Characterization of D10S and K71E Mutants of Human Cytosolic Hsp70

Thavamani Rajapandi,[‡] Chengbiao Wu,[‡,§] Evan Eisenberg, and Lois Greene*

Laboratory of Cell Biology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892

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ABSTRACT: To determine the effect of mutations at the nucleotide-binding site of recombinant Hsp70 on its interaction with protein and peptide substrates, point mutations were made at D10 and K71, two residues at the active site. The D10S mutation weakened both ATP and ADP binding, while the K71E mutation weakened only ATP binding. In binding experiments using Hsp70 with no bound nucleotide, the mutated Hsp70s interacted with clathrin and peptide just like the wild-type Hsp70. However, the D10 mutation completely abolished the effects of both ATP and ADP on peptide and clathrin binding. The K71 mutation also abolished the effect of ATP on substrate binding, but ADP, which still bound tightly, had its normal effect on substrate binding. In addition, the D10S and K71E mutants had greatly reduced ability to uncoat clathrin-coated vesicles at pH 7.0, bind to clathrin baskets at pH 6.0, and undergo polymerization induced by YDJ1 in the presence of ATP. We conclude, first, that nucleotides must bind strongly to Hsp70 to affect substrate binding and, second, that interaction of Hsp70 with DnaJ homologues may also require a strongly bound ATP.

The 70 kDa heat shock proteins (Hsp70s)[1] are a class of highly conserved proteins, present in all organisms, that play an essential role in many cellular processes. The constitutive members of this class, the Hsc70s, are involved in the folding, unfolding, and proteolysis of individual proteins, in the formation and dissociation of protein complexes, and in the translocation of proteins across membranes (*1*−*3*). ATP hydrolysis appears to be involved in all of these functions, suggesting that the conversion of ATP to ADP at the active site of Hsc70 causes major conformational changes in the enzyme.

We and others have shown that one way to detect these conformational changes is to follow the effect of bound ATP and ADP on the interaction of Hsc70 with its protein and peptide substrates (*4*−*8*). These studies suggest Hsc70 occurs in two different conformations, one in the presence of ATP and a second in the presence of ADP. The ATP conformation is characterized by rapid binding and dissociation of protein and peptide substrates. On the other hand, substrate appears to be locked onto the ADP form of the enzyme; i.e., its rate of dissociation is very slow. Nucleotide-free Hsc70, that is Hsc70 with no bound nucleotide, shows hybrid properties (*9*) in that it resembles Hsc70−ATP in its interaction with clathrin, while it resembles Hsc70−ADP in its interaction with peptide, suggesting that the substrate-binding site of nucleotide-free Hsc70 may be flexible.

Interestingly, nucleotide analogues such as AMP-PNP, which bind to Hsc70 2 orders of magnitude more weakly than either ATP or ADP (*10*), have essentially no effect on the properties of nucleotide-free Hsc70 (*9*), suggesting that nucleotides only induce specific conformations on Hsc70 if they bind tightly to the nucleotide-binding site. This, in turn, suggests that there may be a simple explanation for the effects of mutations which weaken the binding of ATP and/or ADP to Hsc70. Rather than affecting the properties of the nucleotide-free Hsc70, these mutations may simply cause the mutated Hsc70 with bound ATP or ADP to act like nucleotide-free Hsc70; that is, they may cause bound ATP or ADP to act like other weakly binding nucleotide analogues.

A number of mutations have been made at the nucleotide-binding site of bovine brain Hsc70 based on the X-ray crystallographic studies of the 44-kDa N-terminal domain of Hsc70 (*11*). These studies (*12*−*14*), for the most part, elucidated both the effect of the mutations on nucleotide binding and ATPase activity as well as the effect of the mutations on the conformation of Hsc70 by diffraction studies. The effect of mutations on the interaction of Hsc70 with peptide and protein substrates has not been examined, except for the study of Huang et al. (*15*) in which they found that mutation of the D10 residue of Hsc70 had no effect on the affinity of peptide for Hsc70. Point mutations at the site of MgATP catalysis, specifically D10, K71, D199, E175, and D206, caused no gross conformational change in the structure of the 44-kDa domain of Hsc70 even though they caused dramatic reductions in ATP binding and/or ATPase activity (*12*−*14*). This is not surprising since X-ray crystallography studies of the 44-kDa N-terminal domain of Hsc70 revealed only subtle differences in the structure of the active

* Address correspondence to this author at the National Institutes of Health, 9000 Rockville Pike, Building 3, Room B1-10, 3,Center Dr., MSC 0301, Bethesda, MD 20892-0301. Fax: 301-402-1519. Email: greenel@helix.nih.gov.

