Sequential action of two hsp70 complexes during protein import into mitochondria

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The mitochondrial chaperone mhsp70 mediates protein transport across the inner membrane and protein folding in the matrix. These two reactions are effected by two different mhsp70 complexes. The ADP conformation of mhsp70 favors formation of a complex on the inner membrane; this 'import complex' contains mhsp70, its membrane anchor Tim44 and the nucleotide exchange factor mGrpE. The ATP conformation of mhsp70 favors formation of a complex in the matrix; this 'folding complex' contains mhsp70, the mitochondrial DnaJ homolog Mdj1 and mGrpE. A precursor protein entering the matrix interacts first with the import complex and then with the folding complex. A chaperone can thus function as part of two different complexes within the same organelle.

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Introduction

Proteins of the 70 kDa stress protein (hsp70) family are involved in a variety of different processes including protein folding, disassembly of oligomeric protein complexes, delivery of proteins to proteases and the translocation of polypeptides across intracellular membranes (reviewed in Hightower *et al.*, 1994; McKay *et al.*, 1994). The members of the hsp70 family are highly conserved (Lindquist and Craig, 1988; Boorstein *et al.*, 1994). One of the family members in *Saccharomyces cerevisiae*, mitochondrial hsp70 (mhsp70), is located in the mitochondrial matrix (Craig *et al.*, 1988; Kang *et al.*, 1990; Scherer *et al.*, 1990). Mhsp70 is essential for viability of yeast and plays a major role in mitochondrial protein import and protein folding (reviewed in Langer and Neupert, 1994; Rassow *et al.*, 1995; Brodsky, 1996; Rospert *et al.*, 1996). Mhsp70 forms a transient complex with the inner membrane protein Tim44 and the mitochondrial nucleotide exchange factor mGrpE (Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994; Horst *et al.*, 1996). This 'import complex' drives the translocation of precursor proteins across the mitochondrial inner membrane. The mechanism by which mhsp70 mediates protein import is not fully understood. Mhsp70 has been proposed to function as a 'Brownian ratchet' (Neupert *et al.*, 1990; Simon *et al.*, 1992; Schneider *et al.*, 1994). In this model,

a polypeptide chain oscillates randomly by diffusion within the translocation channel, but gets trapped on each inward oscillation by mhsp70 which prevents reverse movement. A series of such events could drive complete translocation of the precursor. However, this model cannot easily explain the finding that precursor proteins such as cytochrome *b₂* are actively unfolded by mhsp70 during import into mitochondria (Glick *et al.*, 1993; Voos *et al.*, 1993; Stuart *et al.*, 1994). This observation suggested an alternative model in which mhsp70 undergoes a conformational change while bound both to the translocating precursor chain and Tim44, thereby generating an inward force that could drive unfolding and import of the precursor (Glick, 1995a; Pfanner and Meijer, 1995).

Mhsp70 mediates not only the translocation of proteins across the mitochondrial inner membrane, but also the folding of these precursor proteins in the matrix (Kang *et al.*, 1990; Scherer *et al.*, 1990; Manning-Krieg *et al.*, 1991). This fact correlates with the existence of two different populations of mhsp70, one bound to the inner membrane and the other one soluble in the matrix.

In order to understand how mhsp70 can mediate two different processes within the same organelle, we sought to identify proteins interacting with mhsp70. Therefore, we constructed a yeast strain in which all mhsp70 molecules carried a C-terminal hexahistidine tag. The tagged mhsp70 can be bound selectively to nickel-nitrilo triacetic acid (Ni-NTA)-agarose beads under conditions which should not disrupt interactions with bound partner proteins (Hochuli, 1990; Bolliger *et al.*, 1994; Kronidou *et al.*, 1994).

We found that mhsp70 exists in mitochondria as two different hetero-oligomeric complexes. One complex is associated with the inner membrane, contains mhsp70, its membrane anchor Tim44 and the nucleotide exchange factor mGrpE, and functions as an import complex (Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994). The other complex is localized in the matrix, contains mhsp70, the DnaJ homolog Mdj1 (Rowley *et al.*, 1994) and mGrpE (Bolliger *et al.*, 1994), and appears to function as a folding complex. Nucleotides inducing the ADP conformation of mhsp70 favor formation of the import complex, whereas those inducing the ATP conformation favor formation of the folding complex. Tim44 and Mdj1 thus appear to bind different conformations of mhsp70. A precursor protein entering the matrix interacts first with the import complex and then with the folding complex.

