Identification of *in vivo* substrates of the yeast mitochondrial chaperonins reveals overlapping but non-identical requirement for hsp60 and hsp10

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The mechanism of chaperonin-assisted protein folding has been mostly analyzed *in vitro* using non-homologous substrate proteins. In order to understand the relative importance of hsp60 and hsp10 in the living cell, homologous substrate proteins need to be identified and analyzed. We have devised a novel screen to test the folding of a large variety of homologous substrates in the mitochondrial matrix in the absence or presence of functional hsp60 or hsp10. The identified substrates have an M_r of 15–90 kDa and fall into three groups: (i) proteins that require both hsp60 and hsp10 for correct folding; (ii) proteins that completely fail to fold after inactivation of hsp60 but are unaffected by the inactivation of hsp10; and (iii) newly imported hsp60 itself, which is more severely affected by inactivation of hsp10 than by inactivation of pre-existing hsp60. The majority of the identified substrates are group I proteins. For these, the lack of hsp60 function has a more pronounced effect than inactivation of hsp10. We suggest that homologous substrate proteins have differential chaperonin requirements, indicating that hsp60 and hsp10 do not always act as a single functional unit *in vivo*.

Keywords: chaperone/mitochondria/protein folding/
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Introduction

Chaperonin-assisted folding of proteins *in vitro* is a well-studied process. The crystal structures of the *Escherichia coli* model chaperonins GroEL (cpn60 from *E.coli*) and GroES (cpn10 from *E.coli*) have been solved both for the individual proteins, as well as for the protein complex in the presence of nucleotide (Braig *et al.*, 1994; Hunt *et al.*, 1996; Xu *et al.*, 1997). Detailed cryo-electron microscopy has revealed the structural rearrangements of GroEL upon binding of nucleotide and GroES (Roseman *et al.*, 1996). The basic principles of the folding cycle have become clear, although important details are still under debate (Fenton and Horwich, 1997; Bukau and Horwich, 1998; Horovitz, 1998).

In vitro investigations using a multitude of almost exclusively non-homologous substrate proteins have

revealed two particularly interesting properties of the chaperonin system. First, cpn60 indiscriminately binds to most unfolded proteins *in vitro* and, either alone or in tandem with cpn10, promotes their refolding. Secondly, despite this broad substrate range, different substrate proteins vary in their chaperonin requirements. Depending on the substrate protein and the experimental conditions, the folding yield can be significant or negligible, the rate of folding can be either slowed down or increased, and some proteins are assisted by cpn60 alone while others require cpn60, cpn10 and nucleotide (Lorimer *et al.*, 1993). The latter proteins are the substrates that fold with slow kinetics during multiple rounds of binding and release, and have been central in elucidating the mechanism of chaperonin action (e.g. Goloubinoff *et al.*, 1989; Martin *et al.*, 1991; Weissman *et al.*, 1995). Surprisingly, these slow-folding substrates are all heterologous with respect to the chaperonin system used for *in vitro* refolding. For instance, mouse dihydrofolate reductase (DHFR) has been one of the tight-binding model substrates for *in vitro* refolding studies (e.g. Viitanen *et al.*, 1991; Mayhew *et al.*, 1996). However, *E.coli* DHFR interacts with GroEL only weakly and transiently (Clark and Frieden, 1997). The relevance of these differences to the situation *in vivo* is not known. It is also not clear whether all *in vivo* substrates depend on the complete chaperonin system.

It is not only the properties of the substrate proteins determine the chaperonin–substrate interaction. Chaperonins from different organisms have distinct affinities for a given protein substrate and fold it with different efficiencies (Rospert *et al.*, 1996; Dubaquié *et al.*, 1997). Chaperonins, although closely related in sequence and structure, might have become optimized for their *in vivo* substrates during evolution. Identification and characterization of the natural substrates may thus be a prerequisite for a better understanding of the folding processes *in vitro* and *in vivo*.

We are only beginning to understand the exact role of chaperonins *in vivo*. Most studies so far have focused on the role of the central chaperonin partner cpn60. Early studies had suggested that ~30% of the soluble cytoplasmic proteins in an *E.coli* cell fold via GroEL, and a few potential *in vivo* substrates of GroEL have been assigned (Horwich *et al.*, 1993; McLennan and Masters, 1998). However, theoretical estimates based both on the concentration of GroEL/ES in the cell and the rate of protein synthesis suggest that no more than 2–7% of the cellular proteins can possibly fold with the help of the chaperonins (Ellis and Hartl, 1996; Lorimer, 1996). The general importance of the chaperonin machinery in *E.coli* has recently been reinvestigated. The results indicate that ~15% of newly synthesized soluble *E.coli* proteins become transiently attached to GroEL before acquiring the folded state (Ewalt *et al.*, 1997). The combined data suggest that the

Fig. 1. hsp10P36H fails to bind to hsp60 after incubation at 37°C. The wild-type or hsp10-ts strains were grown on minimal lactate medium at 25°C to mid-log phase. The temperature was shifted to 37°C for 2 h, cells were harvested and mitochondria were isolated. Extracts from these mitochondria were incubated with excess purified hsp60 in the presence of ADP at 25°C. After separation of unbound hsp10 from hsp60 by sucrose density centrifugation, fractions of the sucrose gradient were analyzed by Western blotting for hsp60 and hsp10. The top and bottom fractions of the gradient are indicated in the figure.

majority of proteins fold without the help of chaperonins *in vivo*. This is in agreement with our finding that several proteins were able to fold in the absence of functional hsp60 after import into intact mitochondria of yeast (Rospert *et al.*, 1996).

Less is known about the *in vivo* function of cpn10. The effects of independent inactivation of the two partner chaperonins *in vivo* have not been compared so far, and virtually nothing is known about *in vivo* substrates that require cpn10 for folding. Interestingly, cpn10 seems to provide specificity for at least some substrate proteins. For example, the bacteriophage T4 encodes its own cpn10 on which it strictly depends for propagation (van der Vies *et al.*, 1994; Hunt *et al.*, 1997).

The matrix space of isolated intact mitochondria is an environment that closely resembles the *in vivo* situation. We have developed a method to synthesize a large variety of yeast proteins in a homologous yeast translation system and to import the proteins destined for mitochondria into the isolated organelles. In order to study the effect of the yeast mitochondrial chaperonins hsp60 and hsp10 on the folding of newly imported authentic proteins and to identify homologous substrates we used a temperature-sensitive mutant of each chaperonin. Both mutants can be irreversibly inactivated *in vivo* and allow us to follow selectively the fate of proteins in the absence of functional hsp60 or hsp10, respectively (Cheng *et al.*, 1989; Hallberg *et al.*, 1993; Höhfeld and Hartl, 1994; Dubaquié *et al.*, 1997). The identification of these substrates will help to solve the question of how chaperonins act *in vivo*.

