A Mutational Study of the Peptide-binding Domain of Hsc70 Guided by Secondary Structure Prediction

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The abundant, cytoplasmic molecular chaperones of eukaryotic cells, of which mammalian Hsc70 is a member, have central roles in protein folding pathways in cells. Although substantial information is now available on substrate interactions and ATPase activity, neither the crystal structure of the intact Hsc70 molecule nor its isolated peptide-binding domain is known. Recently, the crystal structure of the isolated peptide-binding domain of an evolutionary relative of mammalian Hsc70, the DnaK protein of Escherichia coli, was solved. We have generated several rat Hsc70 mutants using site-directed and cassette mutagenesis guided by secondary structure predictions to test the hypothesis that the peptide-binding domains of mammalian Hsc70 and DnaK have similar molecular structures. Biochemical properties along with the ATPase and peptide binding activities of the resulting recombinant proteins were determined. Biochemical analyses included one- and two-dimensional gel electrophoresis, electrospray mass spectrometry, and N-terminal amino acid sequencing. The results of our study suggest that the DnaK molecular structure is a useful working model for the mammalian Hsc70 peptide-binding domain. Evidence is provided that (i) small additions to the N terminus of Hsc70 alter the function of the peptide-binding domain, (ii) alterations in the C-terminal tetrapeptide EEVD result in dramatic increases in basal ATPase activity, (iii) polyalanine substitution of a helical connector segment compensates for changes at the C terminus to restore near-normal function, (iv) specific side chain interactions involving this connector segment are not required for peptide-stimulated ATPase activity, and (v) disruption of the cap homologue region inhibits peptide binding by Hsc70.

The molecular chaperone Hsc70 of mammalian cells is an abundant, cytoplasmic protein that binds to nascent polypeptides, accompanies proteins to sites of membrane translocation and assembly, and may also deliver proteins targeted for degradation to lysosomes and proteasomes (1, 2). All of these functions depend upon the ability of Hsc70 to preferentially bind unfolded proteins. Hsc70 selects peptides enriched in large neutral and aromatic amino acid residues from peptide phage display libraries, and these peptides are thought to represent the hydrophobic binding sites on unfolded proteins recognized by Hsc70 (3). A second peptide motif containing hydrophobic and basic residues is also selected from these libraries by Hsc70 and may represent a different kind of chaperoning function (4).

X-ray diffraction studies have revealed an ATPase domain (residues 1–386, 44-kDa fragment) consisting of four subdomains arranged in a bilobed structure with a single ATP/ADP binding site located in a deep cleft between these lobes. The N terminus and the C terminus of the ATPase domain are located close together on one face of this domain, which may interact with the peptide-binding domain (5). However, since the intact protein has not been crystallized, the relationship between the two domains is undefined. That the two domains interact has been clear from the earliest studies showing that clathrin (6, 7) and bound peptide/unfolded proteins stimulate Hsc70 ATPase activity (8, 9). The isolated 44-kDa fragment has elevated, unregulated ATPase activity (10), suggesting that the peptide-binding domain acts as a brake. The conformations as well as the activities of the two domains are coupled (11).

Additional evidence of overall changes in conformation as well as the overall shape of the molecule came from solution small-angle x-ray scattering studies of recombinant bovine Hsc70 and derived fragments (12). These data suggest that the peptide-binding domain is relatively flat and may interact either end-to-end or side-by-side with the ATP-bound form of the ATPase domain. In the model for the ADP-bound form of Hsc70, the molecule is longer with less interaction between the two domains. Much of this analysis was carried out using an N-terminal 60-kDa fragment. In another study, a comparison of tryptophan fluorescence emission spectra of recombinant human Hsp70 and a deletion mutant missing the C-terminal EEVD indicated a change in conformation of the mutant protein. Increases in both Stokes radius and frictional ratio accompanied this change (13), suggesting that the interaction of EEVD with the ATPase domain may limit the full expansion of the 60-kDa fragment modeled in the x-ray scattering studies.

