Regulation of the Heat-shock Protein 70 Reaction Cycle by the 
Mammalian DnaJ Homolog, Hsp40*

Yasufumi Minami†, Jörgh Höhfeld, Kenzo Ohtsuka§, and Franz-Ulrich Hartl¶

From the Howard Hughes Medical Institute and Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10021 and §Laboratory of Experimental Radiology, Aichi Cancer Research Institute, Chikusa-ku, Nagoya 464, Japan

The effects of the human DnaJ homolog, Hsp40, on the ATPase and chaperone functions of the constitutively expressed Hsp70 homolog, Hsc70, were analyzed. Hsp40 stimulates the hydrolysis of ATP by Hsc70, causing a ~7-fold increase in its steady-state ATPase activity. In contrast to the prokaryotic Hsp70 system, ATP-hydrolysis and not the release of bound ADP is the rate-limiting step in the overall ATPase cycle of mammalian Hsc70. The ability to activate the Hsc70 ATPase is partially preserved in a deletion mutant containing the J-domain and the G/F region of Hsp40 but not in a deletion mutant that contains the J-domain alone. As a result of its ATPase stimulating activity, addition of Hsp40 allows Hsc70 to bind peptide in the presence of ATP, whereas in the absence of Hsp40, peptide is efficiently released upon ATP binding to Hsc70. The functional cooperation of Hsp40 with Hsc70 is essential to ensure the ATP hydrolysis-dependent binding of aggregation-sensitive denatured polypeptides, such as thermally denatured firefly luciferase and chemically denatured rhodanese. Binding of these proteins results in the formation of ternary complexes of Hsc70, Hsp40, and substrates.

Hsc70 and Hsp40 cooperate with further factors in protein renaturation, as demonstrated by the finding that luciferase, thermally denatured in the presence of Hsc70, Hsp40, and ATP, refolds upon addition of rabbit reticulocyte cytosol. Our results indicate that Hsp40 has a critical regulatory function in the Hsc70 ATPase cycle that is required for the efficient loading of peptide substrate onto Hsc70.

The folding of many newly synthesized polypeptides in the cell is assisted by molecular chaperones, a class of proteins which function mainly in preventing off-pathway folding reactions that lead to aggregation (1–3). Two major chaperone families, the 70-kDa heat-shock proteins (Hsp70s) and the chaperonins, play a central role in these reactions in the cytosol and within organelles (2, 3). Among other functions, Hsp70 family members have been thought to prevent the misfolding of translocated polypeptides. This is accomplished by their ability to bind extended peptide segments, preferentially 7-mer or 8-mer peptides which exhibit a certain enrichment and pattern of hydrophobic amino acid residues (4–7). Such sequences are probably exposed during translation but are buried within the core of the folded protein. The chaperonins act downstream from the Hsp70s by sequestering partially folded intermediates within a central cavity and promoting their folding to the native state (2, 3).

Hsp70 proteins consist of two domains, a highly conserved N-terminal ATPase domain of ~45 kDa (8, 9) and a C-terminal domain of 25 kDa. An 18-kDa portion of the C-terminal domain adjacent to the ATPase domain contains the polypeptide binding site (10, 11). The chaperone activity of the Hsp70 family is controlled by a reaction cycle of ATP binding, hydrolysis, and nucleotide exchange. The ATP-bound form of Hsp70 binds and releases peptide rapidly, resulting in a low overall affinity, whereas the ADP form binds peptide slowly but more stably (12–15). ATP binding to the ATPase domain causes a conformational change, which in turn results in structural alterations in the C-terminal domain leading to substrate release (12, 16–19). Since the intrinsic ATPase activity of Hsp70 proteins is low, the ATP-bound form with low substrate affinity predominates (12–15). As a consequence, the chaperone activity of Hsp70 depends on its functional regulation by cofactors that catalyze the interconversion between the ATP and ADP states. Among these factors are the DnaJ and GrpE proteins of Escherichia coli (15, 20–24). DnaJ accelerates the rate of ATP-hydrolysis of the bacterial Hsp70, DnaK, whereas GrpE promotes nucleotide exchange (15, 21, 22). Acting in concert, DnaJ and GrpE stimulate the ATPase activity of DnaK by up to 50-fold or more (22). In addition, DnaJ has the ability to bind unfolded polypeptide on its own (20, 21, 23, 24) and may target a substrate protein to DnaK in its ATP-bound state. This leads to the formation of a ternary complex of DnaJ, DnaK (in the ADP state), and polypeptide (21, 23). The complex dissociates upon GrpE-catalyzed ADP release and subsequent ATP rebinding. At this point, substrate protein is set free and has the option to fold, to be transferred to another chaperone system, or to bind back to DnaJ and DnaK (3).

