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# Prevention of Protein Denaturation Under Heat Stress by the Chaperonin Hsp60

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**The increased synthesis of heat shock proteins is a ubiquitous physiological response of cells to environmental stress. How these proteins function in protecting cellular structures is not yet understood. The mitochondrial heat shock protein 60 (Hsp60) has now been shown to form complexes with a variety of polypeptides in organelles exposed to heat stress. The Hsp60 was required to prevent the thermal inactivation in vivo of native dihydrofolate reductase (DHFR) imported into mitochondria. In vitro, Hsp60 bound to DHFR in the course of thermal denaturation, preventing its aggregation, and mediated its adenosine triphosphate–dependent refolding at increased temperatures. These results suggest a general mechanism by which heat shock proteins of the Hsp60 family stabilize preexisting proteins under stress conditions.**

On exposure to various forms of environmental stress, cells generally respond by increasing the rate of synthesis of a set of highly conserved stress, or heat shock, proteins (Hsps) (*1*). Many stress proteins, including members of the Hsp70 and Hsp60 families, are constitutively expressed and fulfill essential functions as "molecular chaperones" (*2*) under normal cellular conditions. The Hsp60s, which are found in bacterial cytosol as well as in mitochondria and chloroplasts, are high molecular mass, double-ring complexes consisting of 14 ~60-kD subunits (*2*). These "chaperonins" (*3*) interact with early intermediates in the protein folding pathway and mediate the acquisition of the native structure of newly synthesized proteins by releasing the substrate in an adenosine triphosphate (ATP)–dependent process (*4–8*). The ATP-hydrolytic activity of chaperonins is regulated by smaller co-chaperonins, ring complexes of seven ~10-kD subunits (*4*, *9*).

Although most conditions that induce the stress response potentially lead to the accumulation of denatured proteins (*10*), little is known about the mechanisms by which Hsps might function in preventing denaturation or in renaturing damaged proteins (*1*). On exposure of *Escherichia coli* to temperatures between 42° and 46°C, the concentration of the bacterial chaperonin GroEL increases five- to tenfold, reaching up to 12% of total cellular protein. Similarly, the concentration of the mitochondrial Hsp60 of yeast increases two- to threefold at 42°C (*8*, *11*), and that of the co-chaperonin Hsp10 of mammalian mitochondria approximately tenfold under these conditions (*12*). Such induction may reflect an increased requirement for these Hsps in protecting preexisting proteins from denaturation.

A large number of polypeptides were bound to mitochondrial Hsp60 when isolated mitochondria of $^{35}$S-labeled *Neurospora crassa* were incubated for 10 min at increased temperatures (Fig. 1). Mitochondrial extracts (*13*) were analyzed by electrophoresis on native polyacrylamide gels, on which Hsp60-bound polypeptides comigrate with the Hsp60 complex. When the bands corresponding to Hsp60 were excised and subjected to electrophoresis on reducing SDS–polyacrylamide gels, a range of polypeptides of 10 to 90 kD that had been associated with Hsp60 was apparent. The amount of bound protein increased significantly after shifting the temperature from 32° to 39° or 46°C (Fig. 1). A small number of proteins comigrated with Hsp60 already at 25°C, an unidentified polypeptide of ~34 kD being most prominent. The total amount of $^{35}$S present in the Hsp60-associated polypeptides corresponded to ~10% of that in Hsp60, which is consistent with the observed capacity of the 14-subunit chaperonin to bind only one to two substrate molecules (*4–6*, *14*). The Hsp60-associated proteins did not represent degradation products of Hsp60 (*15*). Protein binding to Hsp60 occurred in the intact mitochondria because it was not prevented when a large excess of $\alpha_{s1}$-casein was present during preparation of the extracts (Fig. 1); casein binds to the chaperonin despite its stability in solution (*4*). Similar results were obtained with *Saccharomyces cerevisiae* mitochondria (*16*).

