Destabilization of Peptide Binding and Interdomain Communication by an E543K Mutation in the Bovine 70-kDa Heat Shock Cognate Protein, a Molecular Chaperone*

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We have compared 70-kDa heat shock cognate protein (Hsc70) isolated from bovine brain with recombinant wild type protein and mutant E543K protein (previously studied as wild type in our laboratory). Wild type bovine and recombinant protein differ by posttranslational modification of lysine 561 but interact similarly with a short peptide (fluorescein-labeled FYQLALT) and with denatured staphylococcal nuclease-(Δ135–149). Mutation E543K results in 4.5-fold faster release of peptide and lower stability of complexes with staphylococcal nuclease-(Δ135–149). ATP hydrolysis rates of the wild type proteins are enhanced 6–10-fold by the addition of peptide. The E543K mutant has a peptide-stimulated hydrolytic rate similar to that of wild type protein but a higher unstimulated rate, yielding a mere 2-fold enhancement. All three versions of Hsc70 possess similar ATP-dependent conformational shifts, and all show potassium ion dependence. These data support the following model: (i) in the presence of K⁺, Mg²⁺, and ATP, the peptide binding domain inhibits the ATPase; (ii) binding of peptide relieves this inhibition; and (iii) the E543K mutation significantly attenuates the inhibition by the peptide binding domain and destabilizes Hsc70-peptide complexes.

The 70-kDa heat shock-related proteins (Hsp70s)¹ comprise a family of molecular chaperones that bind and release unstructured, hydrophobic segments of polypeptide in an ATP-dependent manner, thereby presumably suppressing intramolecular aggregation and intramolecular misfolding that might otherwise occur. The binding and release of peptide is regulated by nucleotide; with MgADP bound, Hsp70s form stable, long lived complexes with peptides with half-lives on the order of 10³ to 10⁴ s, whereas when MgATP binds, the peptide-Hsp70 complexes become more labile and peptide is released (1–5). The coupling between peptide binding/release and the ATPase cycle also manifests itself through peptide-dependent enhancement of the ATPase activity (1–12).

In addition to binding unstructured segments of polypeptide, Hsp70s self-aggregate into dimers and higher order oligomers (13). Self-aggregation can be reversed by the addition of ATP or the addition of competing peptides and hence is thought to be similar to, and competitive with, the heterologous peptide binding activity of the proteins (8). In certain contexts, some Hsp70s are postranslationally modified; phosphorylation of threonine residues (14–23), ADP-ribosylation (14, 24), and methylation of lysine and arginine (25) have been reported; however, the functional significance of these modifications is not known.

The activities of Hsp70s reside in (at least) two functional and physically separable domains. The ATPase activity resides in the amino-terminal 380–400 residues (26), and the peptide binding activity resides in the carboxyl-terminal part of the molecule (27). The structure of an ATPase fragment of bovine Hsc70 complexed with nucleotide has been solved, revealing a two-lobed molecule with nucleotide buried at the base of a deep cleft (28). The structure of a peptide binding fragment of the Escherichia coli DnaK protein complexed to peptide has also been solved, revealing a subdomain of eight antiparallel β-strands, which form a peptide binding pocket, and a second, α-helical subdomain, one helix of which lies over the peptide binding pocket (29). The level of amino acid similarity within the Hsp70 protein family (e.g. 47% sequence identity between E. coli DnaK and bovine Hsc70) is such that the tertiary fold and many of the intramolecular interactions seen in a three-dimensional structure of one member of the family can be extrapolated safely to other Hsp70s.

Based on their structure of the DnaK peptide binding domain, Hendrickson and co-workers (29) suggested that the helical subdomain acts as a “lid” over the peptide binding pocket and that release of polypeptides would be predicated on displacement of the helix lying over the peptide binding pocket. There are several salt bridges between the “lid” helix and the β-subdomain, suggesting that peptide binding and release is accompanied by formation and breaking of these salt bridges. If this model is correct, then disrupting one or more of these salt bridges should result in greater lability of protein-peptide complexes.

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1 The abbreviations used are: Hsp70, 70-kDa heat shock-related protein; Hsc70, 70-kDa heat shock cognate protein; rHsc70, recombinant Hsc70; bHsc70, bovine brain Hsc70; MOPS, 4-morpholinepropanesulfonic acid; SNase, staphylococcal nuclease; HPLC, high pressure liquid chromatography; Rₚ, radius of gyration.

