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The Tim core complex defines the number of mitochondrial translocation contact sites and can hold arrested preproteins in the absence of matrix Hsp70–Tim44

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Preprotein import into mitochondria is mediated by translocases located in the outer and inner membranes (Tom and Tim) and a matrix Hsp70–Tim44 driving system. By blue native electrophoresis, we identify an ~90K complex with assembled Tim23 and Tim17 as the core of the inner membrane import site for presequence-containing preproteins. Preproteins spanning the two membranes link virtually all Tim presequences of preproteins (Martin et al., 1989; Rassow and Pfanner, 1991; Segui-Real et al., 1993) and components of the general insertion pore (GIP; Tom40 and the three small subunits Tom7, Tom6 and Tom5) (reviewed by Alconada et al., 1995; Lithgow et al., 1995; Lill and Neupert, 1996; Pfanner and Meijer, 1997). The majority of Tom proteins, including the two essential proteins, Tom40 and Tom22, recently were found to associate in a stable complex of ~400K (Dekker et al., 1996; Dietmeier et al., 1997).

The translocase of the inner membrane includes the essential proteins Tim17, Tim23 and Tim44 (Pfanner et al., 1994; Ryan and Jensen, 1995; Schatz and Dobberstein, 1996). Both Tim17 and Tim23 are integral membrane proteins and are thought to be structural members of the translocation channel of the inner membrane (Kübrich et al., 1994; Berthold et al., 1995; Blom et al., 1995). Tim44 is a peripheral membrane protein mainly located on the matrix side of the inner membrane (Blom et al., 1993; Horst et al., 1993) and loosely associates with Tim23 (Berthold et al., 1995; Böner et al., 1997). Two energy sources are required to drive a preprotein across the inner membrane. The membrane potential ΔΨ promotes the initial translocation of the positively charged presequences of preproteins (Martin et al., 1991). Further translocation of preprotein segments requires the action of the matrix heat shock protein 70 (mHsp70), an ATP-dependent molecular chaperone (Kang et al., 1990; Scherer et al., 1990). A fraction of mHsp70 binds to the inner membrane in a nucleotide-sensitive manner. Tim44 has been identified as a major binding site for mtHsp70 (Kronidou et al., 1994; Rassow et al., 1994; Schneider et al., 1994). In addition, the chaperone can also transiently bind to Tim17–Tim23 (Böner et al., 1997). Currently two models for the function of mHsp70 in protein import are discussed (Pfanner and Meijer, 1995; Voos et al., 1996). In the translocation motor model, membrane-bound mHsp70 generates a force that pulls the preprotein in (Glick, 1995; von Ahsen et al., 1995; Horst et al., 1996). In the Brownian ratchet model, movement of the precursor polypeptide in the import channels is driven by Brownian motion and trapping by mHsp70 (Schneider et al., 1994; Ungermann et al., 1994).

In the past years, qualitative information concerning the mitochondrial import process has been accumulated with the use of co-immunoprecipitation and cross-linking experiments. Thereby, mHsp70, Tom and Tim proteins were found in association with a translocating polypeptide (Vestweber et al., 1989; Kang et al., 1990; Scherer et al., 1990; Söllner et al., 1992; Blom et al., 1993; Gambill et al., 1993; Horst et al., 1993, 1995; Ryan and Jensen, 1993; Kübrich et al., 1994; Berthold et al., 1995). These experiments, however, provided only limited information.

Keywords: Hsp70/mitochondria/protein import/ Saccharomyces cerevisiae/Tim complex

Introduction

Preproteins cross the mitochondrial membranes at translocation contact sites, where outer and inner mitochondrial membranes are closely opposed (Schleyer and Neupert, 1985). Transport is mediated by individual translocases in the outer and inner membranes (Tom and Tim, respectively) and a mHsp70-ATP driving system (Kübrich et al., 1995; Lill and Neupert, 1996; Schatz and Dobberstein, 1996; Pfanner and Meijer, 1997). In contrast to the original suggestion, translocation contact sites do not constitute a single, stable channel spanning both membranes (Hwang et al., 1989; Rassow and Pfanner, 1991; Segui-Real et al., 1993), but can be observed when a preprotein links outer and inner membrane translocases (Horst et al., 1995).

The translocase of the outer mitochondrial membrane consists of at least eight different subunits including the preprotein receptors (Tom70, Tom37, Tom20 and Tom22; numbers indicate the apparent molecular mass in kDa) and components of the general insertion pore (GIP; Tom40 and the three small subunits Tom7, Tom6 and Tom5) (reviewed by Alconada et al., 1995; Lithgow et al., 1995; Lill and Neupert, 1996; Pfanner and Meijer, 1997). The majority of Tom proteins, including the two essential proteins Tom40 and Tom22, recently were found to associate in a stable complex of ~400K (Dekker et al., 1996; Dietmeier et al., 1997).

The translocase of the inner membrane includes the essential proteins Tim17, Tim23 and Tim44 (Pfanner et al., 1994; Ryan and Jensen, 1995; Schatz and Dobberstein, 1996). Both Tim17 and Tim23 are integral membrane proteins and are thought to be structural members of the translocation channel of the inner membrane (Kübrich et al., 1994; Berthold et al., 1995; Blom et al., 1995). Tim44 is a peripheral membrane protein mainly located on the matrix side of the inner membrane (Blom et al., 1993; Horst et al., 1993) and loosely associates with Tim23 (Berthold et al., 1995; Böner et al., 1997). Two energy sources are required to drive a preprotein across the inner membrane. The membrane potential ΔΨ promotes the initial translocation of the positively charged presequences of preproteins (Martin et al., 1991). Further translocation of preprotein segments requires the action of the matrix heat shock protein 70 (mHsp70), an ATP-dependent molecular chaperone (Kang et al., 1990; Scherer et al., 1990). A fraction of mHsp70 binds to the inner membrane in a nucleotide-sensitive manner. Tim44 has been identified as a major binding site for mHsp70 (Kronidou et al., 1994; Rassow et al., 1994; Schneider et al., 1994). In addition, the chaperone can also transiently bind to Tim17–Tim23 (Böner et al., 1997). Currently two models for the function of mHsp70 in protein import are discussed (Pfanner and Meijer, 1995; Voos et al., 1996). In the translocation motor model, membrane-bound mHsp70 generates a force that pulls the preprotein in (Glick, 1995; von Ahsen et al., 1995; Horst et al., 1996). In the Brownian ratchet model, movement of the precursor polypeptide in the import channels is driven by Brownian motion and trapping by mHsp70 (Schneider et al., 1994; Ungermann et al., 1994).
on the stoichiometry of protein interactions and the relevance of protein complexes for the translocation process. Here we quantitatively analyzed the interactions between a membrane-spanning preprotein and the transport complexes of mitochondrial translocation contact sites. We identified a complex of ~90K containing Tim23 and Tim17 as the major inner membrane import site for presequence-containing preproteins. Surprisingly, preproteins spanning the mitochondrial membranes connected the bulk of 90K Tim complexes with only ~25% of all 400K Tom complexes in a 600K supercomplex. Apparently, mitochondria contain many more 400K Tom complexes than 90K Tom complexes. Accumulated preproteins were kept stably in the 600K complex in the absence of mtHsp70–Tim44. The findings suggest that the import channel itself is able to hold the translocating chain.

