ACADEMIC PRESS

Biochemical and Biophysical Research Communications 293 (2002) 321–326

BBRC

www.academicpress.com

# Probing the membrane topology of a subunit of the mitochondrial protein translocase, Tim44, with biotin maleimide

Pavel F. Pavlov* and Elzbieta Glaser

*Department of Biochemistry and Biophysics, Arrhenius Laboratories for Natural Sciences, Stockholm University, 10691 Stockholm, Sweden*

Received 19 March 2002

**Abstract**

Tim44 is an essential component of the translocase of the inner mitochondrial membrane (TIM) complex that mediates transport of nuclear encoded mitochondrial precursors across the inner membrane. Here, we have investigated the topology of Tim44 by probing mitochondria with membrane impermeable 3-(*N*-maleimidopropionyl)biocytin (MPB) followed by the specific immunoprecipitation of modified proteins. Our data indicate that a single cysteine residue, Cys-369, located in the C-terminal domain of the yeast Tim44 is exposed to the mitochondrial intermembrane space. 

*Keywords:* Mitochondria; Protein import; Tim44; 3-(*N*-Maleimidopropionyl)biocytin

Mitochondrial biogenesis requires co-ordinated synthesis, transport, and assembly of proteins encoded by the mitochondrial DNA (accounting for less than 5% of the total number of mitochondrial proteins) and nuclear DNA. The outer and inner mitochondrial membranes contain protein complexes that mediate the passage of mitochondrial precursors across the membranes or the insertion of integral membrane proteins. The TIM23 translocase of the inner membrane mediates presequence-dependent protein transport into the mitochondrial matrix (for recent reviews see [1,2]). It consists of two structurally and functionally distinct subcomplexes—a Tim17/23 complex representing the translocation channel core and an associated Tim44/mHsp70 complex, the ''translocation motor'' that utilises the energy from ATP hydrolysis to drive the movement of precursors into the mitochondrial matrix. Two different mechanisms were proposed to explain how vectorial movement of mitochondrial precursors across the membrane occurs [3,4]. The ''Brownian ratchet'' model proposes that spontaneous precursor unfolding on the outer membrane surface accompanied by the sequential binding of mitochondrial heat shock protein 70 kDa (mHsp70) to the preprotein polypeptide chain on the matrix side of the inner membrane provides the driving force for protein translocation. The ''pulling'' model suggests that mHsp70 hydrolyses ATP to provide energy not only for interaction with the translocated precursor chain but also to generate a pulling force that actively unfolds tightly folded protein domains. In both models Tim44 serves as membrane anchor for mHsp70 in close proximity to the import sites.

Tim44 was the first component of the TIM23 complex to be identified. It was isolated using a biochemical approach and independently identified by genetic screening of yeast mutants defective in mitochondrial protein import [5,6]. Early reports suggested that Tim44 spanned the inner membrane with the majority of its N-terminal portion protruding into the mitochondrial matrix. Tim44 was found in the screen of an antibody library raised against right-side out, glutaraldehyde fixed submitochondrial particles because of its ability to quench the inhibition of protein import into right-side out vesicles [7]. A C-myc epitope tag fused to the extreme C-terminus of Tim44 was susceptible to proteolytic degradation in mitoplasts [6]. By cross-linking of preproteins that had been arrested at the import site, it was shown that a segment of extended polypeptide chain 33 amino acid residues long was required to reach Tim44 [8]. About 50 amino acid residues of an extended polypeptide chain of the precursor are required to span

* Corresponding author. Fax: +46-8-153679.
*E-mail address:* pavel@dbb.su.se (P.F. Pavlov).