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Destabilization of Peptide Binding in Hsc70

In this work, we first characterize the differences between Hsc70 protein isolated from bovine brain and recombinant Hsc70 protein expressed in E. coli. We find that the bovine brain protein has at least one posttranslational modification in the peptide binding domain. Additionally, we find that the recombinant protein used in our earlier experiments differs in sequence at one position from the major fraction of bovine brain protein; residue 543 is glutamic acid in bovine protein and lysine in our original recombinant protein. This residue is expected to participate in one of the salt bridges between the "lid" helix and arginine 469 in the β-subdomain of the peptide binding domain; replacing glutamic acid with lysine would disrupt this salt bridge. We compare the activities (ATPase, peptide binding, and the coupling of the two) in recombinant Hsc70 differing at position 543 (lysine or glutamic acid) and further compare these with the activities of Hsc70 isolated from bovine brain. Comparison of bovine and recombinant protein having the same amino acid sequence allows delineation of the effect of the posttranslational modification on activities we measure, while comparison of recombinant proteins with glutamic acid versus lysine at position 543 allows us to test the model of peptide binding and release proposed by Hendrickson and co-workers (29).

MATERIALS AND METHODS

Peptide and Other Reagents—A peptide with the sequence FYQLALT (hereafter referred to as "faf1"; we thank Dr. Anne Fourie for suggesting the sequence (30)) was synthesized, purified, and labeled with fluorescein at the amino terminus using fluorescein isothiocyanate (Molecular Probes, Eugene, OR) as described previously (5). The fluoresceinated peptide is called faf1. ATP and ADP were purchased from Sigma and Boehringer Mannheim; [γ-32P]ATP and [γ-32P]ADP were purchased from Amersham Corp.

Cloning and Mutagenesis—Plasmids for expression of Hsc70 and a 60-kDa truncation of Hsc70 (amino acids 1–554; referred to as the "60-kDa fragment") with lysine at position 543 in E. coli have been described (31, 32). In those publications, the proteins are assumed erroneously to be wild type, but they are now referred to as mutant recombinant Hsc70 (rHsc70 E543K) and mutant truncation (60-kDa fragment E543K).

E. coli strain G2124, derived below, the discovery of an apparent mutation (E543K) in the original cdna clone (33) made it necessary to construct a wild type coding sequence by oligonucleotide-directed mutagenesis. The Hsc70 coding sequence was excised from the pTT7-7 expression construct as an Ndel-SalI fragment and inserted into the polycloning site of pRSETA (Invitrogen), cut with Ndel and XhoI. The resulting plasmid (pRSET-Hsc70-E543K) was used as the starting point for subcloning coding sequences for wild-type Hsc70 and its 60-kDa truncation (both with glutamic acid at position 543).

To subclone the full-length wild type coding sequence, a fragment of the cloned Hsc70 E543K sequence was amplified by PCR using one primer complementary to the polylinker following the stop codon and the PCR product were digested with EcoRI and the fragment was inserted into the plasmid, replacing the 3′–330 base pairs of the coding sequence. To subclone the 60-kDa wild type coding sequence, this same region was excised as an EcoRI-HindIII fragment and replaced with a synthetic fragment introducing glutamic acid at codon 543 and a stop codon after codon 554, both underlined in the top strand oligonucleotide, AATTGCCGCTAAATCTACGGCTTCAAGAGAACTACGTGAGTA (bottom strand oligonucleotide: AGTCTACTAACAAGTGCT-TTCTGTTAGGGGCTAGTATGACCG). Both subclones were confirmed by sequencing between the EcoRI site and the 3′-polylinker.

Protein Expression and Purification—Recombinant wild type and E543K mutant rHsc70 were expressed in E. coli using the plasmids described above and purified to >95% homogeneity as described previously (32) with the following modifications. Cells were lysed by incubation at 4 °C with lysozyme (final concentration of 0.2 mg/ml) in 50 mM Tris (pH 7.5), 200 mM NaCl, 5% glycerol (v/v), 1 mM dithiothreitol, 1 mM phenylmethylsulfonfluoride and by subsequent sonication. Cell debris was removed by centrifugation for 1 h at 15,000 × g. Supernatant was dialyzed extensively against 20 mM MOPS, 4 mM Mg(CH3COO)2, and 0.1 mM EDTA, the pH was adjusted to 7.0 with Tris, and the supernatant was loaded onto a Q-Sepharose (Pharmacia Biotech AB, Uppsala, Sweden) column. Recombinant Hsc70 was eluted with a gradient of 0–1 M KCl and dialyzed extensively against 25 mM HEPES (pH 7.0), 75 mM KCl, and 5 μM EDTA. After the addition of Mg(CH3COO)2 to a final concentration of 10 mM, the protein was subsequently purified on a Phenyl-Sepharose (Sigma) column that had been equilibrated with 25 mM HEPES, 150 mM KCl, and 4.5 mM MgCl2, adjusted to pH 7.0 with KOH. The 60-kDa fragment of Hsc70 was purified following a similar protocol.

