The TIM17·23 preprotein translocase of mitochondria: composition and function in protein transport into the matrix

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We have analysed the structural organization of the TIM17·23 complex, the preprotein translocase of the mitochondrial inner membrane specific for protein targeting to the matrix. The components Tim17, Tim23 and Tim44 are present in this complex in equimolar amounts. A sub-complex containing Tim23 and Tim44 but no Tim17, or a sub-complex containing Tim23 and Tim17 but no Tim44 was not detected. Tim44 is peripherally associated at the matrix side. Tim44 forms dimers which recruit two molecules of mt-Hsp70 to the sites of protein import. A sequential, hand-over-hand mode of interaction of these two mt-Hsp70·Tim44 complexes with a translocating polypeptide chain is proposed.
Keywords: mitochondrial protein import/TIM17·23 complex

Introduction

Import of nuclear-encoded mitochondrial preproteins is mediated by translocation complexes in the outer and inner mitochondrial membranes (Kübrich *et al*., 1995; Schatz and Dobberstein, 1996; Neupert, 1997). Initially, precursor proteins are recognized by receptors of the TOM complex, the translocation machinery in the outer membrane (Pfanner *et al*., 1992; Lithgow *et al*., 1995; Mayer *et al*., 1995). The TOM complex mediates insertion of precursors into the outer membrane and import of some precursors into the intermembrane space. For import of preproteins in the inner membrane and into the matrix, the TOM complex cooperates with translocases of the inner membrane. Two preprotein translocases of the inner membrane have been identified which differ in their substrate specificity (Sirrenberg *et al*., 1996, 1998; Kerscher *et al*., 1997; Koehler *et al*., 1998a,b; Adam *et al*., 1999). The TIM22·54 complex mediates import of a class of inner membrane proteins that contain internal import signals.

In contrast, precursor proteins with a matrix-targeting signal are imported by the TIM17·73 complex (Berthold *et al*., 1995; Horst *et al*., 1995; Sirrenberg *et al*., 1996). Three mitochondrial inner membrane proteins, Tim17,

Tim23 and Tim44, have been identified as essential components for the import of precursor proteins into the matrix (Maarse *et al*., 1992, 1994; Scherer *et al*., 1992; Dekker *et al*., 1993; Emtage and Jensen, 1993; Ryan *et al*., 1994). These components are organized in a complex in the inner membrane (Berthold *et al*., 1995; Blom *et al*., 1995; Bauer *et al*., 1996) and cooperate with the chaperone mt-Hsp70 to drive protein translocation across the inner membrane (Kronidou *et al*., 1994; Rassow *et al*., 1994; Schneider *et al*., 1994, 1996; Berthold *et al*., 1995). The N-terminal targeting sequence of the precursor after passage through the TOM complex is transferred to Tim23, which acts as a receptor for the matrix-targeting signal (Bauer *et al*., 1996). Tim23 forms a dimer in the presence of a membrane potential. The dimerization is mediated by the N-terminal intermembrane space domain of Tim23, which contains a leucine zipper motif. Binding of the presequence causes dissociation of the Tim23 dimer and initiation of translocation of the presequence across the inner membrane (Bauer *et al*., 1996). The membrane-integrated portion of Tim23 shares sequence similarity with Tim17 (Maarse *et al*., 1994; Ryan *et al*., 1994). Both proteins are part of the preprotein-conducting channel across the inner membrane (Berthold *et al*., 1995; Ryan *et al*., 1998).

Tim44 is a hydrophilic protein (Maarse *et al*., 1992). It is associated with the TIM17·73 complex at the matrix side of the inner membrane (Berthold *et al*., 1995). Tim44 plays a central role in preprotein import; it recruits mt-Hsp70 and its co-chaperone Mge1p to the sites of protein import (Kronidou *et al*., 1994; Rassow *et al*., 1994; Schneider *et al*., 1994, 1996). Tim44, mt-Hsp70 and Mge1p were proposed to act as an ATP-dependent molecular ratchet that traps segments of the translocating preprotein as they emerge from the import channel (Schneider *et al*., 1994, 1996; Gaume *et al*., 1998). A conformational change in mt-Hsp70 could even exert a pulling force that facilitates unfolding of tightly folded domains that are about to be translocated across the outer membrane (Glick, 1995; Voos *et al*., 1996; Matouschek *et al*., 1997).

Here, we investigate the structural organization of the TIM17·73 complex. We show that virtually all Tim17, Tim23 and Tim44 components are present in a unique complex containing equimolar amounts of each. Sub-complexes containing Tim23 and Tim44 but no Tim17, or Tim23 and Tim17 but no Tim44 could not be detected. Tim44 is associated at the matrix side with the TIM17·73 complex and may interact with a M_r 14 000 membrane protein. Ionic interactions contribute significantly to the membrane association of Tim44. The protein is not accessible to proteases from the intermembrane space. Tim44 forms dimers and such dimers can recruit two molecules of mt-Hsp70 to the sites of protein import. A

sequential interaction of these two mt-Hsp70 with a translocating polypeptide chain is discussed.

Results

Tim17, Tim23 and Tim44 are organized in one complex in the mitochondrial inner membrane

To estimate the amounts of the components of the TIM17·73 complex, yeast cells were metabolically labelled by growth in the presence of [^{35}S]sulfate and mitochondria were prepared. These were lysed with Triton X-100 and SDS, and Tim17, Tim23, Tim44 and mt-Hsp70 were depleted from the detergent extract by quantitative immunoprecipitation. The immunoprecipitated proteins were analysed by SDS–PAGE and quantified using a phosphoimaging system (Figure 1). Tim17 and Tim23 each comprise ~0.04%, Tim44 0.03% and mt-Hsp70 1.2% of total radiolabelled mitochondrial protein. Taking into account the number of methionyl and cysteinyl residues for each protein, the molar ratio of Tim17:Tim23: Tim44:mt-Hsp70 was found to be ~1:1:1:30.

In order to investigate how the components of the TIM machinery are organized in the inner membrane, mitochondria were solubilized with digitonin and subjected to immunoprecipitation with affinity-purified antibodies directed against Tim17 and Tim23. Both antibodies efficiently precipitated the same set of proteins, which were identified by immunoblotting as Tim17, Tim23, Tim44 and mt-Hsp70 (Figure 2A). The relative amounts of proteins precipitated with each antibody were similar in both cases.