A glutathione S-transferase fusion protein containing the 18-kDa segment (residues 384–543) following the N-terminal ATPase domain had affinities for several peptides similar to intact Hsc70 (14). Therefore, the peptide binding domain of Hsc70 resides in this segment, which consists of two four-stranded anti-parallel β-sheets and a single α-helix, as determined with multidimensional NMR (15). These data rule out a model for the peptide-binding domain of Hsp70 family proteins based on the peptide-binding region of the major histocompatibility complex class I molecules which predicted two α-helices and seven β-strands connected in a different order (16, 17). The secondary structure elements identified by NMR in Hsc70 have also been identified in the crystal structure at 2.0-Å resolution of the peptide-binding fragment (residues 389–607) of DnaK bound to a heptameric peptide (18). The overall structure is about 60 by 40 by 15 Å which agrees well with the shape of the Hsc70 peptide-binding fragment deduced from small-angle scattering measurements (12). The peptide-binding domain
Mammalian Molecular Chaperone Hsc70

consists of two four-stranded β-sheets arranged like the palms of clasped hands with two of the connecting loops holding the peptide and two loops supporting the two binding loops. The core binding site is hydrophobic as expected with some negatively charged surface at each end, which explains why basic residues are allowed and negative residues excluded at the ends of the peptide substrates favored by DnaK. A long, bent helix connects the molecular hands to a cap structure composed of the C-terminal part of the long helix joined by three short helices. A second crystal structure was determined in which the cap structure is tilted up by a kink in the long helix at about position 537, suggesting that movement of the cap is part of the conformational changes that occur in the molecule.

The CD spectra of DnaK and bovine Hsc70 are almost superimposable, indicating substantial overall structural similarity (19). Encouraged by this comparison, we tested several predictions of the DnaK crystal structure using mutated forms of Hsc70. The placement of restriction sites to create these mutants was guided by secondary structure predictions (20) that were subsequently verified in part by NMR studies (15).

EXPERIMENTAL PROCEDURES

Materials—[3H]NaBH₄ (28 Ci/mmol) was from Amersham Corp. γ-[32P]ATP (3000 Ci/mmol) was from NEN Life Science Products. All restriction endonucleases and modifying enzymes were from Boehringer Mannheim.

Plasmid Constructions—The rat hsc70 gene was cloned into the NcoI and HindIII sites of the expression vector pKK233-2 (Pharmacia Biotech Inc.) and was designated pKS1 (see Table I for all plasmid/clone designations). The hsc70 sequence originated from a full-length cDNA in the plasmid pRC62 (21) which was obtained from Dr. Larry Kedes (Stanford University, CA).

The secondary structure of rat hsc70 has been predicted using circular dichroism spectroscopy analyzed with deconvolution methods and the Chou-Fasman and Garnier-Osguthorpe-Robson algorithms (20). The amino acid sequence of each α-helix is shown in bold. Each restriction site is marked, and the new nucleotide and restriction site are shown below.

Fig. 1. The nucleotide and corresponding amino acid sequence of Hsc70 helices α-helix 1 (αh1) and α-helix 3 (αh3), predicted by secondary structure algorithms (20). The amino acid sequence of each α-helix is shown in bold. Each restriction site is marked, and the new nucleotide and restriction site are shown below.

The substitution of amino acids 572–599 deleted (pKS1ah3) was generated by digesting pKS1+ with XbaI and XhoI, two of the unique sites incorporated by mutagenesis. This segment was removed, and the DNA was ligated after filling the 5′ ends with Klenow. This blunt ending resulted in the addition of a valine residue. Site-directed and cassette mutagenesis were carried out essentially as described by others (22, 23).

