

Figure 3. Complementation analysis of Tim23 proteins furnished with an HA tag in the *tim23* null strain MB29. The positions of the HA tags in Tim23 are as indicated in Figure 2. Growth analysis on 5-FOA containing plates of *tim23* null strain MB29 transformed with either YCplac111, YCplac111::*TIM23*, YCplac111::*Tim23-4-HA*, YCplac111::*Tim23-5-HA* or YCplac111::*Tim23-6-HA*. For further experimental details of the shuffling assay, see legend to Table 2. Right panel, growth on 5-FOA containing minimal medium plate of MB29 cells producing the mutant proteins which are indicated in the left panel.

Analysis of the membrane topology of epitope-tagged Tim17 and Tim23

To investigate the membrane topology of Tim17 and Tim23, mitochondria were isolated from the *tim17* or *tim23* null strains harbouring a plasmid encoding a functional Tim17 or Tim23 mutant protein. Mitochondria were isolated from *tim17* null strain MB26 expressing either *Tim17-2*-cmyc or Tim17-*6-cmyc* and from *tim23* null strain MB29 producing either *Tim23-5-cmyc*, *Tim23-4-HA* or *Tim23-5-HA* and mitochondria were subjected to protease accessibility experiments. Mitochondria were converted to mitoplasts by osmotic swelling and the accessibility of the tags to externally added proteinase K was determined (Figure 4, *c-myc* tagged proteins; Figure 5, HA tagged proteins).

Figure 4b shows that mitochondria were isolated with an intact outer membrane, since treatment with proteinase K did not lead to degradation of the IMS exposed domains of the inner membrane protein ADP/ATP carrier (AAC, lane 2). When mitochondria were converted to mitoplasts and then subjected to proteinase K treatment, the *c-myc* signal of *Tim17-2-cmyc*, *Tim17-6-cmyc* and *Tim23-5-cmyc* was lost, indicating that all three *c-myc* epitopes were accessible to proteinase K and therefore exposed to the intermembrane space (Figure 4a, lanes 4). As a control for the integrity of the inner membrane of the mitoplasts, we monitored a decrease of the amount of intact AAC during proteinase K treatment, while the matrix exposed proteins Tim44 and $F_1\beta$ were still protected against proteolysis (Figure 4b, lanes 4). As a control on the intrinsic proteinase K sensitivity of these proteins, Triton X-100 was added to solubilize the membranes, which made all proteins accessible to the protease (Figure 4b, lanes 5).

Figure 5 shows that incubating mitoplasts derived from cells expressing *Tim23-4-HA* with proteinase K generated a proteolytic fragment of ±18 kDa that can be detected with anti-HA antibodies (Figure 5a, lane 4). The appearance of this proteolytic fragment is consistent with the size of a Tim23 fragment of 16 kDa which is generated after protease treatment of mitoplasts of yeast cells expressing the wild type protein and represents a truncated Tim23 from which the IMS-localized N-terminal part is removed [18, 46, 75, 112, 160]. The size of the 18 kDa proteolytic fragment of *Tim23-4-HA* is in agreement with the addition of the 2 kDa HA tag to this 16 kDa Tim23 fragment and furthermore shows that the HA tag in *Tim23-4-HA* is resistant to degradation by proteinase K treatment of mitoplasts. This indicates that the tag is located at the matrix space side of the inner membrane. The fragment of ±18 kDa is also generated when

mitochondria are treated with proteinase K, indicating that the outer membrane of a minor part of the mitochondria has already been ruptured during the isolation procedure (Figure 5a, lane 2). Even in the absence of added proteinase K, a fragment of similar size is generated in mitoplasts, but which migrated at a higher position during gel electrophoresis (Figure 5a, lane 3). This suggests that the N-terminal extension of Tim23 which protrudes into the IMS is very sensitive to either endogenous proteases of small contaminations of proteases in the mitochondria preparation.