To address the question of whether these Tim components and mt-Hsp70 are organized in a single complex or in different sub-complexes immunodepletion experiments with antibodies against Tim17 and Tim23 were performed. To preserve the TIM machinery, mitochondria were solubilized with digitonin and immunoprecipitations were performed (Figure 2B). Tim23
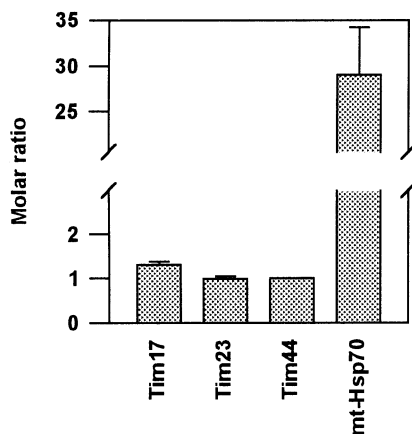
antibodies depleted Tim23 and Tim17 from the detergent extract, indicating that all Tim17 was in a complex with Tim23. Similarly, Tim17 antibodies immunodepleted Tim17 and Tim23. Mitochondria were solubilized with *n*-octyl-β-D-glucopyranoside (octylglucoside), which disrupts the TIM machinery (Berthold *et al*., 1995). Under these conditions Tim23 antibodies depleted Tim23, whereas Tim17 was not co-precipitated. Vice versa, Tim17 was immunodepleted by antibodies against Tim17, whereas Tim23 did not co-precipitate.

These results demonstrate the existence of a unique TIM17·73 complex which contains equimolar amounts of Tim17 and Tim23. In addition, this complex contains approximately equimolar amounts of Tim44 and also of mt-Hsp70 (see below).

Assembly of precursors of Tim17 and Tim23 imported into isolated mitochondria

To assess the assembly of the TIM17·73 complex, precursors of Tim17 and Tim23 were synthesized in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine and imported into isolated yeast mitochondria (Figure 3A). The

Fig. 1. Quantification of Tim17, Tim23, Tim44 and mt-Hsp70 in mitochondria. Yeast cells were metabolically labelled by growth in the presence of [^{35}S]sulfate. Mitochondria were prepared and lysed with 0.05% SDS and 0.5% Triton X-100. Lysates were incubated with affinity-purified antibodies against Tim17, Tim23, Tim44 and mt-Hsp70, which were pre-bound to protein A–Sepharose beads. The beads were collected by centrifugation, and the precipitates were analysed by SDS–PAGE and quantified using a phosphoimaging system. Molar ratios of proteins were calculated taking into account the predicted methionine and cysteine contents of the proteins. The amount of Tim44 was set to 1.
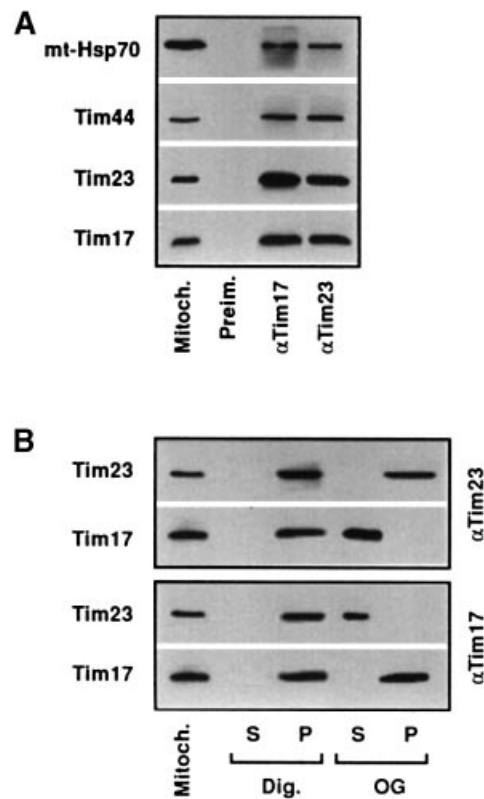
Fig. 2. Tim17, Tim23 and Tim44 are in a complex in the mitochondrial inner membrane. (**A**) Co-immunoprecipitation of Tim17, Tim23, Tim44 and mt-Hsp70. Yeast mitochondria (250 mg) were solubilized with 1% digitonin. Extracts were incubated for 1 h at 4°C with affinity-purified anti-Tim17 (α-Tim17) and anti-Tim23 IgG (α-Tim23) or pre-immune IgG (Preim.), pre-bound to protein A–Sepharose beads. The beads were washed and the immunoprecipitates were analysed by SDS–PAGE and Western blotting. Immunoblots were decorated with antibodies against the antigens indicated. (**B**) Immunodepletion of Tim17 and Tim23. Mitochondria (100 μg) were solubilized in 1% digitonin (Dig.) or 3.5% octylglucoside (OG). The detergent extracts were immunoprecipitated as above with an excess of antibodies. Supernatants (S) and immunoprecipitates (P) were analysed by SDS–PAGE and Western blotting.

samples were then treated with trypsin to remove non-imported precursors. Subsequently the mitochondria were solubilized with digitonin and analysed by gel filtration. The elution profile of newly imported Tim17 and Tim23 differed from that of the endogenous forms (Figure 3B). Endogenous Tim17 and Tim23 co-eluted in one high molecular weight fraction. The radiolabelled imported Tim17 and Tim23 eluted in three fractions. Approximately one-third of the imported Tim17 and one-half of the imported Tim23 co-eluted with the endogenous Tim17 and Tim23, suggesting that the precursors were assembled in a similar manner as endogenous Tim17 and Tim23. Approximately 30% of Tim17 and 10% of Tim23 eluted in a high M_r form ($>400\ 000$) and 30% of both proteins eluted with an M_r of ~100 000, corresponding to that of the unassembled proteins in a detergent micelle (Berthold *et al.*, 1995). For further characterization, radiolabelled Tim23 precursor was
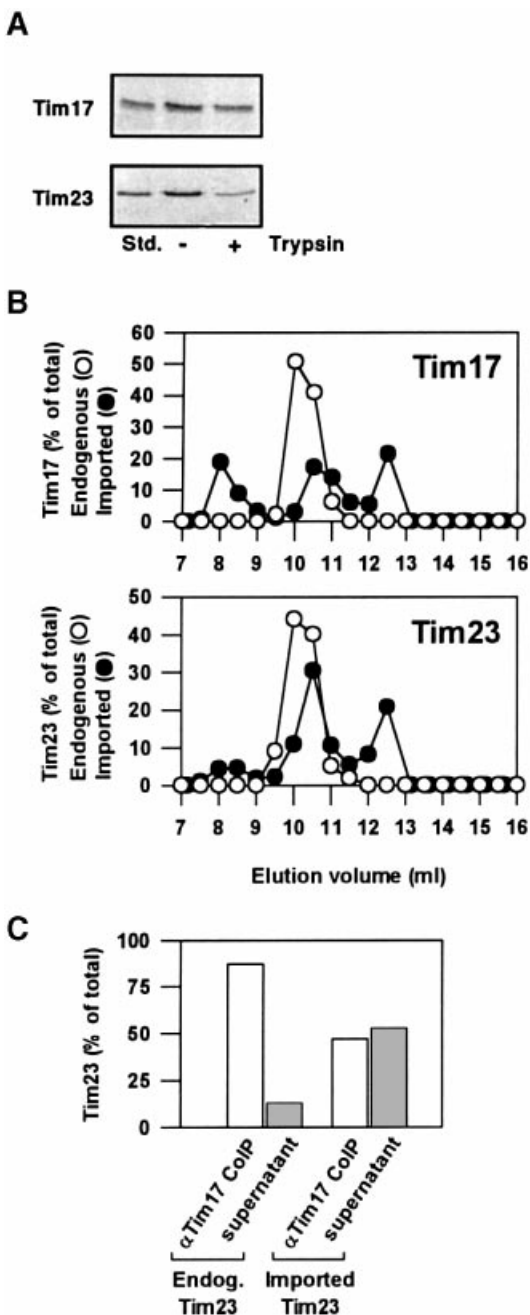
imported into mitochondria. The mitochondria were then treated with trypsin and lysed with digitonin. Subsequently, Tim17 was quantitatively precipitated with affinity-purified antibodies. Under these conditions the anti-Tim17 IgG co-precipitated virtually all of the endogenous Tim23, supporting the theory that Tim23 and Tim17 are in a complex. However, only 30% of the newly imported Tim23 co-precipitated with Tim17, indicating that only a fraction of the imported Tim23 was assembled in a complex with Tim17.