Apparently, Hsp60 recognizes a variety of preexisting mitochondrial proteins as

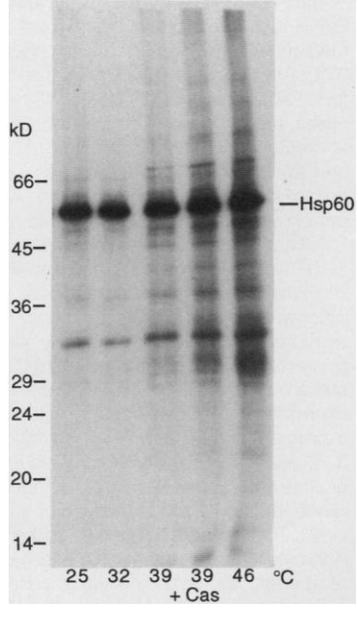


**Fig. 1.** Temperature-dependent association of proteins with Hsp60 in organello. Aliquots (50 μl) of *Neurospora crassa* mitochondria (5 mg of protein per milliliter) isolated from cells grown in the presence of $^{35}SO_4$ (*28*) were incubated for 10 min at the indicated temperatures in buffer A [0.6 M sorbitol, 20 mM MOPS (pH 7.2), 1 mM dithiothreitol (DTT), and 25 mM KCl] containing 4 mM magnesium acetate, 1 mM ATP, and 2 mM reduced nicotinamide adenine dinucleotide (NADH). Mitochondria were lysed in buffer A containing 7.5 mM cyclohexane diamine tetraacetic acid (to prevent ATP hydrolysis by Hsp60) and 0.075% of the nonionic detergent Genapol (Hoechst, Frankfurt, Germany). One sample, incubated at 39°C, received 50 μg of $\alpha_{s1}$-casein (Cas) before lysis. Membranes and insoluble material were removed by centrifugation for 15 min at 30,000*g*. The amounts of total $^{35}$S-labeled protein in pellet fractions (containing mostly membrane protein) increased from ~30 to 40% after a temperature shift from 25 to 46°C. Approximately 95% of total Hsp60 remained soluble upon incubation of mitochondria at the various temperatures. Supernatant fractions were analyzed by nondenaturing polyacrylamide gel electrophoresis (native PAGE) (*7*). The Hsp60-containing band was excised and analyzed by SDS-PAGE and fluorography. The position of Hsp60 is indicated on the right, and the positions of molecular mass standards (in kilodaltons) are indicated on the left.

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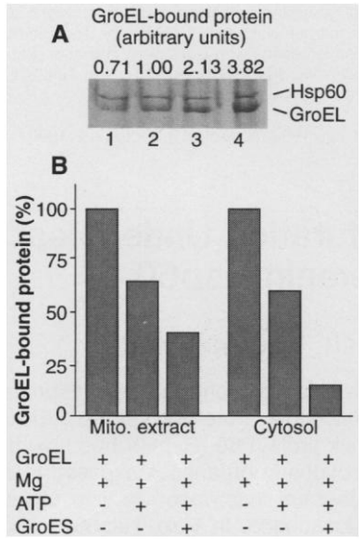


**Fig. 2.** Temperature-dependent binding of $^{35}$S-labeled yeast proteins to GroEL in vitro. (**A**) Soluble mitochondrial protein (25 μg) prepared by sonication of $^{35}$S-labeled yeast mitochondria (*8, 29*) in buffer A was incubated with 8 μg of unlabeled GroEL (0.4 μM) for 12 min at 25°, 37°, or 50°C (lanes 2 to 4, respectively). One reaction received GroEL after incubation at 50°C for 12 min and cooling to 25°C for 4 min (lane 1). The positions of the Hsp60 and GroEL complexes on a fluorograph of the native gel are shown. GroEL-bound proteins were quantified by densitometry. (**B**) $^{35}$S-Labeled proteins of yeast mitochondria (Mito.) and cytosol [a 100,000*g* supernatant of lysed spheroplasts (*29*)] were incubated with GroEL at 40°C as in (A). After cooling to 25°C, each incubation mixture was divided into three portions, which received (i) 5 mM magnesium acetate, (ii) 1 mM ATP and 5 mM magnesium acetate, or (iii) 1 mM ATP, 5 mM magnesium acetate, and 1 μg of GroES (0.57 μM). After incubation for 30 min at 25°C, GroEL-bound protein was analyzed by native PAGE and fluorography and quantified by densitometry. The amount of protein bound at 40°C was taken as 100%.