‡ These two authors contributed equally to this work.

§ Present address: Department of Cell Biology and Neuroscience, University of Texas Southwestern Medical Center, Dallas, TX 75235.

[1] Abbreviations: Hsp70 (Hsc70), 70 kilodalton heat shock protein and its cognate form; HPLC, high-performance liquid chromatography; FPLC, fast protein liquid chromatography; PCR, polymerase chain reaction; BiP, immunoglobulin binding protein; PAGE, polyacrylamide gel electrophoresis; MES; 2-(*N*-morpholino)ethanesulfonic acid; AMP-PNP, adenyl-5′-yl imidodiphosphate.

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site in the presence of ATP, ADP, and AMP-PNP (*13*) even though the nucleotides have very different effects on the binding of substrate to Hsc70. It is possible that different bound nucleotides or mutations at the active site do not cause major changes in the structure of the 44-kDa fragment of Hsc70 because these changes only occur in the intact molecule which contains the 25-kDa C-terminal substrate-binding domain.

In the present study, we made two point mutations at the nucleotide-binding site of full-length Hsp70: D10S in an effort to affect both ATP and ADP binding since D10 interacts with Mg^{2+} which in turn interacts with both the β- and γ-phosphates of ATP (*13*), and K71E in an effort to affect only ATP binding since K71 interacts with the γ-phosphate of ATP by stabilizing an H_2O molecule or an OH^- for nucleophilic attack (*14*). Our results suggest that both ATP and ADP must bind strongly to the nucleotide-binding site of Hsp70 to induce their normal conformational changes; weak binding of ATP and ADP has no effect on the properties of nucleotide-free Hsp70 mutants.

MATERIAL AND METHODS

Materials. ATP, AMP-PNP, ATP-agarose, creatine kinase, creatine phosphate, hexokinase, imidazole, and MES were from Sigma. ADP was from Fluka. [γ-^{32}P]ATP, [^{14}C]-ADP, and [^{14}C]formaldehyde were from New England Nuclear. DnaK antibodies were from StressGen. The SDS gels (4−20%) were from Integrated Separation Systems.

Methods. Clathrin-coated vesicles, mixed assembly protein-clathrin baskets, clathrin, and Hsc70 were prepared from bovine brain as described previously (*16, 17*). The yeast DnaJ homolog, YDJ1, was expressed in *E. coli* strain BL21-(DE3) and purified according to Cyr et al. (*18*).

Hsp70 point mutations were made by polymerase chain reaction. The PCR product was sequenced to verify the point mutation, and then the fragment containing the desired mutation was reinserted into the Hsp70 cDNA. The wild type (WT) and mutant recombinant human Hsp70s were overproduced in *E. coli* BL21(DE3) cells containing a pET11a with the human Hsp70 gene. We found it was necessary to grow the cells at 28 °C to obtain fully active protein in our uncoating assays. The Hsp70 was purified on ATP-agarose. It was further purified on hydroxylapatite for ATPase assays. The extent of DnaK contamination in these Hsp70 preparations was less than 3% as determined using Western blotting analysis with monoclonal anti-DnaK antibodies and a pure DnaK standard.

All experiments were conducted in buffer A consisting of 25 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM magnesium acetate, 20 mM imidazole, pH 7.0, except 20 mM MES (pH 6.0) was used instead of imidazole in the binding studies of Hsp70 to mixed baskets. Clathrin and peptide binding to Hsp70 were measured by FPLC filtration using a Superose 6 and a Superose 12 column, respectively, as described in Gao et al. (*9*). The amount of clathrin-bound Hsp70 was quantified by electrophoresis of the clathrin peak on SDS gels followed by densitometric scanning of the Hsp70 and clathrin bands. Peptide binding was quantified using radioactive peptide which was trace-labeled with [^{14}C]formaldehyde according to the procedure of Jentoft and Dearborn (*19*). To measure the dissociation rate of peptide, peptide exchange was performed by first saturating the Hsp70 with unlabeled peptide and then adding a trace amount of labeled peptide. At given times, the reaction mixture was chromatographed on a Superose 12 FPLC column.