Results

Mhsp70 is present in two different hetero-oligomeric complexes in yeast mitochondria

When mitochondria were depleted of ATP and then solubilized with the non-ionic detergent octyl-polyoxy-

Fig. 1. Identification of two mhsp70 complexes in mitochondria. (A) One mg of mitochondria containing hexahistidine-tagged mhsp70 was ATP depleted, solubilized with the non-ionic detergent OPOE, and the hexahistidine-tagged mhsp70 was isolated on Ni-NTA-agarose beads. This purification yielded the 'import complex' containing mhsp70, Tim44 and mGrpE. The purified complex was subjected to SDS-PAGE and either blotted onto nitrocellulose and immunodecorated with antibodies against mhsp70, Mdj1, Tim44 and mGrpE or stained with Coomassie Blue. Solubilization of the mitochondria upon readdition of ATP yielded the 'folding complex'. Lanes 1 and 2: immunoblots using a mixture of antibodies against mhsp70, Mdj1, Tim44 and mGrpE. Lanes 3 and 4: Coomassie Blue-stained gel. The band marked with an asterisk is a contaminating mitochondrial protein that binds unspecifically to the Ni-NTA-agarose beads, as it was also found in the analogous experiment with wild-type (WT) mitochondria. (B) ATP promotes the formation of the folding complex whereas ADP promotes the formation of the import complex. A 1 mg sample of purified mitochondria containing hexahistidine-tagged mhsp70 was solubilized with the non-ionic detergent OPOE in the absence of added nucleotides (lane 1; NONE) or in the presence of the indicated nucleotides (lanes 2–4). Hexahistidine-tagged mhsp70 together with its bound partner proteins was isolated, subjected to SDS-PAGE and analyzed for mhsp70, Mdj1, Tim44 and mGrpE by immunoblotting as described in (A). In all experiments, ADP and AMP-PNP were purified chromatographically (Horst *et al.*, 1996), and ADP was added together with 10 mM deoxyglucose and 10 U/ml hexokinase to prevent generation of ATP by adenylate kinase.

ethylene (OPOE), mhsp70 was affinity isolated as a complex with its membrane anchor Tim44 and the mitochondrial nucleotide exchange factor mGrpE (Figure 1A,

lanes 2 and 4; see also Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994). The 34 kDa protein seen in the Coomassie Blue-stained gel represents a mitochondrial protein that binds non-specifically to these beads, as it was also found with wild-type mitochondria (asterisk; Figure 1A, lanes 3–6). When mitochondrial ATP levels were restored before solubilization, association of mhsp70 with Tim44 was disrupted, and mhsp70 was recovered as a complex with the mitochondrial DnaJ homolog Mdj1 (Rowley *et al.*, 1994) and mGrpE (Figure 1A, lanes 1 and 3). Essentially the same result was obtained by immunoprecipitation with anti-mhsp70 IgGs (data not shown). Moreover, if the mitochondria contained only untagged mhsp70 (Figure 1, lanes 5 and 6), or if the matrix extract had been immunodepleted of mhsp70 (data not shown), none of these proteins (except the 34 kDa contaminant) were precipitated by the Ni-NTA-agarose beads, suggesting that the co-isolation of Mdj1, Tim44 and mGrpE with mhsp70 reflects a specific interaction of these proteins. ATP thus induces formation of an mhsp70–Mdj1–mGrpE complex, whereas ADP induces formation of an mhsp70–Tim44–mGrpE complex. von Ahsen and co-workers showed that this import complex contains ADP and no ATP (von Ahsen *et al.*, 1995).

When mitochondria were solubilized directly in the absence of added nucleotides, the mhsp70–Tim44–mGrpE complex predominated. This was expected, as purified mitochondria contain only a small amount of ATP (Figure 1B, lane 1, NONE) (Kronidou *et al.*, 1994; Horst *et al.*, 1996). When mitochondria were solubilized after addition of 1 mM ATP, no mhsp70–Tim44–mGrpE complex was detected; instead, a complex containing mhsp70 and Mdj1, but little mGrpE, was isolated (Figure 1B, lane 2). Here, less mGrpE was bound to mhsp70 than in the experiment shown in Figure 1A, lanes 1 and 3 because the ATP concentration during solubilization was now much higher. Addition of the non-hydrolyzable ATP analog 5'-adenylyl-imidodiphosphate (AMP-PNP) generated a complex containing mostly mhsp70 and mGrpE, but very little Mdj1 (Figure 1B, lane 4), whereas addition of ADP yielded a complex containing mhsp70, Tim44 and mGrpE (Figure 1B, lane 3).