Protein Purification—LB supplemented with either ampicillin (100 mg/liter) or kanamycin (25 mg/liter) was used to propagate the plasmid DNA in the host Escherichia coli strain SGI2036 (Agal, Mon-510, sulA). The cells were incubated at 30 °C on a shaker, and protein expression was induced by the addition of 4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) after the A₅₀₀ reached approximately 0.6. The incubation was continued for an additional 3 h after which the cells were collected by centrifugation and washed with ice-cold buffer I (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM dextrose). After re-centrifugation the pellet was resuspended in buffer I supplemented with the following protease inhibitors: 4-(2-aminoethyl)-benzenesulfonylfluoride, phenylmethylsulfonyl fluoride, leupeptin, pepstatin, and bestatin. All protease inhibitors were obtained from Boehringer Mannheim except 4-(2-aminoethyl)-benzenesulfonylfluoride which was obtained from Calbiochem. Unless otherwise specified, all subsequent steps were carried out at 4 °C. Cells were lysed by sonication after treatment with lysozyme. The supernatant was recovered and dialyzed overnight against buffer C (20 mM Hepes, pH 7.0, 10 mM ammonium sulfate, 25 mM KCl, 2 mM magnesium acetate, 0.1 mM EDTA, and 0.1 mM dithiothreitol) without magnesium acetate. This dialysis step was included to facilitate the removal of Mg²⁺/ATP that may be bound to Hsc70, thereby allowing maximum binding to ATP-agarose. Nonfusion proteins were purified by sequential chromatography using ATP attached to agarose. Nonfusion proteins were purified by sequential chromatography using ATP attached to agarose. Nonfusion proteins were purified by sequential chromatography using ATP attached to agarose. Nonfusion proteins were purified by sequential chromatography using ATP attached to agarose.
terminal fusions), 29,300 \text{ m}^{-1} \text{ cm}^{-1} (\text{Hsc70oh1-Ala/N-} \text{ and C-terminal His}), \text{ and } 24,890 \text{ m}^{-1} \text{ cm}^{-1} (\text{Hsc70ah3}). \text{ The molar extinction for native bovine Hsc70 (Hsc70bc) was determined by others (25), and the values for the mutants were obtained by correcting for changes in amino acid composition.}

\textbf{ATPase Assays—}\text{ATPase assays were carried out as described previously (8). Briefly, 50-\mu l reactions were assembled containing 2.5 } \mu \text{g of Hsc70, a final concentration of } 50 \mu \text{M ATP, } 20 \mu \text{g of apocytochrome } c \text{ (apoc), } 2 \mu \text{Ci of } [\gamma-\text{P}]\text{ATP, and } 1 \times \text{ buffer C. The basal rate of ATP hydrolysis catalyzed by Hsc70 was measured in the absence of apoc. Aliquots were removed at 5-min intervals from 0 to 20 min and added to acidic, activated charcoal (50 mM HCl, 5 mM H}_3\text{PO}_4, \text{ and } 7\% \text{ (w/v) activated charcoal). Acidified, activated charcoal effectively quenches the reactions and inhibits the enzyme activity after the sample is removed. During a subsequent 1-h incubation at room temperature, no significant hydrolysis of the } [\gamma-\text{P}]\text{ATP occurred.}

\textbf{Peptide Binding Assays—}\text{The peptide binding assays, carried out essentially as described by others (26), used increasing concentrations of a tritiated fragment of pigeon cytochrome } c \text{ (amino acids 81–104, described below). 50-\mu l reactions contained } 1 \times \text{ binding buffer (50 mM Tris, pH 7.5, 150 mM NaCl, } 0.1 \text{ mM EDTA), } 2 \mu \text{g of purified Hsc70, and } [\text{H}]\text{cytochrome } c \text{ peptide ranging from 300 to 1.56 } \mu \text{Ci with } 2\text{-fold serial dilutions. The reactions were incubated in a } 37 ^\circ \text{C water bath for 30 min after which free peptide was removed with Sephadex G-50 quick spin columns (Pharmacia). These columns were pre-equilibrated with binding buffer and blocked with } 100 \mu \text{l of a solution of bovine serum albumin (1 mg/ml).}

