Separation of structural and dynamic functions of the mitochondrial translocase: Tim44 is crucial for the inner membrane import sites in translocation of tightly folded domains, but not of loosely folded preproteins

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The essential gene TIM44 encodes a subunit of the inner mitochondrial membrane preprotein translocase that forms a complex with the matrix heat-shock protein Hsp70. The specific role of Tim44 in protein import has not yet been defined because of the lack of means to block its function. Here we report on a Saccharomyces cerevisiae mutant allele of TIM44 that allows selective and efficient inactivation of Tim44 in organello. Surprisingly, the mutant mitochondria are still able to import preproteins. The import rate is only reduced by ~30% compared with wild-type as long as the preproteins do not carry stably folded domains. Moreover, the number of import sites is not reduced. However, the mutant mitochondria are strongly impaired in pulling folded domains of preproteins close to the outer membrane and in promoting their unfolding. Our results demonstrate that Tim44 is not an essential structural component of the import channel, but is crucial for import of folded domains. We suggest that the concerted action of Tim44 and mtHsp70 drives unfolding of preproteins and accelerates translocation of loosely folded preproteins. While mtHsp70 is essential for import of both tightly and loosely folded preproteins, Tim44 plays a more specialized role in translocation of tightly folded preproteins. 

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Introduction

The passage of nuclear-encoded preproteins from the cytosol to the mitochondrial matrix is mediated by Tom proteins in the mitochondrial outer membrane and by Tim proteins in the mitochondrial inner membrane (Ryan and Jensen, 1995; Schatz, 1996; Neupert, 1997; Pfanner and Meijer, 1997). The mechanism which drives the preproteins across the outer membrane seems to depend on a stepwise interaction of the positively charged presequences with patches of negatively charged amino acids of the Tom proteins (Dietmeier et al., 1997; Schatz, 1997). Translocation across the inner membrane is driven by two different mechanisms. The first step is mediated by the presequences which are pulled into the translocation channel by the mitochondrial membrane potential (Martin et al., 1991; Bauer et al., 1996). The presequences subsequently are cleaved off and translocation of the mature parts of the preproteins is membrane potential-independent. The final steps of translocation are mediated by mitochondrial Hsp70 (mtHsp70) and are coupled to the hydrolysis of ATP (Kang et al., 1990; Scherer et al., 1990; Hwang et al., 1991; Rassow and Pfanner, 1995a; Ungermann et al., 1996).

The inner membrane proteins Tim44, Tim23 and Tim17 have been identified in the yeast Saccharomyces cerevisiae as components of the machinery which mediates the translocation of presequence-targeted preproteins into the matrix (Maarse et al., 1992, 1994; Scherer et al., 1992, 1994; Schatz, 1997). The presequences are, however, still ambiguous. It has been suggested that both proteins directly participate in the formation of the inner membrane protein import channel (Dekker et al., 1991; 1993; Ryan et al., 1993; Küberich et al., 1994; Bauer et al., 1996; Lohret et al., 1997; Ryan et al., 1998).

Tim44 is encoded by an essential gene (Maarse et al., 1992). A mammalian homologue was identified recently in mice (Wada and Kanwar, 1998). The elucidation of its functions has been hampered by the lack of a means to block its activity. The role of Tim44 in mitochondrial protein import is, therefore, still ambiguous. It has been suggested that Tim44 might participate directly in the formation of a protein import channel since it can be cross-linked efficiently to preproteins in transit across the mitochondrial membranes and co-purified with translocation intermediates (Scherer et al., 1992; Blom et al., 1993; Berthold et al., 1995; Horst et al., 1995, 1996; Kanamori et al., 1997). Interactions of Tim44 with Tim17 and Tim23 have been demonstrated by both biochemical and genetic means (Berthold et al., 1995; Blom et al., 1995; Bömer et al., 1997). However, the contribution of Tim44 to the formation of the channel is difficult to conceive because the primary structure of Tim44 does not show putative membrane-spanning segments, and in fractionations Tim44 behaves like a peripheral membrane protein (Maarse et al., 1992; Blom et al., 1993; Berthold et al., 1995).

According to its accessibility for proteases, Tim44 is
exposed to the matrix side of the inner membrane (Blom et al., 1993; Rassow et al., 1994). In this location, Tim44 serves as a membrane anchor for mtHsp70, and the complex of Tim44 with mtHsp70 was suggested to represent the core of the mitochondrial protein import machinery (Kronidou et al., 1994; Rassow et al., 1994, 1995; Schneider et al., 1994; von Ahsen et al., 1995; Horst et al., 1996; Ungermann et al., 1996). The functional relevance of this interaction was addressed using a mutant form of mtHsp70, Ssc1-2p, that is unable to bind to Tim44 but still binds to preproteins (Voos et al., 1996). However, more recent data indicate that mtHsp70 binds not only to Tim44, but also directly to the Tim17–Tim23 complex. The mutant form Ssc1-2p is defective in the interaction with both membrane-binding sites (Bömer et al., 1997). Therefore, it could not be decided if the defects in protein translocation which were observed with the mutant allele ssc1-2 were indeed caused by the lack of interaction with Tim44. Up to now, the individual functions of the two membrane anchors in the mechanism of protein translocation could not be discerned, and the contribution of Tim44 to the activity of mtHsp70 is unclear.