0006-291X/02/$ - see front matter 
PII: S0006-291X(02)00221-8

both the outer and the inner mitochondrial membranes [9] indicating that a part of the Tim44 faces the intermembrane space of mitochondria. However, there are also results indicating that Tim44 is associated with the Tim17/23 complex only at the matrix side of the inner membrane. Tim44 is synthesised with an N-terminal, cleavable presequence and imported into mitochondria in a membrane potential dependent manner [10]. Sequence analysis revealed predominantly a hydrophilic profile for Tim44 without potential transmembrane regions [6]. Like a peripheral membrane protein, Tim44 could be extracted from the membrane with a solution of a high pH or high ionic strength [10,11]. Wild type Tim44 as well as Tim44 fused at its C-terminus with c-myc epitope, His-tag of dihydrofolate reductase was resistant to proteinase K treatment of mitoplasts [11]. Despite the fact that Tim44 is an essential protein, study of a temperature-sensitive Tim44 mutant revealed that Tim44 depletion did not reduce the number of import sites and had little effect on the import of a subset of mitochondrial precursors [12]. Other studies also demonstrated that Tim44 is not a tranlocation pore forming protein [13]. At present there is a common agreement that the N-terminal bulk of Tim44 is located in the matrix and interacts with mHsp70. The C-terminal domain was shown to interact with acidic phospholipids [14] and can be responsible for the association of Tim44 with the Tim17/23 core complex.

First introduced by Bayer [15], MPB is widely used for cysteine-specific covalent modification of proteins resulting in the coupling of a biotin moiety to the polypeptide chain. Femtomole amounts of available thiol groups can be detected [15]. Due to bulkiness of the biotin moiety and the hydrophilic nature of the linker between maleimide and biotin, MPB is a poor membrane-permeant agent. Topology and orientation of several membrane proteins have been determined by MPB labelling [16–18]. No reaction of biotin maleimides with proteins lacking cysteine has been observed [17]. In the present study we have exploited properties of the membrane impermeable cysteine-selective compound MPB to probe the topology of Tim44 in intact mitochondria.

## Materials and methods

MPB and other chemicals were purchased from Sigma. Antibodies against yeast Tim44, Imp1, Tom40, and Tom70 were obtained from Schatz. Antibodies against α-MPP were obtained from B. Glick, anti-Yta10, and Yta12 antibodies were a gift from W. Neupert. Antibodies against human Tim44 and against DnaK were raised by immunising rabbits with purified recombinant proteins and were manufactured by Antibody AB, Sweden.

*Isolation of mitochondria*. Yeast mitochondria were purified according to [19]. Rat liver mitochondria were isolated by differential centrifugation as described in [20].

*MPB labelling*. Mitochondria (800 μg protein) were resuspended in 1 ml of buffer containing 0.6 M sorbitol, 20 mM Hepes–KOH, pH 7.4 for yeast mitochondria and 0.23 M mannitol, 70 mM sucrose, 20 mM Hepes–KOH, pH 7.2 for rat liver mitochondria. MPB (0.5 mM) was added and the mitochondrial suspension was divided into two aliquots. One sample was incubated with MPB for 30 min, 4 °C. The reaction was stopped by incubation with 2 mM DTT for 10 min, 4 °C. Mitochondria were solubilised in 0.5% Triton X-100 and centrifuged at 14 000 rpm, 10 min. Supernatants were subjected to immunoprecipitation for 3–4 h, 4 °C using antisera against mitochondrial proteins followed by incubation with ProteinA-Sepharose. Pellets were washed four times with TBS-Tween buffer, loaded with Laemmli solubilisation buffer and boiled 5 min. The second aliquot of the mitochondrial suspension (order 2) was treated as the previous sample except that mitochondria were solubilised with Triton X-100 at the beginning of the MPB treatment.

*SDS–PAGE and Western blotting*. Samples were analysed by SDS–PAGE in the presence of 4 M urea. Separated proteins were blotted onto nitrocellulose membrane and probed with respective antibodies followed by horseradish peroxidase-coupled secondary antibodies using enhanced chemiluminiscence (ECL; Amersham Pharmacia). In parallel, samples were probed with avidin-peroxidase conjugate to detect biotinylated proteins.

*Computational analysis*. Prediction of transmembrane regions in the proteins was done using the dense alignment surface (DAS) method [21]. Secondary structure prediction and sequence alignment of Tim44 was done on the ExPASy server (www.expasy.org).