As the Ni-agarose precipitation of the two complexes in the experiment of Figure 1 was not quantitative, the results showed only that Tim44, Mdj1 and mGrpE could each associate with mhsp70. In order to prove that the import complex of mhsp70 contained both mGrpE and Tim44, and that the folding complex of mhsp70 contained both Mdj1 and mGrpE, co-immunoprecipitations with affinity-purified antibodies against mGrpE, Mdj1 and Tim44 were performed (Figure 2). Wild-type mitochondria were ATP depleted, solubilized in the non-ionic detergent OPOE and subjected to immunoprecipitation with antibodies against mGrpE (Figure 2, lane 4) or Tim44 (Figure 2, lane 5). Either antibody immunoprecipitated mhsp70, Tim44 and mGrpE, albeit with different efficiencies. If the mitochondria had been re-energized before solubilization, antibodies against Mdj1 immunoprecipitated mhsp70, Mdj1 and mGrpE (Figure 2, lane 6). Monospecificity of the three antisera was confirmed by immunoprecipitation of SDS-solubilized mitochondria (Figure 2, lanes 1–3).

These results agree with those of an earlier experiment in which yeast mitochondria containing hexahistidine-

Fig. 2. Co-immunoprecipitation of the folding and the import complex. Mitochondria (0.5 mg) from the wild-type strain D273-10B were ATP depleted, solubilized with the non-ionic detergent OPOE and the import complex containing mhsp70, Tim44 and mGrpE was immunoprecipitated using affinity-purified antibodies against mGrpE (lane 4) or against Tim44 (lane 5). Solubilization of mitochondria with OPOE after readdition of ATP yielded the folding complex that could be immunoprecipitated with affinity-purified antibodies against Mdj1 (lane 6). In lanes 1–3, the mitochondria had been solubilized in 2% SDS before immunoprecipitation with the indicated antibodies to show that the antibodies were monospecific. The immunoprecipitated complexes were subjected to non-reducing SDS-PAGE, blotted onto nitrocellulose filters and immunodecorated with a mixture of antibodies against mhsp70, Mdj1, Tim44 and mGrpE. Immunodecorated bands were visualized with [125 I]protein A followed by autoradiography. The lower band (asterisk) of the doublet in lane 6 is a breakdown product of Mdj1.

tagged Tim44 were depleted of ATP, solubilized with non-ionic detergent, and subjected to affinity purification with Ni-agarose beads. This purification yielded a heterotrimeric complex composed of Tim44, mhsp70 and mGrpE (Figure 1D in Kronidou *et al.*, 1994; Figure 4 in Lithgow *et al.*, 1995).

Mhsp70 interacts with Mdj1 and mGrpE *in vitro*

The experiments reported above have shown that the three subunits of each of the two complexes are physically associated with each other. Do they also interact functionally? The mhsp70–Tim44–mGrpE complex has already been shown to mediate the translocation of proteins across the mitochondrial inner membrane (Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994). To show functional interaction of the proteins in the folding complex, we measured the effect of Mdj1 and mGrpE on the ATPase activity of mhsp70 *in vitro*. To this end, we constructed C-terminally hexahistidine-tagged versions of Mdj1 and mGrpE, overexpressed these proteins in *Escherichia coli* and purified them to homogeneity, as judged by SDS-PAGE and Coomassie Blue staining, by binding to Ni-NTA-agarose beads and anion exchange chromatography (Figure 3, lanes 2 and 3). Mhsp70 was purified as described by Horst *et al.* (1996) (Figure 3, lane 1).