In this study, we have examined the function of Tim44, using a temperature-sensitive mutant of Tim44. For the first time, this mutant allows investigation of the effects of a selective inactivation of functional Tim44. We found that the number of translocation sites in the inner membrane is not reduced by depletion of Tim44, suggesting that Tim44 is not a constitutive component of the protein import channel. The mutant mitochondria show a reduced rate of import reactions, most pronounced in the case of tightly folded protein domains. We conclude that Tim23 and Tim17 can form the protein import channel in the absence of Tim44. Tim44 is part of a separate functional unit which acts as the dynamic element of the Tim machinery.

Results

A temperature-sensitive mutant of Tim44

We generated mutated forms of TIM44 by low fidelity PCR and expressed the mutated genes in S. cerevisiae in a single-copy vector under the control of their own promoters. The clone containing the allele tim44-8 was isolated and used in this study (Figure 1A). At 23°C, the mutant strain grows similarly to the wild-type on glucose and on glycerol, whereas at 37°C it grows on glycerol plates at 23 or 37°C, respectively. (B) Aggregation of mutant Tim44 after heat shock. Isolated mitochondria were incubated in import buffer (including 2 mM ATP; BSA was omitted) at 37°C for different time periods as indicated. Subsequently, all samples were shifted to 25°C for 5 min. In parallel, mitochondria were kept on ice and then incubated at 25°C. Triton X-100 was added to a final concentration of 0.1%, and the samples were shaken end-over-end at 8°C for 10 min. Aggregates were pelleted for 1 h at 106 000 gmax and analysed by SDS–PAGE and immunoblotting. (C) Mitochondria were treated as in (B) and tested for aggregation of mtHsp70, Mge1, ADP/ATP carrier (AAC) and cytochrome b2 (Cyt. b2).

Fig. 1. Temperature-sensitive mutant of Tim44. (A) Growth defect of a yeast strain containing the allele tim44-8. Wild-type TIM44 and the mutant allele tim44-8 were cloned into the single-copy vector YCplac111 and expressed in a tim44 deletion strain. The strains were grown on glycerol plates at 23 or 37°C, respectively. (B) Aggregation of mutant Tim44 after heat shock. Isolated mitochondria were incubated in import buffer (including 2 mM ATP; BSA was omitted) at 37°C for different time periods as indicated. Subsequently, all samples were shifted to 25°C for 5 min. In parallel, mitochondria were kept on ice and then incubated at 25°C. Triton X-100 was added to a final concentration of 0.1%, and the samples were shaken end-over-end at 8°C for 10 min. Aggregates were pelleted for 1 h at 106 000 gmax and analysed by SDS–PAGE and immunoblotting. (C) Mitochondria were treated as in (B) and tested for aggregation of mtHsp70, Mge1, ADP/ATP carrier (AAC) and cytochrome b2 (Cyt. b2).

structure which collapses at higher temperature and renders the protein prone to aggregation. Hence, the allele tim44-8 provides the opportunity to deplete mitochondria of functional Tim44. As the mutant phenotype can be induced by a pre-incubation of isolated mitochondria without affecting other known components of the protein import machinery (and does not require a temperature shift in vivo), indirect effects of the inactivation of Tim44 on mitochondrial structure and function are minimized.

The number of translocation sites for preproteins carrying a presequence is not reduced upon depletion of functional Tim44