In summary, when radiochemical amounts of Tim17 and Tim23 are imported into mitochondria, significant fractions of the precursors are correctly assembled with the endogenous forms. Some precursors remain unassembled or are present in high molecular mass complexes, which could represent import intermediates. Radiolabelled Tim proteins imported *in vitro* into isolated mitochondria are therefore not reliable markers for the assembled TIM17·73 complex.

Tim44 is a peripheral membrane protein

Tim44 is located at the inner face of the inner membrane; it is a hydrophilic protein and is lacking putative membrane-spanning segments (Maarse *et al.*, 1992; Blom *et al.*, 1993). It is not understood how Tim44 is associated with the TIM17·73 complex.

To characterize the topology of Tim44 we constructed the fusion protein Tim44DHFR containing mouse dihydrofolate reductase at the C-terminus (Table I). Tim44DHFR was functional as it complemented a disruption of the endogenous *TIM44* gene (Table I). Tim44DHFR was then co-expressed with Tim44 in the yeast strain W334. Mitochondria were prepared from this strain, converted to mitoplasts and then treated with proteinase K (PK). Both Tim44DHFR and Tim44 were resistant to protease treatment of mitoplasts (Figure 4A, left). Tim23, which exposes a domain into the intermembrane space, was protected when mitochondria were treated with PK but degraded in mitoplast preparations. Tim44DHFR and Tim44 were both extracted from the inner membrane by carbonate, pH 11 (Figure 4A, right), indicating that they were peripheral membrane proteins. The ATP/ADP carrier

Fig 3. Assembly of imported Tim17 and Tim23. (**A**) Import of Tim17 and Tim23. Radiolabelled precursor proteins were synthesized in rabbit reticulocyte lysate in the presence of [^{35}S]methionine and imported into isolated mitochondria (0.5 mg) for 15 min at 25°C. Mitochondria were treated with trypsin (50 μg/ml). Aliquots corresponding to 50 μg mitochondria were removed and analysed by SDS–PAGE and autoradiography. The remaining mitochondria were re-isolated in the presence of 500 μg/ml soybean trypsin inhibitor. Std., 20% of the lysate used. (**B**) Gel filtration analysis of endogenous and imported Tim17 and Tim23. Mitochondria containing imported radiolabelled Tim17 or Tim23 (see above) were solubilized with 1% digitonin and subjected to gel filtration on a Superose 12 column. Fractions were analysed by SDS–PAGE and Western blotting to determine endogenous Tim17 and Tim23. Imported Tim17 and Tim23 were quantified by a phosphoimager analysis. (**C**) Co-immunoprecipitation of endogenous and imported Tim23 with antibodies against Tim17. Mitochondria containing imported radiolabelled Tim23 were lysed with digitonin and immunoprecipitation with affinity-purified anti-Tim17 IgG was performed. Immunoprecipitate and the supernatant were subjected to SDS–PAGE and Western blotting. Endogeneous Tim23 was detected by immunoblotting with anti-Tim23 IgG and quantified by densitometry (left columns). Imported Tim23 was quantified with a phosphoimaging system (right columns). The signals obtained from supernatant plus immunoprecipitate were set at 100%.

Table I. Localization of Tim44 fusion proteins

Protein	C-terminal sequence	Complementation of *tim44*::LYS2	PK resistance in mitoplast	Carbonate extraction	Salt extraction
Tim44	WKILEFVRGGSRQFT	+	+	+	+
Tim44DHFR	WKILEFVRGGSR-**DHFR-His6**	+	+	+	+
Tim44Δ3His6	WKILEFVRGGSRHHHHHH	+	+	+	+
Tim44Δ12His6	WKIFRSHHHHHH	−	+	nd	nd
Tim44myc	WKILEFVRGGSR-**c-myc**	+	+[a]	+	+

'nd', not determined.
[a]Analysed with c-*myc* and Tim44 antibodies.

(AAC), an integral membrane protein, was recovered in the membrane fraction. To characterize further the nature of the membrane interaction, mitochondria were incubated in buffers with different ionic strengths and subjected to sonication to allow the release of proteins from the matrix space. Mitochondrial membranes were then sedimented by ultracentrifugation and their protein composition was analysed (Figure 4B). At low salt concentrations, Tim44DHFR and Tim44 were predominantly detected in the membrane pellet, whereas most of both proteins were released from the membranes at high salt concentrations.

We constructed yeast strains which expressed Tim44-myc and Tim44Δ3$_{His6}$, functional versions of Tim44 containing a myc-tag and his-tag, respectively, at their C-terminus (Table I). In addition, we used a yeast strain which contains wild-type Tim44 together with a non-functional his-tagged version, Tim44Δ12$_{His6}$ (Schneider *et al.*, 1994). Mitochondria were prepared from these strains, converted to mitoplasts, and then treated with proteinase K (PK). All versions of Tim44 were resistant to protease treatment of mitoplasts. They were extracted from the inner membrane at alkaline pH and at high salt concentration (Table I).