Results

Assembly of Tim23 and Tim17 into an ~90K complex involved in import of cleavable preproteins

After lysis of mitochondrial membranes with digitonin, Tim23 and Tim17 can be co-precipitated (Berthold et al., 1995; Blom et al., 1995). In order to visualize a protein complex with Tim17 and Tim23 directly, digitonin-lysed Saccharomyces cerevisiae mitochondria were subjected to blue native electrophoresis (BN-PAGE) (Schägger and von Jagow, 1991; Schägger et al., 1994). Immunodecoration with antibodies directed against Tim23 identified an abundant complex of ~90K (Figure 1, lanes 5 and 6). Additionally, complexes of ~140K and ~240K were detected in low amounts. When denatured by SDS prior to BN-PAGE, all Tim23 migrated at the monomer position (Figure 1, lane 4). For comparison, all Tom40 (Figure 1, lanes 2 and 3) and other Tom proteins such as Tom22 are present in an ~400K complex (Dekker et al., 1996). The integrity of the 400K and 90K complexes was not affected when the membrane potential (Δψ) was dissipated prior to lysis of mitochondria (Figure 1, lanes 3 and 6) or by the presence or absence of ATP (see below).

To determine if Tim17 was present in the 90K complex, we synthesized and radiolabeled Tim17 in vitro and imported it into isolated yeast mitochondria. When the import was performed in the presence of a Δψ, Tim17 was transported to the inner membrane (Bömér et al., 1996) and assembled into an ~90K complex (Figure 1, lane 7). As control, radiolabeled Tim17 incubated with isolated mitochondria in the absence of a Δψ was not found in a 90K complex (Figure 1, lane 8). This indicates that assembly of Tim17 into the 90K complex depends on import into the inner membrane and does not occur after lysis of the mitochondria. These results suggest that Tim23 and Tim17 are contained in an ~90K complex in the inner membrane.

To obtain evidence that both Tim proteins are present in the same 90K complex, we analyzed a yeast mutant harboring the mutant allele tim23-2. This mutant is impaired in mitochondrial protein import in vivo (Maarse et al., 1992; Dekker et al., 1993), and a double mutant with tim17-1 is synthetically lethal (Blom et al., 1995). We determined the DNA sequence of the mutant allele and found a single point mutation, leading to substitution of a conserved glycine for a glutamic acid at position 112 of wild-type Tim23. This mutation does not affect mitochondrial import of wild-type and mutant Tim23 after opening of the outer membrane by swelling (Figure 2B, lanes 3 and 4; the antiserum was directed against the N-terminus of Tim23), indicative of a correct membrane insertion of the mutant protein (Kübrich et al., 1994). Import of 35S-labeled precursors of Tim23 and Tim17 into the inner membrane and correct membrane insertion, assessed by formation of the fragments Tim23’ and Tim17’ after swelling and protease treatment (Kübrich et al., 1994; Bömér et al., 1996), were comparable between mutant and wild-type mitochondria (Figure 2B, lanes 9, 10, 15 and 16).

tim23-2 mitochondria contained, however, strongly reduced amounts of the 90K complex (Figure 2C, lane 2). The mutant protein Tim23-2 mainly migrated at the monomer position in BN-PAGE, indicating that the stability of the 90K Tim complex was decreased in the mutant. Assembly of in vitro imported Tim17 into the 90 K complex was strongly reduced compared with wild-type (Figure 2C, lanes 4 and 5). Interestingly, the assembly of in vitro imported wild-type Tim23 into the 90 K complex was enhanced in the mutant mitochondria (Figure 2C, lane 8; compare with lane 7). Evidently, unassembled Tim subunits (like Tim17) are present in increased amounts in the mutant mitochondria and immediately form new 90K Tom complexes with imported wild-type Tim23. Thus, the tim23-2 mutation does not affect mitochondrial import and correct insertion into the inner membrane of Tim23 and Tim17, but reduces the stable assembly of Tim17 into the 600K complex in the absence of mtHsp70–Tim44 complexes with only ~25% of all 400K Tom complexes were detected by autoradiography.