Results

Irreversible inactivation of temperature-sensitive chaperonin mutants

Mif4, a temperature-sensitive mutant of hsp60 was isolated originally in a screen for proteins that confer a defect to mitochondrial function. The mutant protein forms detergent-insoluble aggregates at the non-permissive temperature (Cheng *et al.*, 1989; Rospert *et al.*, 1996). However, the exact mutation that causes temperature-sensitivity had not been determined. We sequenced the temperature-sensitive allele from the original Mif4 strain and identified a single point mutation in hsp60 in which

glycine at position 319 was replaced by aspartic acid. The allele was cloned and introduced into the strain JK9-3d, the parental yeast strain used throughout this study. We termed the new strain hsp60-ts. As with the original Mif4 strain, hsp60-ts fails to grow at 37°C (data not shown). When mitochondria, isolated from hsp60-ts cells pre-incubated for 2 h at 37°C, were solubilized with 1% Triton X-100, >90% of total hsp60 was contained in the detergent-insoluble pellet. Aggregation was irreversible and not observed with wild-type hsp60 (data not shown; Rospert *et al.*, 1996). We have previously characterized a temperature-sensitive mutant of hsp10 *in vitro* in which proline 36 is replaced by a histidine (Dubaquié *et al.*, 1997). The yeast strain expressing hsp10P36H is termed hsp10-ts. After incubation of hsp10-ts at the non-permissive temperature for growth, hsp10P36H fails to bind to hsp60; this loss of function is irreversible (Figure 1). We used mitochondria from wild-type, hsp60-ts and hsp10-ts yeast strains to study the folding of authentic yeast mitochondrial proteins in the matrix.

Import of radiolabeled mitochondrial precursors translated from endogenous mRNA in yeast cell extracts

In order to identify yeast mitochondrial proteins that are significantly influenced by the chaperonin system we developed a novel screen to test the folding of a large number of authentic proteins in the matrix. *In vitro* translation in the presence of [³⁵S]methionine was performed in a yeast cytosolic extract containing endogenous yeast mRNA. The total mixture of radiolabeled proteins obtained was used for an import reaction into isolated wild-type mitochondria. According to the abundance of proteins targeted to mitochondria, ~5–10% of the radiolabeled proteins were imported into mitochondria. After treatment with proteinase K, only the proteins that had crossed the mitochondrial membranes were protected against protease digestion. A comparison of the total labeled protein in the translation extract with the proteins imported into mitochondria indicated that a specific set of proteins was enriched inside the mitochondria (Figure 2, compare STD 5% with Tot). In the absence of ATP and a membrane potential, import was abolished (Figure 2, –ATP –ΔΨ). The smear of low molecular mass material

Fig. 2. Homologous *in vitro* import of a variety of yeast mitochondrial proteins. Total yeast mRNA was translated in a yeast cell-free extract in the presence of [35 S]methionine. The mixture of radiolabeled proteins was subsequently incubated with wild-type mitochondria in the presence or absence of ATP and a membrane potential ('+ATP + $\Delta\Psi$ ' and '-ATP - $\Delta\Psi$ ', respectively). Tot, autoradiography of material bound to the mitochondrial pellet after 60 min of import; Imp 30' and 60', material after proteinase K treatment of the intact organelles after 30 and 60 min of import, respectively.

present even in the absence of a membrane potential decreased after longer incubation times (Figure 2, -ATP - $\Delta\Psi$, 30' and 60'). It is probable that this material is unspecifically attached to the outer mitochondrial membrane as a result of the large quantities of radiolabeled protein used in this experiment.

Identification of chaperonin substrates by two-dimensional (2D) gel analysis

Import of the mitochondrial proteins proceeds with similar efficiency, irrespective of whether mitochondria are isolated from wild-type, hsp60-ts or hsp10-ts (Figure 3, Imp). In order to analyze the folding state of the imported proteins, we solubilized the mitochondria with non-ionic detergent and separated the soluble supernatant from the insoluble aggregates by centrifugation. Analysis on a one-dimensional gel indicated that more proteins became insoluble in hsp60-ts or hsp10-ts than in mitochondria from wild-type (Figure 3, compare Sup and Pel).

In order to identify the proteins that become aggregated in the absence of either hsp60 or hsp10, detergent insoluble pellets from mitochondria of the three different strains were prepared (Figure 3) and supplemented with total, unlabeled, wild-type mitochondrial protein. The samples were run on 2D gels, transferred to PVDF membranes and subjected to autoradiography, followed by Coomassie Blue staining. Figure 4 shows the Coomassie Blue stain of a representative blot (Figure 4A) and the autoradio-

Fig. 3. Solubility of mitochondrial proteins after import into wild-type (wt), hsp60-ts (60-ts) or hsp10-ts (10-ts) mitochondria. Import of radiolabeled yeast mitochondrial proteins was performed in the presence of ATP and NADH as described for Figure 2. Non-imported material was removed by treatment of the intact mitochondria with proteinase K (Imp). After re-isolation, mitochondria were lysed with buffer containing 0.5% Triton X-100. The extracts were centrifuged at 150 000 g and separated into detergent-insoluble pellet (Pel) and supernatant (Sup).

graphs of the detergent-insoluble pellets from wild-type, hsp60-ts or hsp10-ts mitochondria (Figure 4B–D). As expected, we found some proteins in the detergent-insoluble pellet of wild-type mitochondria. These might represent proteins that are subunits of large macromolecular complexes or proteins that aggregate in our *in vitro* import system, e.g. because a partner protein for assembly is not translated or imported in sufficient amounts. Virtually all protein spots visible in autoradiographs from the wild-type were also present in autoradiographs from mutant mitochondria, most of them at higher intensities. However, the spot with the strongest signal from wild-type mitochondria was faint only in the mutant mitochondria (Figure 4B–D, spot labeled with the asterisk). In general, significantly more proteins become detergent-insoluble in mitochondria from hsp60-ts and hsp10-ts, indicating that aggregation is favored when hsp60 or hsp10 is inactivated (Figure 4). The aggregated proteins in hsp10-ts and hsp60-ts varied in their M_r s and isoelectric points. A comparison of aggregates in hsp10-ts and hsp60-ts revealed that similar, but non-identical groups of proteins were affected. In particular, the degree of aggregation varied between hsp60-ts and hsp10-ts; for some proteins aggregation was more severe in mitochondria from hsp60-ts, for others in mitochondria from hsp10-ts.