The ATPase activity of DnaK (the bacterial hsp70

Fig. 3. Purification of mhsp70, Mdj1 and mGrpE. Mhsp70 was purified as described in Horst *et al.* (1996). The hexahistidine-tagged versions of Mdj1 and mGrpE were purified as described in Materials and methods. A Coomassie Blue-stained 12% SDS-PAGE of the purified proteins is shown. Lane 1, purified mhsp70; lane 2, purified hexahistidine-tagged Mdj1; lane 3, purified hexahistidine-tagged mGrpE.

chaperone) is stimulated by the bacterial co-chaperones DnaJ and GrpE (Georgopoulos and Welch, 1993); DnaJ appears to increase ATP hydrolysis directly, whereas GrpE acts as a nucleotide exchange factor (Liberek *et al.*, 1991; McCarty *et al.*, 1995). We used this well-characterized bacterial system as a control for interpreting the effects of Mdj1 and mGrpE on the ATPase of mhsp70. As expected, bacterial DnaJ alone stimulated the ATPase activity of DnaK 9-fold (Figure 4A, compare lanes 5 and 6) whereas the combination of bacterial DnaJ and bacterial GrpE stimulated it 116-fold (Figure 4A, compare lanes 5 and 7). Similar results were obtained with the purified mitochondrial chaperones, except that mhsp70 alone had a significantly higher ATPase activity than DnaK alone (Figure 4A, compare lanes 1 and 5). This ATPase activity was stimulated only weakly by either Mdj1 alone (Figure 4A, compare lanes 1 and 2) or by mGrpE alone (Figure 4A, compare lanes 1 and 3), but was stimulated 30-fold by a combination of Mdj1 and mGrpE (Figure 4A, compare lanes 1 and 4). Mhsp70 thus interacts with Mdj1 and mGrpE *in vitro*, and this interaction resembles that between the corresponding bacterial proteins.

The similarity between the mitochondrial and the bacterial systems was underscored further by the observation that components of the two systems were functionally interchangeable. Combinations of bacterial DnaJ and mGrpE, or of bacterial DnaJ and bacterial GrpE, stimulated the ATPase of mhsp70 as effectively as a combination of the two mitochondrial co-chaperones (compare lanes 3 and 4 of Figure 4B and lane 4 of Figure 4A). The combination of Mdj1 and bacterial GrpE was nearly as effective (Figure 4B, compare lane 2 with lanes 3 and 4). DnaK was more selective than mhsp70. Although its ATPase activity was stimulated strongly by a combination of the two mitochondrial co-chaperones, this stimulation was three times lower than that induced by bacterial DnaJ and GrpE (compare lane 5 of Figure 4B and lane 7 of Figure 4A).

The mhsp70–Mdj1–mGrpE complex functions in protein folding

What is the function of the mhsp70–Mdj1–mGrpE complex? Since the bacterial DnaK–DnaJ–GrpE complex

Fig. 4. Stimulation of the ATPase activity of purified mhs70 by purified Mdj1 and mGrpE. ATPase assays were performed as described in Materials and methods with the purified mitochondrial chaperones mhs70 (Horst *et al.*, 1996), Mdj1 and mGrpE and their purified *E. coli* homologs DnaK, DnaJ and GrpE (Georgopoulos and Welch, 1993; here labeled bGrpE to emphasize the bacterial source). DnaK, DnaJ and bGrpE were purified as described in Schönfeld *et al.* (1995). ATPase activity is expressed as mmol ATP hydrolyzed/mmol protein/min.

mediates protein folding (Georgopoulos and Welch, 1993), and since deletion of Mdj1 impairs folding of imported mitochondrial proteins (Rowley *et al.*, 1994; Prip-Buus *et al.*, 1996), it appeared likely that the mhs70–Mdj1–mGrpE complex functions as a folding mediator. If so, precursor proteins destined for the matrix should first bind to the import complex of mhs70 and then, if they need folding assistance by mhs70–Mdj1–mGrpE, to the folding complex. We tested this hypothesis with a fusion protein containing the first 95 amino acids of the cytochrome *b*₂

Fig. 5. Sequential interaction of the two mhs70 complexes with a precursor protein in transit to the mitochondrial matrix. (A) pCytb₂(1–95)-DHFR was imported into isolated mitochondria in the presence of methotrexate. The mitochondria were then divided into five equal aliquots. One aliquot (INTERMEDIATE) was solubilized and subjected to immunoprecipitation with antibodies against mhs70, Tim44 or Mdj1. In the other four aliquots, the translocation intermediate was chased into the matrix for 0.25, 0.5, 1 and 2 min, the sample was solubilized and analyzed in the same way as the unchased aliquot. STD: 5% of the amount of precursor protein added to each import reaction. The lower band marked with an asterisk is a side-product of the *in vitro* translation reaction which probably reflects the aberrant use of a downstream AUG codon in the mRNA. (B) Samples from each of the four 'chase' aliquots (CHASE) were treated with trypsin to remove unspecifically bound precursor (Rospert *et al.*, 1996). They were then solubilized and checked for the folding state of the imported DHFR domain by limited protease digestion (Rospert *et al.*, 1996). STD: the amount of imported and folded DHFR after a 20 min chase.

