Mitochondrial Protein Import: Molecular Basis of the ATP-dependent Interaction of MtHsp70 with Tim44*

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Protein translocation across the mitochondrial inner membrane is driven by cycles of binding and release of mitochondrial heat shock protein 70 (mtHsp70) in the matrix. The peripheral inner membrane protein, Tim44, recruits mtHsp70 in an ATP-dependent manner to the import sites. We show that DnaK, the closely related Hsp70 of Escherichia coli, when targeted to the matrix of yeast mitochondria, interacts in a specific manner with Tim44. The interaction is, however, not regulated by ATP, and DnaK cannot support protein translocation. We used truncated mtHsp70s and chimeric proteins consisting of segments of mtHsp70 and DnaK to analyze which portions of mtHsp70 bind and functionally interact with Tim44. We show that Tim44 interacts with the β-stranded core of the peptide binding domain of mtHsp70 and of DnaK. The α-helices A and B of the peptide binding domain of mtHsp70 are required to transmit the nucleotide state of the ATPase domain to the peptide binding domain. Tim44, by interacting in this way with the peptide binding domain, is proposed to coordinate the binding of mtHsp70 to the incoming preprotein and the subsequent release of the mtHsp70-preprotein complex from the TIM23 complex, the translocase of the inner membrane.

The major mtHsp70,1 Ssc1p in yeast, mediates translocation of proteins across the inner membrane of mitochondria. Ssc1p cooperates with Tim44, a peripheral inner membrane protein associated on the matrix with the TIM23 complex, which constitutes the preprotein conducting import channel across the inner membrane (1–7). Tim44 is a dimer that recruits two molecules of Ssc1p to the import channel (6, 8). It was proposed that Tim44, Ssc1p, and its co-chaperone Mge1p are all parts of an ATP-dependent molecular motor that drives translocation of preproteins by a “hand over hand” mode (6, 7). Ssc1p binds to segments of a translocating preprotein as they emerge from the import channel. It is then released from the inner membrane, and the Ssc1p-preprotein complex can move further into the matrix. A central question regarding this pathway is how Tim44 can sense when to release mtHsp70 that has bound a preprotein. To address this question it is important to determine which portions of mtHsp70 interact with Tim44.

Molecular chaperones of the Hsp70 family are composed of two domains, an N-terminal ATPase domain of about 45 kDa that is connected by a short linker to a peptide binding domain of ~25 kDa (9). The first part of the peptide binding domain consists of eight anti-parallel β-strands that form the substrate binding pocket (10). The β-stranded core is followed by an α-helical portion consisting of five helices, αA–αE, and a C terminus of unknown structure. αB consists of two halves connected by a flexible hinge. αA and the first half of αB are packed against the β-stranded core. The second half of αB and αC–αE correspond to a compact α-helical subdomain (11). To efficiently close the substrate binding pocket, the α-helical subdomain folds like a lid over the β-stranded core. Hsp70s bind unfolded polypeptides in an ATP-dependent manner (12–17). When Hsp70 is in the ATP form, the substrate binding pocket of Hsp70 is open; the chaperone can rapidly bind and release polypeptide substrates. In the ADP form the substrate binding pocket is closed, and polypeptide substrates can hardly bind to Hsp70, whereas bound polypeptides are tightly held and not released. A cycle driven by ATP hydrolysis allows Hsp70s to associate with an unfolded polypeptide in the ATP form and then hydrolyze ATP to tightly hold the substrate and finally release the bound polypeptide after exchange of ADP by ATP. To bind and release polypeptides on a physiologically relevant time scale, Hsp70s cooperate with co-chaperones that accelerate the nucleotide reaction cycle (15, 18–20). Co-chaperones also tag the relevant substrates and thereby recruit the chaperones for specific tasks (21). Tim44 is such a co-chaperone, and it recruits mtHsp70 for protein import.