\textbf{Peptide Labeling—}\text{The cytochrome } c \text{ peptide was labeled by reductive methylation with tritiated sodium borohydride, chemistry specific for the } \alpha\text{-amino groups of peptides, and } \varepsilon\text{-amino groups of lysyl residues. The amino acid sequence of this peptide is as follows: IFAGIKKKRANAA-DLIAAYLKQATAK. Methylation was carried out essentially as described by others (27). The final sample was purified with Sephadex G-10 (Pharmacia). Fractions that showed a peak of radioactivity coincident with a maximum absorbance at 214 nm were pooled.}

\section{RESULTS}

\textbf{Purification and Activities of Wild-type Hsc70—}\text{Several different rat Hsc70 mutants were created using site-directed and cassette mutagenesis (Table I). All proteins were purified to homogeneity as described in detail under “Experimental Procedures.” Nonfusion proteins were purified in successive steps of ATP-agarose, Q-Sepharose, and size exclusion chromatography (Fig. 2A). To assess the biological activities of the various proteins, ATPase and peptide binding assays were carried out as described under “Experimental Procedures.” Wild-type Hsc70 encoded by DNA containing new restriction sites (Hsc70+1) expressed in E. coli had elevated intrinsic ATPase activity compared with native Hsc70bc, with rates of 10.0 and 0.96 pmol/min/\mu g, respectively (Table II). This elevated intrinsic activity was similar to that observed for recombinant Hsc70bc encoded by DNA without the new restriction sites (data not shown), so the single amino acid change introduced by a new restriction site was not responsible for this elevated activity. Since recombinant Hsc70bc and Hsc70+ were similar with regard to ATPase activity and peptide binding, we used

![Image](328x472 to 543x594)

\begin{table}[h]
\centering
\caption{Plasmid/protein designations and descriptions}
\begin{tabular}{|c|c|c|}
\hline
Plasmid & Protein & Description \\
\hline
pBC62 & Hsc70bc & Bovine brain Hsc70 \\
pKS1 & Hsc70 & Wild-type (wt) \\
pKS1 & Hsc70 & Wild-type \\
pKS1oh1-Ala & Hsc70oh1-Ala & wt, restriction sites + (rs+) \\
pKS1oh3 & Hsc70oh3 & rs+, deletion of \alpha-helix 1512-536 \\
pQ60 & Hsc70/N-His & rs+, \alpha-helix 1 = poly(A), N-terminal His tag \\
pQ60h1-Ala & Hsc70oh1-Ala & wt, rs+, C-terminal His tag \\
pQ60h1-Ala & Hsc70oh1-Ala/C-His & rs+, \alpha-helix 1 = poly(A), C-terminal His tag \\
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\end{tabular}
\end{table}
Cever, the placement of a His 6 tag at the N terminus had a drastic effect on peptide binding. The binding curve (Fig. 3B) had essentially the same plot). C, Hsc70/N-His. D, Hsc70oh1-Ala/C-His. In the axis titles, B = bound peptide concentration and F = free peptide concentration.

GraphPad Prism 2.0 (Fig. 3A). Both Hsc70+ and Hsc70bc had very similar curves that fit a one-site binding model with a $K_D$ of 16.9 $\mu$M, as calculated for Hsc70bc. The dissociation constant ($K_D$) determined by Scatchard analysis for Hsc70+ was 22.2 $\mu$M, essentially the same as for Hsc70bc at 17.5 $\mu$M (Table II and Fig. 3B). Thus, the ATPase and peptide-binding domains of Hsc70+ were active but uncoupled, with no stimulation of ATPase by bound peptide and an elevated, unregulated ATPase activity similar to the 44-kDa fragment described under the Introduction.