To determine the role of Tim44 in mitochondrial protein import, we isolated mitochondria from the tim44-8 mutant and the corresponding wild-type, and imported several different preproteins. To deplete the tim44-8 mitochondria of Tim44, the mitochondria were subjected to a heat shock prior to the import reactions. We synthesized the precursors of the α-subunit of the matrix-processing peptidase (MPP), the β-subunit of the ATP synthase (Fβ), and AAC in reticulocyte lysate in the presence of [35S]methionine/ [35S]cysteine and imported the proteins into the isolated mitochondria (Figure 2). Complete translocation of αMPP and Fβ across the mitochondrial membranes was assayed...
Fig. 2. Mitochondria of the *tim44-8* mutant show efficient import of several preproteins. (A) Import of the α-subunit of the matrix-localized mitochondrial processing peptidase MPP. The preprotein was synthesized in a reticulocyte lysate in the presence of [35S]methionine/[35S]cysteine and incubated with isolated mitochondria from the *tim44-8* mutant strain and the corresponding wild-type (WT) at 25°C for the times indicated. Prior to import, the mitochondria of both strains were incubated at 37°C for 15 min to induce the mutant phenotype. The import reactions were stopped by addition of valinomycin and cooling on ice. The samples were split into halves and treated with proteinase K to remove preproteins outside the mitochondrial membranes (+PK) or left without protease (–PK). After reisolation of the mitochondria by centrifugation, the imported proteins were separated by SDS-PAGE and analyzed using a Phosphorimager. p, precursor protein; m, mature protein. The graphs show the relative amounts of processed proteins. The amount of processed protein obtained after 30 min in the absence of proteinase K was set to 100% (control). (B) Import of the β-subunit of the mitochondrial ATP synthase (*F_1*β). The experiment was performed as described in (A). (C) Import of the ADP/ATP carrier (AAC). Since the AAC does not contain a cleavable presequence, only the protease-protected AAC is shown. For quantification of the Δψ-dependent imported AAC, the amount of protease-protected AAC which accumulated in the absence of a membrane potential was subtracted.

by resistance to externally added protease. In this assay, the import efficiencies in the *tim44-8* mutant mitochondria were reduced by ~20–40% (Figure 2A and B, samples +PK). However, processing of the imported proteins reached 80–95% of the wild-type levels, demonstrating that the inactivation of Tim44 has only minor effects on the translocation of the presequences through the protein import channels of the membranes (Figure 2A and B, samples –PK). Considering that the mutant Tim44 was depleted by >95%, the reduction in the import efficiencies of αMPP and *F_1*β was less pronounced than expected. In particular, the insertion of the presequences into the Tim channel seems to be Tim44-independent. Tim44 appears to act primarily in late stages of protein translocation.

A slight reduction in the import kinetics was also observed with the AAC, the import of which is regarded as completely independent of the Tim17/Tim23/Tim44 machinery (Sirrenberg et al., 1996, 1998; Dekker et al., 1997; Koehler et al., 1998). However, the import of the AAC requires the membrane potential Δψ across the mitochondrial inner membrane. We found that the membrane potential was slightly weaker in the *tim44-8* mutant mitochondria than in the wild-type mitochondria (see below, Figure 6), suggesting that the reduced import efficiencies may be due in part to this.

To study the import defects of the mutant mitochondria in more detail, we used several hybrid proteins containing a DHFR (dihydrofolate reductase) domain which were designed specifically to characterize the mechanisms of mitochondrial protein import. First we imported the hybrid protein Su9(70)–DHFR (containing the presequence of subunit 9 of the ATP synthase fused to the entire DHFR) which is used commonly to characterize the import of matrix-targeted proteins (Figure 3A). The import efficiency was reduced by 20–30%, again demonstrating that the inactivation of Tim44 still allows considerable activity of the import machinery.

The possibility existed that the radiochemical amounts of the preprotein synthesized in the reticulocyte lysate may be imported by a small number of sites still containing intact Tim44. We therefore tested the proximity of the translocating Su9(70)–DHFR to Tim44 by chemical cross-linking (Figure 3B). Using the reagent disuccinimidyl suberate (DSS), the preprotein was cross-linked efficiently to Tim44 in wild-type mitochondria as observed previously (Blom et al., 1993; Kübrich et al., 1994). Two cross-
linking products were formed which could be immunoprecipitated by antibodies raised against Tim44, representing cross-linking to the preprotein and to the processing intermediate (Figure 3B, bands labelled Tim44* and Tim44** in lanes 2 and 9). In contrast, Su9(70)–DHFR imported into the mutant mitochondria was not cross-linked (Figure 3B, lane 5 versus lane 2, and lane 13 versus lane 9). The same result was obtained with ethylene glycolbis succinimidyl-succinate (EGS; Figure 3B, lanes 15–18), a reagent with a longer spacer arm than DSS (1.61 nm versus 1.14 nm). These results thus confirm that preproteins can be imported in the absence of Tim44.

We then asked whether the mitochondria with inactivated Tim44 were able to import large amounts of preproteins. To obtain a soluble preprotein in chemical amounts, we expressed the hybrid protein b2(167)Δ–DHFR in Escherichia coli and purified it in functional form (Dekker et al., 1997). The construct consists of the first 167 residues of cytochrome b2 and complete DHFR (Koll et al., 1992). Due to the deletion (Δ) of a segment within the presequence, the preprotein is targeted to the mitochondrial matrix space (Koll et al., 1992). The preprotein was applied at concentrations close to saturation of the mitochondrial import sites (Dekker et al., 1997). The import kinetics of this protein are shown in Figure 4A. The import efficiency in the mutant mitochondria was reduced to a similar extent as the import of the radiochemical amounts of preproteins from reticulocyte lysate shown in Figures 2 and 3.