Mitochondria were solubilized with digitonin in the presence of low (30 mM) and high (500 mM) concentrations of NaCl, and immunoprecipitation with antibodies against
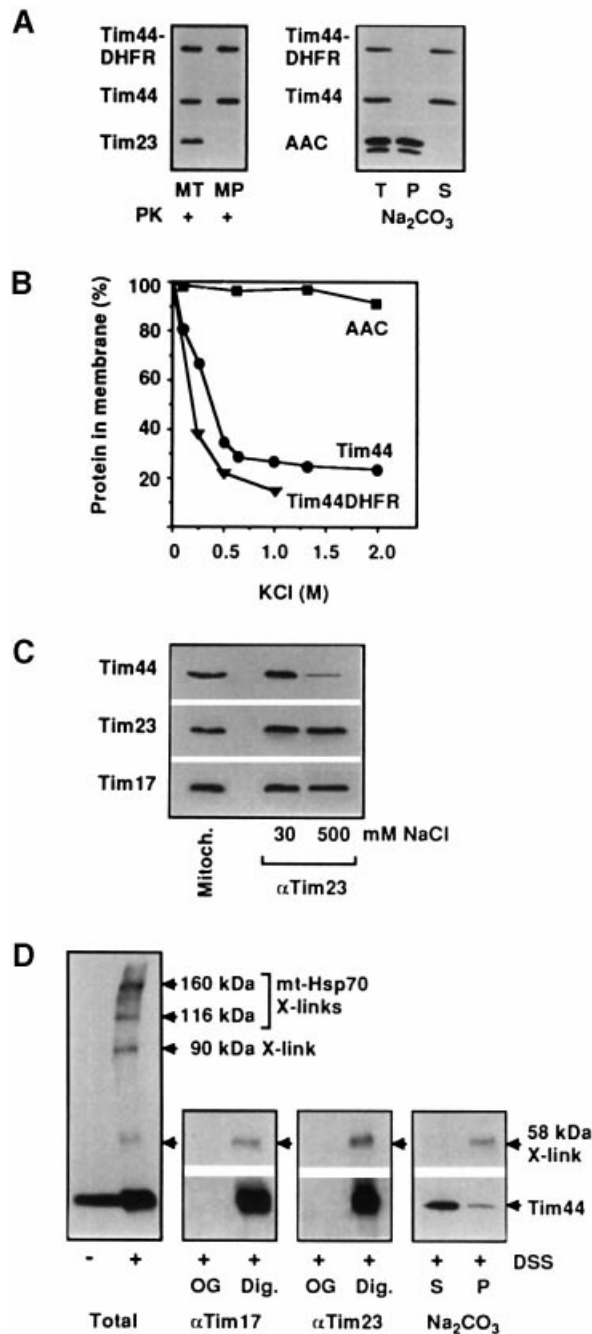

Fig. 4. Tim44 is peripherally associated with the inner membrane and in contact with a 14 000 membrane protein. (**A**) Submitochondrial localization of Tim44. Left: mitochondria (MT) and mitoplast preparations (MP) containing Tim44 and Tim44DHFR were treated with 50 µg/ml PK and analysed for Tim44. Tim23, which is exposed into the intermembrane space is shown for contol. Right: the mitochondria were treated with 0.1 M Na$_2$CO$_3$, pH 11, and membranes were collected by ultracentrifugation (30 min, 100 000 *g*). Total mitochondria (T), membrane fraction (P) and supernatant fraction (S) were analysed. The ADP/ATP carrier (AAC), an integral membrane protein, is shown for control. (**B**) Extraction of Tim44 and Tim44DHFR from mitochondrial membranes at high ionic strength. Mitochondria (200 µg) containing Tim44 and Tim44DHFR were resuspended in 30 mM Tris–HCl, pH 8.3, containing 0.1, 0.65, 1.3 or 2.0 M KCl and subjected to sonication (10× 10 s at 9 min intervals). Membranes were collected by ultracentrifugation and the samples were analysed by SDS–PAGE, Western blotting and immunodecoration with antibodies against Tim44 and AAC. Quantification was by densitometry. (**C**) Release of Tim44 from the TIM17·23 complex at high ionic strength. Mitochondria (250 mg) were solubilized with 1% digitonin in the presence of 30 or 500 mM NaCl. The detergent extracts were immunoprecipitated with anti-Tim23 IgG, and the precipitates were analysed as in Figure 2. (**D**) Tim44 can be cross-linked with a 14 000 membrane protein. Wild-type mitochondria were subjected to cross-linking with 200 µM DSS. The mitochondria were then solubilized with 1% digitonin (Dig.) or 3.5% octylglucoside (OG) and immunoprecipitated with α-Tim17 or α-Tim23 IgG, or they were treated with 100 mM carbonate, pH 11. Tim44 and Tim44-specific cross-links were resolved by SDS–PAGE and detected by immunostaining with anti-Tim44 IgG. The sizes of the cross-links (X-link) are indicated.

3670

A

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S.c.                    MHRSTPIRTSGTSSRTLTARYRSQYTGLLVARVLFSTSTTRAQGGNPRSPLQIFRD___TTFKKEWEKS_____QELQENIKTLQDASG_KLGESEAYKKARE
M.m. MAAAARLRGGWCRCPRRCLGSGIQPFLSSHNLPHGSSYQISRP_GRELTLTKSYSSGS_RK_GFLSGLLDNIKQELAKNKEMKESIKKFRDEAKKLEESDALQEARRKYKSIESETVRTSEA
C.e.                    MLSRICGNGIRLTRTRLQFQPSIVTFRDYSNPAPKR_GGNPRSPLQIFRD___TTFKKEWEKS_____QELQENIKTLQDASG_KLGESEAYKKARE

S.c. AYLKAQRGSTIVGKTLKKTGETMEHIATKAWESELGKNTRKAAAATAKKLDESFEPVRQTKIYKEVSEVIDDGESSRYGGF__ITKEQRRL_KRERDLAAS__GKRHRAVKSNEDAGTAV
M.m. IKKKLGELTGTVKESLDEVSKSDLGRKIKEGVEEAARTA_KQSAESVSKSGEKLGKTAAFKAISQGVESVKKELDESVLGQTGPYRRPERL_RKRTEFAGAAKFKESKVFEANEEALG__
C.e. VKQKIEELSDHMKKMVHEIQKTEAGKKMTEAGAEALKQARK_AAEHVEKVAEKVGDTEVYKHVSTSMKTVKDEIDNIADVR__MYSRPEALTKRTD___GFFDLEKERVVEANDSATD__

S.c. VATNIESKESFGKKVEDFKEKTVVGRSIQSLKNKLWDESENPLI_____VVMRKITNKVGGFFA_ETESSRVYSQFKLMDPTFSNESFTRHLREYIVPEILEAYVKGDVKVLKKWFSEAPF
M.m. VVLHKDSK__WYQQWKDFKDNNVVFNRFFEMKMK_YDESDNVLIRASRALTDKVTDLLGGLFS_KTEMSEVLTEILRVDPTFDKDHFLHQCETDIIPNILEAMISGELDILKDWCYEATY
C.e. VTLHKDSK__WYSGWKNFSESNTYYHKLLDWKIK_YDESDNMAV_____RHMRGVTEKIGSVFSGQNEVSEVLTEIHKIDANFDKQEWLRFCETKIIPNILEAFIRFDLEVLQSWCHERAY