The functional regulation of the eukaryotic Hsp70 system is less well understood. Homologs of DnaJ exist in all compartments which contain Hsp70 (25–30), and several typical family members have been identified in mammalian cells (25, 26, 30). However, a eukaryotic GrpE homolog has so far been found only in mitochondria (31). It has therefore been speculated that the Hsp70 proteins in the eukaryotic cytosol may be regulated differently from DnaK. Here, we have characterized the role of the human DnaJ homolog, Hsp40 (Hdj1), in the reaction cycle of Hsc70, the constitutively expressed Hsp70 in the mammalian cytosol. Our results show that Hsp40 stimulates the Hsc70 ATPase by increasing the rate of ATP hydrolysis. Hsp40 is required to allow the efficient, ATP-dependent binding of polypeptide substrate to Hsc70. Thus, the chaperone function...
Regulation of the Hsc70 Reaction Cycle by Hsp40

MATERIALS AND METHODS

Plasmid Constructions

Nontagged Hsp40—The DNA fragment used to introduce an NdeI site at the initiation methionine of Hsp40 was constructed via a polymerase chain reaction (PCR) using an NdeI primer (5′-CGCCGAGAGGGCATATTGGGTAAGGAC-3′) and an NcoI-primer (5′-GGAGGCTTCATGGAAATGCTG-3′). The latter included an NcoI site corresponding to nucleotide 322 of the human Hsp40 cDNA, pBSII-Hsp40 (25), which was used as the template. The PCR-amplified DNA was digested with NdeI and NcoI, and replaced the NdeI-NcoI region of the above plasmid to create the plasmid pET/Hsp40, expressing nontagged Hsp40 (Fig. 1, Hsp40).

Hi-Hsp40—The DNA fragment used to introduce a BamHI site prior to the initiation methionine in pBSII-Hsp40 was constructed via PCR by using two primers, a BamHI primer (5′-CGCCGAGAGGGCATATTGGGTAAGGAC-3′) and an NcoI-primer. The PCR-amplified DNA was digested with BamHI and NcoI and replaced the BamHI-NcoI fragment of pBSII-Hsp40. The entire coding region of Hsp40 was excised with BamHI and PsI and inserted into the complementary sites in pQE-9 (QIAGEN Inc.) to create the plasmid pQE-9/Hsp40, expressing histidine-tagged Hsp40 (Fig. 1, Hsp40).

Hi-Hsp40[O-G/F]—The DNA fragment used to introduce a SalI site at methionine-125 of Hsp40 was constructed via PCR by using a BamHI primer and a SalI primer (5′-GTCATCAATGTCCAGGCCTCCTC-3′) and pBSII-Hsp40 as a template. The PCR-amplified DNA was digested with BamHI and SalI and inserted into the complementary sites in pQE-9 to create the plasmid pQE-9/Hsp40[O-G/F], expressing the truncated protein Hi-Hsp40[O-G/F] (Fig. 1). All DNA fragments amplified by PCR were sequenced. Standard molecular biology techniques were followed as described previously (32) or recommended by the manufacturers.

Protein Purification

To purify nontagged Hsp40, the plasmid pET/Hsp40 was transformed into BL21(DE3) cells grown at 37°C. After a 2-h incubation with 0.4 mM isopropl-1-thio-β-D-galactoside (IPTG), cells were lysed in a French Press cell (SLM Instruments, Inc.) in buffer A (20 mM Tris-HCl, pH 7.5, 20 mM NaCl, 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride. The cleared lysate was mixed with DEAE-Sephalac (Pharmacia Biotech Inc.) on ice for 1 h. The unbound material was filtered and dialyzed against buffer C (20 mM sodium phosphate, pH 7.6. The column was washed with the same buffer, and Hsp40 was collected, and the resin was washed with buffer A. The flow-through and first wash were combined and loaded into a hydroxyapatite HTP column (Bio-Rad) equilibrated with 100 mM potassium phosphate, pH 7.6. The column was washed with the same buffer, and Hsp40 was eluted with a linear gradient of 0–300 mM potassium phosphate, pH 7.6. Peak fractions were chromatographed on an HTP column after passing them through a DEAE-Sephalac column.