## Results and discussion

Due to the presence of porin, the outer mitochondrial membrane is freely permeable for compounds with a molecular mass $\leqslant$8 kDa [22]. The inner mitochondrial membrane is virtually impermeable for charged or hydrophilic compounds, including protons, unless specific transporters are present. MPB has a molecular mass of 524 Da, therefore, it is ideally suited for non-invasive probing of cysteine residues of proteins outside the inner membrane. Yeast Tim44 contains a single cysteine residue, Cys-369, at the C-terminal part of the protein. Therefore, the accessibility of Cys-369 to biotinylation can be easily monitored using Western blotting with avidin-conjugated horseradish peroxidase. As controls for MPB labelling and of mitochondrial intactness, we have investigated several well-characterised proteins located at the outer membrane, the inner membrane or in the matrix. Some biochemical properties of these proteins are summarised in Table 1. All selected proteins have at least one cysteine. Tom40 represents the core of the outer membrane translocation channel [1,2]. Tom70 serves primarily as an import receptor for a subset of precursor proteins. The large cytosolic domain of Tom70 participates in the recognition of preproteins [1,2]. Imp1 is the yeast homologue of the leader peptidase. It is an integral protein of the inner membrane exposing its catalytic domain in intermembrane space and forming a heterodimer with Imp2 protein [23]. Yta10p and Yta12p belong to the AAA-family of metalloproteases that are integral proteins of the inner membrane, forming the 850 kDa heterodimeric complex with large domains protruding into the matrix [24].

Table 1
Summary of data on proteins used in this study

| Species | Name | Accession number | Mitochondrial location | Molecular mass (kDa) | Number of cysteine residues |
|---|---|---|---|---|---|
| Yeast | Tom40 | NP_013930 | OM | 42 | 4 |
| | Tom70 | PO7213 | OM | 70 | 3 |
| | Impl | NP_013870 | IM | 21 | 3 |
| | Tim44 | NP_012242 | IM | 44 | 1 |
| | YtalO | P39925 | IM | 75 | 4 |
| | Ytal2 | P40341 | IM | 89 | 5 |
| | •-MPP | P11914 | Matrix | 48 | 6 |
| Rat | Tim44 | O35094 | IM/matrix | 44 | 3 |
| | mHsp70 | P48721 | Matrix | 70 | 5 |

Mitochondrial processing peptidase (MPP) in yeast is a soluble heterodimeric complex consisting of α-MPP and β-MPP subunits located in the matrix [2]. The mHsp 70 is located mostly in the mitochondrial matrix and a small fraction of mHsp70 is associated with the import channel on the matrix side of the inner membrane [1,2].

Fig. 1 shows the results of labelling yeast mitochondria with MPB in the absence and in the presence of Triton X-100. After labelling, proteins were subjected to immunoprecipitation with respective antibodies (left column) followed by Western blotting using avidin-peroxidase (right column). Proteins of the outer mitochondrial membrane, Tom40 and Tom70, were readily labelled with MPB in intact mitochondria. The degree of labelling of Tom70 was greater upon solubilisation of mitochondrial membranes with detergent. It has to be mentioned that amounts of immunoprecipitated proteins were equal in both samples as verified by staining of blots with respective antibodies (not shown). Imp1 was labelled in intact as well as in solubilised mitochondria which was in agreement with topology of this protein described previously [23]. Tim44 was another inner membrane protein that could be labelled with MPB in intact mitochondria. The degree of Tim44 labelling by MPB was similar in intact and solubilised mitochondria suggesting that the only present Cys-369 was free and exposed to the intermembrane space. Two other inner membrane proteins, Yta10 and Yta12, as well as matrix located α-MPP were biotinylated only upon solubilisation of mitochondria with Triton X-100 suggesting that the inner membrane was indeed impermeable for MPB. It has to be stressed that at high concentrations (⩾200 μM) MPB was reported to penetrate plasma membrane of mammalian cells [18]. In our experiments with intact mitochondria we did not observe any significant modification (⩽5%) of cysteine residues located in the matrix proteins even at MPB concentration of 500 μM. At lower concentrations of MPB efficiency of protein modification decreased but the pattern of protein labelling in different intra-mitochondrial compartments was similar.