precursor fused to mouse DHFR (dihydrofolate reductase). DHFR fusion proteins interact with soluble mhs70 in the matrix, suggesting that their folding is aided by soluble mhs70 (Rospert *et al.*, 1996). For this experiment, the cytochrome *b*₂ presequence of the construct was mutated twice: first, Arg30 was replaced by glycine to prevent cleavage by the mitochondrial matrix processing peptidase and second, Leu62 was replaced by proline to convert the cytochrome *b*₂ presequence into a matrix targeting presequence (Beasley *et al.*, 1993; Schwarz *et al.*, 1993).

When this precursor is imported into isolated yeast mitochondria in the presence of the folate analog methotrexate (which stabilizes the DHFR domain), it gets stuck in the import site (Eilers and Schatz, 1986): its DHFR domain remains outside the mitochondria, whereas its presequence penetrates across both membranes and interacts with mhs70 on the inner face of the inner membrane (Rassow *et al.*, 1989; Vestweber *et al.*, 1989; A. Matouschek, K. Schmid, A. Azem and G. Schatz, in preparation). In the experiment shown in Figure 5, isolated yeast mitochondria were allowed to accumulate the translocation intermediate and were then divided into five equal aliquots. One aliquot was kept on ice (INTERMEDIATE). The other four aliquots (CHASE) were washed free of methotrexate, re-energized and incubated for the indicated times to chase the stuck import intermediate completely into the matrix (Figure 5A). Samples of each of the four aliquots (CHASE) were treated with trypsin (to remove unspecifically bound precursor), solubilized and checked for the folding state of the DHFR moiety by limited

protease digestion (Figure 5B) (Rospert *et al.*, 1996). The remainder of each of the five aliquots was divided into three sub-aliquots, and each of the sub-aliquots was solubilized and tested for association of the precursor with mhsp70, Tim44 or Mdj1 by co-immunoprecipitation with IgGs monospecific for one of these three proteins (Figure 5A).

As shown in Figure 5A, the arrested translocation intermediate (INTERMEDIATE) was bound to mhsp70 and Tim44, but not to Mdj1. This result confirmed earlier work (Horst *et al.*, 1995) and provided independent evidence that Mdj1 is not a component of the translocation machinery proper (Rowley *et al.*, 1994). When the intermediate was chased into the matrix, it dissociated from Tim44 and associated with Mdj1, but remained bound to mhsp70 (Figure 5A, CHASE, 15", 30", 1'). At the same time, its DHFR moiety started to fold (Figure 5B; CHASE). After a 2 min chase, the intermediate had completely dissociated from Mdj1 and mhsp70 (Figure 5A, 2') and its DHFR moiety had completely folded (Figure 5B, 2'). To rule out any artificial interaction of the chaperones with the partly unfolded precursor after lysis of the mitochondria, radiolabeled precursor was added to solubilized ATP-depleted or fully energized mitochondria. Under these conditions, no precursor was immunoprecipitated with antibodies against mhsp70, Tim44 and Mdj1 (data not shown), suggesting that the interaction of the precursor with the two complexes occurred in intact mitochondria.

These results suggest that a precursor in transit to the matrix first binds to the import complex of mhsp70 and then to the folding complex. Release from the folding complex is paralleled by acquisition of a protease-resistant, native conformation (Figure 6).

Discussion

Proteins of the hsp70 family perform many different functions in prokaryotic and eukaryotic cells (reviewed in Hightower *et al.*, 1994; McKay *et al.*, 1994). Even a given hsp70 such as mhsp70 can mediate processes as different as protein transport across the inner membrane and protein folding in the matrix. Despite their broad substrate specificity (Flynn *et al.*, 1991; Blond-Elguinidi *et al.*, 1993), members of the hsp70 family are usually not functionally interchangeable (Gao *et al.*, 1991; Brodsky and Schekman, 1993; Wiech *et al.*, 1993). This contradiction may be explained by the observation that hsp70s perform most if not all of their different cellular functions with the help of different co-factors (reviewed in Rassow *et al.*, 1995). The co-chaperone seems to determine the specificity of its hsp70 partner.

