The Interplay between Components of the Mitochondrial Protein Translocation Motor Studied Using Purified Components*\[S\]

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The final step of protein translocation across the mitochondrial inner membrane is mediated by a translocation motor composed of 1) the matrix-localized, ATP-hydrolyzing, 70-kDa heat shock protein mHsp70; 2) its anchor to the import channel, Tim44; 3) the nucleotide exchange factor Mge1; and 4) a J-domain-containing complex of co-chaperones, Tim14/Pam18-Tim16/Pam16. Despite its essential role in the biogenesis of mitochondria, the mechanism by which the translocation motor functions is still largely unknown. The goal of this work was to carry out a structure-function analysis of the mitochondrial translocation motor utilizing purified components, with an emphasis on the formation of the Tim44-mHsp70 complex. To this end, we purified Tim44 and monitored its interaction with other components of the motor using cross-linking with bifunctional reagents. The effects of nucleotides, the J-domain-containing components, and the P5 peptide (CALLASAPPR, representing part of the mitochondrial targeting signal of aspartate aminotransferase) on the formation of the translocation motor were examined. Our results show that only the peptide and nucleotides, but not J-domain-containing proteins, affect the Tim44-mHsp70 interaction. Additionally, binding of Tim44 to mHsp70 prevents the formation of a complex between the latter and Tim14/Pam18-Tim16/Pam16. Thus, mutually exclusive interactions between various components of the motor with mHsp70 regulate its functional cycle. The results are discussed in light of known models for the function of the mitochondrial translocation motor.

Only a very small fraction of the estimated ~800–1000 mitochondrial proteins are made in situ (eight in yeast). The rest are encoded by nuclear genes, synthesized in the cytosol, and then delivered to one of the four mitochondrial compartments: the outer membrane, inner membrane, and intermembrane space and the matrix. Each compartment contains essential proteins for the viability of every eukaryotic cell. Thus, functional import systems for nuclear encoded proteins are indispensable for the biogenesis of mitochondria (1).

The import of nuclear encoded proteins into the mitochondria is a multistep process mediated by the coordinated action of translocation machineries localized in the outer and inner mitochondrial membranes (2, 3). The TOM (translocase of the outer mitochondrial membrane) complex is a multimeric oligomer that constitutes the main portal of protein entry into mitochondria. As such, it serves for the recognition, insertion, and delivery of all nuclear encoded mitochondrial precursor proteins (2–5).

Proteins that contain N-terminal targeting signals and that are destined for full translocation across the inner membrane are transferred from the TOM complex to the TIM23 complex (translocase of the inner mitochondrial membrane). The latter complex is composed of two integral inner membrane proteins, Tim17 and Tim23. Although the function of Tim17 in mediating protein import is not yet well understood, Tim23 forms the translocation channel in the inner membrane during the import process. A third protein, Tim50 (6–8), probably serves as a receptor in the mitochondrial intermembrane space for proteins to be handled by the TIM23 complex. Recently, it was shown that Tim50 also maintains the permeability barrier of the mitochondrial inner membrane via a direct interaction with Tim23 (9). A precursor protein that is found in transit in the TIM23 channel requires additional help to be imported completely into the mitochondrial matrix. This final step of translocation across the inner mitochondrial membrane is catalyzed by the function of a translocation motor at whose core stands the matrix-localized, ATP-hydrolyzing, 70-kDa heat shock protein mHsp70; its J-domain-containing co-chaperone complex, Tim14/Pam18-Tim16/Pam16; the nucleotide exchange factor Mge1; and the component that anchors mHsp70 to the TIM23 channel, Tim44 (4, 10, 11). Tim44 is a peripheral membrane protein that is found in close proximity to the precursor protein during its passage in the import channel. Tim44 associates transiently with the TIM23 complex to perform its function, and this association is essential for normal import of matrix-localized proteins by mitochondria (12–14). It was shown in vitro that Tim44 is also able to interact with negatively charged phospholipids, particularly cardiolipin (15). Upon association with the import channel, Tim44 recruits mHsp70 to the channel in a nucleotide-dependent manner. Regulation of this interaction involves the nucleotide exchange factor Mge1 (16, 17) and the J-domain-containing chaperone complex Tim14/Pam18-Tim16/Pam16 (18–20).