Fig. 4. Two-dimensional gel analysis of aggregates formed by newly imported proteins in mitochondria from wild-type, hsp60-ts or hsp10-ts. Import and treatment of the samples was as described in Figure 3 and Materials and methods. Each sample was supplemented with total mitochondrial protein and subjected to isoelectric focusing at pH 3–10, SDS gel electrophoresis and blotting onto PVDF membranes. The arrow indicates the acidic side of the isoelectric focusing gel strip loaded onto the SDS gel. The asterisk labels the most prominent spot in B that shows reduced intensity in C and D. (A) is stained with Coomassie Blue; (B–D) are autoradiographs.

We selected some of the proteins that showed strong aggregation in either hsp60-ts, hsp10-ts or both for further investigation. Mass-spectrometric analysis of the corresponding protein spots allowed their identification in the completed yeast genome database. All identified proteins are abundant components in mitochondria, mainly enzymes of biosynthetic pathways (see Table I and Discussion).

Differential effect of hsp60 versus hsp10 inactivation

The corresponding genes from the identified proteins were cloned for *in vitro* transcription and translation. To confirm and quantify the effects of hsp60/hsp10 inactivation, we performed import experiments using the individual proteins. The analyzed proteins showed increased aggregation in either hsp60-ts or hsp10-ts compared with the wild-type. However, the degree of aggregation was variable, and for most proteins the inactivation of hsp60 had a more severe effect than the inactivation of hsp10. Figure 5 shows representative examples of the results.

Isocitrate dehydrogenase (Idh1p) remained detergent-soluble in the matrix of wild-type mitochondria and in mitochondria from hsp10-ts. However, the protein completely aggregated when imported into mitochondria lacking functional hsp60 (Figure 5A). Thus, inactivation of either hsp60 or hsp10 has a differential effect on the aggregation of this precursor protein. 2,3-dihydroxy acid hydrolase (Ilv3p) and aconitase (Aco1p) behaved differently. Again, hsp60 inactivation resulted in complete

aggregation, but these proteins were also severely affected by inactivation of the smaller partner protein (Figure 5B and C): ~50% of Ilv3p and 80% of Aco1p are recovered in the detergent-insoluble pellet of hsp10-ts mitochondria. The other identified proteins were also affected by the inactivation of one or both chaperonin components, but the effect was less severe (Table I). The data illustrate the variability in chaperonin dependence for authentic substrates.

The observation that hsp60 depends on itself for folding and assembly in the matrix was reported almost 10 years ago (Cheng *et al.*, 1990). We were able to reproduce this result in our system. After 30 min of import into wild-type mitochondria, >80% of the newly imported hsp60 folded into a protease-resistant conformation. When hsp60 was imported into mitochondria from hsp60-ts, its folding was reduced to ~20%. Unexpectedly, the effect of hsp10 inactivation was even more severe than the inactivation of hsp60: <5% of imported hsp60 polypeptides reached a protease-resistant state in hsp10-ts mitochondria (Figure 6). Thus, it is possible that in the absence of functional hsp10, newly imported hsp60 becomes trapped on pre-existing hsp60. According to this theory, a small fraction of newly imported hsp60 would reach the native state in hsp60-ts mitochondria lacking this permanent trap.

Yeast rhodanese and malate dehydrogenase fold in the absence of chaperonins *in vivo*

Two of the most commonly used *in vitro* substrates for chaperonin-assisted protein folding are bovine rhodanese

Table I. Proteins tested for their ability to fold after import into isolated yeast mitochondria.

Gene	Protein (Swiss-Prot.)	Mm (kDa)	Sequenced from 2D gel of:	Oligomeric state	% Pel	
					60-ts	10-ts
<i>ILV3</i>	2,3-dihydroxy acid hydrolase P39522	63	hsp10-ts	homodimer	100	50
<i>ILV5</i>	ketol-acid reductoisomerase P06168	38	hsp60-ts	?	20	<5
<i>IDH1</i>	isocitrate dehydrogenase P28834	38	hsp60-ts	octamer of two non-identical subunits (Idh1p and Idh2p)	95	<5
<i>ACOX</i>	aconitase homolog P39533	87	hsp60-ts	monomer	100	80
<i>ACO1</i>	aconitase P19414	83	—	monomer	100	80
<i>MDH1</i>	malate dehydrogenase P17505	34	—	dimer	<20	<5
<i>RHO1</i>	rhodanese homolog YPD YOR251C	34	—	monomer	<5	<5
<i>HSP60</i>	mitochondrial hsp60 P19882	58	—	tetradecamer	80 ^a	95 ^a
<i>SSC1</i>	mitochondrial hsp70 P12398	68	—	monomer, dimer	<5	<5

Individual radiolabeled proteins were imported into mitochondria of hsp60-ts (60-ts) and hsp10-ts (10-ts), and aggregation was assessed as described in Figure 5. '% Pel' gives the fraction of imported material that is present in the detergent-insoluble pellet. The % Pel in wild-type mitochondria was <5% for all proteins tested here.

^aFolding was determined by protease resistance.

Fig. 5. Aggregation of mitochondrial proteins in intact mitochondria in the presence or absence of functional hsp60 or hsp10. Radiolabeled proteins were imported into the matrix of wild-type, hsp60-ts or hsp10-ts mitochondria. Mitochondria were then solubilized by addition of Triton X-100 and the detergent extract was separated into supernatant and pellet. (A) Isocitrate dehydrogenase (Idh1p); (B) 2,3-dihydroxy acid hydrolase (Ilv3p); and (C) aconitase (Aco1p). Corresponding amounts of each sample were separated by SDS-PAGE and analyzed by autoradiography. Imp, total of the import reaction; Sup, supernatant; Pel, pellet fraction after centrifugation of the detergent extract.

(b-rho) and porcine mitochondrial malate dehydrogenase (p-mdh1). The refolding of the two enzymes is dependent on chaperonins *in vitro* under a variety of conditions (e.g.

Fig. 6. Folding of newly imported hsp60 in mitochondria of wild-type, hsp60-ts and hsp10-ts. Hsp60 was synthesized in reticulocyte lysate and imported into isolated mitochondria. After 5 and 30 min of import, equal samples were withdrawn and non-imported hsp60 precursor was removed as described above. Mitochondria were collected by centrifugation, resuspended in 100 μ l solubilization buffer (see Materials and methods) and disrupted by freeze-thaw sonication. Fifty microliters of the extract were precipitated with 5% TCA (Imp). The remainder was treated with 1 μ g/ml proteinase K for 10 min. Protease was inhibited by addition of 1 mM PMSF, and the samples were precipitated with 5% TCA (Fold) and analyzed by SDS-PAGE and autoradiography.