Bovine brain Hsc70 (bHsc70) was purified from fresh brains as described (34). Three peaks of bHsc70 are separated on Mono-D during the purification. Unless noted otherwise, the third and largest peak (presumably with the lowest pI) was used for these studies. In comparative studies, we have found no functional differences between proteins from the three different Mono-D peaks.

When required, proteins were rendered nucleotide-free using methods described previously (35) with the following modification. After charcoal treatment, proteins were dialyzed against 40 mM HEPES, 150 mM KCl, and 4.5 mM MgCl2, adjusted to pH 7.0 with KOH. For comparison of the effect of K+ with that of Na+ on ATPase kinetics, some of native proteins were dialyzed against 40 mM HEPES, 150 mM NaCl, and 4.5 mM MgCl2, adjusted to pH 7.0 with NaOH.

Protein Mapping—To allow complete proteolytic digestion, samples of bHsc70 and rHsc70 E543K were reduced and alkylated. Reduction was done in 6 M guanidinium hydrochloride, 1 mM EDTA, 0.25 M Tris-HCl, pH 8.5, with 50 μg of dithiothreitol for 2 h at room temperature. Free sulfhydryl groups were then alkylated in the same solution by adding 1 μl of neat 4-vinylpyridine and incubating for an additional 2 h. Samples were desalted by gel filtration on a Fast Desalting column, using a Pharmacia SMART system.

Proteolytic digestion was done in 0.1 M Tris-HCl, pH 9.2, with Achromobacter lyticus protease I (Lys-C-specific endopeptidase, WAKO Chemicals, Dallas, TX) at an approximate substrate:enzyme ratio of 300:1. Samples were incubated overnight at 30 °C, the reaction was stopped by acidification with trifluoroacetic acid, and peptides were isolated by narrow bore reverse phase liquid chromatography on a Vydac 218TPC 5-μm column, using a SMART system. The samples were eluted with a linear gradient of acetonitrile in 0.06% trifluoroacetic acid at a flow rate of 100 μl/min, and peptide fractions were collected automatically.

Selected peptides were subjected to automated Edman degradation with a PE- Applied Biosystems model 494 sequencer, following the manufacturer's guide that coseparated an unidentified amino acid residue was analyzed on a Kratos IV matrix-assisted laser desorption/ionization time of flight mass spectrometer.

Determination of Kinetic Parameters of the ATPase Cycle—Kinetic experiments were carried out either in KCl buffer (40 mM HEPES, 150 mM KCl, 4.5 mM MgCl2, and 50 μg/ml bovine serum albumin, adjusted to pH 7.0 with KOH) or in NaCl buffer, which is identical to the KCl buffer except that K+ is replaced with Na+.

Rate constants of single turnover ATP hydrolysis, P, release, and ADP release were measured for both rHsc70 and bHsc70 at 25 °C, both in the presence and in the absence of peptide faf1, using methods described previously (35). To investigate the effect of peptide binding on ATPase kinetics, Hsc70 was preincubated with faf1 at 25 °C for 10 min prior to measurements. For measurement of ATP hydrolysis rates under single turnover conditions, the final concentration of [α-32P]ATP was 10 μM, and that of protein ranged from 2.3 to 9.1 μM for bHsc70 and from 2 to 8.0 μM for rHsc70. The dependence of the ATP hydrolytic rate on [faf1] was determined at 2.3 μM native Hsc70, 10 nM [α-32P]ATP, and faf1 ranging from 0 to 200 μM. To determine the rate of ADP dissociation, a complex between ADP and Hsc70 was formed by incubating 2.5 μM protein with 2.1 μM of [α-32P]ATP (71 Ci/mmol) at 25 °C for 3 h (bHsc70) or 1.5 h (rHsc70) to allow essentially complete hydrolysis to ADP prior to measurements. Experiments were then started by the addition of unlabeled ADP to a final concentration of 1 μM, and the decrease of protein-bound radioactivity was measured by filter binding.

The rate constant of the steady state ATPase activity of bHsc70 was determined at final protein concentrations of 2.1 μM with ATP concentrations from 55 to 215 μM. The effect of faf1 on the steady state ATPase activity was measured at 2.1 μM bHsc70, 108 μM ATP, and faf1 ranging from 0 to 200 μM. A detailed description of the methods of data analysis has been published previously (35).

Measurements of Hsc70-Peptide Binding and Release—Measurements of apparent equilibrium binding constants for faf1-Hsc70 complexes by HPLC gel filtration and peptide dissociation rates by fluoro-
To find the source of the differences in behavior of bHsc70 and the original recombinant Hsc70, we carried out peptide mapping experiments as described under “Materials and Methods.” After digestion with LysC protease, the product peptides were separated by HPLC (Fig. 1). There are four obvious peaks that differ between the two HPLC profiles. Amino acid sequencing of these peaks gave the following sequences: peak 1, LQGXINDEDK; peak 2, INDEDK; peak 3, NSLEYAFNMK; and peak 4, SYAFNMK. Notably, the carboxyl-terminal amino acid of each peptide is lysine, the residue at which the protease cleaves, demonstrating that each peptide was sequenced through its carboxyl terminus.