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The Mitochondrial Translocation Motor

Two models, the active pulling and the trapping (Brownian ratchet), have been suggested initially to explain how proteins are translocated across the inner membrane and, in particular, the ability of mitochondria to import stably folded proteins. In the pulling model, mHsp70 undergoes a conformational change generating an active pulling force on the polypeptide chain. This pulling force, controlled by ATP binding, drives the unfolding of precursor proteins and their concomitant translocation across the inner membrane. According to this model, the pulling force will be effective only if mHsp70 forms a ternary complex with the imported precursor protein and Tim44. The latter would provide a platform for a lever-like movement of mHsp70 (21).

The trapping model proposes a movement of the polypeptide chain in the translocation channel due to Brownian molecular motion, which is then trapped by interaction of mHsp70 with the matrix-exposed part of the polypeptide. Trapping by mHsp70 leads to vectorial transport, as the polypeptide chain can no longer move backward. According to the ratchet model, the unfolding of the precursor protein is achieved by trapping the conformational changes of the polypeptide chain that occur with natural breathing of the protein (4).

Recently, a third model, the "entropic pulling," was proposed. This model suggests that the bulky mHsp70 bound to the translocating chain reduces the latter's conformational freedom, thereby accelerating protein import by means of entropic pulling (22). The entropic pulling model suggests the presence of active pulling, of entropic origin, but in the absence of a molecular fulcrum. Thus, both the Brownian ratchet and entropic pulling models have similar molecular requirements for functioning during protein import into the matrix.

In this study, we used cross-linking with the bifunctional reagent disuccinimidyl suberate (DSS)3 to study the formation of the translocation motor utilizing purified components. Mechanistic implications of the results are discussed in light of these known models for function of the translocation motor.

**EXPERIMENTAL PROCEDURES**

**Materials**—Nucleotides were purchased from Sigma: ATP (catalog no. A7699), high pressure liquid chromatography-purified ADP (catalog no. A6521), and AMP-PNP (catalog no. A2647).

**Construction of N-terminally Octahistidine-tagged Tim44**—A yeast Tim44 open reading frame lacking 43 N-terminal amino acids (corresponding to the mitochondrial targeting sequence) was amplified using forward primer 5′-TAA GGA TCC CAA GGT GGA AAC CCT CGA and backward primer 5′-TAA GGC GCC GCT CAG GTG AAT TGT CTA GA. The PCR product was subcloned into pGEM-T-Easy (Promega Corp.) and sequenced to confirm the fidelity of the *Tag* polymerase. The fragment was digested with BamHI-NotI and ligated with a double-digested (BamHI-NotI) modified pET-21d(+) vector (Novagen). The resulting recombinant plasmid encodes a Tim44 protein in which the mitochondrial targeting sequence is replaced by an initiation codon followed by a octahistidine tag and a tobacco etch virus (TEV) protease recognition site. The His-tagged Tim44 was overexpressed in *Escherichia coli* strain BL21.

**Purification of Octahistidine-tagged Tim44**—The bacterial transformants were grown in 1 liter of LB medium at 37 °C to an *A*~600~ of 0.5–0.6, and overexpression of Tim44 was induced with 1 mM isopropyl β-d-thiogalactopyranoside for 3 h. The cells were then harvested, suspended in 100 ml of buffer A (50 mM Tris·HCl (pH 7.5), 0.1% Triton X-100, 0.1 mg/ml lysozyme, 2 mM MgCl₂, 2 mM phenylmethylsulfonyl fluoride, 5% (v/v) glycerol, 1500 units of DNase, and protease inhibitor mixture (catalog no. 11873580001, Roche Applied Science)), disrupted using a Microfluidizer (Teta Sense), and centrifuged at 20,000 × g to clear the solution. The supernatant was loaded at 1 ml/min onto a nickel-nitrilotriacetic acid–agarose column (Bio-Rad) that had been pre-equilibrated with buffer B (50 mM Tris·HCl (pH 7.4), 0.4 M NaCl, 10 mM imidazole, and 10% glycerol). The column was washed with 20 ml of buffer B and developed with a linear imidazole gradient (10–500 mM) in buffer B. Tim44 eluted at ~200 mM imidazole. Fractions enriched in Tim44 were pooled, and protein concentration was determined. TEV protease was added at 1:50 (w/w) to the Tim44 eluate and incubated overnight at 4 °C. To purify Tim44 further, the nickel-nitrilotriacetic acid–agarose eluate was concentrated to ~1 ml in Centricon tubes (Vivascience) and further purified using a Superdex 200 gel filtration column (Amersham Biosciences) in buffer C (300 mM NaCl and 20 mM Tris·HCl (pH 7.4)) at a flow rate 1 ml/min. Tim44 eluted at ~100 ml of buffer C and was >95% pure as judged by SDS-PAGE. The Tim44 buffer was exchanged (PD-10, GE Healthcare) into 20 mM Na⁺-HEPES (pH 7.4) and 100 mM NaCl, concentrated to ~10–20 mg/ml in Centricon tubes, frozen in liquid nitrogen, and stored at −80 °C.