Martin *et al.*, 1991; Mendoza *et al.*, 1991; Hutchinson *et al.*, 1994). We have previously shown that b-rho also strictly depends on functional hsp60 for folding in the mitochondrial matrix (Rospert *et al.*, 1996). In order to test whether the corresponding homologs from yeast were also substrates for the chaperonin system we cloned the gene for malate dehydrogenase, y-mdh1, and the gene encoding a protein with high homology to bovine rhodanese. This gene, which we have termed y-rho, is 33% identical to bovine rhodanese on the amino acid level. The catalytically active cysteine is conserved. Both y-mdh1 and y-rho were imported into mitochondria with high efficiency, and folded into a soluble, protease-resistant conformation inside the matrix (Figure 7 and data not shown). Y-rho, like its bovine homolog, does not contain a cleavable presequence (Figure 7A, compare lane 1

Fig. 7. Folding of yeast mitochondrial rhodanese (y-rho) and yeast mitochondrial malate dehydrogenase (y-mdh1). (A) Radiolabeled y-rho was imported into wild-type mitochondria for the times indicated. Import, material that was protease-protected in intact mitochondria; Folding, material that was protease-resistant after solubilization of the mitochondria. (B) Radiolabeled y-mdh1 was imported into mitochondria from wild-type, hsp60-ts and hsp10-ts. Imp, material that is protease-protected in intact mitochondria; Fold, material that is protease-protected after solubilization of the mitochondria; Pel, material that was in the pellet after solubilization followed by centrifugation at 150 000 g.

with lane 2). The half-time for folding of y-rho in the mitochondrial matrix was 4 min (Figure 7A), compared with 15 min for the bovine homolog under the same conditions (Rospert *et al.*, 1996). Folding of y-rho and y-mdh1 in wild-type, hsp60-ts and hsp10-ts mitochondria was tested by two different criteria: first, by monitoring protease resistance of the folded proteins and secondly, by determining the fraction of protein becoming insoluble in non-ionic detergent after import. Y-mdh1 folded into a protease-resistant conformation in the matrix of mitochondria irrespective of whether functional hsp60 or hsp10 was present (Figure 7B). The fraction of imported y-mdh1 that reached the protease-resistant conformation in the matrix was between 70 and 90%. Only a small fraction of y-mdh1 became detergent-insoluble in either of the mutants. We obtained similar results for y-rho (data not shown) and conclude that, in contrast to the homologs from higher eukaryotes, y-rho and y-mdh1 can fold in the absence of functional chaperonins *in vivo*.

Discussion

The mechanism of chaperonin-mediated folding of substrate proteins has been studied extensively *in vitro*. However, the role of chaperonin systems *in vivo* remains less clear (Bukau and Horwich, 1998; Horovitz, 1998). Our work with the yeast mitochondrial system, as well as work with bacterial GroEL/ES, raises the question as to what determines whether a protein becomes an *in vivo* substrate for the chaperonins (Rospert *et al.*, 1996; Ewalt *et al.*, 1997). Previously identified yeast mitochondrial proteins whose folding *in vivo* is affected by inactivation

of hsp60 include the β -subunit of the F₁ATPase and the α -subunit of α -ketoglutarate dehydrogenase (Cheng *et al.*, 1989; Glick *et al.*, 1992; Hallberg *et al.*, 1993). However, results obtained with these potential substrates are open to the criticism that they reflect misfolding of a partner subunit followed by misassembly of the complex. In order to avoid complications by such indirect effects, we have focused our study on proteins that exist either as monomers or as homo-oligomers. To identify authentic substrates of hsp60 and, for the first time, also for hsp10, we have made use of two temperature-sensitive mutants that can be irreversibly inactivated by heat treatment of the mutant yeast strains. A general concern about this approach is how tight the temperature-sensitive phenotype is. This question cannot be answered definitively. However, we have found substrate proteins that completely fail to fold after inactivation of either of the chaperonins. Thus, it is at least safe to conclude that individual proteins show markedly different requirements for folding.

In contrast to our previous results using heterologous substrates (Rospert *et al.*, 1996), we have failed so far to show significant binding of natural substrates to hsp60 (data not shown). We do not know why using a similar experimental approach fails to detect binding of the substrates identified here. In a previous study, <1% of imported Mas2p could be co-immunoprecipitated with antibodies against hsp60 (Manning-Krieg *et al.*, 1991). A possible explanation for the difficulty in detecting significant binding of natural substrates might be that these proteins interact less tightly with their folding helpers than do the commonly used heterologous model substrates. In this respect it is interesting that some non-mitochondrial proteins become irreversibly bound to hsp60 after import (Rospert *et al.*, 1994; Heyrovská *et al.*, 1998). Experimental support for this hypothesis comes also from recent *in vivo* studies in *E. coli* (Ewalt *et al.*, 1997), and from *in vitro* experiments using the *E. coli* chaperonins in combination with an *E. coli* substrate protein (Fisher, 1998).

The three substrates that were most affected by inactivation of hsp60 and hsp10 are 2,3-dihydroxy acid hydrolase (Ilv3p) and the two aconitase homologs Aco1p and AcoXp (Table I and Figure 5). Ilv3p is a homodimeric protein with subunits of 63 kDa, and the aconitase homologs are monomers of 83 or 87 kDa, respectively (Table I). The size of all three proteins considerably exceeds the assumed size limit of the central cavity of GroEL (Fenton and Horwich, 1997) and also that of the yeast homolog hsp60 (unpublished data). The current view of the folding mechanism suggests that substrates become enclosed in the GroEL cavity topped by GroES. This mode of chaperonin action is unlikely for substrates that are larger than ~50 kDa, based on size exclusion. It could be argued that for Ilv3p, Aco1p and AcoXp, the effect of chaperonin inactivation is indirect, either because a smaller partner subunit is required for solubility or another chaperone is inactivated during heat-shock of the mutant strains. However, two observations suggest a direct effect of the chaperonin system on folding of the large proteins. First, the three identified proteins are monomers or homooligomers. Secondly, the other known chaperones present in the mitochondrial matrix (Neupert, 1997) are active after heat-treatment of the mutant strains. In our experimental system a defect in mhsp70 and Mge1p can be excluded.