DnaK, the major prokaryotic hsp70, mediates protein folding in collaboration with two other proteins, DnaJ and GrpE (reviewed in Georgopoulos and Welch, 1993). Recently, the mitochondrial homologs of DnaJ (Mdj1; Rowley *et al.*, 1994) and GrpE (mGrpE; Bolliger *et al.*, 1994; Ikeda *et al.*, 1994; Nakai *et al.*, 1994; Voos *et al.*, 1994) have been identified. We now report that mhsp70 exists in mitochondria of *S.cerevisiae* as a complex with Mdj1 and mGrpE. This mhsp70 complex appears to mediate protein folding in the mitochondrial matrix. The association of mhsp70 with its different partner proteins is regulated by adenine nucleotides. Our experiments

Fig. 6. Two different mhsp70 complexes mediate the import and folding of precursor proteins in mitochondria. Mhsp70 is present in two different protein complexes: an import complex which is associated with the inner face of the inner membrane and which consists of mhsp70, Tim44 and mGrpE; and a folding complex which is localized in the matrix space and which consists of mhsp70, Mdj1 and mGrpE. Nucleotides which induce the ATP conformation of mhsp70 induce the formation of the folding complex; nucleotides which induce the ADP conformation of mhsp70 induce the formation of the import complex. We propose that a precursor protein entering the matrix interacts first with the import complex and then, if it needs folding assistance by mhsp70–Mdj1–mGrpE, with the folding complex.

suggest the following. First, Mdj1 binds to the ATP conformation of mhsp70. Second, stabilization of the mhsp70–Mdj1 complex requires ATP hydrolysis as the mhsp70–Mdj1 complex is only stable in the presence of ATP (Figure 1B). Third, release of mGrpE from mhsp70 requires ATP hydrolysis, in line with recent *in vitro* data on the interaction between purified mhsp70 and purified mGrpE (A.Azem, W.Oppliger, A.Lustig, P.Jenö, B.Feifel, G.Schatz and M.Horst, in press). Fourth, AMP-PNP induces a conformation of mhsp70 which is different from that induced by either ATP or ADP, as the AMP-PNP conformation of mhsp70 binds Mdj1 only weakly, and Tim44 not at all. We propose that Tim44 and Mdj1 have

overlapping binding sites on mhsp70, but further work is required to prove this point.

Each subunit of the import complex is required for viability of yeast, in line with the essential role of this complex in pulling precursor proteins across the inner membrane (Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994). In contrast, Mdj1 is not an essential protein for yeast, at least at temperatures below 37°C (Rowley *et al.*, 1994). Some proteins can thus bypass the folding complex at low temperature, because folding is also mediated by other chaperones, or because it can occur spontaneously (Rospert *et al.*, 1996). At higher temperature, however, Mdj1 is essential for viability, indicating that either at least one essential protein can only fold with the direct or indirect assistance of the folding complex, or that Mdj1 has another function in addition to protein folding (Prip-Buus *et al.*, 1996).

As DnaJ and GrpE are essential for the folding activity of DnaK in *Escherichia coli* (Georgopoulos and Welch, 1993), these proteins appear to form a complex with DnaK. Interaction between DnaK and its co-chaperones has also been suggested by cross-linking experiments (Osipiuk *et al.*, 1993). Furthermore, some indirect evidence for the existence of a complex containing DnaK, DnaJ and a folding intermediate has been described (Langer *et al.*, 1992). In contrast to our present observations with mhsp70 and Mdj1, a stable complex of DnaK and DnaJ has not yet been isolated from *E. coli*; a stable DnaK–DnaJ complex has however, been reported for *Thermus thermophilus* (Motohashi *et al.*, 1994).

This *T. thermophilus* complex (Motohashi *et al.*, 1994), like the mitochondrial folding complex, cannot be assembled *in vitro* from purified components (data not shown). This observation suggests the requirement for an additional component. Such a scaffolding protein for the assembly of the DnaK–DnaJ complex in *T. thermophilus* has been identified recently (Motohashi *et al.*, 1996).

How can the two different mhsp70 complexes co-exist within ATP-sufficient mitochondria? The reason is not clear, but explanations offer themselves. First, it is not the free, but the mhsp70-bound nucleotide that governs formation of one or the other complex. The import complex could thus form even in mitochondria containing relatively high levels of ATP. Second, the import complex is membrane bound whereas the folding complex is in the mitochondrial matrix. The activity of ADP and ATP may be different in these two submitochondrial locations.