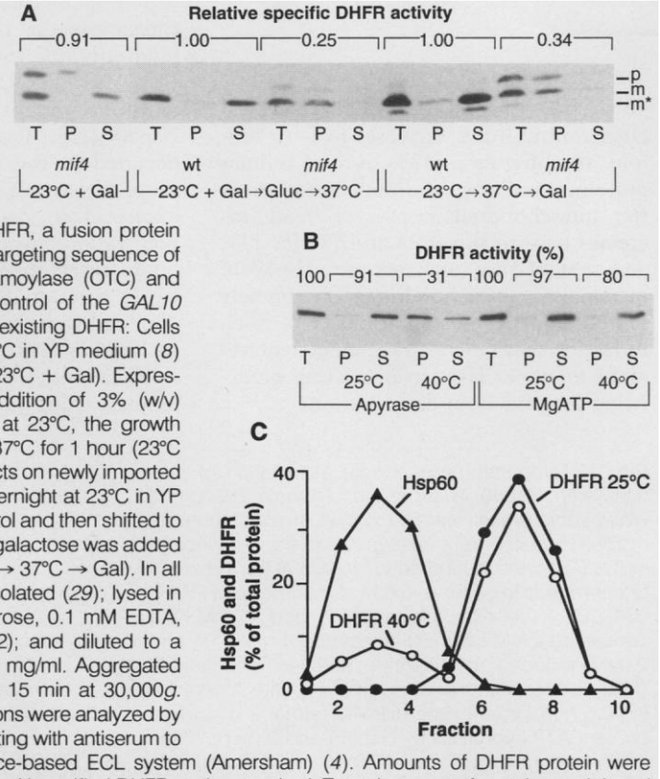


**Fig. 3.** Requirement for Hsp60 and ATP for maintenance of DHFR function in yeast mitochondria. (**A**) *Saccharomyces cerevisiae* strains MC3 (wt) and α143 (*mif4*) (*8*) were transformed with a multicopy plasmid encoding pOTC-DHFR, a fusion protein between the mitochondrial targeting sequence of human ornithine transcarbamoylase (OTC) and mouse DHFR (*19*) under control of the *GAL10* promoter (*8*). Effects on preexisting DHFR: Cells were grown overnight at 23°C in YP medium (*8*) with 1.5% (w/v) galactose (23°C + Gal). Expression was terminated by addition of 3% (w/v) glucose (*20*). After 40 min at 23°C, the growth temperature was shifted to 37°C for 1 hour (23°C + Gal → Gluc → 37°C). Effects on newly imported DHFR: Cells were grown overnight at 23°C in YP medium with 3% (w/v) glycerol and then shifted to 37°C. After 1 hour, 2% (w/v) galactose was added for an additional hour (23°C → 37°C → Gal). In all cases, mitochondria were isolated (*29*); lysed in 0.1% Genapol, 0.25 M sucrose, 0.1 mM EDTA, and 25 mM MOPS (pH 7.2); and diluted to a protein concentration of 2.5 mg/ml. Aggregated protein was sedimented for 15 min at 30,000*g*. Pellet and supernatant fractions were analyzed by SDS-PAGE and immunoblotting with antiserum to DHFR and the luminescence-based ECL system (Amersham) (*4*). Amounts of DHFR protein were determined by densitometry with purified DHFR as the standard. T, total amount of protein precipitated by trichloroacetic acid immediately after lysis of mitochondria; P, pellet fraction; S, supernatant fraction; p, precursor, and m, mature form of OTC-DHFR; m*, a degradation product of OTC-DHFR of the size of authentic mouse DHFR. DHFR activities in total extracts were determined at 25°C (*4, 30*) and are expressed as relative specific activities (activity per microgram of mOTC-DHFR in *mif4*/activity per microgram of mOTC-DHFR in wt). (**B**) Mitochondria (100 μg of protein) isolated from wt cells grown at 37°C with galactose were incubated for 5 min at 25°C in 50 μl of buffer A containing 2 mM magnesium acetate and 2.5 mM succinate. Before incubation for 15 min at 25° or 40°C, either 1 U of apyrase (Sigma; grade VIII) and 2 μM oligomycin or 1 mM ATP were added. Mitochondria were lysed and analyzed as in (A). Total amounts of protein and enzymatic activities measured before incubation are set to 100%. Abbreviations are as in (A). (**C**) After incubation of mitochondria for 15 min at 25° or 40°C in the presence of apyrase (B), soluble extracts were prepared and analyzed on Sephacryl S300 (Pharmacia) gel-filtration columns (*7*). Amounts of Hsp60 and mOTC-DHFR, quantified by densitometry of immunoblots, are shown as a percentage of total protein recovered from the column. Recoveries were 90 to 95%. The elution pattern of Hsp60 at 40°C, which was identical to that at 25°C, is shown.