These two proteins are essential for protein import into the matrix and this process is unaffected in hsp60-ts and hsp10-ts (Figure 3). The amount of folded Mdj1p and its protease resistance in mutant and wild-type mitochondria after heat treatment remains unchanged (data not shown), suggesting that pre-existing Mdj1p is not depleted during incubation of the yeast cells at the non-permissive temperature. It is an open question how the folding of proteins that do not fit into the closed hsp60 cavity are assisted by the chaperonin system. It is possible that only a subdomain enters the cavity and that hsp10 might exert its effect on the opposite, unoccupied ring of hsp60. It is interesting in this respect that the crystal structure of the aconitase homolog from pig heart mitochondria consists of four domains (Robbins and Stout, 1989). The three N-terminal domains are tightly associated with the Fe-S cluster. The fourth domain, consisting of the C-terminal 217 amino acids, is more loosely associated with the protein core by a 23-amino-acid linker peptide that is wrapped around the surface of the folded aconitase.

It is one of the most striking findings of this study that hsp60 can mediate folding of some proteins in the absence of functional hsp10. The recently solved crystal structure of the GroEL–GroES complex (Xu *et al.*, 1997) revealed that binding of GroES renders the surface inside the central cavity more hydrophilic. Release of unfolded proteins sticking to the hydrophobic surface is triggered by this conformational change. Substrates that are released independently of hsp10, e.g. Idh1p, might bind less tightly than substrates, such as Ilv3p, that require hsp10 for release and subsequent folding (Schmidt *et al.*, 1994).

Two proteins, yeast rhodanese and yeast malate dehydrogenase, were tested in our import system because their mammalian homologs have served as chaperonin-dependent model substrates *in vitro*. Remarkably, folding of the yeast proteins does not depend (y-rho), or only slightly depends (y-mdh1) on the functional chaperonins. This is consistent with a number of *in vitro* studies which demonstrate that the chaperonin requirement for closely related proteins can vary significantly (e.g. Gray *et al.*, 1993; Saijo *et al.*, 1994; Mattingly *et al.*, 1995; Widmann and Christen, 1995; Zahn *et al.*, 1996). So far, no obvious relationship between the proteins that require chaperonin assistance for folding has emerged. The findings underscore the importance of investigating the chaperonin-mediated protein folding of authentic substrates *in vitro* and *in vivo*.

Materials and methods

Nomenclature and abbreviations

To prevent confusion when analyzing chaperonins from different sources, we use the following nomenclature: GroEL and GroES are the chaperonins from *E. coli*; hsp60 and hsp10 are the corresponding homologs from *Saccharomyces cerevisiae*; cpn60 and cpn10 designate any member of the chaperonin 60 or chaperonin 10 family, respectively. DTT, dithiothreitol; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; TCA, trichloroacetic acid; DHFR, dihydrofolate reductase.

Materials

Chemicals, enzymes and protease inhibitors were from Fluka or Sigma, unless stated otherwise. RNase inhibitor from human placenta, SP6 RNA polymerase, and creatine kinase from rabbit muscle were from Boehringer Mannheim.

Yeast strains and plasmid construction

For generation of a yeast strain corresponding to mif4 in a JK9-3d background, genomic DNA was isolated from the original Mif4 strain (Cheng *et al.*, 1989). Primers located upstream of the promoter region and downstream of the stop-codon of the mutated hsp60-allele were used for PCR amplification. The mif4 allele was then cloned into pYCplac111 (cen-ARS, LEU2, amp) (Gietz and Sugino, 1988). The hsp60 gene from three independent clones was sequenced and found to contain a point mutation resulting in the single amino acid change of glycine 319 to aspartic acid. The plasmid pYCplac-MIF4 was transformed into the haploid yeast strain JK9-3d (Heitmann *et al.*, 1991), in which the entire hsp60 gene had been replaced by TRP1 in the presence of the plasmid pYEP352–HSP60 (2 μ , URA3, amp, HSP60) (Hill *et al.*, 1986). The resulting yeast strain (Ymif4–JK9-3d) was cured from the wild-type hsp60-containing plasmid by growth on 5-FOA (Boeke *et al.*, 1984) and is referred to as hsp60-ts in this study. The corresponding isogenic wild-type control strain (Yhsp60–JK9-3d) was generated by cloning the wild-type hsp60 gene into pYCplac111.

For generation of the yeast strain hsp10-ts in a JK9-3d background, mutants of hsp10 were generated by low-fidelity PCR (Leung *et al.*, 1989) using the YCplac22-based plasmid pST19 (cen-ARS, TRP1, amp, hsp10) (Gietz and Sugino, 1988) as a template. The resulting mutant plasmids were transformed into a haploid JK9-3d in which the genomic copy of hsp10 had been disrupted by the LEU2 gene while being rescued by the plasmid pST10 (2 μ , URA3, amp, hsp10) (Rospert *et al.*, 1993). The resulting strain was cured from pST10 by growth on 5-FOA. Five hundred independent clones were tested for growth at elevated temperature. Plasmid DNA was re-isolated from temperature-sensitive clones and the hsp10 gene sequenced. All clones contained a single point mutation in the hsp10 gene resulting in an exchange of proline 36 for histidine. The yeast strain that shows temperature-sensitive growth used in this study is termed YTS1–JK9-3d; we refer to it as hsp10-ts.

For cloning of mitochondrial yeast genes into vectors for *in vitro* transcription/translation, the genes corresponding to the proteins of interest were amplified from genomic DNA (strain JK9-3d) using Pfu-Polymerase. All constructs used for transcription/translation are based on the vector pSP65 (Promega). Two independent clones for each protein were tested.

Yeast growth conditions and isolation of mitochondria

Mitochondria were isolated from wild-type, hsp60-ts and hsp10-ts strains after growth on lactate-based medium at 25°C. Before harvest of the cells the temperature was shifted to 37°C for 2 h. Mitochondria were purified as described previously (Glick and Pon, 1995).

Holo-chaperonin complex formation with wild-type and mutant Hsp10

Extracts of purified mitochondria (2.5 mg/ml mitochondrial protein) were prepared in buffer A containing 50 mM Tris–HCl pH 7.4, 20 mM KCl, 20 mM MgCl₂, 1 mM PMSF, leupeptin (1.25 μ g/ml), antipain (0.75 μ g/ml), chymostatin (0.25 μ g/ml), elastinal (0.25 μ g/ml) and pepstatin (5 μ g/ml) by freeze–thaw sonication followed by ultracentrifugation, as described previously (Rospert *et al.*, 1996). The mitochondrial extracts prepared from either wild-type or hsp10-ts mitochondria after heat-shock were mixed with hsp60 (35.7 nM oligomer) in a final volume of 50 μ l in buffer A containing 2 mM ADP. The samples were incubated for 5 min at 25°C. The mixture was layered onto a sucrose cushion consisting of 250 μ l 15% (w/w) sucrose (in buffer A) overlaid with 200 μ l buffer A lacking sucrose. The samples were centrifuged in a Beckman Optima TLX Ultracentrifuge at 356 000 g for 1 h at 25°C. Fractions of 50 μ l were collected from the top with a pipette, precipitated with 5% trichloroacetic acid and subjected to SDS–PAGE on 10% Tris–Tricine gels. Immunoblotting of the fractions was performed with polyclonal antisera raised against hsp60 and hsp10, followed by detection with ¹²⁵I-labeled protein A.