Purification and Activities of Hsc70 Fusion Proteins—To develop a general purification scheme for mutant proteins, regardless of their affinity for ATP-agarose, the various constructs were cloned into vectors encoding a six-residue histidine tag (His6) for nickel chelation chromatography. His-tagged fusion proteins were isolated using a nickel-agarose affinity step followed by Q-Sepharose and size exclusion chromatography (Fig. 2B). The His6 tag, when placed at the N terminus of Hsc70, had little effect on ATPase activity, which remained insensitive to the addition of apoprotein (Table II). However, the placement of a His6 tag at the N terminus had a drastic effect on peptide binding. The binding curve (Fig. 3A) could not be fit to a one-site model and was best fit by a two-phase exponential function. However, the first five points could be fitted to a one-site model with a calculated $K_D$ of 52 $\mu$M, whereas the fit to all seven points (not shown) yielded a calculated $K_D$ of 920 $\mu$M and no indication of saturable binding, suggesting a nonspecific interaction. Scatchard analysis of the binding assays yielded a nonlinear plot for Hsc70/N-His consisting with the presence of either two or more binding sites or multiple forms of Hsc70/N-His with at least 10-fold differences in affinity (Fig. 3C). An explanation for the complex binding curve is proposed under “Discussion.”

Due to the altered peptide binding caused by the His6 tag at the N terminus, a recombinant protein was engineered with the His6 tag at the C terminus. The binding data for this protein, Hsc70/C-His, could be fit to a one binding site model with a calculated $K_D$ of 32 $\mu$M (Fig. 3A), and it had a linear Scatchard plot with a $K_D$ of 62.9 $\mu$M (Fig. 3D), indicating that the C-terminal modification was less disruptive to the peptide-binding domain than the N-terminal modification. Basal ATPase activity was lower than that of Hsc70+, and peptide-binding caused a small stimulation in ATP hydrolysis, suggesting that the two domains were weakly coupled in Hsc70/C-His.

Biochemical Comparisons of Native, Wild-type, and Hsc70 Fusion Proteins—The biological activities of native Hsc70bc and E. coli-expressed Hsc70+ were clearly different. Therefore, we considered the possibility of differences in posttranslational modification of these proteins. To address this question, Hsc70 from rat embryo cell lysates was compared with rat Hsc70+ using two-dimensional polyacylamide gel electrophoresis (Fig. 4). The pI of Hsc70+ was more basic than that of Hsc70 isolated from rat embryo cells.

To investigate further this change in pI and to further characterize the fusion proteins, mass spectrometry and N-terminal sequencing were used (Table III). The molecular mass determinations from electrospray mass spectrometry for Hsc70bc and the C-terminal His6 tagged-proteins agreed with the molecular weights calculated from amino acid compositions. However, the masses of the Hsc70+ and N-terminal His6 tagged-proteins were smaller than expected. The results from protein sequencing indicated that the N termini of these proteins were intact, suggesting that amino acids were missing from the C terminus. This conclusion is consistent with the two-dimensional gel analysis of Hsc70+. The C terminus of native Hsc70 is an acidic EEVD tetrapeptide. Taken together with the mass spectrometry, these data were consistent with the loss of the last three amino acids, including two acidic residues, from Hsc70+ resulting in a more basic pI. The mass spectrometry data further indicated that the addition of a His6 tag to the C terminus of Hsc70+ protected the EEVD tetrapeptide.

The purified proteins were also analyzed using non-denaturating polyacylamide gels (Fig. 5). Purified Hsc70bc was separated into two major bands, previously identified as monomer and dimer (lanes 2 and 9). Hsc70+ had essentially the same pattern (lane 3) as did Hsc70/C-His (lane 4) except that both monomer and dimer of the latter protein migrated more slowly. Hsc70/N-His monomer migrated like Hsc70bc monomer; however, there was no band with the same mobility as Hsc70bc dimer and a slower migrating band was detected instead (lane 7). Thus, the N-terminal His6 tagged-protein was altered in oligomer formation as well as peptide binding.