they become denatured under heat stress. These proteins were found associated with Hsp60 despite the presence of ATP in mitochondria, perhaps cycling between free and Hsp60-bound states at the increased temperature. Consistent with this proposal, the amount of bound protein decreased considerably when the temperature was lowered from 39° to 25°C (*16*); it was unclear, however, whether this protein was released from Hsp60 or whether it was proteolytically degraded. To address this question, we analyzed the interaction of purified chaperonin with $^{35}$S-labeled proteins in extracts of yeast mitochondria and cytosol. GroEL, the *E. coli* homolog of Hsp60, was used in these experiments because both GroEL and its co-chaperonin GroES are easily purified from an overproducing strain of *E. coli*, whereas the mitochondrial GroES is rather difficult to isolate (*9, 12*). Furthermore, GroEL could be distinguished on native gels from the $^{35}$S-labeled Hsp60 in mitochondrial extracts. Again, a wide range of polypeptides bound to GroEL in a temperature-dependent manner, but only when the chaperonin was present during incubation at the increased temperature (Fig. 2A). When GroEL was added to the extracts after a shift from 50° to 25°C, the amount of bound protein was reduced to the level seen before heat treatment. Proteins bound to GroEL at 40°C were at least partially released on incubation with MgATP at 25°C (Fig. 2B). ATP-dependent release was enhanced by the addition of GroES, more efficiently with cytosolic than with mitochondrial proteins (*13*). The discharged proteins were soluble, which suggests that they were in their native conformations (*17*).

Dihydrofolate reductase (DHFR) was chosen as a relatively thermolabile model protein [Gibbs free energy of unfolding ($\Delta G^{\circ}_{unfolding}$) = 5.9 kcal/mol (*18*)] to test whether Hsp60 was able to preserve the functional integrity of proteins during heat shock. DHFR, normally a cytosolic protein, interacts with Hsp60 when it is directed into mitochondria by a targeting sequence fused to the $NH_2$-terminus (*7*). A fusion protein, pOTC-DHFR, containing the cleavable presequence of human ornithine transcarbamoylase (OTC) (*19*), was expressed from a galactose-regulated promoter in wild-type and in *mif4* yeast. In the *mif4* mutant, Hsp60 loses its function at the nonpermissive temperature of 37°C and becomes insoluble (*8*). pOTC-DHFR was efficiently imported into mitochondria and was processed to the mature form (mOTC-DHFR), which contains nine linker residues $NH_2$-terminal to the DHFR sequence (Fig. 3) (*19*). mOTC-DHFR constituted 0.5 to 1% of the total mito-

chondrial matrix protein and possessed ~85% of the specific enzymatic activity of authentic DHFR (*16*). When expressed at the permissive temperature (23°C), DHFR could be extracted from the isolated mitochondria of wild-type and *mif4* cells in a soluble, enzymatically active form (Fig. 3A). In mitochondria of mutant cells grown at 37°C, most of the DHFR protein was inactive and was recovered in the insoluble pellet fraction, whereas in wild-type organelles, DHFR remained soluble and active at 37°C.