Screen for aggregation of yeast mitochondrial proteins

For preparation of yeast translation extract and translation of total yeast RNA, yeast translation extract was prepared essentially as described previously (Garcia *et al.*, 1991). To enable the translation of a comprehensive endogenous protein population, the whole procedure was performed under RNase-free conditions in the presence of RNase inhibitor. The detailed protocol will be published elsewhere.

For import of the mitochondrial protein population synthesized in yeast translation extracts, the translation mixture was added to the import reaction containing import buffer (0.6 M sorbitol, 50 mM HEPES–KOH pH 7.0, 50 mM KCl, 10 mM MgCl₂, 2 mM KH₂PO₄, 5 mM methionine),

1 mg/ml fatty-acid-free bovine serum albumin, 2 mM NADH, 2 mM ATP, 20 mM creatine phosphate, 0.1 mg/ml creatine phosphate kinase and 0.5 mg/ml of either wild-type, hsp10-ts or hsp60-ts mitochondria. Prior to the addition of the translation mixture, the import reaction was incubated at 37°C for 2 min. Incubation was for 1 h at 37°C. Aeration was assured by vortexing the import reaction every few minutes. The reaction was stopped on ice and mitochondria were collected by centrifugation at 4°C. Mitochondrial pellets were resuspended in 1 ml import buffer, transferred to fresh tubes, supplemented with 300 µg/ml proteinase K and incubated for 30 min on ice. Proteinase K was inhibited by addition of 1 mM PMSF, and mitochondria were re-isolated by centrifugation. Mitochondrial pellets were resuspended in 200 µl lysis buffer (20 mM HEPES-KOH pH 7.4, 150 mM NaCl, 1 mM PMSF) and solubilized by addition of 200 µl 1% Triton X-100. The extracts were centrifuged for 30 min at 150 000 g in an airfuge at 4°C (Beckman). Supernatants were removed and pellets were washed once in lysis buffer followed by centrifugation at 150 000 g for 5 min. Pellets were resuspended in 200 µl lysis buffer. Ten percent of each sample (supernatant and pellet) was removed and run on a 10% Tricine gel to compare the efficiencies of the different import reactions. The remainder was precipitated by the addition of 5% TCA (final concentration).

2D gel electrophoresis

For the preparation of 2D gels containing sufficient material for micro-sequencing, each individual detergent-insoluble pellet was supplemented with 0.5 mg total wild-type mitochondrial protein. Samples were resuspended in 100 µl 2D-gel buffer containing 8 M urea, 66 mM CHAPS, 25 mM DTT and 100 µl ampholytes pH 3–10 (Pharmacia Biotech). The samples were run on 18 cm Immobiline DryStrips (Pharmacia Biotech) pH 3–10, according to manufacturer's instructions. The second dimension was on a 10% Tris-Tricine gel (Schägger and von Jagow, 1987). The separated proteins were transferred onto PVDF membranes (Millipore), which were first analyzed by autoradiography and subsequently stained with Coomassie Blue. This method allowed the exact superposition of the Coomassie-Blue stained protein spots (total soluble mitochondrial protein) and the radiolabeled protein species (aggregated protein).

Mass-spectral analysis

Blotted proteins which had been separated by 2D gel electrophoresis were digested with 0.5 µg trypsin. The digests were injected onto a capillary column (100 µm i.d. × 2 cm) and packed with PORS R2, and the resulting fragments were desorbed into the mass spectrometer with a 10 min gradient from 0 to 100% methanol containing 0.0025% acetic acid. Ionization was carried out using a micro-source (Davis *et al.*, 1995). Spray voltages were usually between 1100 and 1400 V. Fragment spectra of eluting peptides were acquired by data-controlled automated switching between precursor ions and daughter ions during a single chromatographic run (Stahl *et al.*, 1996). The daughter-ion spectra acquired were used to identify the proteins with the SEQUEST program (Yates *et al.*, 1995). Mass determinations were carried out on a TSQ7000 triple quadrupole mass spectrometer (Finnigan, San José, CA). For precursor ion scanning, the resolution of the instrument was set to 1 Da. For operation in the MS/MS mode, the resolution of Q1 was set to transmit a mass window of 4 Da and the resolution of Q3 was adjusted to 1.5 Da. Scanning was performed from 50 to 2250 Da in 3.5 s. Argon was used as collision gas at a pressure of 3.0 mTorr.

Import of individual proteins synthesized in reticulocyte lysate

Transcription, translation and import of identified substrate proteins into isolated mitochondria was as described (Rospert and Schatz, 1998). Import into wild-type and mutant mitochondria occurred with similar efficiency. Translation of the individual proteins was in reticulocyte lysate. Import was performed in a 1 ml reaction in standard import buffer at a mitochondrial protein concentration of 0.5 mg/ml for 30 min at 37°C. Import reactions were transferred onto ice and trypsin was added to a final concentration of 100 µg/ml for 30 min. Trypsin was inhibited by the addition of 200 µg/ml soybean trypsin inhibitor and mitochondria were collected by centrifugation at 4°C. Mitochondrial pellets were resuspended in 100 µl solubilization buffer (20 mM HEPES-KOH pH 7.4, 150 mM NaCl, 1 mM PMSF, 20 µg/ml trypsin inhibitor). Fifty microliters of the samples were removed and directly precipitated with 5% TCA (Imp). One percent Triton X-100 in solubilization buffer was added to the remaining 50 µl, followed by centrifugation for 30 min in an airfuge at 150 000 g. The supernatant was removed and precipitated with TCA (Sup). The detergent-insoluble pellet was washed once in

solubilization buffer without detergent and subsequently resuspended in SDS sample buffer (Pel).