**Purification of [35S]Met-radiolabeled Tim44**—Purification was carried out essentially as described above, except that the bacteria overexpressing Tim44 were grown in dilute LB medium (0.25% Bacto-Tryptone, 0.125% yeast extract, and 0.5% NaCl). Production of radiolabeled Tim44 was initiated by addition of 1 mM isopropyl β-d-thiogalactopyranoside and 5 mCi of Redivue L-[35S]methionine (catalog no. AG1094, Amersham Biosciences).

**Construction and Purification of the C-terminal Domain of Tim44**—A yeast Tim44 open reading frame lacking 210 N-terminal amino acids was amplified using forward primer 5′-GGA TCC ACA AAT ATC GAG TCT AAA GAA and backward primer 5′-TAA GCG GCC GCT CAG GTG AAT TGT CTA GA. The PCR product was cloned in a modified pET-21d(+) vector. The resulting plasmid overexpresses Tim44 carrying an octahistidine tag at its N terminus followed by the TEV protease recognition site. The purification procedure was carried out as described above for full-length Tim44.

**Purification of the Tim14/Pam18-Tim16/Pam16 Complex**—A plasmid co-overexpressing a soluble domain of Tim16/Pam16 named Tim16/Pam16c, containing an octahistidine tag at the N terminus, and the soluble domain of Tim14/Pam18 named Tim14/Pam18, was constructed (23). The histidine tag is removable by digestion with TEV protease. The full purification procedure is described in Ref. 24.

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3 The abbreviations used are: DSS, disuccinimidyl suberate; AMP-PNP, 5′-adenyl γ-5′-adenylyl β-5′-adenylyl γ-5′-adenylyl phosphatase; TEV, tobacco etch virus.
Mutagenesis—Site-directed mutations were created using the QuikChange mutagenesis kit (catalog no. 0720099, Stratagene). PCR amplification of mutant Tim44 was performed with forward primer 5′-GAA TGG GAG AAG TCT CCT GCA CTG CAG GAG AAC and backward primer 5′-GTT CTC CGG TGC GAG ACT TCT CTC CCC ATT C. Recombinant N-terminally hexahistidine-tagged Tim44 cloned in the pGEM-T-Easy vector was used as a template. Following sequence analysis to confirm the mutation, the PCR product was digested with BamHI-NotI and cloned into the modified sequence analysis to confirm the mutation, the PCR product was digested with BamHI-NotI and cloned into the modified reagents and is carried out as follows. [35S]Met-radiolabeled protein was digested with TEV protease cleavage and subsequent gel filtration, Tim44 was purified and incubated with increasing concentrations of mHsp70, representing a complex of Tim44 bound to mHsp70 (Fig. 1A, lanes 2–6). Maximal formation of the Tim44-mHsp70 complex was reached at 3 μM mHsp70, which was close to the Tim44 concentration (2 μM) present in the reaction mixture. A second minor form of the Tim44-mHsp70 complex was also observed (Fig. 1, asterisks). The latter form probably represents higher oligomers of the Tim44-mHsp70 complex. It was suggested previously that the functional form of the import channel is dimeric (28). Thus, we cannot exclude the possibility that the Tim44-mHsp70 complex itself has a tendency to form dimers. Notably, much less Tim44-mHsp70 complex was observed in the presence of ATP (Fig. 1B). Similar results (i.e. weaker binding in the presence of ATP) were also observed in pulldown experiments using Tim44 carrying a histidine tag (supplemental Fig. S2).

In the presence of Mge1, we observed a new cross-linked form representing a complex composed of Tim44-mHsp70-Mge1 (Fig. 1C). Similar to the complex formed in the absence of Mge1, much less Tim44-mHsp70-Mge1 complex was observed in the presence of ATP (Fig. 1D). We conclude that ATP destabilizes the interaction between mHsp70 and Tim44.