To purify the His-tagged proteins, the plasmids pQE-9/Hsp40 and pQE-9/Hsp40[O-G/F] were transformed into S.G31009 cells and grown at 37°C. After a 1-h induction with 0.1 mM IPTG, cells were lysed as above in buffer B (50 mM HEPES-KOH, pH 7.5, 300 mM NaCl) containing 1 mM phenylmethylsulfonyl fluoride. The cleared lysate was loaded onto a Ni2+-NTA agarose column (QIAGEN Inc.) equilibrated with buffer B. The column was washed with buffer B, and proteins were eluted with a linear gradient of 0–500 mM imidazole in buffer B. Peak fractions of Hi-Hsp40 were collected and further purified by a combination of hydroxyapatite and DEAE-Sephalac as described above. Purified Hsp40, Hi-Hsp40, and His-Hsp40[O-G/F] were concentrated by ultrafiltration and dialyzed against buffer C (20 mM sodium phosphate, pH 7.5, 100 mM NaCl, 0.1 mM EDTA). The purified Hi-Hsp40 domain (residues 1–77; Hsp40) was provided by D. McColl (52).

ATPase Assay

Hsc70 and DnaK were incubated for 30 min at 30°C in 50-mM reaction mixtures containing 10 mM MOPS-KOH, pH 7.2, 50 mM KCl, 10 mM MgCl2, and 50 mM [γ-32P]ATP (2.5 μCi; 3000 Ci/mmol, Ames Corp. or DuPont NEN). 2-μl samples were assayed for ADP formation by thin layer chromatography on Polygram CEL 300 polyethyleneimine cellulose plates (Merck). The plates were visualized with autoradiography and quantitated and their radioactivity determined by liquid scintillation counting to determine rates of ATP hydrolysis. To analyze the effects of Hsp40 on the nucleotide-bound state of Hsc70, Hsc70-32P-nucleotide complexes were formed as follows. 17.5 μM Hsc70 was incubated in a 100-μl reaction mixture containing 100 μM [γ-32P]ATP (10 μCi) for 10 min at room temperature and then loaded onto a 1-ml Sephadex G-50 spin column (34). Hsc70-32P-nucleotide complexes were recovered by centrifugation for 2 min and divided into two portions (containing about 3 μM Hsc70), to which Hsp40 (5 μM) and buffer C were added, respectively. After a 1-min incubation at room temperature, Hsc70-bound nucleotide was again separated from free nucleotide by spin-column chromatography. To analyze the dissociation of ADP bound to Hsc70 or DnaK, Hsc70- or DnaK-32P-nucleotide complexes were formed as described above. Within a 1-min incubation of the respective complex with Hsp40 (4.6 μM) or DnaK (4.8 μM) at room temperature, all the nucleotide was converted to ADP (data not shown; cf. Fig. 2C). The obtained Hsc70- or DnaK-[γ-32P]ADP complexes were incubated with 0.1 mM ATP at room temperature for the times indicated, immediately followed by spin-column chromatography to recover the [γ-32P]ADP bound to Hsp40 or DnaK.