The preprotein b2(167)Δ–DHFR is processed in two stages. Immediately after translocation across the inner membrane, the first part of the presequence is cleaved off and the processing intermediate i is formed. In a subsequent step, ~8 additional amino acids are removed, yielding the intermediate i*, indicating a further maturation step of the preprotein (Laloraya et al., 1995). Interestingly, the formation of the i* intermediate is reduced drastically in the tim44-8 mitochondria while the formation of the i intermediate is less affected (Figure 4A, upper panel). The same effect has been observed with mutants of the mtHsp70/Mge1 system (Voos et al., 1993, 1994; Laloraya et al., 1995), suggesting that Tim44 and the mtHsp70
possibility of accumulating the $b_2(167)\Delta$–DHFR in translocation sites by import in the presence of the DHFR ligand methotrexate (MTX). It has been shown previously that the number of translocation intermediates which can be accumulated by this method is limited by the number of available import sites in the inner membrane (Dekker et al., 1997). We now titrated the maximal number of translocation intermediates in parallel for the $\text{tim44-8}$ mutant mitochondria and the corresponding wild-type mitochondria. Surprisingly, we found similar numbers for mitochondria from both strains (Figure 4B). About 20 pmol of preprotein could be accumulated per mg of mitochondrial protein, in agreement with previous studies with wild-type mitochondria (Vestweber and Schatz, 1988; Rassow et al., 1989; Dekker et al., 1997). We conclude that Tim44 is not an essential factor in the formation of functional protein translocation channels. The involvement of Tim44 increases the efficiency of protein import, but the amount of Tim44 does not determine the number of import sites.

**Function of Tim44 in unfolding and import of a tightly folded protein domain**

To assess the features of a preprotein which determine its dependence on Tim44, we investigated the import of cytochrome $b_2$–DHFR hybrid proteins and compared constructs which differ in their requirement for unfolding of defined protein domains (Figure 5A). We first tested two different constructs, containing N-terminal parts of cytochrome $b_2$, residues 1–220 or 1–85, respectively. The presequences of both constructs are identical; however, the longer construct [b$_2$(220)–DHFR] contains the complete haem-binding domain of cytochrome $b_2$ (residues 81–180) which is tightly folded and requires unfolding to allow translocation across the mitochondrial membranes. The haem-binding domain is missing in b$_2$(85)–DHFR. Thus, both preproteins differ in the requirement for energy-dependent unfolding of a distinct protein domain; the rate of import is limited by the efficiency of unfolding of the domain (Gambill et al., 1993; Glick et al., 1993; Voos et al., 1993, 1996). The DHFR domain is less stably folded and, in the absence of MTX, does not cause effective restrictions in import reactions (Pfanner et al., 1990; Voos et al., 1996). We found that both preproteins were imported efficiently into mitochondria of the wild-type (Figure 5B, lanes 1–4). In contrast, in mitochondria of the $\text{tim44-8}$ mutant, the import of b$_2$(220)–DHFR was considerably delayed while the import of b$_2$(85)–DHFR showed only a minor reduction (Figure 5B, lanes 6–9). The effect was most pronounced after treatment of the mitochondria with protease (+ PK). The comparison of the import efficiencies of both constructs thus demonstrates a role for Tim44 in the unfolding of the cytochrome $b_2$ haem-binding domain. The unfolding and translocation of more loosely folded protein domains, as in b$_2$(85)–DHFR and b$_2$(167)Δ–DHFR, Figure 4A], is far less affected.

The preprotein b$_2$(220)–DHFR contains an intact cytochrome $b_2$ presequence. This presequence directs the processed proteins to the intermembrane space (Guiard, 1985). A substitution of alanine in position 63 by proline entails mistargeting of the imported proteins into the mitochondrial matrix (Beasley et al., 1993). We now tested whether in $\text{tim44-8}$ mitochondria the defect in
K-protected protein were quantified.

fluorography. The relative amounts of processed and proteinase (– PK), reisolated and analysed by SDS–PAGE and cytochrome b

Valinomycin. After the import reaction, the mitochondria were treated indicated. To dissipate the membrane potential, two samples received

of processed and proteinase K-protected protein were determined using proteins; i, processing intermediate; m, mature protein. (C) Import of

preprotein. 35S-labelled b 2(220)A63P–DHFR was synthesized in reticulocyte lysate and imported into isolated mitochondria for the times indicated. Due to the amino acid substitution in the presequence, the preprotein (p) is only processed to the intermediate (i). The mitochondria were treated with proteinase K (+ PK) or left without protease (– PK), reisolated and analysed by SDS–PAGE and fluorography. The relative amounts of processed and proteinase K-protected protein were quantified.