Here we show that DnaK, the Hsp70 of Escherichia coli, when targeted to the matrix of yeast mitochondria, is also recruited by Tim44 to the inner membrane import sites. The interaction with Tim44, however, is not modulated by nucleotides, and DnaK does not drive protein translocation. We constructed a series of chimera between Ssc1p and DnaK to identify the segments in Ssc1p that are necessary for a functional interaction with Tim44. We show that Tim44 interacts with the β-stranded core of the peptide binding domain of Ssc1p and of DnaK but not via the substrate binding pocket. The interaction is independent of nucleotides. The α-helices A and B of the peptide binding domain of Ssc1p, but not of DnaK, modulate the interaction between the β-stranded core and Tim44 in a nucleotide-dependent fashion. Implications of the mechanism of protein translocation are discussed.

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1 The abbreviations used are: mtHsp70, mitochondrial heat shock protein 70; PK, proteinase K; DHFR, dihydrofolate reductase; MTX, methotrexate; WT, wild type; Ni-NTA, nickel-nitritotriacetic acid.
A 1.3-kb fragment encoding the ATPase domain and the transposase domain of Ssc1p was amplified by PCR using the primers 5'-GGGGAATTCACCATGGGTGGTGTTTTCACAAGA-3' and 5'-CCCGAATTCTTATTTTTTGTTTAGTCC-3'. The 0.7-kb fragment encoding the peptide binding domain of DnaK was amplified by PCR using the primers 5'-CCCGAATTCGCC-3' and 5'-CCCGGAACCTTTGGATCCCTCAAGGATGATTACAAACCAGAAGAACCGG-3'. The 0.6-kb fragment encoding the helices of Ssc1p peptide binding domain was amplified by PCR using the primers 5'-CCCCCGAGCTCCTTAGTTTCACCA-3' and 5'-CCCCCGAATCTTTTTTTACTGCTTAGTTTCACCA-3'.

To target ATP (Fig. 2A), the mitochondrial homologue of the bacterial co-chaperone GrpE, in an ATP-dependent manner and to drive protein translocation in reticulocyte lysate. After a 5-min incubation at 25 °C, samples were transferred on ice and split into two aliquots. One aliquot remained untreated, and the other was treated with 50 μg/ml PK or trypsin for 25 min on ice. Protease digestion was stopped by the addition of 1 mM phenylmethylsulfonyl fluoride or 0.5 mg/ml soybean trypsin inhibitor, respectively, and mitochondria were resolated by centrifugation. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and detected by autoradiography.

**RESULTS**

**Expression of E. coli DnaK in Yeast and Targeting to Mitochondria**—To target E. coli DnaK into the matrix of yeast mitochondria, the open reading frame was fused to the presence of the ATPase subunit 9 of N. crassa (pSu9) (Fig. 1A). A His tag was introduced between the presence and the N-terminus of DnaK. This construct was expressed under control of a galactose-inducible promoter in the temperature-sensitive yeast strain ssc1-3, which carries a mutation in the major mtHsp70 (23). DnaK was processed to the mature form and localized in the mitochondrial matrix (Fig. 1B). The amount of DnaK that could be isolated by Ni-NTA chromatography was comparable with the amount of Ssc1-3p in control mitochondria as estimated from Coomassie Blue-stained gels (data not shown). This finding indicates that DnaK was expressed at a physiologically relevant level. The mitochondria-targeted DnaK bound to ATP agarose (Fig. 1C), suggesting that it was properly folded in the matrix of the mitochondria. However, when yeast cells were shifted to nonpermissive temperature, DnaK in the mitochondrial matrix did not rescue the temperature-sensitive phenotype of the ssc1-3 strain (data not shown).

**DnaK**

DnaK interacted with Mge1p, the mitochondrial homologue of the bacterial co-chaperone GrpE, in an ATP-dependent manner (Fig. 2A). Using an antibody against Tim44, DnaK coimmunoprecipitated with Tim44 in the presence and absence of ATP (Fig. 2B). Apparently, the interaction between DnaK and Tim44 was not regulated by ATP. Furthermore, DnaK did not support import of pSu9-(1–69)DHFR into mitochondria that were preincubated at 37 °C in order to inactivate the mutant Ssc1-3p (data not shown).