S.c. _NVYAAQQKIFKEQDVYADGRILDIRGVEIVSAKLLAPQDIPVLVVGCRAQEINLYRKKKTGEIAAGDEANILMSSYAMVFTRDPEQIDDDETEGWKILEFVRGGSRQFT
M.m. SQL_AHPIQQAKALGFQFHSRILDISNVDLAMGKMM__EQGPVLIVTFQAQVVMVIKNSK_GEVYDGDPDKVQRMLYVWALCRDQEELNPYA__AWRLLDISASSTEQIL
C.e. TQLSTVVKEYQKMHFSTKDSRIIDINKVEMATGKMM__EQGPVLIISFQVYMINVTKNAD_GKVVEGDPDNPKRINHIWVLCRDVEEYNPAL__AWKLLEVHMQETPLAL
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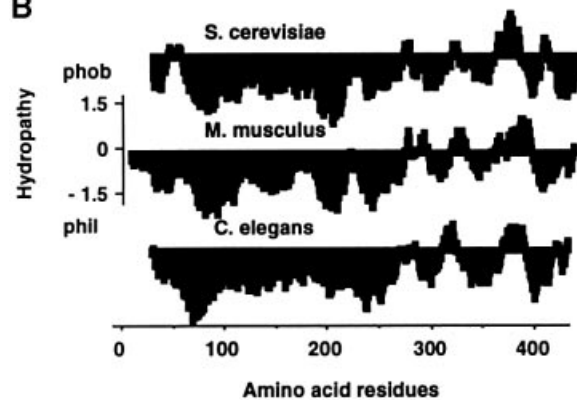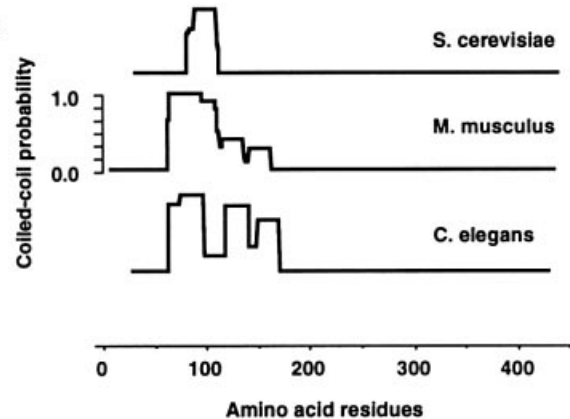
B

C

Fig. 5. Structural characteristics of Tim44. (**A**) Sequence alignment of Tim44 from *S.cerevisiae*, *C.elegans* and *M.musculus*. Similar amino acid residues conserved in all three sequences are indicated by grey boxes. (**B**) Hydropathy profiles. (**C**) Coiled-coil prediction (Lupas, 1996) using a window size of 14 residues.

Tim23 was performed. At the low salt concentration Tim44 was efficiently co-precipitated and ~80% of the protein was recovered with the TIM17·73 complex (Figure 4C). However, at the high salt concentration, the amount of Tim44 in association with the TIM17·73 complex was reduced to ~10%. The interaction between Tim17 and Tim23 was not affected by ionic strength. Similar results were obtained when immunoprecipitations were performed with antibodies against Tim17 (not shown).

To characterize components of the TIM17·23 complex that interact with Tim44, mitochondria were subjected to chemical cross-linking with disuccinimidyl suberate (DSS). Specific Tim44 adducts were detected with anti-Tim44 IgG by Western blotting (Figure 4D). Adducts with apparent M_r of 160 000, 116 000, 90 000 and 58 000 were generated. The 160 000 and 116 000 species were identified previously and represent cross-links between Tim44 and mt-Hsp70 (Schneider *et al.*, 1994; data not shown). The 90 000 M_r species does not contain mt-Hsp70 and corresponds to a Tim44 dimer (see below). The 58 000 adduct was not recognized by antibodies against Tim17, Tim23 or Tim11/ATPase e (Tokatlidis *et al.*, 1996; Arnold *et al.*, 1997, 1998). When mitochondria were subjected to cross-linking and solubilized with digitonin, which preserves the integrity of the TIM17·73 complex, the 58 000 adduct as well as Tim44 were co-precipitated with antibodies against Tim17 and with antibodies against Tim23. This indicates that Tim44 was cross-linked to a 14 000 M_r component in association with the TIM17·73

complex. However, when the TIM17·73 complex was disrupted by solubilization of mitochondria with octyl-glucoside, neither the 58 000 M_r adduct nor Tim44 were precipitated by antibodies against Tim17 or Tim23. When mitochondria were treated with carbonate, pH 11, Tim44 was released from the membranes, whereas the 58 000 M_r adduct was not extracted. This indicates that Tim44 was cross-linked to an integral membrane protein.

In conclusion, Tim44 is a peripheral inner membrane protein. Ionic interactions contribute to its association with the TIM17·73 complex. Tim44 is associated with the TIM17·73 complex and it is in contact with a membrane protein of M_r ~14 000.

Tim44 forms a dimeric complex

The predicted amino acid sequences of Tim44 proteins from three different species, *Saccharomyces cerevisiae*, *Mus musculus* and *Caenorhabditis elegans*, share significant similarity in their C-terminal portions but to a lesser extent in the N-terminal portions (Figure 5A). Despite an apparent lack of high sequence similarity, the N-terminal portions appear to be structurally conserved as indicated by the similarity of the hydropathy profiles (Figure 5B). In addition, the N-terminal portions contain segments with a high predicted probability to form coiled coils (Figure 5C), structures which are involved in protein–protein interactions and which are often found in oligomeric proteins (Lupas, 1996).

We asked whether Tim44 forms dimers. Mitochondria

were lysed with octylglucoside in the absence or presence of ATP in order to preserve or disrupt the interaction of Tim44 with mt-Hsp70 (Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994, 1996). The samples were then analysed by gel filtration on a Superose 12 column (Figure 6A). In the absence of ATP, Tim44 eluted in a complex with an M_r of ~250 000. When ATP was included in the lysis buffer, Tim44 eluted in a fraction corresponding to an M_r of ~100 000. The same results were obtained when Triton X-100 was used instead of octylglucoside, indicating that the apparent molecular masses of Tim44 and the mt-Hsp70·Tim44 complex were not affected by the micellar size of the detergent. When the M_r 100 000 fraction was subjected to cross-linking with DSS, a Tim44-specific adduct of M_r 90 000 was detected (Figure 6B). These observations suggest that Tim44 forms dimers which elute from a gel filtration column with an apparent M_r of 100 000.