Materials and methods

Purification of the mhsp70 complexes

Mitochondria from a yeast strain in which all mhsp70 molecules carried a C-terminal hexahistidine tag were purified by Nycodenz gradient centrifugation (Glick and Pon, 1995), then depleted of ATP in 1 mg samples (Glick, 1995b) and finally divided into two aliquots; in one aliquot the level of ATP in the matrix was restored by incubating for 5 min at 25°C with 5 mM α -ketoglutarate (+ATP). The other aliquot was kept ATP depleted by incubation for 5 min at 25°C in the presence of 10 U/ml apyrase (–ATP). The mitochondria were re-isolated and solubilized in buffer S (containing 1% OPOE instead of Triton X-100; Horst *et al.*, 1996). The hexahistidine-tagged mhsp70 (together with any bound partner proteins) was then bound to Ni-NTA-agarose beads (Hochuli, 1990; Bolliger *et al.*, 1994). Finally, the bound proteins were eluted from the beads with buffer S containing 150 mM imidazole and analyzed by SDS–PAGE and either Coomassie Blue staining or immunoblotting for mhsp70, Mdj1, Tim44 and mGrpE.

Construction of hexahistidine-tagged mGrpE and Mdj1

The mitochondrial mGrpE gene (Bolliger *et al.*, 1994; Ikeda *et al.*, 1994; Laloraya *et al.*, 1994) was isolated by PCR using a forward primer corresponding to base pair 72–87 and a reversed primer corresponding to base pair 668–683 of the mGrpE gene. The forward primer contained a restriction site for NcoI upstream of the coding sequence and the reverse primer contained a restriction site for BamHI downstream of the coding sequence. To add a hexahistidine tag to the C-terminus, plasmid pQE60 (Qiagen Co.) was used. The PCR product as well as the pQE60 vector were cut with NcoI and BamHI and the purified fragments were ligated with T4 DNA ligase (Boehringer Co., Mannheim). The resulting recombinant plasmid pMGRPE-6HIS encodes an mGrpE in which the mitochondrial targeting sequence (residues 1–24) has been replaced by Met–Ala, and in which the C-terminus has been extended by the sequence Gly–Ser–Arg–Ser–(His)₆. The tagged mGrpE was overexpressed in a protease-deficient, *E. coli* strain BL21-DE3 (Novagen, Phillips *et al.*, 1984).

The Mdj1 gene (Rowley *et al.*, 1994) was isolated by PCR using a forward primer corresponding to base pair 166–189 and a reversed primer corresponding to base pair 1509–1533 of the Mdj1 gene. The forward primer contained a restriction site for AflIII upstream of the coding sequence and the reverse primer contained a restriction site for BglIII downstream of the coding sequence. To add a hexahistidine tag to the C-terminus, plasmid pQE60 was used. The PCR product was cut with AflIII (ends generated by AflIII are compatible with those generated by NcoI) and BglIII. pQE60 was cut with NcoI and BglIII and the purified fragments were ligated with T4 DNA ligase. The resulting recombinant plasmid pMDJ1-6HIS encodes an Mdj1 in which the mitochondrial targeting sequence has been replaced by Met–Ser and in which the C-terminus has been extended by Arg–Ser–(His)₆.

Purification of hexahistidine-tagged mGrpE

The bacterial transformants were grown in 1 l of Super-rich medium (2.5% Bacto-tryptone, 1.5% Bacto yeast extract, 0.5% NaCl) at 30°C. Once the culture had reached an OD₆₀₀ of 0.6–0.7, expression of mGrpE was induced by adding isopropyl- β -thiogalactoside (IPTG) to 0.5 mM and continuing growth for 3 h. The cells were then harvested, suspended in 20 ml of buffer A [20 mM K⁺-HEPES pH 7.5, 250 mM KCl, 20 mM imidazole, 2 mM mercaptoethanol, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1.25 mg/ml leupeptin, 0.75 mg/ml antipain, 0.25 mg/ml chymostatin, 0.25 mg/ml elastin, and 5 mg/ml pepstatin] and disrupted by ultrasound (Heat Systems Ultrasonics; W-375 sonicator tip; 75% duty cycle). To remove unbroken cells and membranes, the solution was cleared by 30 min centrifugation at 20 000 g. The supernatant was mixed with 0.5 ml of a 1:1 aqueous suspension of Ni-NTA-agarose and incubated for 90 min at 4°C. The beads were collected by centrifugation and washed three times with buffer A. mGrpE was eluted with buffer A containing 300 mM imidazole, diluted 10-fold in buffer B (20 mM K⁺-HEPES pH 7.5, 3 M urea, 2 mM mercaptoethanol, 5% glycerol), incubated for 10 min, and loaded onto a Mono Q column (HR5/5, Pharmacia, Sweden) that had been pre-equilibrated with buffer B at a flow rate of 0.5 ml/min. The column was washed with 10 ml of buffer B and developed with a linear KCl gradient (0–500 mM) in buffer B. mGrpE eluted at ~200 mM KCl and was >95% pure as judged by SDS–PAGE (Figure 3). Urea was removed by dialysis against buffer C (20 mM K⁺-HEPES pH 7.5, 100 mM NaCl, 10% glycerol). All purification steps were performed at ~4°C.