**DnaK/Ssc1p Chimeric Proteins**—What are the functional elements that allow Ssc1p, but not DnaK, to interact with Tim44 in an ATP-dependent manner and to drive protein translocation? DnaK and Ssc1p are the two members of the Hsp70 protein family that share the highest degree of sequence similarity. The N-terminal ATPase domain and the β-stranded
portion of the peptide binding domain are highly conserved between DnaK and Ssc1p (65 and 68% amino acid sequence identity, respectively). The α-helical portion, which comprises the lid and the C-terminal domain, is significantly less conserved (22% identity). Based on the sequence alignment with Ssc1p and on the crystal structure of the peptide binding domain of DnaK (10) (Fig. 3A and B), we divided the chaperones into four structural elements: (i) the ATPase domain; (ii) the β-stranded core constituting the substrate binding pocket (β1-β8); (iii) helix A plus helix B (αA–αB), the latter containing the flexible hinge; and (iv) the C-terminal lid domain (αC–αE plus the C terminus). Combining these four structural elements, we constructed chimeric Hsp70s (Fig. 3C). The elements originating from Ssc1p and DnaK are indicated by “C” and “K,” respectively, in the appropriate position of a four-letter acronym.

When the chimeric chaperones were expressed in the ssc1–3 strain, none of the chimeric Hsp70s was able to rescue the temperature-sensitive growth phenotype of the ssc1–3 strain (data not shown). Similarly, the chimeric Hsp70s did not complement a disruption of the Ssc1 gene (data not shown). As estimated from Coomassie Blue-stained gels, the expression levels of the chimera were significantly lower (∼25%) than those of Ssc1p in mitochondria from WT cells yet were comparable with those of Ssc1-3p (data not shown). Thus, it is not clear whether the failure of the chimera to substitute for Ssc1p is because of the lower expression levels or reflects functional deficiencies of the chimera.

For purposes of functional characterization, mitochondria from ssc1–3 cells harboring DnaK or one of the chimeric Hsp70s were lysed with Triton X-100 in the absence or the presence of ATP, and the His-tagged chimera were adsorbed to Ni-NTA beads. WT mitochondria containing a C-terminally His-tagged version of Ssc1p were used as a control. Like DnaK and Ssc1p, the chimeric Hsp70s interacted with Mge1p in a nucleotide-dependent manner (data not shown), demonstrating that all Hsp70s underwent physiologically relevant nucleotide-dependent conformational changes.

To study the interaction of the chimeric chaperones with Tim44, immunoprecipitation with affinity-purified antibodies against Tim44 was performed in the presence and absence of ATP (Fig. 3D). In the absence of ATP, Ssc1p, DnaK, and each of the chimeric Hsp70s coprecipitated in a complex with Tim44. In the presence of ATP, the complexes of Tim44 with DnaK, CKKK, and KKKK were stable. In contrast, the complexes of Tim44 with KKCC, KCCC, and Ssc1p dissociated in the presence of ATP, indicating a nucleotide-dependent interaction of these Hsp70s with Tim44. Thus, the α-helices A and B of the peptide binding domain of Ssc1p appear to be necessary to transmit the nucleotide-induced conformational changes from the ATPase domain to Tim44 and thereby regulate the interaction of the chaperones with Tim44 in an ATP-dependent manner.

To characterize the interaction of Ssc1p with Tim44 in more detail, we constructed C-terminally truncated versions of Ssc1p (Fig. 4A, left). The deleted structural elements are indicated by
a "0" in the corresponding position of the acronym. CC00 lacks the α-helical portion and C-terminal domain, and C000 consists only of the ATPase domain. In addition, we constructed DHFR-0CCC and DHFR-0KKK, fusion proteins between the chimeric preprotein pSu9-(1–69)DHFR and the entire peptide binding domain of Ssc1p and DnaK, respectively. To study the interaction of the chimera with Tim44, radiolabeled precursors were synthesized in vitro and imported into isolated mitochondria. The radiolabeled precursors of Ssc1p and pSu9-(1–69)DHFR were imported for control. After the import reactions the samples were treated with PK to remove precursors associated with the surface of mitochondria. All precursors were efficiently imported into the matrix and processed to their mature forms (data not shown). Subsequently the mitochondria were lysed with Triton X-100 in the absence or presence of ATP. The clarified lysates were subjected to immunoprecipitation with antibodies against Tim44. Ssc1p and DnaK were detected by immunostaining of Western blots with antibodies against DnaK. Ssc1p was detected with Ssc1p antibodies.