Peptide 1 from bHsc70 matches residues 558–567 of the published cDNA-derived peptide sequence (33), except for one unknown residue (X). Residue X was tentatively identified through mass spectrometry of the peptide. Subtraction of the predicted mass of the nine known residues from the mass of peptide 1 gave a mass of 171 Da for unknown residue X. This residue of bHsc70 occupies the position of LysX in the cDNA-derived sequence (residue mass, 128 Da). The mass difference of 43 Da is consistent with acetylation, carbamylation, or trimethylation of the lysine residue. Each of these modifications would block cleavage by LysC protease. Since trimethylation of one lysine residue per Hsc70 chain has been reported (25), it seems likely that residue 561 of bHsc70 is trimethyllysine. Cleavage at unmodified LysX in original recombinant Hsc70 yielded peptide 2, which matches the carboxyl-terminal six residues of peptide 1.

Peptide 4 spans residues 544–550 of the original recombinant Hsc70. The production of this peptide by LysC protease identifies lysine at position 543, in agreement with the cDNA-derived sequence. Peptide 3 covers the same sequence as peptide 4, sharing the carboxyl terminus but including an additional four amino acids at the amino terminus. The longer peptide arises from the occurrence of glutamic acid at position 543 in bHsc70. In contrast to lysine at this position in original recombinant Hsc70. Apart from this difference, peptide 3 from bHsc70 corresponds with residues 540–550 of the cDNA-derived sequence.

Alignment of >50 amino acid sequences for eukaryotic cytosolic and endoplasmic reticulum Hsp70-related proteins (Swiss-Prot release 34.0, April, 1996) reveals complete conservation of glutamic acid at position 543 in a segment of polypeptide that otherwise shows only modest conservation. Among
organellar and bacterial Hsp70 proteins, aspartic and glutamic acid predominate in this position. The recent crystal structure of the peptide binding domain of E. coli DnaK protein shows that this residue participates in a salt bridge to an arginine (29). This suggests that the difference we observe at position 543 is most likely a cloning artifact, although it is possible that the original bovine brain cDNA clone represents a minor isoform of Hsc70. Regardless of the basis of the difference, our original recombinant material has an E543K amino acid difference relative to the major fraction of bHsc70 purified from bovine brain. Hence, we now consider the original recombinant Hsc70 to be an E543K mutant protein (rHsc70 E543K), and we henceforth refer to recombinant Hsc70 with glutamic acid at position 543 as rHsc70.

Since these experiments revealed two differences between bHsc70 and our original rHsc70 E543K, we have carried out a series of experiments characterizing bHsc70 and wild type rHsc70. Comparison of these wild type proteins shows whether any differences in activity resulted from posttranslational modification of Lys561 (and possibly of other residues not yet identified as modified). Comparison of these results with our earlier characterization of rHsc70 E543K shows differences in activities due to the mutation at position 543.

Binding and Release of a Peptide (faf1) and a Denatured Protein (Staphylococcal Nuclease-(136–149)) in the Presence of MgADP—In the presence of MgADP, bHsc70 displays a mixture of monomer, dimer, and higher order oligomer on gel filtration, with ~70–80% of the protein monomeric, in agreement with earlier reports of Schmid and co-workers (13). Wild type rHsc70 displays a similar mixture of oligomers; typically ~50% of the protein is monomeric. For both proteins, incubation with MgATP results in dissociation of the oligomers, and essentially 100% monomeric protein is found on gel filtration. In contrast, incubation of rHsc70 E543K with MgATP did not result in dissociation of oligomeric species.

The deletion mutant SNase-(136–149) is unfolded in the absence of inhibitor or substrate and was used as a representative denatured protein. When 2 μM wild type bHsc70 or rHsc70 was incubated with 50 μM SNase-(136–149) in the presence of MgADP, as described under “Materials and Methods,” complexes between the nuclease and Hsc70 were detected by gel filtration. Typically, approximately equal fractions of Hsc70 were present as monomer and as complexes with SNase-(136–149), and the amount of dimeric Hsc70 present was reduced substantially. However, when rHsc70 E543K was incubated with SNase-(136–149) under these or other conditions, no appreciable amount of complex could be detected. The mutant rHsc70 E543K fails to form stable complexes with SNase-(136–149).