Fig. 1. Tim23 and Tim17 are present in a 90K protein complex. Mitochondria were pre-incubated for 5 min in the absence (lanes 1, 2, 4, 5 and 7) or presence of valinomycin (lanes 3, 6 and 8) to disrupt Δψ. For lanes 7 and 8, mitochondria were incubated for 15 min at 25°C with [35S]Tim17. Mitochondria were re-isolated, lysed with digitonin buffer (lanes 2, 3 and 5–8) or 2% SDS (lanes 1 and 4) and analyzed by BN-PAGE as described in Materials and methods. Tom and Tim complexes were detected by immunodecoration with anti-Tom40 (lanes 1–3) and anti-Tim23N (lanes 4–6) antibodies, respectively. Tim17-containing complexes were detected by autoradiography.
Fig. 2. An intact 90K Tim complex is required for import of presequence-containing preproteins. (A) Determination of the amino acid sequence of Tim23-2. The conserved Gly112 in the first putative membrane-spanning segment of the S.cerevisiae (S.c.) Tim23 protein is changed to glutamic acid in Tim23-2. Homology to S.c.Tim17, S.c.Tim22 and A.t. (Arabidopsis thaliana) Tim23 is boxed. Putative transmembrane segments in Tim23 are hatched. (B) 35S-labeled Tim23 (lanes 5–10) or Tim17 (lanes 11–16) were imported into wild-type and tim23-2 mitochondria at 25°C in the presence or absence of a Δψ. For lanes 1–4 no import was performed. Samples were split in two and one half was kept on ice. The other half was swollen to disrupt the outer membrane and treated with proteinase K (PK). Mitochondria were re-isolated, washed and analyzed by SDS–PAGE. Endogenous Tim23 was detected by immunodecoration with anti-Tim23N antibodies [generated against a peptide corresponding to the 14 amino-terminal residues of Tim23 (Kübrich et al., 1994)] (lanes 1–4). Imported Tim23 and Tim17 were detected by autoradiography (lanes 5–16). Tim23', Tim17', proteolytic fragments of Tim23 and Tim17, respectively. (C) 35S-labeled Tim23 (lanes 3–5) or Tim17 (lanes 6–8) were imported into wild-type and tim23-2 mitochondria in the presence or absence of Δψ. Mitochondria were re-isolated, washed and lysed with digitonin buffer and analyzed by BN-PAGE. Endogenous Tim23 was detected by immunodecoration with anti-Tim23N antibodies (lanes 1–2). Imported Tim23 and Tim17 was detected by autoradiography (lanes 3–8). (D) Assessment of Δψ in wild-type (WT) and tim23-2 mitochondria. Fluorescence is measured in time at 670 nm. The presence of a membrane potential across the mitochondrial membranes leads to a drop in fluorescence. The difference in fluorescence in the absence and presence of valinomycin is taken as assessment of Δψ. Mito., mitochondria added; Val., valinomycin added. (E) Radiolabeled F1β (lanes 1–3) and ADP/ATP carrier (AAC; lanes 4–6) were imported into wild-type and tim23-2 mitochondria in the presence or absence of Δψ. Samples were split and incubated with or without PK after swelling. Import was analyzed by SDS–PAGE and autoradiography. Protease-resistant material was quantified (mF1β and AAC bound to wild-type mitochondria before PK treatment was set at 100%). p, precursor form of F1β; m, mature F1β.
functional 90K Tim complex for efficient import and membrane insertion, whereas cleavable mitochondrial preproteins require the assembly of Tim23 and Tim17 into an intact 90K complex.

**Preproteins spanning both mitochondrial membranes quantitatively associate with the 90K Tim complexes and one in four Tom complexes**

In order to address if the 90K Tim complex associated with a precursor polypeptide in transit, chemical amounts of a mitochondrial preprotein were accumulated in mitochondrial import sites. The fusion protein b$_2$Δ-DHFR, consisting of the first 167 amino acid residues of the cytochrome b$_2$ preprotein (except for a deletion of 19 residues in the intermembrane space sorting signal) and the entire dihydrofolate reductase (Koll et al., 1992), was expressed in *Escherichia coli* cells (Figure 3A, lane 2). b$_2$Δ-DHFR was purified from *E. coli* lysates such that it was >95% pure (Figure 3A, lane 3) and fully soluble. b$_2$Δ-DHFR was added to energized yeast mitochondria and analyzed by SDS–PAGE. Immunodecoration with specific antibodies demonstrated that the preprotein was processed twice (Figure 3B, lane 1). The processed forms were protected against treatment of the mitochondria with proteinase K (Figure 3B, lane 6). Import depended on the generation of a membrane potential Δψ across the mitochondrial inner membrane (Figure 3B, lanes 2 and 7). The ligand methotrexate stabilizes the tertiary structure of the DHFR domain and thereby prevents its translocation across the mitochondrial membranes (Eilers and Schatz, 1986; Rassow et al., 1989). When b$_2$Δ-DHFR was imported in the presence of methotrexate, it accumulated as a translocation intermediate spanning both mitochondrial membranes at translocation contact sites: the presequence was processed to the first intermediate form (Voos et al., 1994) but remained accessible to externally added protease (Figure 3B, lanes 3 and 8). In order to test if the methotrexate intermediate was on the correct import pathway, mitochondria were re-isolated and washed to remove the ligand. After a second incubation in the absence of methotrexate, the processed preprotein became protease resistant (Figure 3B, lane 10). We conclude that chemical amounts of b$_2$Δ-DHFR can be reversibly accumulated in mitochondrial translocation contact sites.

We then tested the effect of b$_2$Δ-DHFR on the 90K Tim complex. After accumulation of b$_2$Δ-DHFR in the presence of methotrexate and lysis of the mitochondria under non-denaturing conditions (digitonin), Tim23 was found mainly at ~600K (Figure 3C, lane 4). A small amount of Tim23 migrated at an even higher molecular weight (>800K). The small amounts of Tim23 observed at 140K and 240K after long exposures of the blots (see Figure 1, lanes 5 and 6) also quantitatively shifted to the high molecular weight area (>600K) (not shown). The occurrence of Tim23 in the high molecular weight area strictly depended on the accumulation of b$_2$Δ-DHFR across both membranes. When the preprotein was accumulated at the outer membrane in the absence of a Δψ (Figure 3C, lane 3) or completely imported into the matrix in the presence of a Δψ, and absence of methotrexate (Figure 3C, lane 2), Tim23 was only found in the low molecular weight area (mainly the 90K complex). Similarly, the vast majority of assembled Tim17 shifted to the >600K area in the presence of the two-membrane-spanning intermediate (Figure 3D, column 3). The molecular weight shift to the 600K area suggested that the 90K Tim23–Tim17 complex efficiently associated with the translocation intermediate.