absence but also in the presence of ATP. This supports the conclusion (see above) that the α-helices A and B are required for the ATP dependence of the Tim44-Ssc1p interaction. CC00 (ATPase domain) was not coprecipitated with Tim44, indicating that it either did not bind stably to Tim44 or that it could not efficiently compete with the endogenous Ssc1p. This result contradicts earlier findings by Krimmer et al. (24). Possible explanations for the apparent discrepancy are discussed. Interestingly, DHFR-0CCC and DHFR-0KKK interacted with Tim44, whereas imported DHFR did not coprecipitate with Tim44 (Fig. 4B). This indicates that the peptide binding domains of Ssc1p and DnaK interact with Tim44. The interaction of both chimeras with Tim44 was not modulated by ATP. In particular, the β-stranded portion of the domain is required for the interaction with Tim44, whereas the helices αA and αB are not required for binding per se but modulate the interaction in response to nucleotides.

To obtain further evidence that Tim44 interacts with the peptide binding domain of Ssc1p in a specific manner, we constructed DHFR-0CCC1–2, a fusion protein between pSu9-
Fig. 4. Tim44 interacts with the β-stranded core of the peptide
binding domains of Ssc1p and DnaK. A, the precursors of Ssc1p, CC00, and C000 and the precursors of DHFR-0CCC, DHFR-0K KK, and
DHFR, which carry the pSu9 presequence, are indicated schematically. The DHFR moiety is indicated by a dotted box. Portions of DnaK
and Ssc1p are indicated as white and black boxes, respectively. The radio-
labeled chimeric preproteins were imported for 15 min at 25 °C into WT
mitochondria. Samples were treated with 1 μM valinomycin and 50
μg/ml trypsin. Mitochondria were resolubilized, washed twice in import
buffer, and then lysed with Triton X-100 in the absence or presence of
1 mM ATP. The lysates were subjected to immunoprecipitation with
antibodies against Tim44. Immunoprecipitates (IP) were analyzed by
SDS-PAGE and autoradiography. pSu9-(1–69)DHFR was used as a con-
trol. B, interaction of Tim44 with the peptide binding domain of Ssc1-2p
is temperature-sensitive. The precursors DHFR-0CCC1–2, which con-
tains the peptide binding domain of Ssc1-2p, is shown in comparison
with DHFR-0CCC derived from WT Ssc1p. The position of the amino
acid exchange in the peptide binding domain of Ssc1-2p is indicated
(P442S with respect to the Ssc1-2p precursor). The radiolabeled precur-
sors were imported into WT mitochondria. For immunoprecipitations,
antibodies against Tim44 that were bound to protein A-Sepharose
beads were used. Incubation with the beads was for 15 min at 4 or at
37 °C, and then they were washed twice at 4 °C. The immunoprecipi-
tates were resolved by SDS-PAGE and analyzed by autoradiography.

(1–69)DHFR and the peptide binding domain of Ssc1-2p (Fig.
4B, left). The conditional mutant protein Ssc1-2p contains a mutation in the β-stranded core of peptide binding domain (Pro
to Ser in position 442 of the precursor) (23). At nonpermissive
temperature, Ssc1-2p interacts with substrate proteins in mi-
 tochondria but no longer interacts with Tim44 (3, 25). Purified
Ssc1-2p protein in the ADP form displays a reduced on-rate for
peptide (26), suggesting that the flexibility of the closed peptide
binding domain is compromised because of the mutation.
DHFR-0CCC1–2 and, for control, DHFR-0CCC were imported
into mitochondria, and immunoprecipitation with antibodies
against Tim44 was performed (Fig. 4B, right). To induce the
temperature-sensitive phenotype associated with the ssc1–2
mutation, the immunoprecipitates were incubated at 37 °C or
at 4 °C for control. DHFR-0CCC was detected in the immuno-
precipitate at 4 and at 37 °C. In contrast, the complex between
Tim44 and DHFR-0CCC1–2 was stable at 4 °C but dissociated
at 37 °C. This indicates that the mutation in Ssc1-2p affects the
conformation of the peptide binding domain independently of the ATPase domain. Because Ssc1-2p binds substrate proteins
at permissive and nonpermissive temperature, the observation
demonstrates that Tim44 is not bound like a substrate by the
chimera DHFR-0CCC1–2.