Analysis of Hsc70 Constructs to Test Predictions Based on the DnaK Molecular Structure—The peptide-binding domain of DnaK is comprised of two subdomains. The first of these is formed by two sets of four anti-parallel $\beta$-strands. Each set forms a sheet and together they form a $\beta$-sandwich. The second subdomain contains five $\alpha$-helices, two of which together serve as a connector and three form a cap that covers the substrate bound in a channel created mainly by two loops of the $\beta$-sandwich. The first construct that was generated contained a polyalanine substitution for amino acids 512–533 (residues 509–530 of helices $\alpha$A-$\alpha$B in DnaK), a segment corresponding to the
TABLE III

| Protein            | Massa | Mass spectrometryb | N terminusc | Conclusion              |
|--------------------|-------|--------------------|-------------|-------------------------|
| Hsc70bc            | 71,241| 71,235             | ND          | As expected             |
| Hsc70              | 70,832| 70,482             | Intact      | C-terminal processing   |
| Hsc70/N-His        | 72,254| 71,884             | Intact      | C-terminal processing   |
| Hsc70/C-His        | 71,921| 71,794             | Missing N-terminal Met | As expected without NMet |
| Hsc70h1-Ala/C-His  | 70,622| 70,669             | ND          | As expected             |

a Molecular mass based on expected amino acid sequence.
b Electrospray mass spectrometry.
c N-terminal amino acids as determined by N-terminal amino acid sequencing; ND, not done.

![Fig. 5. Composite of 2-15% non-denaturing gels comparing native and mutant Hsc70. Lane 1, bovine serum albumin monomer/dimer standard; lane 2, Hsc70bc standard; lane 3, Hsc70+; lane 4, Hsc70/C-His; lane 5, Hsc70oh1-Ala/N-His; lane 6, Hsc70oh1-Ala/C-His; lane 7, Hsc70N-His; lane 8, Hsc70ah3; lane 9, Hsc70bc standard; lane 10, bovine serum albumin monomer/dimer standard.](image)

two α-helices which serve as a connector between the cap and β-sandwich of DnaK. This segment, marked α-helix 1 in Fig. 1, was predicted using a secondary structure algorithm (20) and later confirmed by NMR analysis (15). A polyanaline segment was chosen as a replacement because alanine residues occur frequently in α-helices and they are good helix formers (28). Therefore, substituting with polyanaline should retain the helical structure of the connector while eliminating ionic and polar side chain interactions. If the only role of this segment is to hold the cap in proximity to the peptide binding site in both Hsc70 and DnaK, then this relatively flexible polyanaline segment might allow at least partial function. Circular dichroism (CD) spectra were almost identical for the polyanaline substitution mutants and Hsc70bc (data not shown), consistent with the interpretation that one helical segment was replaced by another. Second prediction that the polyanaline connector and cap are less constrained conformationally than the normal segment appears to explain the behavior of these proteins on nondenaturing gels. They migrate more slowly than Hsc70bc and in a diffuse band, consistent with a less compact, more flexible molecular structure (Fig. 5, lanes 5 and 6).

The activities of N- and C-terminal His-tagged proteins containing the polyanaline segment, Hsc70oh1-Ala/N-His and Hsc70oh1-Ala/C-His, were assessed (Table II). The peptide binding and ATPase activities for Hsc70oh1-Ala/N-His were similar to Hsc70/N-His. Peptide binding remained altered, and the domains remained uncoupled. However, Hsc70oh1-Ala/C-His had a Kd of 27 μM for the pigeon cytochrome c peptide, similar to that of Hsc70bc and Hsc70+ (Table II). Not only was peptide binding almost normal, but the ATPase domain was coupled to peptide binding, as shown by an approximately 2-fold stimulation of ATP hydrolysis in the presence of apoc.

A second mutant had a deletion of a segment predicted as α-helix in Hsc70 corresponding to two helices in the cap of DnaK. This mutant protein (Hsc70αh3) lacked residues 572–599, marked as α-helix 3 in Fig. 1 (corresponding to residues 567–591 of helices αC–αD in DnaK). This deletion had no effect on ATPase activity compared with Hsc70+, but it had no detectable peptide binding activity (Table II). On nondenaturing gels, this mutant had two major bands, presumably monomer and dimer, that migrated more slowly than the corresponding forms of Hsc70bc.