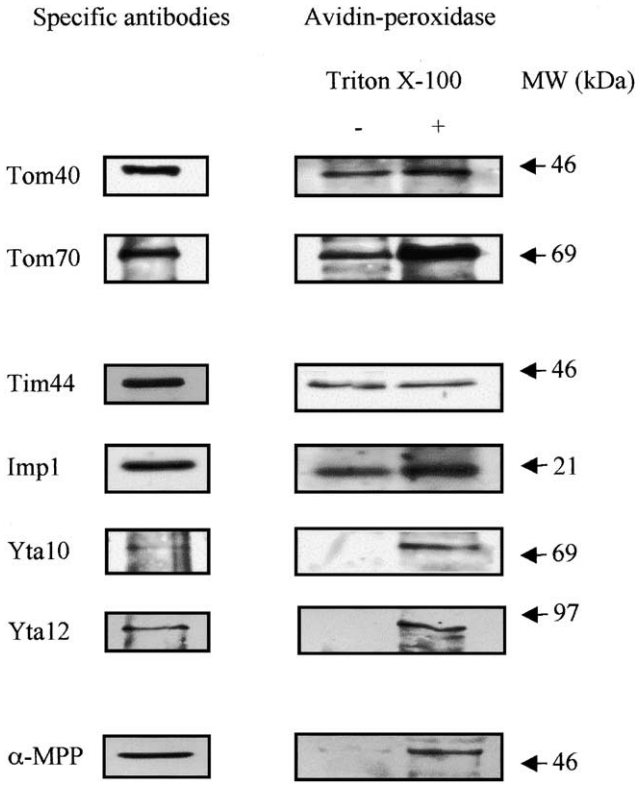


Fig. 1. Analysis of yeast mitochondrial proteins labelled with MPB. Labelling and detection were performed as described in Materials and methods. Left column presents Western blotting results of yeast mitochondrial proteins separated by SDS–PAGE and probed with different antibodies. Right column presents Western blot results of MPB labelling of these proteins, immunoprecipitated with their respective antibodies. Addition of Triton X-100 is described in Materials and methods. Arrows indicate positions of molecular weight standards.

Rat Tim44 contains three cysteine residues and one of these residues is located close to the Tim44 C-terminus. We also probed the Tim44 topology in isolated rat liver mitochondria. Under the conditions used rat Tim44 could not be modified with MPB even in solubilised mitochondria indicating that all cysteines were inaccessible to MPB. Rat mHsp70 was strongly labelled with MPB after solubilisation of the mitochondrial mem-

branes, moreover, labelled mHsp70 was coimmunoprecipitated with anti-Tim44 antibodies (data not shown). Previously, we have observed that the in vitro synthesised precursor of *Nicotiana plumbaginifolia* ATP-synthase $F_1\beta$ subunit could be modified with MPB only when the precursor was unfolded on the mitochondrial surface [25]. Cysteines hidden in the folded protein were not available for modification due to the hydrophilic nature and bulkiness of MPB. This might be the case for rat Tim44. Alternatively, cysteine groups can form inter or intra-molecular disulfide bridges also preventing their modification. Sequence alignment of yeast and rat Tim44 showed no conservation in the position of cysteines at their C-terminal domain (Fig. 2A). Yeast mitochondrial protein import shows no sensitivity to treatment with sulfhydryl-specific reagents in contrast to plant and mammalian mitochondrial protein import systems [25,26]. Taken together, these data suggest different characteristics of cysteine residues in Tim44 from yeast and other species.