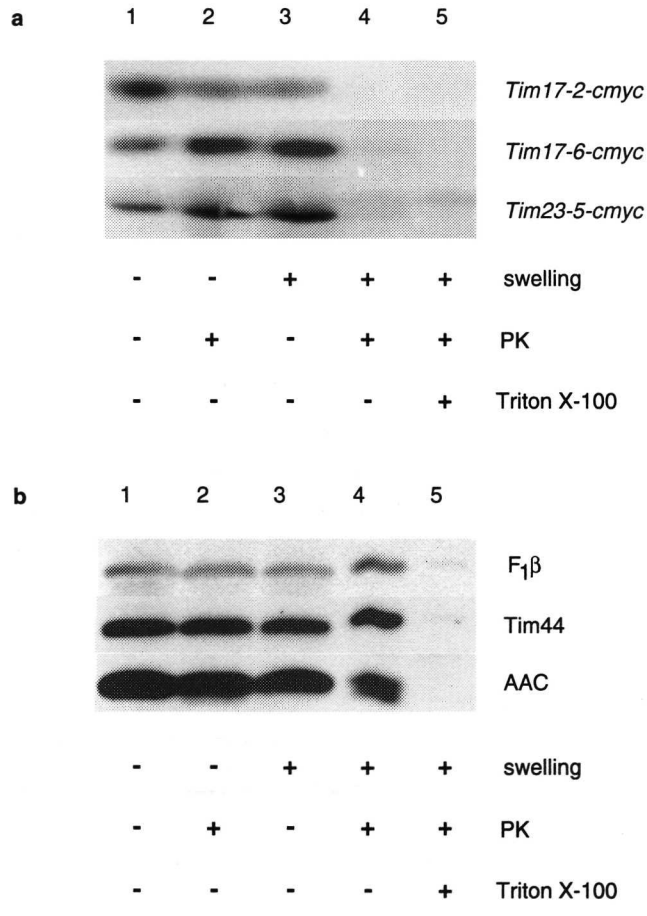

Figure 4. Accessibility to proteinase K of Tim17 and Tim23 *c-myc* tagged proteins. Mitochondria (lanes 1 and 2) or mitoplasts (lanes 3, 4 and 5) were incubated with proteinase K and Triton X-100 as indicated. Protein samples were run on a 15% SDS-PAGE and analyzed by Western blotting with antibodies directed against: **a,** *c-myc*; **b,** $F_1ß$, ß-subunit of the F_1-ATPase (matrix side of the inner membrane); AAC, ADP/ATP carrier (inner membrane); Tim44, subunit of the Tim complex (matrix side of the inner membrane).

When mitochondria harbouring *Tim23-5-HA* are converted to mitoplasts and treated with proteinase K, proteolytic fragments of about 9 and 11 kDa are generated, which were both recognized by anti-HA antibodies (Figure 5a, lane 4). The absence of the 18 kDa proteolytic fragment suggests that in this case not only the N-terminal region of the mutant protein is degraded, but that other, previously protease-insensitive domains of the protein have become accessible to proteinase K as well. This indicates that insertion of the HA tag in the predicted IMS localized loop of Tim23 renders this loop susceptible to proteases, giving rise to the observed smaller proteolytic fragments.