Function of Tim44

We then investigated the possible role of Tim44 in the Δψ-independent steps of the import process. The preprotein Su9(70)–DHFR can be accumulated spanning both mitochondrial membranes by import in the presence of MTX (Schwarz et al., 1993; Ungermann et al., 1994). An active protein import machinery is capable of pressing the MTX-stabilized DHFR domain against the outer membrane, rendering the intermediate resistant to externally added proteases, even after subsequent dissipation of the membrane potential (the DHFR domain of the hybrid protein

Fig. 5. Import of the cytochrome b 2 haem-binding domain is inhibited in tim44-8 mitochondria. (A) Cytochrome b 2–DHFR fusion proteins. In contrast to b 2(85)–DHFR, the preprotein b 2(220)–DHFR contains a complete haem-binding domain (residues 81–180). In b 2(220)A63P–DHFR, the intermembrane space sorting signal is inactivated by substitution of the alanine in position 63 for proline. (B) The preproteins b 2(85)–DHFR and b 2(220)–DHFR were synthesized in reticulocyte lysate in the presence of [ 35S]methionine and imported into isolated mitochondria for the times indicated. To dissipate the membrane potential, two samples received valinomycin. After the import reaction, the mitochondria were treated with proteinase K (+ PK) or left without protease (– PK), reisolated and analysed by SDS–PAGE and fluorography. The relative amounts of processed and proteinase K-protected protein were quantified.

Function of Tim44 in a translocation step after insertion of the presequence into the inner membrane

We then wondered if the strong effect of the tim44-8 mutation on the translocation of the haem-binding domain could be caused by a weakening of the mitochondrial membrane potential Δψ and a differential Δψ dependence of the preproteins used. For this purpose, we compared the membrane potential of isolated wild-type and mutant mitochondria (Figure 6A). As an assay, we used the quenching of the fluorescent dye 3,3′-dipropylthiadicarbocyanine iodide [DiSC 3(5)]. We found a minor reduction in the membrane potential in the mutant mitochondria which may account for the slightly reduced import rate of preproteins in the Δψ-dependent steps (shown in Figures 2 and 3). In order to exclude that differential effects of the tim44-8 mutation were mediated indirectly via a differential Δψ dependence of the preproteins, we determined the influence of the membrane potential on the import rate of several preproteins (Figure 6B). We compared the import of the preproteins in the presence of increasing concentrations of the uncoupling reagent carbonyl cyanide m-chlorophenyl-hydrazone (CCCP; Figure 6B). The preprotein F 1 β showed a strong reduction in import already at low concentrations of CCCP, demonstrating a strong dependence on Δψ. In contrast, the import of the fusion proteins b 2(220)A63P–DHFR and b 2(85)–DHFR revealed only a weak dependence on the membrane potential; the AAC showed an intermediate behaviour. There was no obvious correlation between these differences in the CCCP titrations and the differences observed in the import experiments with tim44-8 mitochondria shown in Figures 2 and 5. In particular, the construct b 2(220)A63P–DHFR, which in the import experiments was strongly affected by the inactivation by Tim44, showed the highest resistance to the reduction of Δψ in the CCCP titrations. We conclude from these data that the membrane potential does not account for the delayed translocation of the haem-binding domain into the tim44-8 mitochondria. The characterization of the import defects as shown in Figures 2–6 suggests that Tim44 is not a structural part of the import channel but acts in later steps of import.
Inhibition of import of the cytochrome b$_2$ haem-binding domain in tim44-8 mitochondria is not caused by a reduced membrane potential. (A) Assessment of mitochondrial membrane potential $\Delta \psi$. Wild-type mitochondria (WT) and tim44-8 mitochondria (200 $\mu$g of protein) were preincubated at 37°C for 15 min and subsequently incubated with the membrane potential-sensitive dye DiSC$_3$(5). The fluorescence changes were recorded by a fluorescence spectrophotometer at 25°C. Valinomycin (Val) was added as indicated to dissipate the membrane potential. The difference between the fluorescence prior to and after the addition of valinomycin represents a relative assessment of the membrane potential. (B) Sensitivity of import efficiency to reduction of the membrane potential. The preproteins were imported as described in the legend to Figure 2A except that increasing amounts of CCCP were included to reduce the mitochondrial membrane potential. All import reactions were stopped after 4 min and analysed by SDS–PAGE and fluorography. The amounts of processed protein were quantified using a phosphorimager, the amount of processed preprotein in the absence of CCCP was set to 100% (control).