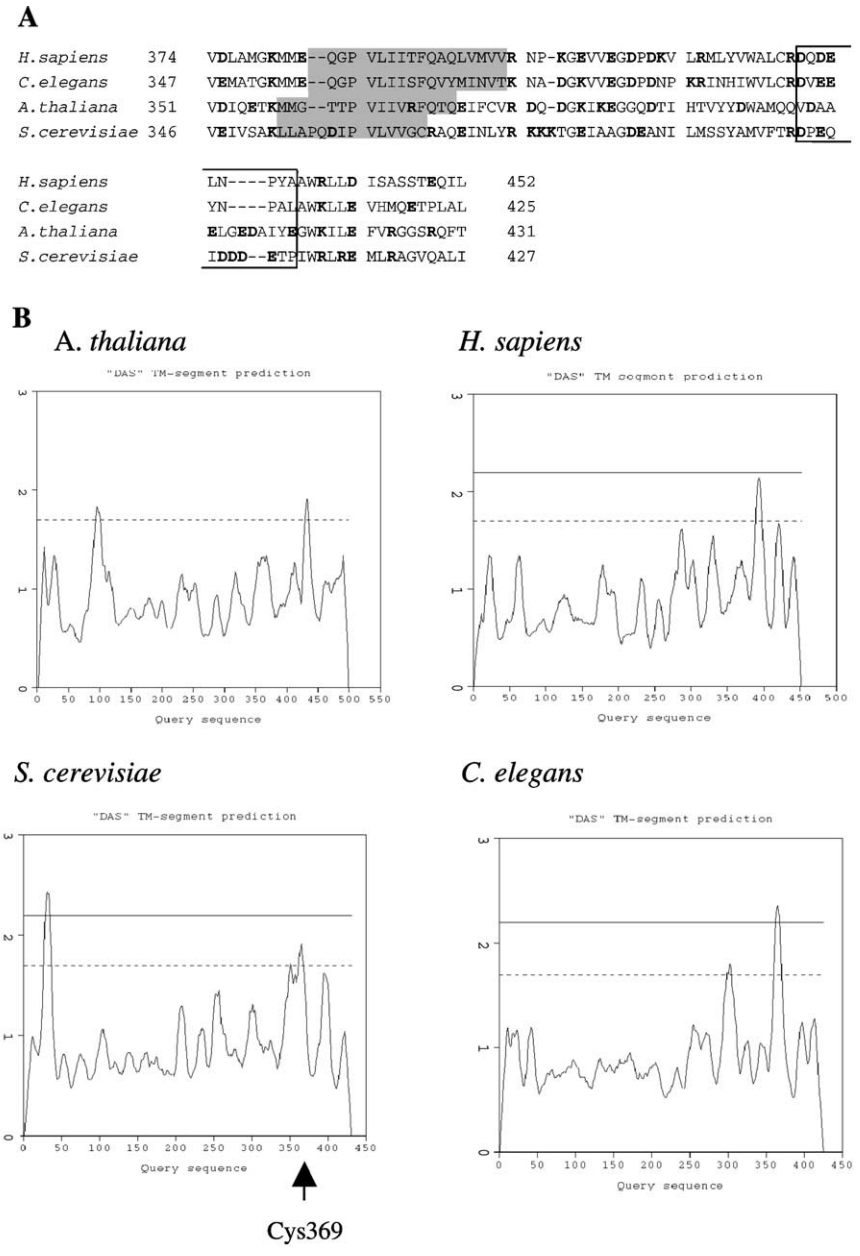


Fig. 2. (A) Clustal multiple sequence alignment of the C-terminal part of Tim44 from *A. thaliana*, *H. sapiens*, *C. elegans*, and *S. cerevisiae*. Protein sequences were obtained from public databases. Accession numbers are NP_181151, NP_006342, NP_012242, and O02161 for *A. thaliana*, *H. sapiens*, *S. cerevisiae*, and *C. elegans* Tim44 proteins, respectively. Hydrophobic segments are shadowed, charged amino acids are presented in bold and regions of negatively charged amino acids are boxed. (B) Analysis of Tim44 family protein sequences with the DAS transmembrane segment prediction program. Note that precursor forms of Tim44 were analysed by the DAS prediction program. Arrow indicates relative position of yeast Cys-369. Dashed horizontal lines show the loose cut off prediction score, solid lines indicate the strict cut off.