The aggregated DHFR in *mif4* cells could have been derived from preexisting protein that denatured at 37°C or from newly imported protein that was unable to fold. The following experiments were performed to address these possibilities. In the first experiment, pOTC-DHFR synthesis was induced by galactose at 23°C, and then expression was repressed by adding glucose (*20*). Subsequently, the culture was shifted to 37°C to induce expression of the *mif4* phenotype. Mostly insoluble mOTC-DHFR was detected in the mitochondria (Fig. 3A). Compared to *mif4* cells that were maintained at 23°C, the amount of DHFR protein in an equivalent amount of mitochondrial protein was reduced by ~30 to 50%, probably because of degradation. The specific activity of mOTC-DHFR in *mif4* mitochondria at 37°C was 25% of that in the wild-type control. Apparently, some aggregated DHFR renatured upon dilution in the presence of substrates. In the second experiment, pOTC-DHFR was expressed after the cells were shifted to the nonpermissive temperature. Both precursor and mature protein were then present in the mitochondria in an insoluble form (Fig. 3A). Under these conditions, DHFR-specific activity was approximately one-third of the wild-type activity when equal amounts of mature-size protein were compared. The accumulation of uncleaved precursor in *mif4* mitochondria suggested that the newly imported proteins adopted misfolded conformations that were not readily accessible to proteolytic processing. Again, there was considerably less total DHFR in *mif4* mitochondria under these conditions than in wild-type organelles. Assuming similar levels of expression, either the mutant mitochondria had a reduced efficiency of protein import at 37°C or, more likely, misfolded protein was degraded (*21*). This degradation may explain why *mif4* mitochondria do not accumulate large amounts of protein aggregates at 37°C when cell growth ceases (*8*). In summary, these results indicate a requirement for functional Hsp60 for maintaining preexisting, folded DHFR in an active conformation at increased temperatures, in addition to Hsp60's function in folding the newly imported protein (*7*) (Fig. 3A).