In the cell, mHsp70 is expected to be in complex with either ADP or ATP. Therefore, the formation of the Tim44-mHsp70 complex was examined in the presence of ADP as well. As shown in Fig. 2, the Tim44-mHsp70 complex obtained in the presence of ADP was the strongest. Although the complex with ADP was consistently more stable than that lacking nucleotide, we were not able to demonstrate that this phenomenon is statistically significant (supplemental Fig. S3). Significant binding was also observed in the presence of AMP-PNP. Thus, the Tim44-mHsp70 interaction is modulated by nucleotides in a manner similar to what has been observed with solubilized mitochondria (12–14). Similar results were obtained in the presence of Mge1 as well (data not shown).

Several observations exclude the possibility that Tim44 was associated with mHsp70 as an unfolded substrate. First, when the purified Tim44 was examined by CD spectroscopy, its spectrum was consistent with that of a folded protein, with a T_m of −51 °C (supplemental Fig. S4). Second, Tim44 did not affect the ATPase activity of mHsp70 under any conditions examined (data not shown), as would be expected from an unfolded substrate (27, 29). Third, when similar experiments were carried out in the presence of DnaK, only a minute amount of Tim44 was detected bound to DnaK under all conditions examined (Fig. 2). This result is consistent with previous observations showing that DnaK does not complement a deletion of yeast mHsp70 (30). Lastly, a Tim44 mutant (E67A) was found to be impaired in its interaction with mHsp70 (see below).

Mapping the mHsp70-binding Site on Tim44—We have shown previously that yeast Tim44 contains a tightly folded domain that is located at the C terminus of the molecule (~25 kDa) (15). Recently, the crystal structure of the Tim44 C-terminal domain was solved (31) and confirmed our previous predictions. We wanted to determine whether the tightly
folded C-terminal domain of Tim44 is able to interact with mHsp70. To this end, the radiolabeled C-terminal domain of Tim44 was overexpressed in bacteria and purified. Next, its ability to bind mHsp70 was examined using cross-linking with DSS. The results presented in Fig. 3 show that the C-terminal domain of yeast Tim44 was less able to bind mHsp70 in comparison with full-length Tim44, indicating that the N terminus of Tim44 may play an important role in the interaction with mHsp70.

Using site-directed mutagenesis, we found that mutating amino acid 67 of Tim44 from Glu to Ala was lethal for yeast cells (data not shown). Because the E67A mutation is located at the N terminus of Tim44, we examined whether the lethal phenotype is due to an impaired interaction of Tim44 with mHsp70. For this purpose, the radiolabeled Tim44 mutant was purified, and its interaction with mHsp70 was examined using cross-linking. As shown in Fig. 4A, in the absence of Mge1, E67A mutant Tim44 bound mHsp70 slightly less compared with wild-type Tim44 in the presence of both ADP and ATP. Notably, in the presence of Mge1, the binding of E67A mutant Tim44 was strongly impaired under all conditions tested (Fig. 4B). Thus, Glu67, located at the N terminus of Tim44, may play an important role in the binding of Tim44 to mHsp70.

Effect of the P5 Peptide on the Formation of the Tim44-mHsp70 Complex—The translocation motor binds precursor proteins during its functional cycle. The P5 peptide, derived from the mitochondrial targeting signal sequence of aspartate aminotransferase, is known to bind Hsp70 chaperones, including mHsp70 (27, 32, 33). Therefore, the effect of the P5 peptide on the formation of the Tim44-mHsp70 complex was examined. Notably, under all conditions examined, the P5 peptide triggered dissociation of the Tim44-mHsp70 complex (Fig. 5). A control peptide (LEEDLRGYM-SWI) did not trigger dissociation of the Tim44-mHsp70 complex (data not shown). Thus, the binding of Tim44 and the P5 peptide to mHsp70 is mutually exclusive: mHsp70 cannot bind Tim44 when a peptide is bound to it. From a mechanistic point of view, the results presented here suggest that precursor binding by mHsp70 causes instant dissociation from its complex with Tim44, which consequently cannot serve as a fulcrum for the function of mHsp70.