Substrate Binding Assay

Peptide C from the vesicular stomatitis virus glycoprotein (KLIGV-LSSLFPRK) was [35S]labeled by reductive methylation. Peptide C (0.8 mg) in 120 μl of 170 mM potassium phosphate, pH 8.0, was incubated with 0.7 μg of [14C]formaldehyde (58 mCi/mmol, DuPont NEN) and 0.2 μl of 1 mM NaBH3CN for 3 h at room temperature. The reaction mixture was subjected to two rounds of gel filtration on a 1-ml Sephadex G-10 spin column to resolute the labeled peptide C. The specific activity of the labeled peptide was 42 cpm/μmol. Hsc70 (5.1 μM) was incubated with the C-peptide C (56 μM) in buffer D (10 mM MOPS-KOH, pH 7.2, 150 mM KCl, 3 mM MgCl2) in the presence of the indicated combinations of Hsp40 (10 μM) and nucleotide (30 μM) for 30 min at 37°C. Hsc70-peptide C complexes were separated from free peptide by gel filtration on 1-ml Sephadex G-50 spin columns. Amounts of Hsc70 were quantitated by densitometry of Coomassie-stained SDS-polyacrylamide gels (PAGE). Hsc70-bound peptide C was quantitated by liquid scintillation counting.

Aggregation Assay by Centrifugation

Purified firefly luciferase (0.1 μM; Sigma) was incubated in buffer E (10 mM MOPS-KOH, pH 7.2, 50 mM KCl, 3 mM MgCl2, 2 mM dithiothreitol) containing the indicated combinations of chaperones (Hsc70, 4.7 μM; Hsp40, 3.2 μM) and ATP (2 mM) for 5 min at room temperature, followed by a 10-min incubation at 42°C. Reaction mixtures were centrifuged at 16,000 × g for 5 min at 4°C. Supernatants, precipitated by trichloroacetic acid, and pellet fractions were analyzed by SDS-PAGE, and the amounts of luciferase were quantitated by densitometry.

Light Scattering Assay

Rhdanese from bovine liver (Sigma) was denatured at 46 μM in a buffer containing 6 mM guanidine-HCl, 30 mM MOPS-KOH, pH 7.2, and 2 mM dithiothreitol by incubation for 30 min at room temperature. Denatured rhodanese was dialyzed 100-fold (0.46 μM final concentration) into buffer E containing the indicated combinations of chaperones (Hsc70, 2.8 μM; Hsp40, 1.8 μM) and nucleotide (2 mM ATP or AMP-PNP). Aggregation was monitored by measuring the turbidity of the solution at 320 nm for 10 min at room temperature.

Complex Formation between Hsc70, Hsp40, and Luciferase

Luciferase (1.1 μM) was incubated in buffer D containing 2 mM dithiothreitol and the indicated combinations of chaperones (Hsc70, 4.7 μM; Hsp40, 3.5 μM) and nucleotides (2 mM ATP, ADP, AMP-PNP, or ATP-γ-S) for 5 min at room temperature followed by a 10-min incubation at 42°C. After removal of large aggregates by centrifugation, supernatants were mixed with 20 μl of Ni2+-NTA-agarose beads on ice for 30 min. Beads were washed sequentially with 500 μl of buffer B containing 3 mM MgCl2 and buffer B containing 3 mM MgCl2 and 50 mM imidazole. When present during complex formation, the respective nucleotides were also added to the wash buffer. Proteins retained on the beads were
were constructed, corresponding to the J-domain plus the G/F
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ulation of Hsp40 to bovine Hsc70 caused a significant stimulation
with His-Hsp40 being equally effective (Fig. 2). The maximum stimulation by Hsp40 was approximately 7-fold

A mixture of [α-32P]ATP- and [α-32P]ADP-bound Hsc70 was isolated (Fig. 2C). When this reaction was incubated with Hsp40 for 1 min, almost all of the Hsc70-bound ATP was converted to ADP (Fig. 2C, lane 3). Reisolation of the Hsc70 revealed that the ADP generated was still bound to the same extent as in the control reaction that had not been incubated with Hsp40 (Fig. 2C, compare lanes 2 and 3 to lanes 4 and 5, respectively). We conclude that Hsp40 does not significantly increase the rate of nucleotide dissociation from Hsc70 but rather catalyzes the hydrolysis of Hsc70-bound ATP to ADP. Thus, Hsp40 functions in a manner similar to E. coli DnaJ (22).