We have shown previously (with rHsc70 E543K and the short peptide faf1) that peptide binding and release in the presence of MgADP is a two-step process but that it can be adequately parameterized with an apparent dissociation rate constant $k_{d}^{app}$ (measured by fluorescence) and an equilibrium binding constant $K_{a}$ (measured by gel filtration) (5). Values measured for the wild type rHsc70 and bHsc70 are shown in Table 1. It is clear that the affinities of rHsc70 and bHsc70 for faf1 are equal within experimental error and are 3-fold tighter than rHsc70 E543K. Similarly, the apparent dissociation rate constants are equal within error and are 4.5-fold slower than rHsc70 E543K. The E543K protein has a severalfold lower affinity for faf1, primarily as a result of the accelerated peptide release rate.

**Table 1**

| Hsc70 Protein          | $K_{d}$ (μM) | $k_{d}^{app}$ (s$^{-1}$) |
|------------------------|--------------|--------------------------|
| bHsc70 wild type       | 1.26 (±0.05) | 0.00047 (±0.00002)       |
| rHsc70 wild type       | 1.31 (±0.16) | 0.00047 (±0.00021)       |
| rHsc70 E543K           | 4.3 (±0.9)$^{a}$ | 0.0021 (±0.0001)$^{a}$ |

*Reported previously in Ref. 5.*

First, the stability of nucleotide-free bHsc70 was determined by measuring the steady state ATPase rate over the protein concentration range 50 nM to 20 μM. In the presence of 50 μg/ml bovine serum albumin, bHsc70 is stable at a concentration ≥2 μM for at least 7 h at 25 °C (data not shown); consequently, protein concentrations ≥2 μM were used in all subsequent kinetic experiments.

To find suitable peptide concentrations for kinetic experiments, the dependence of ATPase activity on [faf1] was measured. Steady state ATPase and single turnover ATP hydrolysis activities were measured over a faf1 concentration range of 0–200 μM. The concentration of bHsc70 was 2.1–2.3 μM. For steady state measurements, the Hsc70:ATP molar ratio was 1:27, while for single turnover measurements, the ratio was 230:1. Results are shown in Fig. 2. In absence of peptide, the steady state ATPase and single turnover ATP hydrolysis rate constants are essentially equal (0.0041 ± 0.00008 s$^{-1}$ and 0.0005 ± 0.0001 s$^{-1}$, respectively). The rates remain indistinguishable over the [faf1] range 0–50 μM, indicating that hydrolysis is the rate-limiting step of the kinetic cycle under these conditions; measurements of the ADP and P$_{i}$ release rates described below corroborate this observation. Above 50 μM peptide, the steady state rate is slower than the single turnover rate, indicating that another step of the ATPase cycle must be contributing to the overall ATPase rate under these conditions; measurements described below identify P$_{i}$ release as the major contributor. The steady state ATP hydrolysis rate of bHsc70 plateaus around 50 μM [faf1] at 5.4 times the basal rate (0.0022 ± 0.0001 s$^{-1}$). The single turnover ATPase activity of bHsc70 does not reach a maximum until the peptide concentration is ≥150 μM (0.0048 ± 0.0002 s$^{-1}$, average of three determinations at 152 μM peptide). Consequently, experiments done “with peptide” have to include [faf1] ≥150 μM.

The dependence of the single turnover ATP hydrolysis rate of bHsc70 was measured as a function of protein concentration, both in the absence and the presence of peptide (Fig. 3). It is clear that the rate reaches a plateau at a protein concentration...
of −2 μM; similar behavior was observed with rHsc70 E543K previously. Hence, [Hsc70] = 2 μM was used in subsequent experiments.

Rate constants for individual steps of the ATPase cycle were then measured for bHsc70, rHsc70, and rHsc70 E543K, using methods that have been described. Results are summarized in Table II; for comparison, previous results for rHsc70 E543K are included. In this and previous work, we have observed pre-to-prep differences of as much as 2–3-fold in the measured values of rate constants; representative measurements from two different preps of rHsc70 are included in Table II to illustrate the largest variation we have seen. In this context, we emphasize the effect of peptide on the relative rate constants within protein from a single prep as being more informative than the differences of the absolute values of constants from different preps.

In the presence of K⁺, the basal rates (k₂) of ATP hydrolysis (i.e. the rates of hydrolysis in absence of peptide) of bHsc70 and wild type rHsc70 are approximately equal, and both are substantially slower than k₂ of rHsc70 E543K. The hydrolysis rates of wild type proteins are stimulated substantially by 152 μM faf1 (−9-fold for bHsc70; 6–8-fold for rHsc70). In contrast, the hydrolysis rate of the mutant protein is stimulated only −2-fold by peptide. The maximum peptide-stimulated hydrolysis rates we have observed are approximately equal for all three proteins. There is no substantial difference between wild type bovine and recombinant protein in the basal rate of ATP hydrolysis in the absence of peptide or the extent to which it is enhanced by faf1 peptide. The primary difference between wild type and E543K mutant protein is that the latter has a higher basal rate of ATP hydrolysis.