Protein Import Facilitated by Chimeric Hsp70s—To investigate
whether the chimeric Hsp70s facilitate preprotein trans-
location, mitochondria were first preincubated at 37 °C to in-
activate endogenous Ssc1-3p and then incubated at 25 °C with
the radiolabeled precursor pSu9-(1–69)DHFR (Fig. 5A). pSu9-
(1–69)DHFR was not imported into the matrix of mitochondria from
the ssc1–3 strain, demonstrating that the mutant chaperone was efficiently inactivated by the preincubation. Rather,
as observed previously, low amounts of the intermediate form,
which was processed only once by the matrix-processing peptidase, accumulated as a dead-end translocation intermediate
in the intermembrane space (data not shown). Likewise, no
import above background was observed with mitochondria har-
bor ing the chimera CKKK, KKKC, or KKCC. In contrast, pSu9-
(1–69)DHFR was imported into mitochondria harboring KKCC
where it was efficiently processed to the mature form. The import
efficiency was lower than that of mitochondria harboring
Ssc1p, indicating that the chimera KKCC was not fully functional and/or not present at the same level as WT Ssc1p.

For further analysis mitochondria were preincubated at
37 °C, and then the kinetics of import were measured at either
25 or 37 °C (Fig. 5B). At 25 °C, mitochondria harboring KKCC
imported the precursor efficiently, yet at a slower rate than WT
mitochondria. This difference was even more pronounced when
import was carried out at 37 °C; import of pSu9-(1–69)DHFR into WT
mitochondria was faster at 37 °C than at 25 °C, whereas import into mitochondria containing KKCC was slower at 37 °C than at 25 °C, suggesting that the chimera
KKCC is temperature-sensitive.

We asked whether the chimera KKCC interacts directly with
translocation intermediates that are arrested in a membrane-
spanning fashion at the import site. To generate translocation
intermediates, mitochondria were pretreated at 37 °C and sub-
sequently incubated with pSu9-(1–69)DHFR in the presence of
MTX to stabilize the folded DHFR moiety and thereby prevent
unfolding and complete import of the precursor (27). Mitochon-
dria were then lysed with Triton X-100 in the absence of ATP,
and the His-tagged chimeric Hsp70s were precipitated with
Ni-NTA beads (Fig. 5C). The arrested translocation intermediate
coprecipitated with KKCC, whereas no interaction of pSu9-
(1–69)DHFR with DnaK or KKCC was observed.

Finally, we asked whether KKCC was able to tightly hold an
arrested precursor in the import channel. Mitoplasts were pre-
pared and incubated with pSu9-(1–69)DHFR in the presence of
MTX (Fig. 5D). In mitoplasts the arrested translocation inter-
mediate spans the inner membrane and is processed to the
intermediate form, iSu9-(1–69)DHFR (5), which readily allows
identification of arrested species. In mitoplasts generated from
WT mitochondria iSu9-(1–69)DHFR was resistant to treatment
with PK. This indicates that the MTX-stabilized DHFR
moiety was tightly apposed to the outer surface of the inner
membrane because of interaction of Ssc1p with segments of the
precursor exposed into the matrix. In mitoplasts from the
ssc1–3 mutant (after preincubation at 37 °C) and in ssc1–3
mitoplasts harboring DnaK, little iSu9-(1–69)DHFR was gen-
erated, and its resistance to protease treatment was very low
(Fig. 5D). Thus, Ssc1-3p and DnaK did not interact with the
membrane-spanning intermediate. In mitoplasts containing
KKCC iSu9-(1–69)DHFR was generated, but it was largely
sensitive to added PK (Fig. 5D). This indicates that KKCC was
translocation intermediates. Mitoplasts were isolated from ssc1–3 cells containing indicated forms of Hsp70 and preincubated for 10 min at 37 °C. Radiolabeled pSu9(1–69)DHFR was added in the presence of MTX and incubated at 25 °C for 10 min. Samples were transferred to ice, treated with 50 µg/ml PK, and analyzed by SDS-PAGE. The intermediate form containing indicated forms of Hsp70 was quantified using a phosphorimaging system. The columns are the means of two measurements. The error bars show the range of measurements.