The strong stimulation of the Hsc70 ATPase by Hsp40 alone suggested that the dissociation of ADP may not be the rate-limiting step in the overall ATPase cycle. This was confirmed by the observation that the ADP bound to Hsc70 in the presence of Hsp40 was exchanged for ATP about three times more rapidly than ADP that was bound to DnaK in the presence of DnaJ. The half-time for the dissociation of ADP from Hsc70 was less than 30 s as compared to approximately 90 s in the case of DnaK (Fig. 2D). This explains why Hsp40 alone is able to stimulate the ATPase of Hsc70 without the aid of a GrpE-like factor. Indeed, in the eukaryotic system the dissociation of ADP from Hsc70 is even subject to negative regulation. A novel protein termed Hip (Hsc70 interacting protein) has recently been described that binds the ATPase domain of Hsc70, thereby slowing the dissociation of ADP from Hsc70 (34). Taken together, the rate of nucleotide exchange by Hsc70 appears to be sufficiently fast so that a nucleotide-releasing factor, such as GrpE, may be dispensable.

The interaction between DnaJ proteins and their respective Hsp70 partners is thought to be mediated mainly by the ~70-amino acid residue J-domain of DnaJ proteins. However, as shown for E. coli DnaJ, the J-domain alone is necessary but not sufficient to stimulate the ATPase of DnaK (38, 39). This function requires the G/F-rich segment in addition to the J-domain. Similar observations were made here for the interaction between Hsp40 and Hsc70. Whereas the J-domain of Hsp40 was inefficient in stimulating the hydrolysis of ATP by Hsc70, a mutant protein containing the J-domain plus the G/F-domain caused a significant activation of the ATPase equivalent to about 50% of that seen with full-length Hsp40 (Fig. 3).

Stabilization of Hsc70-Peptide Complexes by Hsp40—Addition of Hsp40 to bovine Hsc70 caused a significant stimulation of the Hsc70 ATPase in a dose-dependent manner (Fig. 2A). The maximum stimulation by Hsp40 was approximately 7-fold with His-Hsp40 being equally effective (Fig. 2B, lanes 2, 8, and 9). DnaJ of E. coli increased the ATPase activity of bovine Hsc70 at lower concentrations than Hsp40 (Fig. 2A), although the maximum stimulation reached with both proteins was similar (Fig. 2B, lanes 2 and 3). Interestingly, the inverse reaction was not observed; Hsp40 hardly stimulated the ATPase activity of DnaK (Fig. 2B, lane 5).

In agreement with previous observations, the steady-state ATPase activity of DnaK was stimulated by DnaJ only 3-fold (Fig. 2B, lane 6), as compared to the 7-fold stimulation of the Hsc70 ATPase by Hsp40. Since full activation of the DnaK ATPase requires the cooperation of DnaJ and the nucleotide exchange factor GrpE (15, 21, 22), it was of interest whether Hsp40 acts on Hsc70 by accelerating the step of ATP hydrolysis or nucleotide exchange, or both. Hsc70 was incubated with [α-32P]ATP for 10 min and then removed from free nucleotide by rapid gel filtration. A mixture of [α-32P]ATP- and [α-32P]ADP-bound Hsc70 was isolated (Fig. 2C). When this reaction was incubated with Hsp40 for 1 min, almost all of the Hsc70-bound ATP was converted to ADP (Fig. 2C, lane 3). Reisolation of the Hsc70 revealed that the ADP generated was still bound to the same extent as in the control reaction that had not been incubated with Hsp40 (Fig. 2C, compare lanes 2 and 3 to lanes 4 and 5, respectively). We conclude that Hsp40 does not significantly increase the rate of nucleotide dissociation from Hsc70 but rather catalyzes the hydrolysis of Hsc70-bound ATP to ADP. Thus, Hsp40 functions in a manner similar to E. coli DnaJ (22).

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Significantly, efficient binding of peptide C to Hsc70 was detected in the presence of ATP upon simultaneous addition of Hsp40. Under the experimental conditions, 40% of the binding capacity of Hsc70 was reached (Fig. 4, lane 5). Hsp40 itself showed no measurable affinity for peptide C in the presence or absence of nucleotide and it seems unlikely that it acquires such an affinity in the presence of Hsc70. It appears more likely that the stabilization of the Hsc70-peptide complex is the result of the stimulation of the Hsc70 ATPase by Hsp40, which generates the ADP-bound state of Hsc70 with high peptide affinity.