Purification of hexahistidine-tagged Mdj1

The *E. coli* transformants were grown in 1 l of LB medium at 37°C. When the culture had reached an OD₆₀₀ of 0.6–0.7, expression of Mdj1 was induced by adding IPTG to 0.5 mM. After an additional 3 h of growth, the cells were harvested, suspended in 20 ml of buffer A without KCl and disrupted by a sonication as described above. The sonicated cell suspension was centrifuged for 30 min at 20 000 g. The pellet was resuspended in 20 ml of buffer A containing 1% Triton X-100, 500 mM KCl, 3 M urea and incubated for 3 h at 4°C. After removing insoluble material by centrifugation for 30 min at 20 000 g, the supernatant was mixed with 0.5 ml of a 1:1 aqueous Ni-NTA-agarose suspension and incubated for 90 min at 4°C. The beads were washed once with 10 ml of buffer A containing 1% Triton X-100, 3 M urea, once with 10 ml of buffer A containing 0.5% Triton X-100, 2 M urea and finally once with 10 ml of buffer A. Mdj1 was eluted with 2.5 ml of K⁺-HEPES pH 7.4, 10% glycerol, 100 mM KCl, 300 mM imidazole. The protein was >95% pure as judged by SDS–PAGE (Figure 3). All purification steps were carried out at 4°C.

ATPase assay

The ATPase activity of mhsp70 and DnaK was measured (Viitanen *et al.*, 1990) at room temperature in 50 mM K⁺-HEPES buffer pH 7.4, 5 mM MgCl₂, 150 mM KCl, 0.1 mM [γ -³²P]ATP (0.04 Ci/mmol) and 1 mM each of the indicated proteins. The reaction was terminated after 30 min by perchloric acid, Na-Pi. Liberated ³²Pi was extracted into organic solvents and measured by its Cerenkov radiation. The correction for zero time values was always <1.5%. ATPase activity is expressed as mmol ATP hydrolyzed/mmol protein/min. The assays were carried out in the linear range of hydrolysis (data not shown).

Chase and co-immunoprecipitation of the precursor

A pCytb₂(1–95)-DHFR precursor was imported (Glick *et al.*, 1992) into 4 mg of wild-type yeast mitochondria (strain D273-10B) for 5 min at 25°C in the presence of 3 mM methotrexate. The mitochondria were depleted of ATP (Glick, 1995b) and divided into five 800 µg aliquots. One aliquot was re-isolated and solubilized in buffer S lacking imidazole and containing 100 mM NaCl. The other four aliquots were re-isolated, washed twice with 1 ml of import buffer, re-energized by adding α -ketoglutarate (Glick, 1995b) and incubated at 25°C for the indicated periods of time. The chase was stopped by placing the reactions on ice and immediately adding OPOE and apyrase to final concentrations of 1% and 10 U/ml. Each of the aliquots was then divided further into three sub-aliquots which were subjected to immunoprecipitation with 20 mg of affinity-purified IgG against mhsp70, Tim44 or Mdj1. After incubation for 3 h at 4°C, the beads were washed three times with 1 ml of buffer S and bound proteins were eluted with SDS-containing sample buffer and analyzed by SDS-PAGE and fluorography.

Miscellaneous

Published methods were used for SDS-PAGE, fluorography, immunoblotting using [¹²⁵I]protein A, and measurement of mitochondrial protein concentration (Glick *et al.*, 1992; Glick and Pon, 1995). The concentration of the purified proteins was determined with the BCA procedure (Pierce Chemical Co.), which was calibrated against standards whose concentration had been determined by quantitative amino acid analysis.

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