When probed for Tom40 and Tom22, a molecular weight shift of part of the 400K Tom complex to the same mobility as Tim23 and Tim17 was observed (Figure 3C, lane 8 and D, columns 5 and 7). The requirements for the shift of Tom40 and Tom22 to the high molecular weight area were identical to that of Tim23–Tim17, i.e. strictly dependent on the accumulation of b$_2$Δ-DHFR in translocation contact sites. This indicates that the translocation intermediate spans the transport machineries of both outer and inner membranes and that the 600K band contains the 400K Tom complex, the 90K Tim complex and the methotrexate-bound preprotein. Indeed, co-immunoprecipitation experiments confirmed the association of Tom40 and Tim23 after accumulation of b$_2$Δ-DHFR (unpublished data).

Quantification revealed that 80–100% of Tim17 and Tim23, but only 20–25% of Tom22 and Tom40, were present in the 600K supercomplex (Figure 3D). This unexpectedly implied that the 400K Tom complex was more abundant than the 90K Tim complex. We therefore determined the total amount of Tim23 and Tom40 in mitochondria by standardized immunoblotting of mitochondrial Tim23 and Tom40 in comparison with purified Tim23 and Tom40 expressed in *E. coli* (not shown). Indeed, yeast mitochondria were found to contain ~20 pmol of Tim23 and ~250 pmol of Tom40 per mg of mitochondrial protein. The size of the 600K supercomplex suggests that apparently one 400K Tom complex is linked to one or two 90K Tim complex(es) by the translocation intermediate. Thus, only about one in four Tom complexes are connected to the 90K Tim complexes in translocation contact sites.

To investigate the size of the translocation complex formed by the preprotein and the Tim complex alone, b$_2$Δ-DHFR was added to swollen mitochondria with opened outer membrane (mitoplasts), thereby allowing direct access of the preprotein to the Tim machinery (Ohba and Schatz, 1987; Hwang et al., 1989). After accumulation of b$_2$Δ-DHFR in mitoplasts in the presence of methotrexate, Tim23 was found preferentially in an ~130K complex (Figure 3E, lower panel, column 3) and only to a minor extent in the 600K complex (Figure 3E, lower panel, column 4). To detect the preprotein directly, we imported *in vitro* synthesized $^{35}$S-labeled b$_2$Δ-DHFR into mitoplasts in the presence of methotrexate. The preprotein was processed efficiently to the intermediate sized form, demonstrating that it spanned the inner membrane. Similarly to Tim23, the preprotein was found mainly in a 130K complex in mitoplasts (Figure 3E, upper panel, column 3). With intact mitochondria, the processed b$_2$Δ-DHFR, like Tim23, was found mainly in the 600K complex (Figure 3E, columns 2). The size of the 130K complex of mitoplasts can accommodate one 90K Tim complex and one preprotein, indicating that the 90K Tim complex represents the core unit of the inner membrane import machinery. Since the 600K supercomplex is almost absent when import occurred into mitoplasts (Figure 3E, columns 4), its formation depends on the linkage of the 400K Tom complex with a functional 90K Tim complex by a translocation intermediate spanning both membranes.
Accumulation of $b_2\Delta$-DHFR in translocation contact sites strongly inhibits import of cleavable preproteins, but not preproteins with internal targeting information

To address the role of translocation contact sites and the 90K Tim complex in import of different classes of preproteins, $b_2\Delta$-DHFR was arrested in the import sites in the presence of methotrexate and the mitochondria were re-isolated and incubated with various in vitro synthesized preproteins. The import of cleavable preproteins was assessed by monitoring their proteolytic processing and transport to a protease-protected location (Figure 4A); the import of non-cleavable preproteins was assessed by protease protection, in the case of integral inner membrane
Mitochondrial translocation contact sites

Fig. 4. Accumulation of \( b_2\Delta\text{-DHFR} \) blocks translocation of presequence-containing preproteins. (A and B) Mitochondria were pre-incubated for 10 min at 25°C in the absence (left parts) or presence (right parts) of \( b_2\Delta\text{-DHFR} \) and methotrexate (MTX). Mitochondria were re-isolated and washed with SEM + MTX to remove unbound \( b_2\Delta\text{-DHFR} \). \( \text{\textsuperscript{35}S} \)-labeled F1\( \beta \) (A; left panel), \( \alpha \)-subunit of matrix processing peptidase (MPP) (A; middle panel), cytochrome \( b_2 \) (A; right panel), porin (B; left panel), Tom40 (B; middle panel) or AAC (B; right panel) were added, samples were incubated at 25°C and aliquots were removed at the indicated time points. Import was stopped directly by addition of valinomycin on ice. Samples were divided in two, and to one half proteinase K (PK) was added. For the AAC samples, mitochondria were swollen before PK treatment.

After 15 min incubation on ice, mitochondria were re-isolated and washed. Samples were analyzed by SDS–PAGE and autoradiography. Import was quantified with ImageQuant software. Protease-resistant material accumulated after 15 min in the absence of \( b_2\Delta\text{-DHFR} \) was set to 100%. p, precursor; i, intermediate; m, mature. (C) Mitochondria were pre-incubated for 10 min at 25°C without (left panel) or with (right panel) \( b_2\Delta\text{-DHFR} \) and MTX. Membrane potential was assessed directly after the import reaction for 10 min as described in Materials and methods and the legend to Figure 2B. Mito., mitochondria added; Val., valinomycin added. (D) Mitochondria (25 \( \mu \text{g} \)) were pre-incubated for 10 min at 25°C with the indicated amounts of \( b_2\Delta\text{-DHFR} \) and MTX. Mitochondria were re-isolated and washed to remove unbound \( b_2\Delta\text{-DHFR} \). Samples were divided; one part was used directly for import of \( \text{\textsuperscript{35}S} \)-labeled F1\( \beta \) and Tim23 before swelling and re-isolation (lanes 9–16). The other part was first swollen, re-isolated and then used in the import reaction (lanes 1–8). Samples were again divided and one part was treated with PK (lanes 5–8 and 13–16). After re-isolation, mitochondria were analyzed by SDS–PAGE and autoradiography. p, precursor of F1\( \beta \); m, mature F1\( \beta \); Tim23\( 9 \), fragment of Tim23 lacking the N-terminal domain.