With methods used previously to measure the Pᵢ release rate (k₄) of rHsc70 E543K (e.g. release of ³²P from [γ-³²P]ATP after hydrolysis (35)), it was not possible to quantify the Pᵢ release rate of the wild type proteins in the absence of peptide; Pᵢ release was too rapid compared with the ATP hydrolysis rate under these conditions. When ATP hydrolysis was stimulated with peptide, the difference between hydrolysis and the subsequent release of Pᵢ generated from ATP was discernible (Fig. 4). Values of k₄ for wild type recombinant and bovine proteins under conditions of maximal stimulation by peptide are equal to each other and approximately 2-fold more rapid than the value determined previously for rHsc70 E543K.

ADP release (k₅) is more rapid than hydrolysis and Pᵢ release for both wild type proteins and is enhanced only slightly (less than 2-fold) by peptide. This is the same trend that has been observed with rHsc70 E543K.

In summary, we do not observe any major differences between bHsc70 and wild type rHsc70 in the kinetics of the ATPase cycle. The mutant E543K differs from wild type protein, most notably in the basal rate of ATP hydrolysis, where k₂ for E543K protein is severalfold higher than for wild type protein. This results in a lower apparent factor of stimulation of hydrolysis by peptide, although the observed maximal rates of hydrolysis for wild type and mutant proteins are approximately equal. As discussed below, the E543K mutation reduces the ability of the peptide binding domain to inhibit the ATPase activity.

Monovalent Ion Effect on the ATPase Activity—It has been reported that the competency of Hsp70 proteins to release denatured polypeptides requires the presence of K⁺ preferentially over Na⁺ (38). In addition, the ATPase activities of rHsc70 E543K and its (wild type) ATPase fragment are an order of magnitude higher in the presence of 0.1 M K⁺ than in the presence of smaller monovalent ions such as Na⁺ (39). We have measured the monovalent ion dependence of the ATPase activity of ATPase fragment protein derived by proteolysis from bHsc70 and have found its behavior to be similar to that of recombinant ATPase fragment (data not shown). Additionally, we have extended these experiments to bHsc70; we have measured both the ATP hydrolysis rate (k₂) and the steady state turnover rate (k_cat) in the presence of 0.15 M NaCl (Table II; Fig. 5). For bHsc70, the basal rates are similar with K⁺ and with Na⁺. The major difference is in peptide-stimulated rates of hydrolysis and turnover. We see severalfold stimulation of ATP hydrolysis by faf1 when K⁺ is present; in contrast, we see only 2-fold stimulation in the presence of Na⁺ (Table II). Similarly, there is less than 2-fold stimulation of steady state turnover with Na⁺ (0.0027 ± 0.00003 s⁻¹ versus 0.0043 ± 0.0005 s⁻¹). The observed effects do not depend on protein or ATP concentration (Fig. 5). A similar result is seen for the hydrolytic rate of rHsc70, although the basal rate of ATP hydrolysis of rHsc70 with Na⁺ appears to be somewhat slower, and stimulation is slightly greater (Table II). In the presence of Na⁺, the coupling between peptide binding and ATPase activity is impaired for bHsc70 and rHsc70.

Solution Small Angle X-ray Scattering—Solution small angle x-ray scattering studies have shown that rHsc70 E543K and its (mutant) 60-kDa subfragment undergo a substantial (3–5 Å) decrease in R_g when MgATP binds in the presence of K⁺ (32). A probable interpretation of the ∆R_g is that the protein undergoes a large ATP-induced conformational change. When we attempted to extend these experiments to full-length wild type proteins, we found that the self-association of rHsc70 precluded accurate measurement of R_g in the presence of MgADP, since the measurement relies on having a monodisperse solution throughout the time course of the data collection (−20 min). However, since the 60-kDa fragment of rHsc70 is less prone to self-association, we were able to make measurements on it in the presence of MgADP as well as MgATP (Fig. 6). The values of R_g derived by extrapolation to zero concentration are 32.2 ± 0.2 Å with MgADP and 28.6 ± 0.3 Å with MgATP, yielding a ∆R_g of 3.6 ± 0.4 Å. Furthermore, the pair distribution function P(r) (as defined in Ref. 32) computed for rHsc70 showed a shift from an elongated molecule (in the presence of ADP) to a more compact shape (with ATP). Similar P(r) distributions and ∆R_g (4.9–5.4 Å) were obtained with rHsc70 E543K previously (32), demonstrating that the MgATP-induced conformational shift is not an artifact of the E543K mutation and that the mutation does not prevent the change.