Prevention of Protein Aggregation by Hsc70 and Hsp40—The effect of Hsp40 on the binding of denatured polypeptides by Hsc70 was analyzed with thermally unfolded firefly luciferase and chemically denatured rhodanese as the substrates. In the absence of Hsc70 and Hsp40, more than 95% of luciferase aggregated during a 10-min incubation at 42 °C, whether ATP was present or not (Fig. 5A, lanes 7 and 8). However, when both Hsc70 and Hsp40 were present, aggregation was efficiently prevented. Significantly, this chaperone effect was ATP-dependent (Fig. 5A, lanes 1 and 2). Either Hsc70 or Hsp40 alone moderately increased the concentration of soluble luciferase and this effect was enhanced by the addition of ATP (Fig. 5A, lanes 3–6). However, only the luciferase that had been stabilized by both Hsc70 and Hsp40 was competent for subsequent reactivation (see below).

The cooperative effect of Hsc70 and Hsp40 in stabilizing unfolded polypeptide was more clearly observed with rhodanese, a protein that aggregates rapidly upon dilution from guanidinium HCl (43, 44). The combination of Hsc70 and Hsp40 effectively prevented the aggregation of rhodanese, as measured by light scattering (Fig. 5B). This effect was apparently dependent on ATP hydrolysis, because the nonhydrolyzable analog AMP-PNP could not substitute for ATP. Significantly, Hsc70 alone prevented the aggregation of rhodanese only slightly, whereas Hsp40 alone was without a stabilizing effect. We conclude that Hsp40 and ATP are required for the efficient binding of unfolded polypeptide by Hsc70, in particu-
lar with substrates that undergo rapid aggregation. Formation of a Ternary Complex of Hsc70, Hsp40, and Luciferase in the Presence of ATP—The requirement of Hsp40 for efficient substrate binding by Hsc70 suggested the possibility that Hsp40 may induce the formation of ternary complexes containing Hsc70, Hsp40, and unfolded substrate protein. Purified 6His-tagged Hsp40 served to test this hypothesis. Upon incubation at 42 °C, His-Hsp40, Hsc70, and luciferase formed a complex that could be isolated on Ni\textsuperscript{2+}-NTA-agarose (Fig. 6A, lanes 1 and 2). Complex formation was dependent on ATP. His-Hsp40 alone did not stably bind luciferase (Fig. 6A, lane 4) and in the absence of His-Hsp40 neither Hsc70 (data not shown) nor luciferase (Fig. 6A, lane 3) was retained on Ni\textsuperscript{2+}-NTA-agarose. Efficient complex formation apparently required the hydrolysis of ATP, although the complex formed with approximately 40% efficiency in the presence of AMP-PNP. ADP and the nonhydrolyzable analog ATP\textsubscript{gamma}S were without effect (Fig. 6B). Formation of a ternary complex was also observed by size-exclusion chromatography on Sephacryl S-300; in the presence of ATP, a substantial amount of luciferase cofractionated with Hsc70 and Hsp40 as a high molecular mass complex larger than thyroglobulin (M, 670,000) (data not shown), suggesting the presence of small aggregates of luciferase in association with the chaperones. It is possible that in these complexes Hsp40 interacts not only with Hsc70 but also with luciferase.

Reactivation of Thermally Inactivated Luciferase—Although purified Hsc70 and Hsp40 cooperated in an ATP-dependent manner in preventing the formation of insoluble aggregates of luciferase at 42 °C, we did not observe a regain of luciferase activity upon temperature downshift to 30 °C (Fig. 7A, closed triangles). Efficient reactivation with a half-time of \( t_{1/2} \approx 10 \) min occurred, however, when rabbit reticulocyte cytosol was added after the thermal inactivation step. As a prerequisite for successful reactivation, Hsc70, Hsp40, and ATP had to be present during thermal inactivation (Fig. 7A, closed circles). Bovine serum albumin could not substitute for the chaperones during inactivation (Fig. 7B, lane 6). Neither was efficient reactivation observed when the omitted component(s) was added back after thermal inactivation (Fig. 7C). Purified Hip did not show a significant effect on the interaction between Hsc70/Hsp40 and thermally denatured luciferase (data not shown). We conclude that Hsc70, Hsp40, and ATP are necessary during thermal inactivation to maintain luciferase in a soluble, refolding-competent state. A preliminary characterization of the additional
the ribosome or for the efficient binding of Hsc70 to a polypeptide that has been denatured under heat stress.