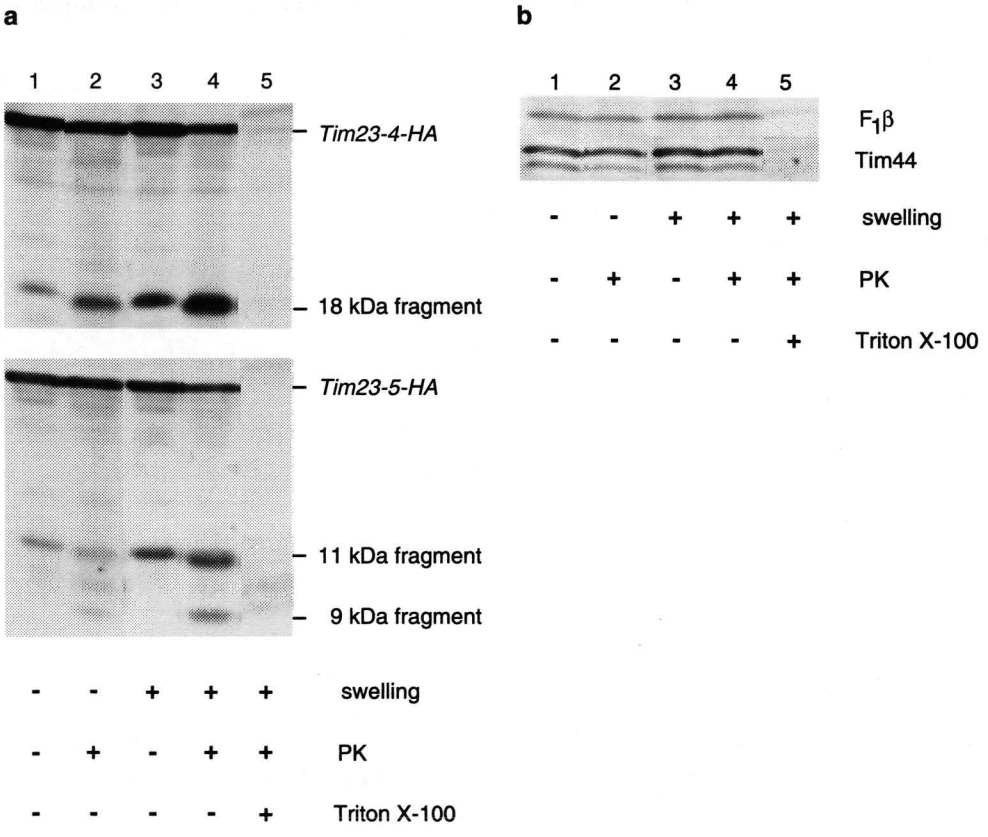

Figure 5. Proteinase K accessibility of HA tagged Tim23 proteins. Mitochondria (lanes 1 and 2) or mitoplasts (lanes 3, 4 and 5) were incubated with proteinase K and Triton X-100 as indicated. Protein samples were run on a 15% SDS-PAGE and analyzed by Western blotting with antibodies directed against: **a,** HA; **b,** Tim44, subunit of the Tim complex (matrix side of the inner membrane); $F_1ß$, β–subunit of the F_1-ATPase (matrix side of the inner membrane).

These results suggest that the HA tag in *Tim23-5-HA* is exposed to the IMS side of the inner membrane, which is consistent with results presented here with the *c-myc* epitope tag and with previous results ([41]; Heiko Martin, personal communication). The data are also in agreement with results obtained with another Tim23 mutant (*Tim23-5-Xa*) which showed that introduction of a cleavage site for factor Xa protease in the proposed IMS localized second loop of Tim23, renders it susceptible to proteinase K degradation (SvW, unpublished results). Minor amounts of the 9 kDa and 11 kDa proteolytic fragments also appear in mitochondria treated with proteinase K, indicating that a small fraction of the mitochondria has already been converted to mitoplasts (Figure 5b, lane 2). In the absence of proteinase K, a proteolytic fragment is visible in mitochondria and mitoplasts, which migrates at a higher position during gel electrophoresis compared to the 11 kDa proteolytic fragment, suggesting that these fragments are not identical. As a control for the integrity of the inner membrane of the mitoplasts, we monitored that the matrix exposed proteins Tim44 and $F_1ß$ were still protected against proteolysis (Figure 4b, lanes 4).

Discussion

Here we report a study of the membrane topology of Tim17 and Tim23 of the protein translocase of the mitochondrial inner membrane from *Saccharomyces cerevisiae*. Computer analysis of the protein sequences and experimental data have led to a model for the membrane topology of Tim17 and Tim23 in which the N- and C-termini of both proteins are localized in the IMS and the proteins span the inner membrane four times. This model has been generally assumed for the past years, although no direct evidence has been provided so far showing that the protein has four membrane spanning domains, one loop facing the IMS and two loops facing the matrix space.

To investigate the membrane topology of Tim17 and Tim23, a *c-myc* epitope tag was introduced at locations in both proteins that were predicted to form IMS and matrix space facing loops and a *c-myc* tag was introduced at the N-terminus of Tim17. Complementation analysis of the mutant proteins in *tim17* and *tim23* null strains indicated that Tim17 and Tim23 proteins with the *c-myc* tag inserted into the proposed matrix localized loops were not functional. According to the 'positive-inside rule' [61], lack of functional complementation may be due to the presence of the three negatively charged glutamic acid residues in the *c-myc* tag, since these residues may hamper the correct translocation of the tagged protein sequences across the inner membrane. By replacing the *c-myc* tag with an HA tag, which contains two negatively charged aspartic acids but no glutamic acids, we showed that all proteins, even those with an HA tag inserted in a predicted matrix localized loop, were functional in the corresponding null strains.