Effect of Tim14/Pam18-Tim16/Pam16 Co-chaperones on the Formation of the Tim44-mHsp70 Complex—A membrane-associated complex of co-chaperones, Tim14/Pam18-Tim16/Pam16, was shown to be a vital component of the translocation motor. It has been suggested that the role of Tim14/Pam18, similar to other J-domain-containing chaperones, is to enhance the ATPase activity of mHsp70 and to promote a conformation that is strongly associated with peptide (18–20, 34, 35). A subsequent study showed that Tim16/Pam16 acts to antagonize the enhancing effect of Tim14/Pam18 by reducing it by half (36). Nevertheless, another work has suggested that enhancement of the ATPase activity by Tim14/Pam18 is not essential for the in vivo function of the co-chaperone complex (23). Previous studies carried out using solubilized mitochondria...
demonstrated that both Tim14/Pam18 and Tim16/Pam16 function in vivo as one stable complex (18, 19, 34). Therefore, in this study, we focused primarily on the effect of the Tim14/Pam18-Tim16/Pam16 complex on the Tim44-mHsp70 interaction. The concentrations of Tim44 and the Tim14j/Pam18j-Tim16s/Pam16s complex (soluble domains of the complex) were kept equal (1.2 μM each), and the formation of the Tim44-mHsp70 complex was examined. The results presented in Fig. 6A show that the presence of the Tim14j/Pam18j-Tim16s/Pam16s complex had very little effect on the formation of the Tim44-mHsp70 complex (10% less bound at 2 μM mHsp70).

FIGURE 2. Effect of nucleotides on the formation of the Tim44-mHsp70 complex. The experiment was carried out as described for Fig. 1 in the presence of the indicated nucleotides at 1 mM.

| Lane no. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|----------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|
| DnaK [μM] | 0 | 2 | 3 | - | - | 2 | 3 | - | - | 2 | 3 | - | - | 2 |
| mHsp70 [μM] | - | - | 2 | 3 | - | - | 2 | 3 | - | - | 2 | 3 | - | - | 2 |

Properties of the Tim44-mHsp70 Complex—Cross-linking with DSS showed that Tim44 interacts with mHsp70, forming a hetero-oligomer. A dimer of the Mge1 nucleotide exchange factor associates with the complex, leading to the formation of a heterotrimeric complex. We demonstrated previously that when examined by the protease resistance assay, the C terminus of Tim44 (~25 kDa) forms a tightly folded domain (15). We have shown here that mHsp70 interacts very weakly with the purified C-terminal domain of Tim44. However, because of the fact that we have not been able to purify the N-terminal domain, we did not demonstrate its direct binding to mHsp70. Thus, in vitro mHsp70 was detected as binding either to Tim44 or to the Tim14/Pam18-Tim16/Pam16 complex, but not to both simultaneously (supplemental Fig. 2S).

DISCUSSION

The aim of this work was a structure-function analysis of the mitochondrial translocation motor with an emphasis on interactions between various components of the motor. For the purpose of the study, we developed a novel method using cross-linking with DSS to monitor the interaction between the various purified partner proteins. The advantage of using cross-linking to investigate protein-protein interactions is that the complexes are stabilized with no disruption of the equilibrium in the system, which enables us to determine the steady-state levels of various complexes.

We demonstrated previously that when examined by the protease resistance assay, the C terminus of Tim44 (~25 kDa) forms a tightly folded domain (15). We have shown here that mHsp70 interacts very weakly with the purified C-terminal domain of Tim44. However, because of the fact that we have not been able to purify the N-terminal domain, we did not demonstrate its direct binding to mHsp70. Thus, we cannot exclude another interesting possibility, viz. that the N-terminal domain affected the folding of the C-terminal domain slightly and altered the binding of the latter to mHsp70. Additional work is needed to demonstrate which possibility is correct. Finally, we found that a single point mutation (E67A) of Tim44 that is lethal for yeast leads to a significant reduction in the formation of the Tim44-mHsp70 complex in particular in the presence of Mge1. Why is the effect of the E67A mutation more pronounced in the presence of Mge1? It is possible that Mge1 induces a conformation of mHsp70 that is less tightly bound to Tim44. This view is supported by the observation that at low

A very significant result is the fact that Tim14/Pam18-Tim16/Pam16 did not form a complex with Tim44 (Fig. 6A) or with the Tim44-mHsp70 complex (Fig. 6A, lanes 2 and 3). One explanation for this result would be that mHsp70 can form a complex either with the Tim14/Pam18-Tim16/Pam16 complex or with Tim44 but has a much higher affinity for Tim44. To examine this possibility, complexes were formed, stabilized by cross-linking with DSS, separated by SDS-PAGE, and detected by staining with Coo massie Blue. Indeed, when incubated alone, mHsp70 and Tim14/Pam18-Tim16/Pam16 associated to form a complex (Fig. 6B). Thus, in vitro mHsp70 was detected as binding either to Tim44 or to the Tim14/Pam18-Tim16/Pam16 complex, but not to both simultaneously (supplemental Fig. 2S).
mHsp70 concentrations, less Tim44 was associated with mHsp70 in the presence of Mge1 than in its absence (Fig. 1, C versus A) (29, 37). Overall, the results suggest that Glu67, located at the N terminus of Tim44, is probably involved in the formation of a complex with mHsp70.