DISCUSSION

What are the determinants in mtHsp70 that enable it to interact with Tim44 and to drive ATP-dependent protein translocation into the matrix of mitochondria? To answer this question we targeted E. coli DnaK to the mitochondrial matrix of yeast cells. This DnaK could not substitute for Ssc1p, the authentic mtHsp70, in vivo. In particular, DnaK in the matrix was properly folded but did not facilitate mitochondrial protein translocation, a process essential for the viability of yeast under all growth conditions. What are the determinants that enable Ssc1p but not DnaK to drive protein translocation? DnaK in the matrix interacted with Mge1p in an ATP-dependent manner and was assembled into a specific complex with Tim44. However, the interaction between Tim44 and DnaK was not regulated by ATP. Accordingly, the defect in protein import may be due to the deficiency of DnaK to interact with Tim44 in an ATP-dependent manner.

We used this situation to study which domains of Ssc1p interact with Tim44 and what the elements are that regulate the interaction in response to ATP. To this end we analyzed chimeric Hsp70s in which portions of DnaK were replaced by corresponding segments of Ssc1p. The result of these domain-swapping experiments is that the helices αA and αB of the peptide binding domain of Ssc1p are required for a nucleotide-dependent interaction of Hsp70 with Tim44. This finding is in agreement with the observation that a C-terminally truncated version of Ssc1p lacking the helices αD and αE was reported to interact with Tim44 in an ATP-dependent manner (24). We showed that Tim44 interacted with a C-terminally truncated version of Ssc1p that lacked the entire α-helical subdomain in a nucleotide-independent manner. Thus, the helices αA and αB are required for the nucleotide-dependent regulation of the interaction but not for the interaction per se.

The chimeric fusion proteins in which the ATPase domains of Ssc1p or DnaK were replaced by DHFR were isolated in a stable complex with Tim44. This clearly demonstrates that not fully competent to hold iSu9-(1–69)DHFR tightly apposed to the inner membrane and to efficiently prevent retrograde movement of the preprotein through the import channel.

In summary, DnaK and the chimeric Hsp70s specifically interacted with Tim44, indicating that Tim44 recruits the Hsp70s via a conserved interface. Two of the chimeras interacted with Tim44 in an ATP-dependent manner, but only KCCC, a construct that contained the ATPase domain of DnaK and the whole peptide binding domain of Ssc1p, interacted with a preprotein in transit and supported import in vitro. Yet, KCCC was not fully functional; it supported import at reduced rates and in a temperature-sensitive manner, and it could not efficiently hold a translocation intermediate in the import channel. The hybrid protein was temperature-sensitive in function; expression levels in vivo were significantly lower than those of Ssc1p. Thus, despite the striking similarity between DnaK and Ssc1p, KCCC could not substitute for Ssc1p in vivo. Fine tuning of the interaction between the ATPase and the peptide binding domain appears to be necessary to drive translocation at rates sufficiently high to support biogenesis of mitochondria in vivo.
Tim44 interacted with the peptide binding domains of Ssc1p and DnaK. A corresponding construct was not investigated by Krimmer et al. (24). We did not detect a complex of Tim44 with a truncated Ssc1p consisting only of the ATPase domain. In contrast, Krimmer et al. (24) could coprecipitate newly imported ATPase domain with Tim44. In both approaches immunoprecipitation with Tim44 antibodies was used to assess binding. In addition to the strong interaction of Tim44 with the peptide binding domain demonstrated here, an additional weak interaction of Tim44 with the ATPase domain cannot be ruled out on principle grounds. However, characterization of the interaction of Tim44 with the conditional mutant protein Ssc1-2p provides further evidence suggesting that Tim44 does not or only weakly interact with the ATPase domain. Ssc1-2p carries a single mutation in the β-stranded core of the peptide binding domain. When the mutant phenotype is induced, Ssc1-2p remains soluble and interacts with nucleotides in a binding domain. When the mutant phenotype is induced, the truncated version of Ssc1p consisting of the ATPase domain with Tim44 independently of ATP, a putative interaction of the ATPase domain with Tim44 must also be independent of ATP. Thus, the small structurally related to actin (31), and Ssc1p was shown to have a single mutation in the ATP-regulated Mitochondrial Protein Import Motor

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