DISCUSSION

To separately delineate the functional consequences of (i) the posttranslational modification (presumably trimethylation) of Lys⁵⁶¹ of bHsc70, and (ii) the E543K mutation found in our original recombinant protein, we have compared bHsc70, rHsc70, and rHsc70 E543K proteins in their interaction with a
Values measured for single turnover kinetic parameters of ATPase cycle

Parameters are defined under "Materials and Methods." Values are determined at 25 °C in 40 mM HEPES, 4.5 mM MgCl₂, 0.15 mM KCl or NaCl (as specified in column 2), and 50 μg/ml bovine serum albumin adjusted to pH 7.0 with KOH or NaOH. For bHsc70, protein from pool 3 of the chromatofocusing column was used in measurements.

| Protein   | Monovalent cation | [fəf1] | μM  | s⁻¹  | k₂  | k₂/κ₂ (no peptide) | k₃  | k₄  |
|-----------|-------------------|-------|-----|------|-----|--------------------|-----|-----|
| bHsc70    | K⁺                | 0     | 0.0005 (±0.0001) | 6.6 | ND   | 0.054 (±0.002)     | ND  |
|           |                   | 80    | 0.0033 (±0.0006) | 9.6 | 0.011 (±0.001)     | 0.090 (±0.010)     | ND  |
| rHsc70    | K⁺                | 152   | 0.0048 (±0.0002) | 50  | ND   | 0.020 (±0.009)     | 0.090 (±0.010)     | ND  |
|           |                   | 152   | 0.0018 (±0.0001) | 6.4 | ND   | 0.034 (±0.012)     | ND  |
| rHsc70    | K⁺                | 152   | 0.0074 (±0.0013) | 7.9 | 0.010 (±0.003)     | 0.022 (±0.001)     | 0.019 (±0.003)     |
| E543K     | K⁺                | 152   | 0.0036 (±0.0005) | 1.6 | ND   | 0.045 (±0.002)     | ND  |
|           |                   | 0     | 0.0030 (±0.0003) | 1   | ND   | 0.028 (±0.008)     | ND  |
|           |                   | 0     | 0.0028 (±0.0003) | 1   | ND   | 0.031 (±0.009)     | 0.055 (±0.004)     |
| bHsc70    | Na⁺               | 50    | 0.0064 (±0.0006) | 2.3 | 0.0056 (±0.0017)   | 0.05 (±0.004)      | ND  |
| rHsc70    | Na⁺               | 152   | 0.0010 (±0.0001) | 2.1 | ND   | ND                 | ND  |
|           |                   | 152   | 0.00009 (±0.00003) | 3.1 | ND   | ND                 | ND  |

* ND, not determined.

* Values measured with protein from preparation 1 of recombinant Hsc70.

* Protein from preparation 2 of recombinant Hsc70.

* Reported previously in Ref. 35; determined at 25 °C in 40 mM HEPES (pH 7.0), 4.5 mM Mg(CH₃COO)₂, 0.075 mM KCl.

* Reported previously in Ref. 5; determined at 25 °C in 20 mM HEPES (pH 7.0), 3.0 mM Mg(CH₃COO)₂, 0.15 mM KCl.

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![Graph](image)

**Fig. 4. ATP hydrolysis and Pᵢ release by bHsc70 in the presence of 152 μM fəf1.** In both experiments, [bHsc70] was 2 μM, and [ATP] was 10 mM. C, fraction, θ, of ATP not hydrolyzed versus time (determined under single turnover conditions with [α⁻³²P]ATP). ○, fraction of [α⁻³²P]Pyr ATP remaining bound to protein (as ATP or Pᵢ) versus time, determined by filter binding assay described under "Materials and Methods." Curves are computed by fitting two exponentials to the data, with one rate constant equal to k₂, as summarized in Table II and the second rate constant (k₃) variable.

short peptide and a denatured protein, in the kinetics of their ATPase cycles, and in the coupling of peptide binding to the ATPase kinetics. We find substantial differences between wild type and E543K mutant proteins. We find only subtle differences between wild type bHsc70 and rHsc70; the posttranslational modification has minimal influence on the activities we have examined.

We find that both bovine and recombinant wild type proteins form relatively stable complexes with a representative denatured protein, SNase-(Δ136-149), while rHsc70 E543K does not. In accord with this result, quantitative measurements of the binding and dissociation constants of a short, 7-residue peptide show that peptide release from rHsc70 E543 is more than 4-fold faster than from the wild type proteins. The E543K mutation significantly reduces the lifetime of complexes of Hsc70 with peptides and polypeptides, while the absence of posttranslational modifications in wild type rHsc70 has a negligible effect.