**DISCUSSION**

We have expressed, purified, and characterized several mutant rat Hsc70 proteins to gain insight into important features of the peptide-binding domain. Analysis of these mutant proteins has provided a test of the hypothesis that the peptide-binding domains of mammalian Hsc70 and DnaK share major structural features. An initially surprising finding was that E. coli-expressed rat Hsc70+ had elevated ATPase activity relative to native Hsc70bc that was unaffected by peptide binding. Despite this difference, Hsc70+ and Hsc70bc had similar affinities for a cytochrome c fragment, indicating that both domains were functional but that they were uncoupled. In fact, the two domains had properties similar to those described previously for an N-terminal 44-kDa fragment of Hsc70 (10) and an isolated 18-kDa peptide-binding domain (14). Sequencing of the insert in pKS+ (data not shown) confirmed that the primary amino acid sequence of Hsc70+ was identical to rat Hsc70 with the expected exception of the conservative Ser to Thr change at position 537. Peptide sequencing showed that the N terminus of Hsc70+ was intact and not blocked. However, the molecular mass of Hsc70+, as determined by electrospray mass spectrometry, was lower than Hsc70bc by an amount equivalent to approximately three amino acids. The PI of Hsc70+ was more basic than Hsc70bc, consistent with a loss of two acidic residues from the C-terminal EEVD tetrapeptide. We speculate that this modification was caused by a carboxypeptidase activity in E. coli lysates.

Previously, Wang and Lee (30) carried out a detailed characterization of E. coli-expressed rat Hsc70. They also found that their purified protein had an elevated basal ATPase activity, but this activity was stimulated by peptide substrates severalfold. Unlike Hsc70bc, their recombinant Hsc70 was not blocked at the N terminus and did not contain methylated basic amino acid residues. It was suggested that these differences might be responsible for the differences in basal ATPase activity between native and recombinant Hsc70. Interestingly, an N-terminal 60-kDa fragment of their recombinant Hsc70 and the intact protein had virtually the same basal and stimulated ATPase activities and affinities for peptides (31). However, this fragment could not form a stable complex with a larger substrate, S-carboxymethyl α-laetalbumin, and it was suggested that the C-terminal 10-kDa fragment is needed for complexing with larger, unfolded proteins. Peptide-stimulated ATPase activity is maintained in the 60-kDa fragment, indicating that neither EEVD nor the rest of the 10-kDa C-terminal segment is required for domain coupling. However, our results with Hsc70+ suggest that changes in EEVD can cause a dramatic increase in the basal activity of the ATPase domain that re-
sembles the effect on ATPase activity when the two domains are proteolytically cleaved to form the N-terminal 44-kDa fragment. Further support for this conclusion came from analysis of a recombinant Hsc70 containing a His\textsubscript{9} C-terminal addition (Hsc70/C-His). The added histidines protected EEVD, which remained intact, and the basal ATPase activity was lower than for Hsc70+ and showed a small peptide-stimulated activity. Thus, Hsc70/C-His behaved more like Hsc70bc, although this construct had a modest loss of binding affinity. Freeman and co-workers (13) found that deletion of EEVD in recombinant human Hsc70 and human-inducible Hsp70 lowered binding affinity for reduced, carboxymethylated lactalbumin, although the effects on Hsc70 were considered weaker. The most dramatic changes were obtained with mutants in which AAAD and AAAA were substituted for EEVD in Hsp70. These mutants had basal ATPase activities (7–8 pmol/min/μg) similar to our Hsc70+/− and only a 50% stimulation by reduced, carboxymethylated lactalbumin. Deletion of EEVD resulted in a marginal increase in ATPase activity which could be stimulated 2-fold by reduced, carboxymethylated lactalbumin. Thus, in agreement with Hu and Wang (31), EEVD is not required for coupled domains, and in agreement with our studies, certain changes in this tetrapeptide result in dramatic increases in basal ATPase activity. Unfortunately, the crystal structure of the DnaK peptide-binding domain is of little help since DnaK does not have the EEVD tetrapeptide, the C terminus was not part of the crystallized fragment in any case, and interpretation of this result will require information on the relative positions of the two domains. Interestingly, Freeman and co-workers (13) found that EEVD is required for the activity of Hsp70 in luciferase refolding and for stimulation of Hsp70 ATPase activity by HDJ-1.