To analyse further the oligomeric state of Tim44, mitochondria were lysed with Triton X-100 in the presence of ATP to dissociate the mt-Hsp70·Tim44 complex. The samples were then subjected to native gel electrophoresis at neutral pH (Figure 6C). To introduce a negative charge shift on the native proteins the sample buffer was supplemented with either Coomassie Brilliant Blue G250 (CB-G250) or taurodeoxycholate (TDC) (Schägger and von Jagow, 1991). CB-G250 was reported to preserve the native structure of many oligomeric proteins, whereas TDC has the tendency to disrupt protein complexes (Schägger and von Jagow, 1991; Schägger *et al.*, 1994). As a reference for the electrophoretic mobility of non-native, monomeric Tim44 we used sample buffer supplemented with SDS. Under these conditions Tim44 migrated in a single band (Tim44-L). As related to marker proteins the electrophoretic mobility of Tim44-L would correspond to an M_r of 140 000. The low electrophoretic mobility of Tim44-L is possibly due to its basic character (pI 9.0). Basic proteins bind less of the charged dye/detergent and migrate with a low mobility in the native gel system used (Schägger *et al.*, 1994). Thus, Tim44-L possibly represents monomeric protein. When the sample buffer was supplemented with CB-G250 instead of SDS, Tim44 migrated corresponding to a higher apparent M_r of ~280 000 (Tim44-H), suggesting that native Tim44 is a dimer. With TDC in the sample buffer, only Tim44-L was recovered indicating that the detergent destabilized the Tim44 dimer. Both species, Tim44-L and Tim44-H, were detected when the sample buffer was supplemented with a mixture of 0.1% TDC and 0.05% CB-G250. Apparently, the two compounds compete for binding to Tim44; CB-G250 preserves dimeric Tim44, whereas TDC disrupts the dimers. To produce cross-linked Tim44, mitochondria were lysed with Triton X-100 and incubated with DSS. The oligomeric state was then analysed by native electrophoresis in the presence of TDC to disrupt Tim44 dimers which were not cross-linked (Figure 6D). A significant fraction of Tim44 migrated as a band with the same apparent molecular mass as Tim44-H. This indicates that Tim44 forms dimers that can be efficiently cross-linked.

Next we isolated the mt-Hsp70·Tim44 complex by immunoadsorbtion with affinity-purified antibodies against Tim44 that were covalently coupled to protein A–Sepharose (Figure 6E). As expected, mt-Hsp70 co-adsorbed with Tim44 only in the absence of ATP. No other proteins were detected. Accordingly, the 100 000 M_r species

of Tim44 recovered from gel filtration and Tim44-H detected by native gel electrophoresis are oligomers of Tim44. Likewise the M_r 250 000 mt-Hsp70∘Tim44 complex is composed of oligomers of Tim44 and mt-Hsp70, presumably two molecules of Tim44 and two of mt-Hsp70.

We reasoned that if Tim44 forms dimers, the associated mt-Hsp70s might also be in close contact and undergo cross-linking to dimers upon addition of DSS. The mt-Hsp70·Tim44 complex was therefore subjected to cross-linking with DSS and then isolated by immunoadsorbtion with antibodies against Tim44. mt-Hsp70 was then eluted from the affinity column with ATP. The eluate contained monomeric mt-Hsp70 and mt-Hsp70-specific adducts of M_rs 140 000 and ~280 000 (Figure 6E). The cross-linked species did not contain Tim44 (not shown), suggesting that they represent dimeric (140 000) and oligomeric forms of mt-Hsp70. The adducts were specific to mt-Hsp70 in complex with Tim44 since they were not observed upon cross-linking of mt-Hsp70 which was not in association with Tim44 (not shown).

In summary, the data show that Tim44 forms dimers. These Tim44 dimers recruit two molecules of mt-Hsp70, resulting in a complex with an M_r of ~250 000.

Discussion

As we show here, the mitochondrial inner membrane contains equimolar amounts of Tim17, Tim23 and Tim44, whereas mt-Hsp70, located mainly in the matrix, is ~30 times more abundant. Using immunodepletion we found virtually all Tim23 complexed with Tim17 and vice versa. Tim44 is peripherally associated with this complex. Ionic interactions contribute significantly to this association. As a consequence, the amount of Tim44 that is recovered by immunoprecipitation in association with Tim23 and Tim17 depends on the salt concentration. At a low salt concentration, >80% of Tim44 are found in complex with Tim23 and Tim17. Although the apparently labile interaction of Tim44 with TIM17·73 may reflect a dynamic interaction of functional significance, it seems possible that the majority of Tim44 is present in a complex with Tim17 and Tim23, and that this complex contains equimolar amounts of the three components. The complex is referred to as the TIM17·73 complex (according to the integral membrane proteins) to distinguish it from the TIM22·54 complex, the recently identified mitochondrial translocase specific for import and insertion of a class of inner membrane proteins that are synthesized without cleavable presequence (Sirrenberg *et al.*, 1996, 1997, 1999; Kerscher *et al.*, 1997; Koehler *et al.*, 1998a,b; Adam *et al.*, 1999).

Recently, the presence of two functional pools of Tim23 in the inner membrane was suggested; one sub-complex containing Tim23 and Tim17 but no Tim44 and a second sub-complex containing Tim44 and Tim23 but no Tim17 (Bömer *et al.*, 1997). This is in apparent contradiction to observations reported here, showing that there is one type of complex that contains essentially equimolar amounts of Tim17, Tim23 and Tim44. The conclusions by Bömer *et al.* were based on the analysis of radiochemical amounts of Tim precursors which were newly imported into isolated mitochondria. Thus, distinction of translocation intermediates from fully assembled precursors was not possible. Here we show that newly imported Tim17 and Tim23 exhibit

properties different from those of the endogenous, assembled Tim components. Therefore, the experiments with radiochemical amounts of Tim23 and Tim17 imported from reticulocyte lysate into isolated mitochondria cannot be taken as evidence for the existence of assembled sub-complexes of functional significance. No endogenous pool of Tim proteins corresponding to these species was observed. The strongest argument in favour of the presence of essentially one assembled pool of Tim23 and Tim17 is that endogenous Tim23, but not newly imported Tim23, can be immunodepleted with antibodies against Tim17 and vice versa.

Tim44 is an essentially hydrophilic protein and does not contain a predicted membrane-spanning segment. It is associated with the TIM17·73 complex at the matrix side of the inner membrane. Tim44 is extracted from the inner membrane at alkaline pH and is resistant to protease treatment of mitoplasts (Blom et al., 1993; this study). In addition, we find that Tim44 is extracted from the inner membrane at high ionic strength. Thus, Tim44 meets all the criteria of a peripheral membrane protein that is associated with the TIM17·73 complex at the inner face of the inner membrane. Yet, the topology of Tim44 is not entirely clear. In contrast to Tim44, a myc-tagged Tim44 was reported to

be accessible to protease added to mitoplasts (Maarse et al., 1992). We show that such a myc-tagged Tim44 is protected from protease added to mitoplasts. Similarly, C-terminally his-tagged versions of Tim44 and a Tim44DHFR fusion protein, which were able to functionally replace wild-type Tim44 in vivo, were found to be protected from attack by protease added to mitoplasts. Antibodies against Tim44 did not inhibit protein import into mitoplasts (Berthold et al., 1995). However, antibodies generated against a 40 000 M_r proteolytic fragment of Tim44 inhibited protein import into inner membrane vesicles which were generated by sonication of mitoplasts (Horst et al., 1993). By cross-linking of an arrested preprotein it was shown that a segment of 33 amino acid residues, i.e. ~12 nm of an extended polypeptide chain, is required to reach Tim44 (Kanamori et al., 1997). As the thickness of a lipid bilayer is ~3.5 nm, a polypeptide segment with a length of 12 nm may well be sufficient to span the outer and inner membrane at translocation contact sites and reach a peripheral protein at the inner side of the inner membrane. However, the possibility is not excluded that a portion of Tim44 might penetrate into the proteinaceous TIM17·73 complex.