Our analysis revealed interesting differences in the ATPase cycles of the eukaryotic and prokaryotic Hsp70 systems. Since the spontaneous release of ADP from Hsc70 is more rapid than from DnaK (Fig. 2D), the stimulation of ATP-hydrolysis by Hsp40 is alone sufficient to produce a significant acceleration of the steady-state ATPase of Hsc70. In contrast, the rate-limiting step in the DnaK ATPase cycle is the dissociation of ADP (15, 21, 22), explaining the dependence of the prokaryotic Hsp70 system on an additional nucleotide exchange factor, GrpE. Despite considerable effort, such a factor has not been identified in the eukaryotic cytosol. In fact, the dissociation of ADP from eukaryotic Hsp70 is even subject to negative regulation. The recently identified cochaperone of Hsc70, Hip, stabilizes the ADP-state of Hsc70, thereby probably slowing the dissociation of Hsc70-substrate complexes (34). These observations suggest that Hsp40 may be sufficient to accelerate the Hsc70 ATPase for efficient binding of substrate proteins. Other eukaryotic DnaJ homologs, including Saccharomyces cerevisiae Ydj1p (45–47) and human Hsj1 (48), have also been reported to stimulate the ATPase activity of Ssa1p and Hsc70, respectively, without the aid of a GrpE-like factor. The nearly complete S. cerevisiae genome contains no sequence homolog of GrpE except for mitochondrial GrpE. Likewise, a recently published list of human cDNA sequences contains only one sequence coding for a putative GrpE homolog, which probably resides in mitochondria (49). In light of the apparent dispensability of a GrpE-like protein in the cytosol, it may be relevant that the cytosolic Hsp70s of eukaryotes contain the conserved C-terminal tetrapeptide EEVD, which has been implicated in the intramolecular regulation of Hsc70 function and in its interactions with Hsp40 (18).

The N-terminal J-domain is the signature domain of all members of the DnaJ family and is thought to be largely responsible for the interaction between DnaJ homologs and their Hsp70 partners (26–29). The three-dimensional structure of the J-domain consists of a scaffolding of four α-helices with a conserved tripeptide His-Pro-Asp located in a solvent-accessible loop region (36, 37, 52). Substitutions within this tripeptide abrogate the interaction with DnaK (38). Interestingly, Hsp40 was unable to stimulate the ATPase of DnaK, whereas E. coli DnaJ has the capacity to activate the ATP-hydrolytic activity of mammalian Hsc70. The structural basis for this differential behavior is not yet understood. The J-domain of Hsp40 alone was not sufficient to activate the ATPase of Hsc70. A similar finding has been made for DnaK with the J-domain of DnaJ (38, 39). Only when the J-domain was combined with the adjacent ~30 residue G/F region could the ATP-hydrolysis stimulating activity be measured (this study) (38, 39). On the other hand, an internal deletion of the G/F region from DnaJ did not abolish its ATPase stimulating activity (50). Furthermore, the NMR solution structures of the J-domain of DnaJ both with and without the G/F region revealed no significant conformational differences in the J-domain (36, 37). Thus, the G/F region may have a more unspecific stabilizing effect on the J-domain, maintaining it in a conformation suitable for the interaction with Hsc70.

The stimulation of the Hsc70 ATPase by Hsp40 resulted in a significant enhancement of the substrate binding activity of Hsc70 in the presence of ATP. Rapid peptide binding is known to occur in the ATP-bound state of Hsp70, but the complex is only stable when the ADP-state of Hsp70 is generated (this study) (12, 15, 21, 23). Whereas free peptide could also form a complex with the ADP-bound or nucleotide-free forms of Hsc70 directly, the full requirement of ATP and Hsp40 for substrate
Regulation of the Hsc70 Reaction Cycle by Hsp40

binding became apparent with completely unfolded polypeptide substrates that undergo rapid aggregation. Rapid binding of Hsp70 may be particularly relevant during the exposure of cells to elevated temperatures, as shown here for firefly luciferase. The ADP-bound and nucleotide-free states of Hsp70 were not capable of preventing the aggregation of the unfolded protein, apparently because their on-rate for binding is too slow. We conclude that the Hsp40-catalyzed conversion of Hsc70-bound ATP to ADP is absolutely critical for the binding of aggregation-prone substrates to Hsc70 and thus for the physiological function of the Hsc70 system, both during translation and under conditions of cellular stress.