Tim44 from different organisms shows a low degree of primary sequence conservation, however, its secondary structure and hydrophobicity profiles exhibit striking similarities even between distantly related species. We have analysed Tim44 protein sequences from a variety of species using the DAS transmembrane prediction program (Fig. 2B). It has been previously reported that Tim44 is a hydrophilic protein without potential transmembrane regions [6]. More detailed analysis of hydrophobicity profiles revealed conservation of a short hydrophobic segment near the C-terminus of Tim44 in different organisms. Analysis of this hydrophobic region revealed a short stretch (8–16 residues) of hydrophobic or uncharged residues predicted to be mostly in an extended conformation. Cys-369 of yeast Tim44 was found within this hydrophobic segment (Fig. 2B).

Does the C-terminal portion of Tim44 protrude into the intermembrane space? The apparent lack of a predicted membrane spanning region in the Tim44 molecule would suggest that the short C-terminal hydrophobic segment avoids direct contact with membrane lipids and rather interacts with Tim17/23 proteins only. In a hydrophilic environment inside the translocation channel this segment can be mobile and susceptible to extraction with high pH or salt solution. It has been shown that Tim44 interacts with preproteins at an early stage of the translocation [10]. Analysis of the C-terminal part of Tim44 revealed the presence of clusters of negatively charged amino acids (Fig. 2A). Together with acidic domains of Tim23 these clusters (for example $^{405}$**DPEQIDDDETE**$^{415}$ in yeast Tim44) might participate in the initial recognition of precursors on the inner membrane surface.

In most reports concerning the yeast and mammalian import systems, Tim44 was found to be resistant to proteinase K treatment of mitoplasts [11,20,27]. After inactivation of a temperature-sensitive mutant of Tim44, yeast mitochondria were still able to import loosely folded preproteins suggesting that the Tim17/23 complex alone can perform the recognition and translocation of a subset of mitochondrial precursors [12].

The movement of proteins across membranes requires that there be large pores or channels. Electrophysiology studies have revealed that mitochondrial membranes contain channel activities capable of large conductance. One of these, the multiple conductance channel (MCC), has been implicated in protein import. Electrophysiology studies of proteoliposome reconstituted yeast membrane proteins revealed that the Tim17/23 complex displays an equivalent activity to the MCC [28,29]. It can be assumed that the import channel of intact mitochondria must be closed when the precursor is not translocated through it. Importantly, in the absence of mitochondrial presequence peptides, the reconstituted MCC displays a high probability of being in an open state. We hypothesise that Tim44 might be the missing part of the liposome reconstituted TIM complex which causes the MCC to be in an open state. The results discussed above may suggest a labile interaction between Tim44 and the Tim17/23 core of the channel. A dynamic interaction between the core of the TIM23 channel and Tim44 can explain the conflicting results obtained using protease treatment of mitoplasts.

In conclusion, we have shown that in the intact yeast mitochondria a single cysteine, Cys-369 located in the C-terminal domain of the Tim44 was labelled with membrane-impermeable maleimide, MPB. The C-terminal portion of Tim44 from a variety of species has been predicted to have positionally conserved stretch of hydrophobic amino acids. In yeast Tim44, this segment contains Cys-369 accessible from the outside of the inner membrane. Taken together these results suggest that the C-terminal part of Tim44 is exposed to the mitochondrial intermembrane space.

## Acknowledgments

We are grateful to Prof. W. Neupert, Prof. G. Schatz, and Prof. B. Glick for providing antibodies. This research was supported by grants from the Swedish Research Council and the Swedish Foundation for Cancer Research.