Discussion

Tim44 was the first protein identified as a component of the protein translocation machinery of the mitochondrial inner membrane (Maarse et al., 1992; Scherer et al.,...
in the presence of \([35S]\text{methionine}/[35S]\text{cysteine}\) and imported into mitochondria from a temperature-sensitive mutant strain and of the corresponding wild-type in the presence of MTX. Subsequently, the mitochondria containing the accumulated translocation intermediate were washed and then incubated at 25°C in the presence of valinomycin (to dissipate the membrane potential \(\Delta\psi\)) and MTX (to stabilize the native folding state of the DHFR domain). After the times indicated, the mitochondria were cooled on ice and treated with proteinase K (PK). The mitochondria were reisolated and analysed by SDS–PAGE and fluorography. The activity of the mitochondrial protein import machinery leads to pressing of the DHFR domain against the outer membrane and thus to resistance to the externally added protease. Reduced activity of the import machinery allows access for the protease. The presence of Su9 contains two sites for processing, at position 35 (i-form) and at position 66 (m-form). p, precursor protein; i, processing intermediate; m, mature protein. The relative amounts of the protease-resistant pSu9(70)–DHFR translocation intermediates were determined using a phosphorimager. The amounts retained after the first incubation in the presence of the membrane potential and reisolation, prior to dissipation (time 0), were set to 100% (control).

Surprisingly, the inactivation of Tim44 allows quite efficient import of many preproteins, even at concentrations close to saturation of import sites. This observation thus challenges the concept both of Tim44 as a component of the import channel and of Tim44 as an essential part of the import motor. Indeed we found that the number of translocation sites was not affected by the inactivation of Tim44, indicating that Tim44 is not required for the formation of the protein import pore. This conclusion is corroborated by the observation that the formation of the 90 kDa complex of Tim proteins which is tightly associated with translocating polypeptides also is not reduced by the depletion of Tim44 (unpublished data). The formation of the inner membrane protein import channel seems to be the function of proteins other than Tim44; candidates are Tim23 and Tim17 which are intrinsic components of the 90 kDa complex (Bauer et al., 1996; Dekker et al., 1997; Lohret et al., 1997).

The insertion of Tim23 and Tim17 into the inner membrane does not require mthsp70 or Tim44 (Bömer et al., 1997; Káldi et al., 1998). However, we cannot rule out the possibility that Tim44 may play a role in the import of other proteins which may be involved in initial steps of channel formation. Once established, the maintenance of functional import channels appears to be completely Tim44-independent.
Our functional data obtained with the *tim44-8* mutant indicate that a preprotein has to traverse the inner membrane and reach the matrix side to encounter the consequences of an inactivation of Tim44. Considering that Tim44 is not a structural component of the protein import pore and that the amino acid sequence of Tim44 shows a hydrophilic protein which does not reveal any potential membrane-spanning segments, we suggest that Tim44 acts at the inner side of the inner membrane, in cooperation with other Tim proteins which form the import channel.

Excluding a role for Tim44 as a pore-forming protein, what is its role in mitochondrial protein import? After it had been observed that Tim44 can form a complex with mtHsp70, it was speculated that Tim44 might be required to deliver mtHsp70 to the translocating polypeptide (Kronidou et al., 1994; Rassow et al., 1994; Schneider et al., 1994). It was known from other studies that mtHsp70 binds to translocating preproteins and is an essential component of the import machinery (Kang et al., 1990; Scherer et al., 1990). However, it was not possible to investigate whether Tim44 actually plays a role in this process. We now find that several preproteins which are strictly mtHsp70-dependent in their translocation (Gambl et al., 1993; Glick et al., 1993; Voos et al., 1993; Stuart et al., 1994) are still imported with reasonable efficiencies into *tim44-8* mitochondria. We conclude that the transfer of mtHsp70 to the polypeptides emerging at the import sites is not entirely dependent on Tim44. At least to some degree, Tim44-independent binding of mtHsp70 seems to be possible, and this binding appears to be functional in the mechanism of protein import.

However, in testing for the consequences of a depletion of active Tim44 in protein import, we observed a drastic reduction in the import of a tightly folded protein domain. In contrast to the import of more loosely folded domains, the unfolding and translocation of a tightly folded domain require the involvement of Tim44. This result is remarkable in the context of data which were obtained previously with mutant forms of mtHsp70 (von Ahsen et al., 1993; Voos et al., 1996; Bömer et al., 1997). The mtHsp70 which is encoded by the allele *ssc1-2* shows strong binding to preproteins but impaired binding to Tim44 and to Tim17–Tim23. This defect correlates with a delayed import of tightly folded protein domains, raising the question of whether the complex of mtHsp70 with Tim44 or the complex with Tim17–Tim23 is involved in the import reaction. The results with the *tim44-8* mutant demonstrate that it is Tim44 which is required in the translocation of the tightly folded domain, and they show that the mtHsp70–Tim17–Tim23 complexes of mitochondria do not substitute for the mtHsp70–Tim44 complexes after the inactivation of Tim44. We conclude that the complexes of mtHsp70 with Tim17–Tim23 and with Tim44 play different roles in the protein import mechanism. The strong ‘pulling’ function which is required for unfolding of tightly folded domains and has been attributed to mtHsp70 (Glick, 1995; Pfanner and Meijer, 1995; Schatz, 1996; Matouschek et al., 1997) is not exerted by the Tim17–Tim23-bound mtHsp70 but by mtHsp70 in cooperation with Tim44. Our data do not exclude, however, that the mtHsp70–Tim17–Tim23 complex could be involved in the formation of a weaker pulling activity required for import of more loosely folded preproteins (Glick et al., 1993; Voos et al., 1996; Matouschek et al., 1997).