proteins the outer membrane was opened by swelling before the protease treatment (Figure 4B) (Alconada et al., 1995). The import of all cleavable preproteins tested was strongly inhibited, independently of their final location in mitochondria, including F1\( \beta \) (matrix side of the inner membrane) (Figure 4A, left panel), the \( \alpha \)-subunit of the matrix processing peptidase (\( \alpha \)-MPP) (Figure 4A, middle panel), cytochrome \( b_2 \) (intermembrane space) (Figure 4A, right panel), cytochrome \( c_1 \), Tim44, CoxVa (all inner membrane) and Hsp60 (matrix) (unpublished data). The import of non-cleavable preproteins was only slightly inhibited, including that of the outer membrane proteins porin (Figure 4B, left panel) and Tom40 (Figure 4B, middle panel), and of the inner membrane proteins AAC (Figure 4B, right panel), phosphate carrier (unpublished data) and Tim23 (see below, Figure 4D). The \( \Delta \psi \) of mitochondria with accumulated \( b_2\Delta\text{-DHFR} \), as assessed by use of DiSC\(_3\)(5), was comparable with that of control mitochondria (Figure 4C).

We conclude that the accumulation of \( b_2\Delta\text{-DHFR} \) in
translocation contact sites inhibits the import of cleavable preproteins. The minor inhibition of import of outer membrane proteins suggests that most Tom complexes are not blocked, in agreement with the quantification reported in Figure 3D. The slight import inhibition of non-cleavable inner membrane proteins is most likely due to the partial inactivation of Tom complexes. In agreement with the results obtained with the \textit{tim}23-2 mutant, a functional 90K Tim complex is not required for the efficient import of proteins lacking a cleavable presequence.

If the strong inhibition of import of presequence-containing proteins is caused by the saturation of the 90K Tim channels, the generation of mitoplasts after \textit{b}2\textit{Δ-DHFR} accumulation should not alleviate the inhibition. Mitochondria were pre-incubated with \textit{b}2\textit{Δ-DHFR} in the presence of methotrexate, re-isolated and swollen. The resulting mitoplasts were used in a second import reaction with the precursors of \textit{F}_{\text{β}} and \textit{Tim}23 (Figure 4D, lanes 1–8). As a control, \textit{F}_{\text{β}} and \textit{Tim}23 were imported into mitochondria, carrying accumulated \textit{b}2\textit{Δ-DHFR}, before swelling (Figure 4D, lanes 9–16). The accumulation of \textit{b}2\textit{Δ-DHFR} led to an inhibition of import of the precursor of \textit{F}_{\text{β}} in both cases. The degree of import inhibition was indistinguishable between mitoplasts (Figure 4D, lanes 2–4 and 6–8) and mitochondria (Figure 4D, lanes 10–12 and 14–16). Insertion of \textit{Tim}23 into the inner membrane was not or only slightly affected, as assessed by formation of the fragment \textit{Tim}23′ by protease treatment of mitoplasts (Figure 4D, lanes 6–8 and 14–16). We conclude that accumulation of \textit{b}2\textit{Δ-DHFR} in translocation contact sites of mitochondria blocks the inner membrane import sites for cleavable preproteins. No additional import sites for cleavable preproteins are revealed after opening of the outer membrane. This strongly suggests that the 90K Tim complex is the core translocase and limits the number of import sites for presequence-containing preproteins.

\textbf{The 600K supercomplex contains only sub-stoichiometric amounts of mtHsp70}

An intriguing observation during the course of this study was that the 600K translocation complex was stable during the isolation procedure, including a 4 h electrophoretic run, indicating that \textit{b}2\textit{Δ-DHFR} is held stably in the transport channels and does not slip back into solution. Previously it has been shown that mtHsp70 has the ability to interact with incoming preproteins (Kang \textit{et al.}, 1990; Scherer \textit{et al.}, 1990; Gambill \textit{et al.}, 1993; Voos \textit{et al.}, 1993; Schneider \textit{et al.}, 1994; Horst \textit{et al.}, 1995). To examine the abundance of mtHsp70 in the 600K complex, we accumulated \textit{b}2\textit{Δ-DHFR} with methotrexate, lysed mitochondria in the presence of EDTA and separated the different membrane complexes by BN-PAGE. We included a urea–SDS–PAGE as second dimension such that the migration of mtHsp70, Tom and Tim proteins could be determined simultaneously. Similarly to the one-dimensional analysis (Figure 3C and D), the accumulation of \textit{b}2\textit{Δ-DHFR} led to an efficient shift of \textit{Tim}23 and a partial shift of \textit{Tom}40 and \textit{Tom}22 into the 600K complex (Figure 5A, compare \textit{−b}2\textit{Δ-DHFR} with +\textit{b}2\textit{Δ-DHFR}). The amount of protein in the 600K complex was found to be almost 20 pmol/mg for \textit{Tim}23, ~60 pmol/mg for \textit{Tom}40 and ~15 pmol/mg mitochondrial protein for \textit{b}2\textit{Δ-DHFR} (Figure 5C, left panel) (\textit{b}2\textit{Δ-DHFR} in the 600K complex was quantified by standardized immunoblotting in comparison with purified \textit{b}2\textit{Δ-DHFR} protein).

The total amount of mitochondrial mtHsp70 was determined to be ~100 pmol of mtHsp70/mg protein. About six or seven molecules of mtHsp70 are, therefore, available per molecule of incoming \textit{b}2\textit{Δ-DHFR} preprotein. In other words, at least 15% of total mtHsp70 should migrate at the 600K position if it were in a stoichiometric interaction with \textit{b}2\textit{Δ-DHFR}. The mtHsp70 spot at the position of the 600K complex (Figure 5A, see arrowhead), however, does not contain more than 0.5 pmol of mtHsp70 per mg of mitochondrial protein (Figure 5C, left panel). This means that <5% of all stably associated \textit{b}2\textit{Δ-DHFR} translocation intermediates interact with mtHsp70 under these conditions. This value is similar to the efficiency of co-immunoprecipitation of membrane-spanning preproteins with mtHsp70 (Ungermann \textit{et al.}, 1994). It should be emphasized that the condition used for analysis (the presence of EDTA) stabilizes the interaction of mtHsp70 with preproteins and permits a quantitative recovery of mtHsp70–preprotein complexes (Rospert \textit{et al.}, 1996). Apparently, stoichiometric amounts of mtHsp70 are not required for holding of translocation intermediates in the isolated translocation complex.