## References

[1] M.F. Bauer, S. Hofmann, W. Neupert, M. Brunner, Trends Cell Biol. 10 (2000) 25–31.
[2] N. Pfanner, A. Geissler, Nat. Rev. Mol. Cell Biol. 2 (2001) 339–349.
[3] C. Ungermann, W. Neupert, D.M. Cyr, Science 266 (1994) 1250–1253.
[4] M. Horst, W. Oppliger, B. Feifel, G. Schatz, B.S. Glick, Protein Sci. 5 (1996) 759–767.
[5] M. Horst, P. Jeno, N.G. Kronidou, L. Bolliger, W. Oppliger, P. Scherer, U. Manning-Krieg, T. Jascur, G. Schatz, EMBO J. 12 (1993) 3035–3041.
[6] A.C. Maarse, J. Blom, L.A. Grivell, M. Meijer, EMBO J. 11 (1992) 3619–3628.
[7] P.E. Scherer, U.C. Manning-Krieg, P. Jeno, G. Schatz, M. Horst, Proc. Natl. Acad. Sci. USA 15 (1992) 11930–11934.
[8] T. Kanamori, S. Nishikawa, I. Shin, P.G. Schultz, T. Endo, Proc. Natl. Acad. Sci. USA 21 (1997) 485–490.
[9] J. Rassow, F.U. Hartl, B. Guiard, N. Pfanner, W. Neupert, FEBS Lett. 275 (1990) 190–194.
[10] J. Blom, M. Kubrich, J. Rassow, W. Voos, P.J. Dekker, A.C. Maarse, M. Meijer, N. Pfanner, Mol. Cell Biol. 13 (1993) 7364–7371.
[11] F. Moro, C. Sirrenberg, H.C. Schneider, W. Neupert, M. Brunner, EMBO J. 18 (1999) 3667–3675.
[12] U. Bomer, A.C. Maarse, F. Martin, A. Geissler, A. Merlin, B. Schonfisch, M. Meijer, N. Pfanner, J. Rassow, EMBO J. 17 (1998) 4226–4237.
[13] P.J. Dekker, F. Martin, A.C. Maarse, U. Bomer, H. Muller, B. Guiard, M. Meijer, J. Rassow, N. Pfanner, EMBO J. 16 (1997) 5408–5419.
[14] C. Weiss, W. Oppliger, G. Vergeres, R. Demel, P. Jeno, M. Horst, B. de Kruijff, G. Schatz, A. Azem, Proc. Natl. Acad. Sci. USA 96 (1999) 8890–8894.

[15] E.A. Bayer, M.G. Zalis, M. Wilchek, Anal. Biochem. 149 (1985) 529–536.
[16] R.G. Zhen, E.J. Kim, P.A. Rea, J. Biol. Chem. 269 (1994) 23342–23350.
[17] S. Wakabayashi, T. Pang, X. Su, M. Shigekawa, J. Biol. Chem. 275 (2000) 7942–7949.
[18] T.W. Loo, D.M. Clarke, J. Biol. Chem. 270 (1995) 843–848.
[19] G. Daum, P.C. Bohni, G. Schatz, J. Biol. Chem. 257 (1982) 13028–13033.
[20] N. Ishihara, K. Mihara, J. Biochem. 123 (1998) 722–732.
[21] M. Cserzo, E. Wallin, I. Simon, G. von Heijne, A. Elofsson, Prot. Eng. 10 (1997) 673–676.
[22] L.S. Zalman, H. Nikaido, Y. Kagawa, J. Biol. Chem. 255 (1980) 1771–1774.
[23] A. Schneider, W. Oppliger, P. Jeno, J. Biol. Chem. 269 (1994) 8635–8638.
[24] K. Leonhard, J.M. Herrmann, R.A. Stuart, G. Mannhaupt, W. Neupert, T. Langer, EMBO J. 15 (1996) 4218–4229.
[25] E.M. von Stedingk, P.F. Pavlov, V.A. Grinkevich, E. Glaser, Plant. Mol. Biol. 35 (1997) 809–820.
[26] G. Wright, K. Terada, M. Yano, I. Sergeev, M. Mori, Exp. Cell. Res. 263 (2001) 107–117.
[27] M.F. Bauer, K. Gempel, A.S. Reichert, G.A. Rappold, P. Lichtner, K.D. Gerbitz, W. Neupert, M. Brunner, S. Hofmann, J. Mol. Biol. 289 (1999) 69–82.
[28] T.A. Lohret, K.W. Kinnally, J. Biol. Chem. 270 (1995) 15950–15953.
[29] T.A. Lohret, R.E. Jensen, K.W. Kinnally, J. Cell Biol 137 (1997) 377–386.