Tim44 can be cross-linked to a component with an M_r of ~14 000. This component is not Tim11/ATPase e (Tokatlidis et al., 1996) which was recently shown to be a novel subunit of the F_1F_0-ATPase rather than a Tim protein (Arnold et al., 1997, 1998). The 14 000 M_r component might be Tim14, which was proposed to be a component of the TIM17·73 complex (Berthold et al., 1995). Whether Tim14 mediates the association of Tim44 with the TIM17·73 complex remains to be determined.

Tim44 forms dimers. The N-terminal portion of Tim44 contains a heptad leucine repeat motif which has a predicted probability to form a coiled-coil. The probability to form coiled-coils is conserved in the N-terminal portions of
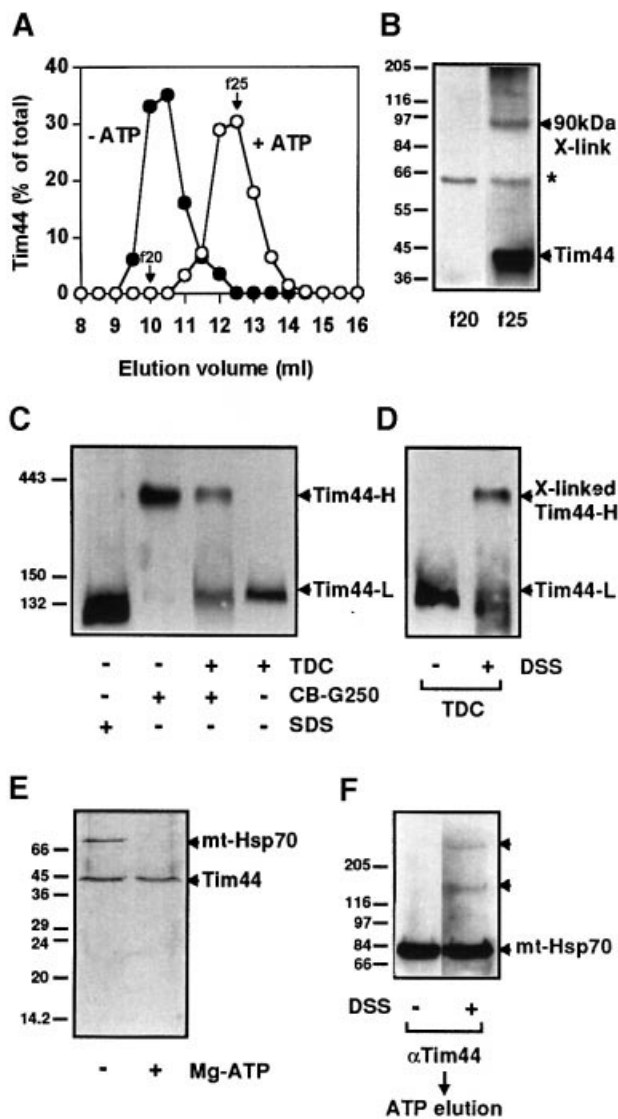

Fig. 6. Tim44 forms a dimer. (A) Gel filtration analysis of Tim44. Yeast mitochondria (2 mg/ml) were lysed with 3.5% octylglucoside in the presence or the absence of Mg-ATP, and 0.2 ml were applied onto a Superose 12 gel filtration column. Fractions were analysed for Tim44. (B) Tim44 yields a 90 000 M_r adduct upon cross-linking. Fraction 25 (f25) from the gel filtration (+ATP) was cross-linked with 200 μM DSS. Tim44 and the Tim44-containing adduct (90 000 X-link) are indicated. Fraction 20 (f20), which does not contain Tim44 was subjected to cross-linking as a control. The cross-reacting band is indicated by an asterisk. (C) Native Tim44 dimer is preserved in presence of CB-G250 but is disrupted by anionic detergents. Mitochondria (200 μg) were lysed with Triton X-100 and subjected to native electrophoresis. The sample buffer was supplemented with SDS (0.5%), CB-G250 (0.05%) and TDC (0.1%) when indicated. Tim44-L and Tim44-H indicate low and high molecular mass species of Tim44, respectively. (D) Cross-linking of Tim44-H. Mitochondria (200 μg) were lysed with Triton X-100 and subjected to cross-linking and native electrophoresis in presence of 0.1% TDC. (E) Immuno-precipitation of Tim44 and the mt-Hsp70·Tim44 complex. Mitochondria (1 mg) were lysed with 0.2% Triton X-100 in the presence or absence of 2 mM Mg-ATP. The detergent extracts were subjected to immunoprecipitation with affinity-purified anti-Tim44 IgG covalently coupled to protein A–Sepharose beads. The immuno-precipitates were resolved by SDS–PAGE under non-reducing conditions and stained with Coomassie Blue. (F) Mt-Hsp70 in complex with Tim44 can be cross-linked to oligomeric forms. Mitochondria were solubilized with 0.2% Triton X-100 in the absence of Mg-ATP and subjected to cross-linking with 200 μM DSS when indicated. The mt-Hsp70·Tim44 complex was then immunopurified as described in (E). The protein A–Sepharose beads were then eluted with buffer containing 2 mM Mg-ATP and the eluate was analysed by SDS–PAGE and immunoblotting with antibodies against mt-Hsp70.
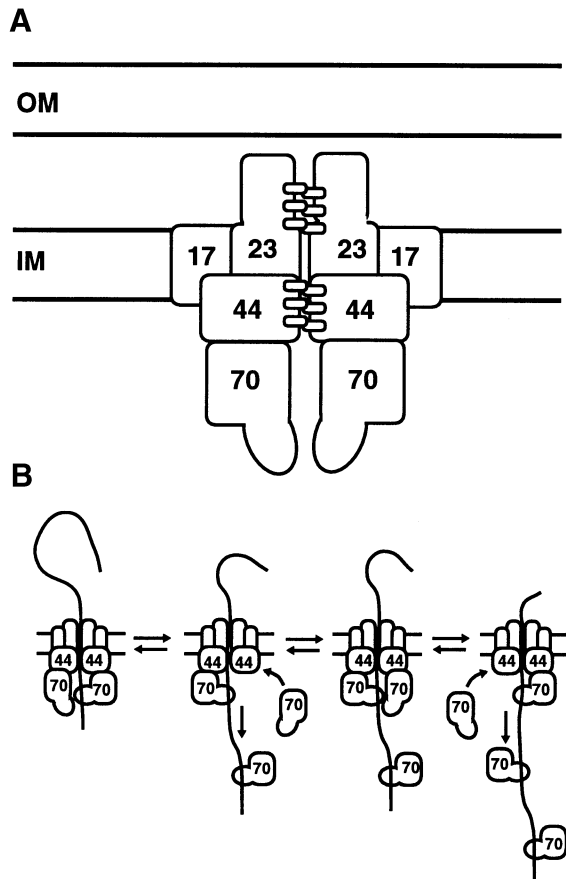
A

B

Fig. 7. Working model for structure and function of the TIM17·73 complex. (**A**) The dimeric structure of the TIM17·73 complex is schematically outlined. Putative coiled-coil interactions mediating dimerization of the TIM17·73 complex via Tim23 on the outside of the inner membrane and via Tim44 on the inside of the inner membrane are indicated. (**B**) Sequential interaction of two molecules of mt-Hsp70 with segments of a preprotein emerging from the import channel facilitated by dimeric Tim44 ('hand-over-hand model').