The stability of the Su9–DHFR translocation intermediates in import sites shows a striking dependence on the presence of Tim44. We suggest that Tim44 strongly facilitates the transfer of mtHsp70 to the translocating polypeptide directly at the membrane. In the presence of Tim44, the mobility of the accumulated translocation intermediates is reduced drastically, suggesting that polypeptide segments which emerge at the import sites immediately receive a mtHsp70 molecule. This high Tim44-dependent fidelity of the import motor appears to be required in the unfolding and import of tightly folded domains. In the absence of Tim44 function, mtHsp70 can drive the import of loosely folded preproteins even though the bound mtHsp70s are more distant from the membrane (the model protein which shows the high mobility in the import channel in the experiment of Figure 7B is the same preprotein that is imported efficiently in Figure 3A). Our results suggest that in mitochondria the functions of channel formation and the generation of a strong force in vectorial protein movement are mediated by different subcomplexes of the import machinery. Tim23 and Tim17 seem to constitute the import pore; Tim44 together with mtHsp70 appears to be the dynamic element of the import machinery. A similar situation has emerged in the elucidation of the protein transport mechanisms of the endoplasmic reticulum. Here, the corresponding functions are exerted by complexes of Sec61p or Sec63p, respectively (Brodsky and Schekman, 1993; Panzner et al., 1995; Corsi and Schekman, 1997; Lyman and Schekman, 1997).

In summary, we find that Tim44 is not a structural constituent of the inner membrane protein import channel. Tim44 rather acts at the inner side of the inner membrane and is an element of an independent motor unit driving unfolding and translocation of preproteins. Mitochondrial Tim44 plays only a stimulatory role in import of loosely folded preproteins, but is crucial for pulling in folded domains.

Materials and methods

Construction of plasmids and manipulation of *S.cerevisiae* strains

Temperature-sensitive alleles of *TIM44* were generated by a low fidelity PCR technique as published previously (Rassow et al., 1994). The mutant alleles were cloned into the single-copy vector YCplac111 and introduced into the diploid strain MB2-22 (Maarse et al., 1992). After random sporulation, haploid cells with a disrupted nuclear *TIM44* gene and harbouring a rescuing plasmid were tested for temperature sensitivity. One of the isolated alleles was designated *tim44-8*. A corresponding strain containing the wild-type *TIM44* gene was used as the wild-type strain. Hybrid proteins containing N-terminal parts of Su9 fused to murine DHFR (Viebrock et al., 1982; Ungermann et al., 1994) were obtained by *in vitro* transcription/translation using cDNA constructs cloned into the vector pGEM4 (Promega). To assemble Su9(58)–DHFR, Su9(76)–DHFR and Su9(81)–DHFR, the corresponding Su9 parts were obtained by PCR and ligated into a pGEM4 plasmid containing a DHFR cDNA, using the BamHI site of the cDNA upstream of the DHFR start site and the *EcoRI* site of the vector. Su9 and DHFR parts are thereby connected by a linker of four residues (Gly, Ser, Gly, Ile). The construct Su9(70)–DHFR was described previously (Pfanner et al., 1987).