To analyze the translocation complex in the absence of mtHsp70, we lysed mitochondria in the presence of 5 mM Mg\textit{ATP}, which efficiently dissociates mtHsp70 from preproteins (Voos \textit{et al.}, 1994). \textit{Tim}23, however, efficiently shifted to the 600K position under these conditions (Figure 5B). Indeed, \textit{b}2\textit{Δ-DHFR} was still stably associated with the import complex after lysis in Mg\textit{ATP}-containing buffer, irrespective of the temperature of incubation of the complex (4–25°C) (Figure 5B and C). Furthermore, no mtHsp70 could be detected in the 600K supercomplex (see Figure 5B, arrowhead, and C), indicating that the molecular chaperone is not required for the stable holding of the translocation intermediate in the isolated import channel.

\textit{Tim}44 did not migrate as a discrete band in BN-PAGE, but was found in a range from 70K to 250K in the absence of \textit{b}2\textit{Δ-DHFR} (Figure 5A, left panel). After accumulation of \textit{b}2\textit{Δ-DHFR}, \textit{Tim}44 did not shift to the 600K area; in fact, the distribution of \textit{Tim}44 shifted more to the lower molecular weight area (<100K) (Figure 5A, right panel). It is therefore unlikely that \textit{Tim}44 is a structural component of the 600K translocation complex.

\textbf{A membrane-spanning preprotein stabilizes the 90K Tim complex}

The stable interaction of the \textit{b}2\textit{Δ-DHFR} preprotein with the import apparatus in the absence of mtHsp70–\textit{Tim}44 suggests that the 90K Tim channel itself might be involved in holding incoming preproteins, rather than just being a passive diffusion channel. To obtain further evidence for an active interaction of preproteins and Tim complex, we investigated the influence of a spanning preprotein on the constitution of the 90K Tim complex. As shown above (Figures 1 and 2C), the 90K Tim complex is dynamic in nature: newly imported subunits can assemble into the 90K complex and are thereby exchanged for endogenous subunits. A subsequent accumulation of membrane-spanning \textit{b}2\textit{Δ-DHFR} leads to an efficient shift of \textit{in vitro
assembled Tim17 and Tim23 into the 600K translocation complex (Figure 3D, and Figure 6, columns 6 and 12).

We wondered if newly imported Tim proteins could also assemble directly into the 600K translocation complex. First b_2Δ-DHFR was accumulated in the presence of methotrexate, then radiolabeled Tim17 or Tim23 were imported. The import and insertion into the inner membrane of these Tim proteins was not inhibited by the membrane-spanning b_2Δ-DHFR (see Figure 4D). However, the assembly into the 600K translocation complex was blocked; only background levels were observed (Figure 6, columns 3 and 9). The accumulation of a translocation intermediate apparently stabilizes the Tim complex and prevents substitution of endogenous subunits for newly imported Tim proteins. Moreover, since the assembly into the 600K complex strongly depended on the order of addition of b_2Δ-DHFR and radiolabeled Tim23–Tim17 to mitochondria, it can be excluded that the 600K complex was formed after lysis of mitochondria with detergent.

We conclude that the Tim complex is dynamic in nature, but is stabilized by the presence of a preprotein. The Tim complex is not a passive diffusion channel, but seems to play an active role in the translocation of presequence-containing proteins across the mitochondrial inner membrane.

Discussion

We report the identification of an ~90K complex in the mitochondrial inner membrane with assembled Tim17 and Tim23. Accumulation of a preprotein directly across the inner membrane of mitoplasts leads to formation of a 130K complex consisting of the 90K complex and the preprotein, indicating that the 90K complex forms the core unit of the Tim machinery. A single amino acid exchange in a membrane-spanning segment of Tim23 destabilizes the 90K complex and thereby selectively inhibits the import of cleavable preproteins into the mitochondrial matrix. The results presented here show that the 90K Tim complex represents the major inner membrane import site for presequence-containing preproteins, and we thus term it the ‘Tim core complex’.

Accumulation of a translocation intermediate of a cleavable preprotein across both mitochondrial membranes links the 90K Tim complexes and 400K Tom complexes into a supercomplex of ~600K. Determination of the constitution of the 600K complex led to several unexpected findings. (i) Mitochondria contain significantly more Tom complexes than Tim core complexes. Only ~25% of the 400K Tom complexes participate in translocation of cleavable preproteins. (ii) The Tim core complexes are stabilized by accumulation of a translocation intermediate, preventing exchange of Tim subunits. (iii) The translocation intermediate is kept stably in the 600K complex in the virtual absence of mtHsp70 and Tim44.