The kinetics of folding of precursor proteins [yeast rhodanese (y-rho), malate dehydrogenase (y-mdh1)] that fold into a protease-resistant conformation were tested as described previously (Rospert *et al.*, 1996). Y-rho and y-mdh1 were synthesized in reticulocyte lysate, precipitated with ammonium sulfate and subsequently resuspended in buffer containing 8 M urea. The denatured proteins were imported into wild-type mitochondria (0.5 mg/ml mitochondrial protein) in import buffer at 37°C. At the times indicated, samples were withdrawn and diluted 2-fold in ice-cold stop buffer (import buffer lacking ATP and NADH but containing 50 µM carbonylcyanide, *p*-trifluoromethoxyphenylhydrazine FCCP, 5 µM oligomycin, 2 µg/ml efrapeptin and 40 U/ml apyrase). After 5 min on ice, 100 µg/ml trypsin was added and incubation was continued for 30 min. Trypsin was inhibited by addition of soybean trypsin inhibitor (SBTI) to 200 µg/ml. After 5 min, the mitochondria were re-isolated by centrifugation for 5 min at 10 000 g and resuspended in sonication buffer (20 mM HEPES-KOH pH 7.4, 1 mM EDTA, 100 mM NaCl, 50 µg/ml SBTI). Half of each sample was directly precipitated with 5% TCA (Import). In the remainder, the mitochondria were disrupted by freeze-thaw sonication and proteinase K was added to a final concentration of 100 µg/ml on ice. After 15 min, the protease was inactivated by the addition of 1 mM PMSF and the sample was precipitated with TCA (Folding). Analysis was carried out on a 10% Tris-Tricine gel followed by autoradiography.

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References

- Boeke, J.D., LaCroute, F. and Fink, G.R. (1984) A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol. Gen. Genet.*, **197**, 345–346.
- Braig, K., Otwinowski, Z., Hegde, R., Boisvert, D.C., Joachimiak, A., Horwich, A.L. and Sigler, P.B. (1994) The crystal structure of the bacterial chaperonin GroEL at 2.8 Å. *Nature*, **371**, 578–586.
- Bukau, B. and Horwich, A.L. (1998) The Hsp70 and Hsp60 chaperone machines. *Cell*, **92**, 351–366.
- Cheng, M.Y., Hartl, F.-U., Martin, J., Pollock, R.A., Kalousek, F., Neupert, W., Hallberg, E.M., Hallberg, R.L. and Horwich, A.L. (1989) Mitochondrial heat-shock protein hsp60 is essential for assembly of proteins imported into yeast mitochondria. *Nature*, **337**, 620–625.
- Cheng, M.Y., Hartl, F.-U. and Horwich, A.L. (1990) The mitochondrial chaperonin hsp60 is required for its own assembly. *Nature*, **348**, 455–458.
- Clark, A.C. and Frieden, C. (1997) GroEL-mediated folding of structurally homologous dihydrofolate reductases. *J. Mol. Biol.*, **268**, 512–525.
- Davis, M.T., Stahl, D.C., Hefta, S.A. and Lee, T.D. (1995) A microscale electrospray interface for on-line, capillary liquid chromatography/tandem mass spectrometry of complex peptide mixtures. *Anal. Chem.*, **67**, 4549–4556.
- Dubaquie, Y., Looser, R. and Rospert, S. (1997) Significance of chaperonin10-mediated inhibition of ATP hydrolysis by chaperonin60. *Proc. Natl Acad. Sci. USA*, **94**, 9011–9016.
- Ellis, J.R. and Hartl, F.-U. (1996) Protein folding in the cell: competing models of chaperonin function. *FASEB J.*, **10**, 20–26.
- Ewalt, K.L., Hendrick, J.P., Houry, W.A. and Hartl, F.U. (1997) *In vivo* observation of polypeptide flux through the bacterial chaperonin system. *Cell*, **90**, 491–500.
- Fenton, W.A. and Horwich, A.L. (1997) GroEL-mediated protein folding. *Protein Sci.*, **6**, 743–760.
- Fisher, M.T. (1998) GroE chaperonin-assisted folding and assembly of dodecameric glutamine synthetase. *Biochemistry (Mosc.)*, **63**, 382–398.
- Garcia, P.D., Hansen, W. and Walter, P. (1991) *In vitro* protein translocation across microsomal membranes of *Saccharomyces cerevisiae*. *Methods Enzymol.*, **194**, 675–682.
- Gietz, R.D. and Sugino, A. (1988) New yeast-*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base pair restriction sites. *Gene*, **74**, 527–534.