Import of preproteins into isolated mitochondria and cross-linking to Tim44

Yeast cells were grown in YPG medium (1% Bacto-yeast extract, 2% Bacto-peptone, 3% glycerol) at 30°C. Mitochondria were isolated...
essentially according to the protocol of Daum et al. (1982), with modifications as published by Gambill et al. (1993). To induce the tim44-8 phenotype, the mitochondria were pre-incubated at 37°C for 15 min and then cooled to 25°C for subsequent import experiments. Preproteins were synthesized in rabbit reticulocyte lysate in the presence of [35S]methionine/[35S]cysteine (Amersham) after in vitro transcription by SP6 RNA polymerase (Stratagene). To import the preproteins, the reticulocyte lysates were diluted with import buffer [3% (w/v) bovine serum albumin (BSA, fatty acid-free; Sigma), 250 mM sucrose, 60 mM KCl, 5 mM MgCl2, 5 mM sodium malate, 2 mM ATP, 20 mM potassium phosphate, 10 mM MOPS-KOH, pH 7.2] and isolated mitochondria were added to a final concentration of 25 μg of mitochondrial protein per 100 μl assay. Under these conditions, the amount of mitochondria was limiting in the import experiments. Some samples received 1 μM valinomycin from a 100-fold concentrated stock solution in ethanol to dissipate the membrane potential. The samples were then incubated at 25°C for up to 30 min. To accumulate translocation intermediates, hybrid proteins containing a DHFR domain were imported in the presence of 2 μM MTX (Sigma). For treatment of the mitochondria after the import reactions, proteinase K was used at a final concentration of 40 μg/ml for 10 min at 0°C. Mitochondria were reisolated by centrifugation at 16 000 g for 10 min. Proteins were analysed by SDS-PAGE and autoradiography. Relative amounts of [35S]-labelled proteins were quantified using a storage PhosphorImaging system (Molecular Dynamics Inc.). Chemical amounts of the soluble preprotein b2(167)A-DHFR were purified after expression in E.coli as published previously (Dekker et al., 1997). The amount of imported protein was determined by immunoblotting, using an antisera raised against the DHFR moiety of the purified preproteins (Dekker et al., 1996, 1997). Blue native electrophoresis of mitochondrial proteins was performed following a published procedure (Schägger and von Jagow, 1991; Dekker et al., 1996, 1997).

For cross-linking experiments, the hybrid protein Su9(70)–DHFR was accumulated in import sites by import into ATP-depleted mitochondria. Mitochondria (in the presence of 20 μM oligomycin to inhibit the F0-F1-ATPase) and reticulocyte lysate were ATP depleted by incubation with apyrase (10 U/ml; Sigma) for 10 min at 0°C; the import was performed in the presence of 20 μM oligomycin. The mitochondria were washed by centrifugation through a sucrose cushion (500 mM sucrose, 1 mM EDTA, 10 mM MOPS, pH 7.2) and resuspended in 250 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH pH 7.2 (SEM buffer). For cross-linking with EGS (Pierce), the mitochondria (50 μg protein) were incubated at 0°C for 20 min in 1 ml of SEM containing EGs at a final concentration of 200 μM. The reaction was stopped by addition of 100 mM Tris–HCl pH 7.2 and a second incubation of 20 min at 0°C. Cross-linking by DSS (Pierce) was performed following basically the same protocol. DSS at a final concentration of 400 μM, details of the procedure were published previously (Blom et al., 1993; Kührich et al., 1994). Proteins were precipitated by addition of 10% trichloroacetic acid in the presence of 0.0125% deoxycholate. For immunoprecipitation, the precipitates were lysed in 1% SDS, 60 mM Tris–HCl pH 6.8. The samples were diluted 40-fold by addition of 1% (w/v) Triton X-100, 0.3 M NaCl, 10 mM Tris–HCl pH 7.5 and the antibodies were added bound to protein A–Sepharose (Pharmacia).

Assessment of the mitochondrial membrane potential

The Δψ of isolated yeast mitochondria was assessed by recording the fluorescence decrease of the voltage-sensitive dye DiSC3(5) (Molecular Probes) essentially as described previously (Simms et al., 1974; Gärster et al., 1995). An aliquot of 200 μg of mitochondrial protein was used per assay. The mitochondria were pre-incubated at 37°C for 15 min to induce the phenotype of the tim44-8 mutant. The measurements were then performed at 25°C.

Co-immunoprecipitations

All antibodies used in this study were polyclonal antibodies raised in rabbits against isolated proteins. For co-immunoprecipitations, antibodies (6 μl of serum) were pre-bound to protein A–Sepharose (10 μl wet volume; Pharmacia Biotech Inc.) for 1 h in 480 μl of lysis buffer [1% digitonin (1× recrystallized from ethanol), 10% (w/v) glycerol, 50 mM NaCl, 2 mM EDTA, 30 mM HEPES-KOH, pH 7.4]. In parallel, a preprotein containing a DHFR domain was synthesized in a reticulocyte lysate and imported in the presence of MTX into isolated mitochondria (20 μg of mitochondrial protein). After washing with SEMP buffer [250 mM sucrose, 1 mM EDTA, 10 mM MOPS–KOH, pH 7.2, 0.2 mM freshly added phenylmethanesulfonyl fluoride (PMSF)], the mitochondria were resuspended in lysis buffer supplemented with protease inhibitors (2 μg/ml antipain, 5 μg/ml aprotinin, 0.25 μg/ml chymostatin, 1.25 μg/ml leupeptin, 0.5 μg/ml pepstatin A, 0.2 mM PMSF) and shaken end-over-end for 10 min at 8°C. Insoluble material was removed by ultracentrifugation (30 min at 100 000 g). The supernatants were incubated for 45 min at 8°C by end-over-end shaking with antibodies pre-bound to protein A–Sepharose. After three washing cycles with lysis buffer, the protein A–Sepharose pellets were boiled in sample buffer and analysed by SDS–PAGE.

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References


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