Since previous models suggested that the Tim complexes

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**Fig. 5.** MtHsp70 is not required for holding of a translocation intermediate in the isolated import channel. (A and B) Mitochondria were pre-incubated for 10 min at 25°C in the absence (left panel) or presence (right panel) of b_2Δ-DHFR and methotrexate. After re-isolation and washing, mitochondria were lysed in digitonin buffer containing 5 mM EDTA (A) or 5 mM MgCl_2 and 5 mM ATP (B). After a clarification, protein complexes were separated by BN-PAGE (first dimension, from right to left). Individual lanes were cut out of the gel and separated by a second dimension urea–SDS–PAGE (from top to bottom). Proteins were blotted on nitrocellulose membranes, and individual proteins were detected by immunodecoration with antibodies against mtHsp70, cytochrome b_2 (for b_2Δ-DHFR), Tim44, Tom40, Tim23 and Tom22. The position of the 90K, 400K and 600K complexes is indicated. The arrowhead points to the position of the 600K complex in the immunoblot for mtHsp70. The 600K complex was stable independently of whether the mitochondrial lysate was incubated at 4 or 25°C. (C) Quantification of the absolute amount of protein migrating in the 600K area of panels (A) (EDTA) and (B) (MgATP). The intensity of immunoreactivity of protein spots on the two-dimensional gels was compared with the immunoreactivity of a standard range of purified b_2Δ-DHFR and mtHsp70. The amount of Tim23 and Tom40 migrating at the 600K position was estimated from a comparison with the total amount of these proteins present in mitochondria.
are more abundant than the Tom complexes (summarized in Hwang et al., 1989; Pfanner et al., 1992), we analyzed the unexpected finding that membrane-spanning preproteins connect the Tim core complexes with only a quarter of Tom complexes, both quantitatively and functionally. A direct comparison of the total amounts of Tim23 and Tom40 supported the conclusion that mitochondria contain more Tom than Tim complexes. Approximately 15 pmol of membrane-spanning preproteins per mg of mitochondrial protein were accumulated in the 600K complex. We conclude that mitochondria contain ~15 pmol of Tim core complexes per mg of protein. Since virtually all Tim23 (20 pmol/mg) associates with membrane-spanning preproteins, the Tim complex contains one or two copies of Tim23 per 90K unit. Bauer et al. (1996) reported that (at least part of) Tim23 is able to form homodimers in the presence or absence of methotrexate (MTX) and valinomycin as indicated. b2-DHFR was then added to reactions 4–6 and 10–12, [35S]Tim23 to reactions 1–3 and [35S]Tim17 to reactions 7–9, and incubation was continued for another 15 min at 25°C. Mitochondria were re-isolated, washed and protein complexes were analyzed by BN-PAGE. [35S]Tim23 and [35S]Tim17 in the 600K area were quantified. [35S]Tim23 and [35S]Tim17 accumulated at 600K in reactions 6 and 12 respectively was set at 100%.

Fig. 6. Accumulation of b2-DHFR stabilizes the Tim complex. Mitochondria were pre-incubated for 15 min at 25°C with b2-DHFR (reactions 1–3 and 7–9), [35S]Tim23 (reactions 4–6) or [35S]Tim17 (reactions 10–12) in the presence or absence of methotrexate (MTX) and valinomycin as indicated. b2-DHFR was then added to reactions 4–6 and 10–12, [35S]Tim23 to reactions 1–3 and [35S]Tim17 to reactions 7–9, and incubation was continued for another 15 min at 25°C. Mitochondria were re-isolated, washed and protein complexes were analyzed by BN-PAGE. [35S]Tim23 and [35S]Tim17 in the 600K area were quantified. [35S]Tim23 and [35S]Tim17 accumulated at 600K in reactions 6 and 12 respectively was set at 100%.

Genetic analysis and co-immunoprecipitation experiments showed multiple interactions between Tim44, Tim23, Tim17 and mtHsp70; thereby Tim44 was found to interact with mtHsp70 and with Tim23 (Blom et al., 1995; Berthold et al., 1995; Bömer et al., 1997). Only a minor amount of Tim44, however, is present at the 90K position, suggesting that the 90K Tim complex does not contain stoichiometric amounts of Tim44 [the total amount of Tim44 in mitochondria is five to ten times lower than that of Tim23 or Tim17 (Blom et al., 1995)]. Since Tim44 migrates in several peaks in a broad range from 70K to 250K, an assignment of complexes with interacting partners is not yet possible. It is interesting to note, however, that a small fraction of Tim23 was found in 140K and 240K Tim complexes (Figure 1) that participated in protein translocation as evidenced by their shift to the 600K position after accumulation of preproteins. Tim44, however, shifted more to the low molecular weight range (<100K) after accumulation of membrane-spanning preproteins. A possible scenario may thus be that Tim44 interacts with a fraction of Tim23 in the 140K and 240K complexes in the absence of translocating polypeptides; insertion of preproteins could cause dissociation of the complexes such that Tim23–Tim17 shifts to the 600K position, whereas Tim44 is set free.

The exchange of subunits of the Tim core complex can take place in the absence, but not in the presence of a membrane-spanning preprotein. The accumulation of a
clevable preprotein apparently changes the dynamic behavior of the Tim core complex such that the interactions between Tim17 and Tim23 are stabilized. We propose that the Tim core complex is not a passive diffusion channel, but that an active interaction with the preprotein in transit can hold the import intermediate in the import channel and prevent retrograde translocation. Interactions between targeted polypeptides and translocation complexes have also been observed for the preprotein translocases of the E. coli plasma membrane, the endoplasmic reticulum and the chloroplast membranes (Joly and Wickner, 1993; Schnell et al., 1994; Walter and Johnson, 1994; Jungnickel and Rapoport, 1995; Lubeck et al., 1996). A direct scanning of translocating polypeptides may be important for the proofreading of sorting signals (Singer and Yaffe, 1990; Borel and Simon, 1996; Do et al., 1996) such as the recognition of intra-mitochondrial sorting signals and their subsequent lateral release into the inner membrane.

A preprotein is kept stably in the isolated translocation complexes in the presence of only sub-stoichiometric amounts of mtHsp70 or even no mtHsp70 at all. It should be emphasized that the preprotein employed (b₂₃-A-DHFR) is one of the ‘classical’ preproteins used to demonstrate a requirement for mtHsp70 for protein import (Schneider et al., 1994; Stuart et al., 1994; Voos et al., 1993, 1994, 1996). This result supports the view that mtHsp70 performs a regulatory effect on the function of the Tim core complex. Upon release of mtHsp70–Tim44, the import channel may thus include a facilitation of opening of the Tim channel in addition to the currently discussed functions in biased diffusion or pulling of preproteins.

Materials and methods

Import of preproteins into isolated mitochondria

Yeast cells were grown in YPG medium (1% yeast extract, 2% peptone, 3% glycerol) and mitochondria were prepared according to published procedures (Daum et al., 1982; Gambill et al., 1993). Radiolabeled preproteins were synthesized in rabbit reticulocyte lysate in the presence of [³⁵S]methionine/[³⁵S]cysteine (Amersham) after in vitro transcription by SP6 or T7 polymerase from transcription vector pGEM4Z (Promega) containing the gene of interest. Reticulocyte lysates were used directly in import reactions, or (when import was analyzed by BN-PAGE) first precipitated with 60% saturated (NH₄)₂SO₄ to remove free label. Pellets were resuspended in the original volume of SEM buffer (250 mM sucrose, 10 mM MOPS–KOH, pH 7.2, 1 mM EDTA), before use in import reactions.