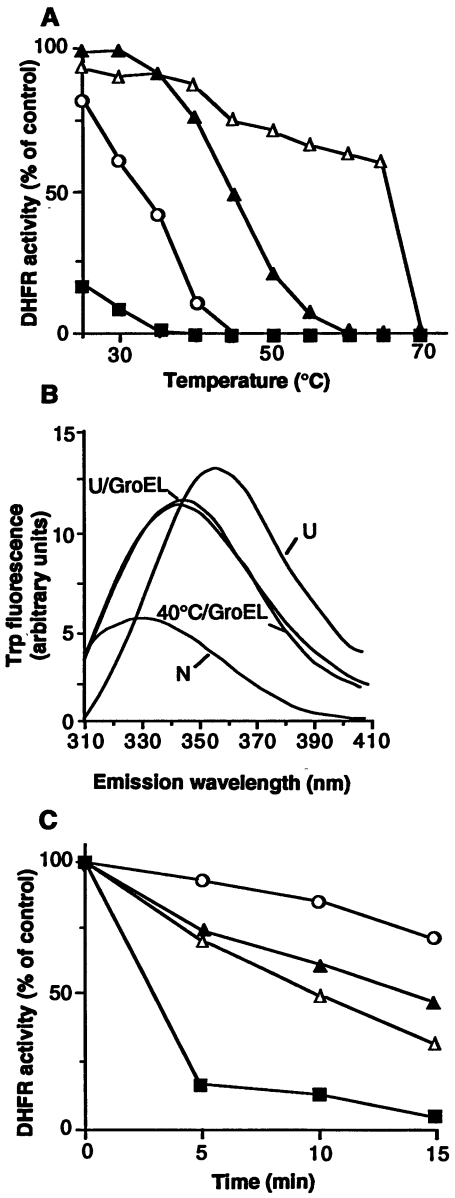


**Fig. 4.** (**A**) Thermal inactivation of DHFR and GroEL-mediated reactivation in vitro. Purified chicken DHFR (*4*) [0.2 μM in 20 mM tris (pH 7.7), 50 mM KCl, 5 mM magnesium acetate, and 2 mM DTT] was incubated for 5 min at temperatures between 25° and 70°C in the absence (▲) or presence (○, △, ■) of the 14-subunit GroEL complex (0.5 μM) (*4*). After cooling to 25°C, 1 mM ATP and 0.6 μM GroES were added to one reaction mixture (△). DHFR activities were determined immediately (▲, ○) or after a further incubation for 25 min at 25°C (△, ■). (**B**) Tryptophan fluorescence of GroEL-bound DHFR. N, native DHFR (0.6 μM) in buffer B [20 mM tris (pH 7.5), 50 mM KCl, and 2 mM DTT]; U, DHFR in buffer B containing 6 M guanidinium chloride; U/GroEL, DHFR bound to 1.2 μM GroEL after 100-fold dilution from 6 M guanidinium chloride into buffer B; 40°C/GroEL, GroEL-bound DHFR after incubation of 0.6 μM DHFR with 1.2 μM GroEL for 10 min at 40°C. Spectra (excitation at 295 nm) were recorded at 23°C and corrected for residual GroEL fluorescence (~20% of total fluorescence) (*4*, *23*). (**C**) GroEL- and GroES-dependent stabilization of DHFR. DHFR (0.1 μM in buffer B) was incubated at 40°C either without further addition (△) or with 0.3 μM GroEL (■); 0.3 μM GroEL, 10 mM ATP, and 12.5 mM magnesium acetate (▲); or 0.3 μM GroEL, 10 mM ATP, 12.5 mM magnesium acetate, and 0.4 μM GroES (○). DHFR activities were measured at 40°C at the times indicated (*30*).

Several authentic proteins of the mitochondrial matrix, although more stable than DHFR, were similarly affected in *mif4* cells at 37°C. Without specifically distinguishing between protein that had been imported before and after the shift to 37°C, we recovered ~20 to 30% of total malate dehydrogenase (MDH) and citrate synthase in Genapol-insoluble forms after 1 hour at the nonpermissive temperature (*16*).

The function of Hsp60 in protein folding generally requires ATP hydrolysis (*4*–*7*). Thus, it seemed likely that maintenance of DHFR in an active state would also be dependent on ATP. In isolated, energized wild-type mitochondria, 80% of DHFR remained soluble and active after incubation for 15 min at 40°C (Fig. 3B). In contrast, only 30% of the original DHFR activity was measured in ATP-depleted mitochondria; ~70% of total DHFR had aggregated. Aggregated DHFR was not bound to Hsp60, which remained soluble. A significant amount of the soluble DHFR was present in a high molecular mass complex with Hsp60 (Fig. 3C) and could be co-immunoprecipitated with antibodies to Hsp60 (*16*). No interaction with Hsp60 was observed at 25°C. The effects of the 15-min heat treatment on authentic mitochondrial proteins were variable; for example, 30% of citrate synthase and 15% of MDH formed insoluble aggregates in an ATP-dependent manner (*16*). DHFR remained soluble at 40°C in ATP-depleted mitochondria that were incubated in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and dihydrofolate (*16*, *22*). Substrate binding is known to stabilize the

native conformation of DHFR (*18*). Thus, small differences in the stability of the native state appear to determine the fate of a protein at the upper end of the physiologically relevant temperature scale.