From their structure of the peptide binding domain of DnaK, Hendrickson and colleagues (29) suggest that a long helix that lies over the peptide binding pocket of the domain acts as a "lid" and that several salt bridges between the helix and the β-subdomain that forms the peptide binding pocket act to "latch" the helix in place (29). Our results support the model. We expect that the structure of the peptide binding domain of Hsc70 is similar to that of DnaK, in which case the E543K mutation would disrupt a salt bridge between Arg⁴⁶⁹ in the β-subdomain and Glu⁵⁴³ in the helix of the wild type structure (equivalent to Arg⁶⁷⁷ and Asp⁵⁴⁰ of DnaK), thereby destabilizing the "latch." The higher peptide release rate of the mutant protein is consistent with a reduced stability for the closed conformation of the "lid" helix, which blocks movement of the peptide in and out of the binding pocket.

Although the E543K mutation lies outside the ATPase domain, it affects the ATPase activity. Specifically, the mutation appears to impair coupling of the ATPase and peptide binding activities. The maximal peptide-enhanced ATP hydrolysis rates are approximately equal for all three proteins and are further equal to the rate measured previously for the isolated ATPase fragment. In contrast, the basal (i.e., in the absence of peptide) ATP hydrolysis and turnover rates are markedly different for mutant and wild type proteins, being about 2-fold below peptide-enhanced rates for rHsc70 E543K, while they are 6–10-fold lower for the wild type proteins. These results suggest a model in which the coupling of the two activities is inhibitory: (i) the peptide binding domain inhibits the intrinsic ATPase activity of the ATPase domain, and (ii) binding of peptide removes the inhibition, resulting in an apparent peptide-dependent enhancement of the activity. In this model, the E543K mutation would impair the inhibitory effect of the peptide binding domain on the ATPase activity, resulting in a higher basal (inhibited) activity.

A monovalent ion dependence of the bHsc70 ATPase activity is apparent in the presence of peptide but not in its absence. Without peptide, the measured ATP hydrolysis and turnover rates are approximately the same when either Na⁺ or K⁺ is included as a monovalent ion. However, with peptide, the rates are substantially higher with K⁺ (6–10-fold) than with Na⁺ (which shows minimal enhancement). Again, it is notable that the ATPase activities of the full-length protein in the presence of peptide parallel those of the isolated ATPase fragment, for which the turnover rates are 5–10-fold higher with K⁺ than...
ATP hydrolysis ( distinguishable from the impairment/inhibition of activity when rHsc70, where a similar change of measurements have been extended to the 60-kDa fragment of proteins undergo an ATP-induced conformation change (32). These proteins, a plausible interpretation of which is that the proposed model for coupling peptide binding and ATPase activity, if the inhibition of ATPase activity in the mutant peptide is present, we suggest that the apparent lack of a peptide binding domain inhibits the ATPase domain unless the mutation altering the global conformation of the peptide binding domain. The functional consequences of the E543K mutation of residue 561, most likely trimethylation of lysine, has no significant influence on these activities. The E543K mutation affects both the peptide binding activity and its coupling with the ATPase activity. Since work we reported previously on the kinetics of peptide binding and release (5) and the kinetic framework of the ATPase cycle (35, 40) was done with E543K mutant protein, values for kinetic constants should be amended with those reported here for wild type protein. The model previously proposed for an ATP-induced conformational change (3, 32, 40) is consistent with data from the wild type protein as well as the E543K mutant and hence does not need to be amended or modified.

In summary, we find that in the activities we have examined, rHsc70 mimics bHsc70 protein; the posttranslational modification of residue 561, most likely trimethylation of lysine, has no significant influence on these activities. The E543K mutation affects both the peptide binding activity and its coupling with the ATPase activity. Since work we reported previously on the kinetics of peptide binding and release (5) and the kinetic framework of the ATPase cycle (35, 40) was done with E543K mutant protein, values for kinetic constants should be amended with those reported here for wild type protein. The model previously proposed for an ATP-induced conformational change (3, 32, 40) is consistent with data from the wild type protein as well as the E543K mutant and hence does not need to be amended or modified.

Since residue 543 is predicted to participate in a salt bridge that forms a “latch” closing the peptide binding groove, it seems likely that the mutation’s primary effect is on peptide binding and conformation of the peptide binding domain, while the difference in ATPase rates is a secondary effect, perhaps transmitted through the wild type coupling of peptide binding/release with the enzymatic ATPase cycle. It is possible that this is achieved through a direct interaction between the ATPase domain and the helix of the peptide binding domain on which Glu543 resides; alternatively, the effect may be indirect, with the mutation altering the global conformation of the peptide binding domain. The functional consequences of the E543K mutation support the model of Hendrickson and co-workers (29) for peptide binding and release. The significance of the posttranslational modification at residue 561 of Hsc70 activity remains unclear.

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