Import reactions were performed as described (Alconada et al., 1995) by incubation of reticulocyte lysate with 20 µg of isolated yeast mitochondria in import buffer [3% (w/v) bovine serum albumin (BSA), 250 mM sucrose, 80 mM KCl, 10 mM MOPS–KOH, pH 7.2, 5 mM MgCl₂, 2 mM NADH, 2 mM ATP] for 15 min at 25°C, unless stated otherwise. Samples with denatured membranes were recovered by 1 (1 mM valinomycin, 8 µM antimycin A and 20 µM oligomycin) prior to the incubation. Import reactions were stopped by the addition of VAO.

For generation of mitoplasts by hypotonic swelling, mitochondria were diluted with 9 volumes of EM buffer (10 mM MOPS–KOH, pH 7.2, 1 mM EDTA) and left on ice for 15 min. PK-treated samples were incubated with 20 µg/ml protease K for 15 min on ice. Phenylmethyl-sulfonyl fluoride (PMSF) was added until 1 mM before re-isolation of mitochondria by centrifugation at 14 000 g for 10 min and washing with SEM buffer. Import was analyzed by urea–SDS–PAGE and a storage phosphor imaging system (Molecular Dynamics Inc.). Quantification was performed using ImageQuant software.

For accumulation of b₂₃-DHFR across the mitochondrial membranes, 50 µg of mitochondria were incubated for 15 min at 25°C in import buffer with 1 µM of purified b₂₃-DHFR protein in the presence of 2 µM methotrexate. Mitochondria were re-isolated by centrifugation, washed with SEM and used in import reactions or directly analyzed by BN-PAGE.

Isolation and purification of b₂₃-DHFR

The E. coli strain BMHT1-18 with the plasmid pUHE 73-1 (expressing a fusion protein consisting of the N-terminal 167 residues of the cytochrome b₂ precursor encompassing the mitochondrial targeting signal, but lacking residues 47–65 of the intermembrane space sorting signal, fused to the complete mouse DHFR protein, under control of the lacZ promoter (Koll et al., 1992)) was grown at 37°C in LB medium (1% Bacto lactose, 0.5% yeast extract, 0.5% NaCl) to an OD₆₀₀ of 1. Then 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added, and shaking at 37°C was continued for another 2 h.

Cells were collected by centrifugation, and the pellet was washed with 30% sucrose, 20 mM KP, pH 8.0, 1 mM EDTA and with 10 mM dithiothreitol (DTT), 1 mM PMSF. Cells were resuspended in buffer A (20 mM MOPS–KOH, pH 8.0, 1 mM EDTA) with 10 mM DTT, 1 mM PMSF and a protease inhibitor mix (1.25 µg/ml leupeptin, 2 µg/ml antipain, 0.25 µg/ml chymostatin, 0.25 µg/ml elastilin, 5 µg/ml pepstatin). Lysozyme was added to 1 mg/ml and the suspension was kept on ice for 10 min and stirred occasionally. Then 0.1% (w/v) Triton X-100 was added and the suspension was kept on ice for another 10 min. The suspension was sonified with a Branson Sonifier at 3°C, 500 pulses (40% duty cycle, micro tip setting 7). The cell lysate was centrifuged for 15 min at 20 000 g, the supernatant filtered through a cellulose acetate membrane (0.22 µm pores) and loaded on a Mono S column (Pharmacia). The column was washed with three times the loading volume of buffer A, and bound proteins were eluted with a NaCl gradient (in buffer A).

Blue native gel electrophoresis

Mitochondria (50–100 µg) were lysed in 50 µl of ice-cold digitonin buffer (1% digitonin, 20 mM Tris–HCl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10% glycerol, 1 mM PMSF) (Blom et al., 1995). Unsolubilized material was removed by centrifugation for 15 min at 100 000 g. After addition of 5 µl of sample buffer [5% (w/v) Coomassie brilliant blue G-250, 100 mM Bis-Tris, pH 7.0, 500 mM 6-aminopurinic acid], the supernatant was analyzed directly by BN-PAGE (Schneller and von Jagow, 1991; Schaeffer et al., 1994; Dekker et al., 1996). In short, a 6–15% polyacrylamide gradient gel (with 4% stacking gel) was run for 4 h at 500 V in a cooled (4°C) gel chamber (Hoefr, SE600). The cathode buffer containing b₂₃-DHFR, glycerol was added to 50% and the protein was stored in small aliquots at –80°C until use.
dimension urea–SDS–polyacrylamide gel. After electrophoresis, proteins were blotted to nitrocellulose membranes and analyzed by immunodetection.

Analysis of the tim23-2 mutation
Chromosomal DNA of MB3-46 was digested with BamHI and cloned into YEplac195 (Gietz and Sugino, 1988). Several plasmids bearing the tim23-2 allele were identified by colony hybridization to a 53P-labeled 996 bp SnuBI fragment containing the wild-type allele. Sequence analysis of two independent subclones containing the mutant SnuBI fragment in an otherwise wild-type environment revealed a single G to A transitional mutation in the coding sequence, leading to a substitution of the conserved glycine residue at position 112 in Tim23 for a glutamic acid residue.

Miscellaneous
Yeast strains used in this study were MB3-46 (MATα ade2-101 his3-Δ200 leu2-3,112 lys2-801 ura3-1 Δlys2-1::UAS2 tim23-2) Dekker et al., 1993; Blom et al., 1995) and the isogenic wild-type MB3 (MATα ade2-101 his3-Δ200 leu2-3,112 lys2-801 ura3-1 Δlys2 tim23-2) from the experiments described in Figure 2. The wild-type strain PK82 (MATα his3-112 lys2 ura3-52 sso11 leu2-3,112) (Gambill et al., 1993) was used in all other experiments.

Standard methods were used for urea–SDS gel electrophoresis, Western blotting and detection of immune complexes by enhanced chemiluminescence (Amersham). The membrane potential Δψ was assessed by using the fluorescent dye DiSC3(5) (Sims et al., 1974; Gätter et al., 1995).

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Mitochondrial translocation contact sites