- Glick, B.S. and Pon, L.A. (1995) Isolation of highly purified mitochondria from *Saccharomyces cerevisiae*. *Methods Enzymol.*, **260**, 213–223.
- Glick, B.S., Brandt, A., Cunningham, K., Müller, S., Hallberg, R.L. and Schatz, G. (1992) Cytochromes c1 and b2 are sorted to the intermembrane space of yeast mitochondria by a stop-transfer mechanism. *Cell*, **69**, 809–822.
- Goloubinoff, P., Christeller, J.T., Gatenby, A.A. and Lorimer, G.H. (1989) Reconstitution of active dimeric ribulose biphosphate carboxylase from an unfolded state depends on two chaperonin proteins and Mg-ATP. *Nature*, **342**, 884–889.
- Gray, T.E., Eder, J., Bycroft, M., Day, A.G. and Fersht, A.R. (1993) Refolding of barnase mutants and pro-barnase in the presence and absence of GroEL. *EMBO J.*, **12**, 4145–4150.
- Hallberg, E.M., Shu, Y. and Hallberg, R.L. (1993) Loss of mitochondrial hsp60 function: nonequivalent effects on matrix-targeted and intermembrane-targeted proteins. *Mol. Cell. Biol.*, **13**, 3050–3057.
- Heitmann, J., Movva, N.R., Hiestand, P.C. and Hall, M.N. (1991) FK 506-binding protein proline rotamase is a target for the immunosuppressive agent FK 506 in *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA*, **88**, 1948–1952.
- Heyrovská, N., Frydman, J., Höfheld, J. and Hartl, F.U. (1998) Directionality of polypeptide transfer in the mitochondrial pathway of chaperone-mediated protein folding. *Biol. Chem.*, **379**, 301–309.
- Hill, J.E., Myers, A.M., Koerner, T.J. and Tzagoloff, A. (1986) Yeast/*E.coli* shuttle vectors with multiple unique restriction sites. *Yeast*, **2**, 163–167.
- Höfheld, J. and Hartl, F.U. (1994) Role of the chaperonin cofactor Hsp10 in protein folding and sorting in yeast mitochondria. *J. Cell Biol.*, **126**, 305–315.
- Horovitz, A. (1998) Structural aspects of GroEL function. *Curr. Opin. Struct. Biol.*, **8**, 93–100.
- Horwich, A.L., Low, K.B., Fenton, W.A., Hirshfield, I.N. and Furtak, K. (1993) Folding *in vivo* of bacterial cytoplasmic proteins: role of GroEL. *Cell*, **74**, 909–917.
- Hunt, J.F., Weaver, A.J., Landry, S.J., Gierasch, L. and Deisenhofer, J. (1996) The crystal structure of the GroES co-chaperonin at 2.8 Å resolution. *Nature*, **379**, 37–45.
- Hunt, J.F., van der Vies, S.M., Henry, L. and Deisenhofer, J. (1997) Structural adaptations in the specialized bacteriophage T4 co-chaperonin Gp31 expand the size of the Anfinsen cage. *Cell*, **90**, 361–371.
- Hutchinson, J.P., El-Thaher, T.S.H. and Miller, A.D. (1994) Refolding and recognition of mitochondrial malate dehydrogenase by *Escherichia coli* chaperonins cpn60 (GroEL) and cpn10 (GroES). *Biochem. J.*, **302**, 405–410.
- Leung, D.W., Chen, E. and Goeddel, D.V. (1989) A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction. *Technique*, **1**, 11–15.
- Lorimer, G.H. (1996) A quantitative assessment of the role of the chaperonin proteins in protein folding *in vivo*. *FASEB J.*, **10**, 5–9.
- Lorimer, G.H., Todd, M.J. and Viitanen, P.V. (1993) Chaperonins and protein folding: unity and disunity of mechanisms. *Phil. Trans. R. Soc. Lond. B*, **339**, 297–304.
- Manning-Krieg, U.C., Scherer, P.E. and Schatz, G. (1991) Sequential action of mitochondrial chaperones in protein import into the matrix. *EMBO J.*, **10**, 3273–3280.
- Martin, J., Langer, T., Boteva, R., Schramel, A., Horwich, A.L. and Hartl, F.U. (1991) Chaperonin-mediated protein folding at the surface of GroEL through a 'molten globule'-like intermediate. *Nature*, **352**, 36–42.
- Mattingly, J.R., Iriarte, A. and Martinez-Carrion, M. (1995) Homologous proteins with different affinities for GroEL. *J. Biol. Chem.*, **270**, 1138–1148.
- Mayhew, M., da Silva, A.C.R., Martin, J., Erdjument-Bromage, H., Tempst, P. and Hartl, F.U. (1996) Protein folding in the central cavity of GroEL–GroES chaperonin complex. *Nature*, **379**, 420–426.
- McLennan, N. and Masters, M. (1998) GroE is vital for cell-wall synthesis. *Nature*, **392**, 139.
- Mendoza, J.A., Rogers, E., Lorimer, G.H. and Horowitz, P.M. (1991) Chaperonins facilitate the *in vitro* folding of monomeric mitochondrial rhodanese. *J. Biol. Chem.*, **266**, 13044–13049.
- Neupert, W. (1997) Protein import into mitochondria. *Annu. Rev. Biochem.*, **66**, 863–917.
- Robbins, A.H. and Stout, C.D. (1989) The structure of aconitase. *Proteins*, **5**, 289–312.
- Roseman, A.M., Chen, S., White, H., Braig, K. and Saibil, H.R. (1996) The chaperonin ATPase cycle: Mechanism of allosteric switching and movements of substrate-binding domains in GroEL. *Cell*, **87**, 241–251.
- Rospert, S. and Schatz, G. (1998) In Celis, J.E. (ed.), *Protein Translocation into Mitochondria*. Vol. 2. Academic Press, San Diego, CA.
- Rospert, S., Junne, T., Glick, B.S. and Schatz, G. (1993) Cloning and disruption of the gene encoding yeast mitochondrial chaperonin 10 the homolog of *E.coli* GroES. *FEBS Lett.*, **335**, 358–360.
- Rospert, S., Müller, S., Schatz, G. and Glick, B.S. (1994) Fusion proteins containing the cytochrome b2 presequence are sorted to the mitochondrial intermembrane space independently of hsp60. *J. Biol. Chem.*, **269**, 17279–17288.
- Rospert, S., Looser, R., Dubaquié, Y., Matouschek, A., Glick, B.S. and Schatz, G. (1996) Hsp60-independent protein folding in the matrix of yeast mitochondria. *EMBO J.*, **15**, 764–774.
- Saijo, T., Welch, W.J. and Tanaka, K. (1994) Intramitochondrial folding and assembly of medium-chain acyl-CoA dehydrogenase (MCAD). Demonstration of impaired transfer of K304E-variant MCAD from its complex with hsp60 to the native tetramer. *J. Biol. Chem.*, **269**, 4401–4408.
- Schägger, H. and von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.*, **166**, 368–379.
- Schmidt, M., Bücheler, U., Kaluza, B. and Buchner, J. (1994) Correlation between the stability of the GroEL–protein ligand complex and the release mechanism. *J. Biol. Chem.*, **269**, 27964–27972.
- Stahl, D.C., Swiderek, K.M., Davis, M.T. and Lee, T.D. (1996) Data-controlled automation of liquid chromatography/tandem mass spectrometry analysis of peptide mixtures. *J. Am. Soc. Mass Spectrom.*, **7**, 532–540.
- van der Vies, S.M., Gatenby, A.A. and Georgopoulos, C. (1994) Bacteriophage T4 encodes a co-chaperonin that can substitute for *Escherichia coli* GroES in protein folding. *Nature*, **368**, 654–656.
- Viitanen, P.V., Donaldson, G.K., Lorimer, G.H., Lubben, T.H. and Gatenby, A.A. (1991) Complex interactions between the chaperonin 60 molecular chaperone and dihydrofolate reductase. *Biochemistry*, **30**, 9716–9723.
- Weissman, J.S., Hohl, C.M., Kovalenko, O., Kashi, Y., Chen, S., Braig, K., Saibil, H.R., Fenton, W.A. and Horwich, A.L. (1995) Mechanism of GroEL action: productive release of polypeptide from a sequestered position under GroES. *Cell*, **83**, 577–587.
- Widmann, M. and Christen, P. (1995) Differential effects of molecular chaperones on refolding of homologous proteins. *FEBS Lett.*, **377**, 481–484.
- Xu, Z., Horwich, A.L. and Sigler, P.B. (1997) The crystal structure of the asymmetric GroEL–GroES–(ADP)7 chaperonin complex. *Nature*, **388**, 741–750.
- Yates, J.R., III, Eng, J.K., McCormack, A.L. and Schieltz, D. (1995) Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database. *Anal. Chem.*, **67**, 1426–1436.
- Zahn, R., Lindner, P., Axmann, S.E. and Plückthun, A. (1996) Effect of single point mutations in citrate synthase on binding to GroEL. *FEBS Lett.*, **380**, 152–156.

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