Nucleotides Modulate the Tim44-mHsp70 Interaction—The function of Hsp70 chaperones is modulated by nucleotides. Nucleotide-dependent formation of the Tim44-mHsp70 complex has also been demonstrated using solubilized mitochondria (12–14). Similarly, we found in this study that in vitro nucleotides differentially affect the formation of the Tim44-mHsp70 complex. The strongest formation of the Tim44-mHsp70 complex was achieved in the presence of ADP. In contrast, in the presence of ATP, very little mHsp70 complex was observed. Thus, mHsp70 alternates between at least two forms in a nucleotide-dependent manner. One form, in the presence of ADP, has a high affinity for Tim44, whereas the second form, in the presence of ATP, has a weak affinity for Tim44. Similar modulation by nucleotides has been observed by several groups in solubilized mitochondria (4, 38). However, another study carried out in vitro demonstrated no modulation of the Tim44-mHsp70 complex by nucleotides (37). We conjecture that the contradictory results are due to the different methods used in the respective studies to detect complex formation.

Mutually Exclusive Interactions of the Peptide and Tim44 with mHsp70—An essential difference between models describing the function of the translocation motor is the need for a fulcrum to enable active unfolding of precursor proteins by mHsp70. Such a fulcrum would be provided for mHsp70 by its membrane anchor, Tim44. Notably, under all conditions examined here, we found that binding of the P5 peptide to mHsp70 triggers the dissociation of the Tim44-mHsp70 complex. Dissociation of the Tim44-mHsp70 complex was not affected by the type of nucleotide added or the presence of the Tim14/Pam18-Tim16/Pam16 complex. However, active pulling by the mitochondrial translocation motor requires that mHsp70 be anchored to Tim44 while being simultaneously bound to precursor proteins. Thus, the results that we obtained in this study are difficult to reconcile with active pulling, in its classical version (21), and are compatible with a function via Brownian ratchet (4) or active pulling as suggested by the entropic pulling mechanism (22).

Role of Tim14/Pam18-Tim16/Pam16 Co-chaperones in the Functional Cycle of the Motor—The results of this study show that, despite the fact that the Tim14/Pam18-Tim16/Pam16 complex is able to form a complex with mHsp70, the interaction between mHsp70 and Tim44 is much stronger. In other
words, when the Tim14/Pam18-Tim16/Pam16 complex and Tim44 are both present, mHsp70 associates only with Tim44. In light of these results, one could suggest possible roles for the Tim14/Pam18-Tim16/Pam16 complex in the functional cycle of the motor, as follows. (i) One role would be to enhance the ATPase activity of mHsp70 and to endorse tight locking of substrate in the binding site of mHsp70. Such an effect has not been demonstrated yet because the Tim14/Pam18-Tim16/Pam16 complex itself does not affect the ATPase activity of mHsp70. However, we cannot exclude the possibility that enhancement of the ATPase activity of mHsp70 by the Tim14/Pam18-Tim16/Pam16 complex requires the context of the translocation channel. (ii) The observation that mHsp70 favors association with Tim44 rather than with the Tim14/Pam18-Tim16/Pam16 complex suggests that the latter may serve to recruit mHsp70 to the translocation motor prior to its transfer to Tim44. This would ensure the presence of several mHsp70 molecules that are in close proximity to the import channel, thereby increasing the local concentration of mHsp70.

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FIGURE 6. Formation of the Tim44-mHsp70 complex in the presence of the Tim14/Pam18-Tim16/Pam16 proteins. A, 1.2 μM [35S]Met-radiolabeled Tim44 was incubated with the indicated components and 1.2 μM purified Tim14/Pam18-Tim16/Pam16, and subjected to cross-linking as described for Fig. 1. B, the Tim14/Pam18-Tim16/Pam16 complex was incubated with the indicated concentrations of mHsp70, and complexes were stabilized by cross-linking with 1 mM DSS. The cross-linking products were separated by 4–16% SDS-PAGE and stained with Coomassie Blue.

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