Tim44 from different species. These putative coiled-coil domains may mediate the dimerization of Tim44. We have recently shown that the N-terminal domain of Tim23 has the capacity to form dimers. This domain is exposed to the intermembrane space and contains a putative coiled-coil motif. The coiled-coil domain of Tim44 may serve a similar role. Dimerization of Tim23 is affected by the membrane potential $\Delta\psi$ (Bauer *et al*., 1996). The dimeric state of Tim44 appears to be independent of $\Delta\psi$ (unpublished observation). Both proteins, Tim23 on the outer side of the membrane and Tim44 on the inner side, may contribute to a dimeric or even higher oligomeric organization of the TIM17·73 complex in the inner membrane (Figure 7A).

Tim44 dimers recruit two molecules of mt-Hsp70 to the sites of protein import. Cooperative sequential binding of these mt-Hsp70s to incoming segments of a preprotein in transit could promote forward movement with highly increased efficiency compared with an import site with only a single mt-Hsp70 reacting with Tim44 (Figure 7B). Such a 'hand-over-hand' mode of action of mt-Hsp70 might efficiently prevent retrograde movements of the translocating polypeptide chain.

Materials and methods

Affinity purification of antibodies
Recombinant his-tagged Tim17 and MBP fusion proteins with Tim23 and Tim44 (Schneider *et al*., 1994; Berthold *et al*., 1995) were coupled to CNBr-activated Sepharose beads (5 mg protein/ml bead volume) in 30 mM HEPES–KOH, pH 7.2, 2.5% SDS. Antiserum against the corresponding protein (5 ml) was diluted with 5 ml Tris-buffered saline (TBS) and applied to the respective affinity column. Antibodies were eluted as described previously (Schneider *et al*., 1996) and the buffer was changed to 50 mM KCl, 30 mM HEPES–KOH, pH 7.2, 0.1% bovine serum albumin (BSA) using PD-10 columns (Pharmacia).

Immunoprecipitation
Mitochondria (1 mg/ml) were solubilized for 30 min at 4°C in IP buffer (20 mM Tris–HCl, pH 7.4, 150 mM sodium acetate, 2 mM EDTA, 4 mM EGTA, 1 mM PMSF) containing 1% (w/v) digitonin, 3.5% octylglucoside or 0.2% Triton X-100. After a clarifying spin, the detergent extracts were incubated with gentle shaking for 2 h at 4°C with the corresponding antibody or pre-immune serum bound to protein A–Sepharose beads (Pharmacia). Immunoprecipitates were washed three times for 5 min with 1 ml of solubilization buffer and analysed by SDS–PAGE.

Construction of Tim44 C-terminal fusion proteins
Tim44DHFR: pGem4-Tim44 (Schneider *et al*., 1994) was linearized with *Xba*I and the sticky ends were filled in with Klenow in a reaction that did not contain dGTP. The DNA was then cleaved with *Hin*dIII. pQE16 was linearized with *Bam*HI and the sticky ends were filled in a reaction that did not contain dCTP. The DNA was then cleaved with *Hin*dIII; the DHFR-encoding fragment was subcloned into the pretreated pGem-Tim44, yielding Tim44DHFR. Tim44DHFR was then subcloned into Yep51. Tim-44myc, a double-stranded, phosphorylated oligonucleotide with sticky *Xba*I and *Hin*dIII ends encoding the c-*myc* epitope (ENKLISEEDLN), was ligated into pGem4-Tim44. Tim44myc was then subcloned into Yep51. The diploid *S.cerevisiae* strain MB2-22 (Maarse *et al*., 1992) was transformed with Yep51–Tim44DHFR, Yep51–Tim44myc, Yep51–Tim44Δ3$_{His6}$ and Yep51–Tim44Δ12$_{His6}$ (Schneider *et al*. 1994) and subjected to sporulation and tetrad analysis.

Import of precursor proteins into isolated mitochondria
Precursor proteins were synthesized by coupled transcription/translation in reticulocyte lysate in the presence of [^{35}S]methionine (Pelham and Jackson, 1976). Mitochondria were isolated as described previously (Berthold *et al.*, 1995). Import reactions were carried out at 25°C in import buffer (600 mM sorbitol, 3 mg/ml BSA, 80 mM KCl, 10 mM MgCl$_2$, 2.5 mM EDTA, 2 mM KH$_2$PO$_4$, 2 mM ATP, 5 mM NADH, 2.5 mM malate, 2.5 mM succinate, 50 mM HEPES–KOH, pH 7.2). Protease treatments were carried out on ice for 20 min.

Gel filtration analysis
Mitochondria (0.5 mg) were solubilized with 1% digitonin or 0.2% Triton X-100 in 250 µl of column buffer (20 mM Tris–HCl, 150 mM sodium acetate, 2 mM EDTA, 4 mM EGTA, 1 mM PMSF, pH 7.4). When ATP was included during solubilization no chelators were used, and 2 mM ATP and 2 mM MgCl$_2$ was added. After a clarifying spin (30 min at 109 000 *g* in a Beckman TLA45 rotor), 200 µl of the sample were applied onto a Superose 12 HR 10/30 gel filtration column (Pharmacia). The column was eluted at a flow rate of 0.2 ml/min with 30 ml of column buffer including the corresponding detergent. Fractions of 0.5 ml were collected.

Chemical cross-linking in intact mitochondria
Intact or solubilized mitochondria (1–4 mg/ml) were subjected to cross-linking. Disuccinimidyl suberate (DSS, Pierce) was added from a 50-fold stock solution in dimethyl sulfoxide (DMSO). After incubation for 30 min on ice, the cross-linker was quenched with 0.1 M glycine, pH 8.0.

Native electrophoresis
Native electrophoresis was carried out as described elsewhere (Schägger and von Jagow, 1991). The original protocol for blue native electrophoresis was modified. The cathode buffer contained 0.05% taurodeoxycholate (TDC) instead of Coomassie G250 (CB-G250), and the samples were loaded in the presence of 0.05% CB-G250, 0.1% TDC or 0.5% SDS.

Acknowledgements

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