We reproduced in vitro the chaperonin function in stabilizing DHFR under heat stress with purified GroEL and GroES proteins. The effects of GroEL were first analyzed in the absence of ATP, which allowed protein binding but not release. Native DHFR was incubated with a twofold molar excess of GroEL for 5 min at temperatures between 25° and 70°C, and enzyme activities were determined after cooling to 25°C (Fig. 4A). The thermal deactivation curve of DHFR was lowered by ~15°C in the presence of GroEL. Apparently, GroEL bound to thermally destabilized DHFR, thus shifting the equilibrium from the folded to the unfolded state. In contrast, a recent study demonstrating the binding of GroEL to thermally denatured α-glucosidase in vitro showed no influence of GroEL on the kinetics of denaturation (*23*). The thermal denaturation of DHFR observed in the absence of the chaperonin was irreversible (*18*); subsequent addition of GroEL and MgATP (with or without GroES) did not result in reactivation (*16*). However, DHFR that had been incubated with GroEL at temperatures up to 65°C (GroEL itself denatured between 65° and 70°C) could be reactivated by incubation with MgATP at 25°C (Fig. 4A). Omission of MgATP during the second incubation at 25°C resulted in further inactivation of DHFR because of GroEL binding (6). DHFR that had associated with GroEL at 40°C showed intrinsic tryptophan fluorescence properties very similar to those described for the GroEL-associated protein bound after dilution from denaturant (Fig. 4B) (*4*). Both of the chaperonin-associated forms of DHFR exhibited an equally high sensitivity toward protease (*16*).

Finally, we investigated whether the chaperonin system was able to maintain DHFR in an enzymatically active state at 40°C, simulating the heat shock conditions applied in organello. Free DHFR lost its activity with a half-time of ~10 min (Fig. 4C), whereas in the presence of GroEL the rate of deactivation was increased. Upon incubation with GroEL and MgATP, DHFR was only slightly more stable than in the absence of the chaperonin. GroEL, MgATP, and GroES were required to efficiently preserve the activity of DHFR at 40°C, thus reproducing the chaperonin function observed in intact mitochondria. Even substoichiometric concentrations of GroEL versus DHFR had a significant stabilizing effect. In contrast, equivalent molar concentrations of bovine serum albumin did not stabilize DHFR at 40°C (*16*).

Partial folding of DHFR can occur in close association with GroEL or Hsp60 in an environment shielded from other unfolded polypeptides (*4, 7*). This mechanism may also underlie the chaperonin function in preserving the active state of proteins under heat stress. GroEL and Hsp60 appear to bind to (partially) unfolded protein molecules, which are relatively more abundant at increased temperatures, thus preventing their (irreversible) aggregation. A folding step before release would reduce the concentration of these unfolded species. If this reaction does not result in productive folding—for example, at high temperature—the proteins may rebind to GroEL or Hsp60. They might renature in a chaperonin-dependent reaction after more favorable conditions are established, or they might be channeled into degradative pathways (*24*). Such a function of the chaperonin system would explain its required presence during thermal denaturation. Some proteins may rely on an interaction with GroEL or Hsp60 even under nonstressful conditions, undergoing repeated unfolding and refolding. The chaperonins appear to be without effect after protein aggregates have formed (*4, 6*). In contrast, the Hsp70 homolog DnaK has the capacity to renature proteins when added after thermal inactivation (*25*). Clearly, the Hsp70 in the mitochondrial matrix, the function of which is required for protein import and subsequent folding (*26*), was not sufficient to maintain the folded state of DHFR at 37°C or to mediate its de novo formation in the *mif4* mutant. Notably, the mitochondrial Hsp70 remains functional in the *mif4* strain (*8, 26*). Nevertheless, it seems possible that the Hsp70 and Hsp60 systems cooperate in preventing protein denaturation under stress conditions. A sequential pathway of Hsp70 and Hsp60 action in de novo protein folding has already been described (*27*).

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