Further modification of Hsc70/C-His, by complete replacement of amino acids 512–533 with polyalanine, produced a very interesting mutant, Hsc70h1-Ala/C-His. This mutant had an even lower basal ATPase activity than Hsc70/C-His which was stimulated 2-fold by apoc and had near-native peptide-binding affinity. In effect, it was closer to Hsc70bc than any construct we tested. The polyalanine segment acted like a compensatory change that corrected the alterations caused by addition of His\textsubscript{9} to the C terminus. Reasoning from the DnaK model, changes in both the C terminus and the region of Hsc70 homologous to the long, bent connector helix of DnaK could affect the position and movement of the molecular cap structure. Modification of the C terminus may result in an aberrant positioning of the cap in the ATP-bound configuration of Hsc70, resulting in elevated basal ATPase activity. Substituting the polyalanine segment for the normal connector is likely to produce a more compact and flexible helix, since it would not be constrained by side chain interactions. This compensation may have allowed a more correct positioning of the cap. We can also conclude that side chain interactions in the 512–533 segment are not required for peptide-stimulated ATPase activity. The analogy to the DnaK structure is strengthened by the fact that the polyalanine substitution had only a small effect on peptide binding, and the corresponding segment in DnaK is neither part of the immediate peptide-binding structure nor part of the cap structure.

The mutants in which N termini were modified by His\textsubscript{9} were less informative. As expected, this modification did not protect the C-terminal EEVD, and these mutants had elevated basal ATPase activity with little or no additional stimulation by peptide. The N terminus lies at the surface in the crystal structure of the Hsc70 ATPase domain. The 12 amino acids added to the N terminus had little effect on ATPase activity, but they resulted in altered affinities for peptide substrate, suggesting that they project out of the ATPase domain and impinge upon the peptide-binding domain to alter its conformation. This implies that the side of the ATPase domain containing the N terminus is close to the peptide-binding domain, and in support of this suggestion, the C terminus lies very near the N terminus in the crystal structure of the 44-kDa fragment. Both the binding curve and subsequent Scatchard analysis are consistent with the presence of a binding site with a nearly wild-type affinity and a second, very low affinity site. We suggest that there may be two conformations of Hsc70/N-His in these preparations, an abundant one in which the ATPase and peptide-binding domains are closely apposed giving maximal interference by the N-His tag, and a less abundant conformation in which the two domains are farther apart with only minor interference by the N-His tag. The existence of two conformations of Hsc70bc with different degrees of domain interaction is supported by solution small-angle x-ray scattering studies (12). Hsc70/N-His was altered in oligomer formation as well as peptide binding. This observation fits well with the recent report from Ladjimi and co-workers (29) that the peptide-binding domain is essential for self-association of Hsc70. Interestingly, inclusion of the same polyalanine substitution as described above in this mutant (Hsc70oh1-Ala/N-His) lowered the basal ATPase activity, again acting like a compensatory change for alterations in the C-terminal EEVD. Peptide binding remained aberrant, presumably due to the N-terminal tag.

The second Hsc70 mutant used to test predictions of the DnaK model was Hsc70h1-Ala3, which has a deletion from amino acids 572 to 579. This deletion is predicted to remove a segment of Hsc70 corresponding to most of two short helices that are part of the cap structure of DnaK. This deletion did not affect ATPase activity, which was the same as Hsc70+. However, it was the only mutant tested in which no peptide binding could be detected. The crystal structure of DnaK shows that the cap is positioned over the bound peptide, but it does not directly contact it. Instead it interacts with the loops that hold the peptide, apparently contributing to the stability of the interaction. Our interpretation of the behavior of this Hsc70 mutant is that the cap does not fold properly without the missing helices, and the unfolded segment collapses onto the peptide binding site, blocking access by incoming peptides. The alternative interpretation that the deleted segment is directly involved in peptide binding in Hsc70 is ruled out by the fact that it is not part of the 18-kDa peptide-binding fragment.

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