E. coli DnaJ (21, 23, 24, 39) and the DnaJ homolog Ydj1p of S. cerevisiae (51) have the capacity to bind unfolded proteins and are thus classified as molecular chaperones. It has been proposed that during the DnaK/DnaJ/GrpE reaction cycle DnaJ binds first to the unfolded polypeptide substrate and presents it to ATP-bound DnaK (15, 21). Interestingly, the substrate binding specificity of DnaJ differs from that of DnaK in that DnaJ is apparently unable to bind extended peptide segments but rather interacts with protein folding intermediates that expose hydrophobic surfaces (23). We were unable to convincingly demonstrate a similar capability of Hsp40 to bind purified unfolded polypeptide (Figs. 5–7). On the other hand, nascent polypeptide chains have been communoprecipitated with antibodies against Hsp40 from a reticulocyte lysate translation, even under conditions where Hsc70 was relatively depleted (35). It is thus likely that Hsp40 has at least a weak affinity for unfolded polypeptide that may explain the observed formation of ternary complexes between unfolded luciferase, Hsp40 and Hsc70 (Fig. 6). In support of this notion, the deletion mutant of Hsp40 containing the J-domain and the G/F region retained a partial activity to stimulate the Hsc70 ATPase but has lost the ability to form a ternary complex with substrate protein and Hsc70. Thus, the C-terminal region of Hsp40 is likely responsible for this activity. This part of the molecule is distinct from the corresponding region of DnaJ in that it lacks the ~80 residue cysteine-rich zinc finger domain that has recently been shown to mediate binding of unfolded polypeptides (39). This may be responsible for the strongly reduced activity of Hsp40 to stabilize unfolded polypeptide in the absence of Hsc70. It is of interest in this respect that Hdj2, the other DnaJ homolog in the mammalian cytosol (26), contains a cysteine-rich central domain. The functional cooperation between Hsc70 and Hdj2 has not yet been analyzed but we speculate that Hdj2 may have chaperone properties similar to those of E. coli DnaJ.

We have recently reported that Hsc70 and Hsp40 can cooperate with Hip in refolding denaturant-unfolded luciferase in an ATP-dependent manner (34). Increasing the concentrations of Hsc70 and Hsp40 relative to luciferase can compensate for the requirement of Hip. Hip has been shown to mediate binding of unfolded polypeptides (39). This may be responsible for this activity. This part of the molecule is distinct from the corresponding region of DnaJ in that it lacks the ~80 residue cysteine-rich zinc finger domain that has recently been shown to mediate binding of unfolded polypeptides (39). This may be responsible for the strongly reduced activity of Hsp40 to stabilize unfolded polypeptide in the absence of Hsc70. It is of interest in this respect that Hdj2, the other DnaJ homolog in the mammalian cytosol (26), contains a cysteine-rich central domain. The functional cooperation between Hsc70 and Hdj2 has not yet been analyzed but we speculate that Hdj2 may have chaperone properties similar to those of E. coli DnaJ.

Although Hsc70 and Hsp40 protected luciferase from aggregation at elevated temperature by forming a ternary complex, the bound luciferase was unable to refold upon temperature

2 Y. Minami and F.-U. Hartl, unpublished results.

omitted component(s) (after thermal inactivation). Luciferase activities were measured 60 min after addition of 2.5% reticulocyte lysate and 2.5 mM ATP.
downshift (Fig. 7). Only upon addition of reticulocyte cytosol was the efficient renaturation of this protein observed. Preliminary experiments to purify this factor showed that the refolding-stimulating activity fractionates at ~600 kDa on a size-exclusion column, distinct from the large chaperonin TRiC. It may represent a new chaperone activity or a combination of known chaperone components that remains to be defined.

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