Mitochondria were isolated from the *tim17* or *tim23* deletion strains expressing functional *c-myc*- and HA-tagged proteins (*Tim17-2-cmyc* and *Tim17-6-cmyc*; *Tim23-5-cmyc*, *Tim23-4-HA* and *Tim23-5-HA*). Determination of the accessibility of the epitope tags of these proteins to proteinase K in mitoplasts revealed that all three *c-myc* tags are exposed to the IMS. This illustrates that, in analogy to Tim23, the N-terminus of Tim17 is exposed to the IMS (*Tim17-2-cmyc*) and that the proposed IMS localized loop of both proteins is indeed facing the IMS (*Tim17-6-cmyc* and *Tim23-5-cmyc*). Proteinase K treatment of mitoplasts harbouring *Tim23-4-HA* generated a Tim23 proteolytic fragment of about 18 kDa which can be detected with anti-HA antibodies. The appearance of this specific proteolytic fragment indicates that this tagged protein has adopted its proper topology in the inner membrane and that the HA tag is localized in the matrix space. Proteinase K treatment did not convert all *Tim23-4-HA* to the 18 kDa proteolytic fragment. This may indicate that the proteinase K treatment was insufficient to digest all *Tim23-4-HA* protein (Figure 5a, lane 4), although Tim44 and $F_1ß$ were completely degraded at this protease concentration when Triton X-100 was added (Figure 5b, lanes 5). Increasing the amount of proteinase K to a final concentration of 200 µg/ml did not degrade more *Tim23-4-HA* (data not shown). Inefficient proteolysis of *Tim23-4-HA* may perhaps be caused by aggregation of Tim23 proteins that have been misincorporated into the inner membrane. The complementation analysis illustrated that *Tim23-4-HA* is functional, suggesting that the protein is therefore likely to have adopted its proper membrane topology. Functionality of the protein was determined by the growth of cells expressing this protein on 5-FOA containing plates. However, the growth monitored may reflect that of only a percentage of the cells harbouring *Tim23-4-HA* in its functional, authentic membrane topology, whereas the remaining cells may contain a misincorporated tagged protein.

Protease treatment of mitoplasts harbouring *Tim23-5-cmyc* or *Tim23-5-HA* indicates that both tags in these mutant Tim23 proteins are accessible to proteinase K and that this loop is exposed to the IMS, which is in agreement with previous results ([41]; Heiko Martin, SvW, unpublished results). Proteinase K treatment of mitoplasts containing the HA tagged *Tim23-5* protein results in the formation of two proteolytic fragments, which can both be detected with anti-HA antibodies. These fragments are not detected with anti-*c-myc* antibodies when mitoplasts harbouring *Tim23-5-cmyc* are treated with this protease. This difference cannot be explained by the cleavage specificity of proteinase K, since both epitope tags contain several cleavage sites for this protease. However, it may be explained by differences in the detection methods that were used in both experiments. In the case of the protease accessibility experiments with *Tim23-5-cmyc*, immunodecorated bands were detected with the HRP color development reagent method. However, for *Tim23-5-HA*, detection was performed with the ECL (Enhance ChemiLuminescence System), which is claimed to be at least ten times more sensitive than other detection systems and therefore may explain why in this case proteolytic fragments could be

detected. As for *Tim23-4-HA*, proteinase K treatment of mitoplasts did not convert all *Tim23-5-HA* protein to the 9 kDa and 11 kDa proteolytic fragments.

The results presented here suggest that Tim17 and Tim23 are embedded in the mitochondrial inner membrane according to the previously proposed topology model [45]. Both the amino- and carboxy-terminus are exposed to the IMS and the protein spans the membrane four times, exposing two loops to the matrix space and one to the IMS. The results presented in this study provide the first experimental evidence that one of the proposed matrix localized loops (at position 4, Figure 2) of Tim23 is indeed oriented toward the matrix space. Tim17 also contains four potential membrane-spanning domains and it was previously shown that its C-terminus protrudes into the IMS. The results presented here indicate that both the N-terminus (*Tim17-2-cmyc*) and the proposed IMS localized loop (position 6, Figure 2) of Tim17 are oriented towards the IMS. Considering the high homology between Tim17 and Tim23, the previously obtained experimental data and the results presented in this study, we propose that Tim17 has a membrane topology similar to that of Tim23.

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