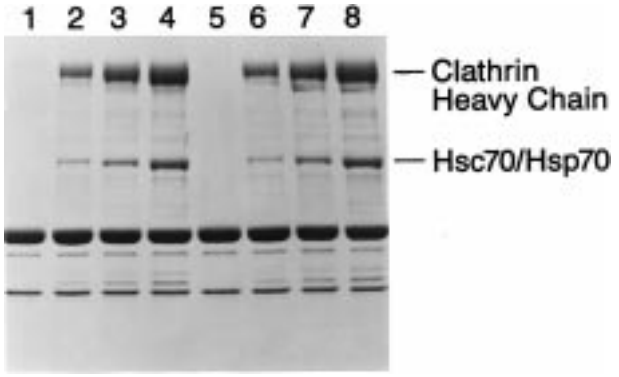

FIGURE 1: Comparison of the uncoating of clathrin-coated vesicles by bovine brain Hsc70 and recombinant human Hsp70. Clathrin-coated vesicles (0.5 μM) were incubated with 0, 0.3, 0.6, and 1.2 μM of either bovine Hsc70 (lanes 1−4) or recombinant Hsp70 (lanes 5−8) in the presence of 1 mM ATP and an ATP-regenerating system consisting of 30 units/mL creatine kinase and 15 mM creatine phosphate at 25 °C for 15 min. The reaction mixture was centrifuged, and the dissociated clathrin was analyzed on 4−20% SDS−PAGE.

ATPase activity was determined from the amount of [^{32}P]-P_i released from [γ-^{32}P]ATP (*20*). HPLC analysis of the bound nucleotide was determined according to Gao et al.-(*21*). Equilibrium dialysis was done using [^{14}C]ADP in the presence and absence of an ATP-regenerating system according to Gao et al. (*10*). The [^{14}C]ADP−Hsp70 was made by exchanging radioactive ADP with the bound ADP, followed by dialysis (*10*). Nucleotide-free forms of the WT and K71E Hsp70 were prepared by exchanging the bound nucleotide of Hsp70 with AMP-PNP, followed by extensive dialysis (*21*).

Clathrin uncoating experiments were performed as described in Greene and Eisenberg (*16*). The binding of Hsp70 to mixed assembly protein−clathrin baskets at pH 6.0 was performed by incubating these proteins for 15 min at 25 °C, followed by centrifugation and SDS gel analysis of the supernatant (*22, 23*). Polymerization of Hsp70 by YDJ1 was quantified by FPLC chromatography as described in King et al. (*24*).

RESULTS

Our previous studies on the interaction of Hsc70 with nucleotide and substrate were primarily carried out with bovine brain Hsc70. However, despite many attempts to purify active recombinant bovine brain Hsc70, the recombinant Hsc70 was never fully active in its ability to uncoat clathrin-coated vesicles. In contrast, we were able to prepare fully active recombinant human Hsp70 but only by growing the *E. coli* at 28 °C.

Figure 1 shows that recombinant human Hsp70 purified from *E. coli* grown at 28 °C releases clathrin from clathrin-coated vesicles with the same stoichiometry as the bovine brain Hsc70. Recombinant human Hsp70 isolated from *E. coli* grown at 37 °C only showed about half as much uncoating under the same conditions although it did not appear to be aggregated based on column chromatography

Table 1: Nucleotide-Binding Properties and ATPase Activity of WT and Mutant Hsp70s[a]

protein	dissociation constants (μM) ATP	ADP	V_{max} (s^{-1})
Hsc70	0.02[b]	0.02[b]	6×10^{-4}
WT Hsp70	<0.1[c]	<0.1[c]	1×10^{-3}
D10S Hsp70	4	16	2×10^{-4}
K71E Hsp70	2	<0.1[c]	8×10^{-5}

[a] Dissociation constants were determined by equilibrium dialysis, and ATPase activity was measured using 25 μM [γ-^{32}P]ATP. [b] Measurements obtained for bovine brain Hsc70 (*21*). [c] Upper limit values were determined using Hsp70 with bound [^{14}C]ADP as described by Gao et al. (*10*).

experiments (data not shown). We also found that recombinant human Hsp70 prepared at 28 °C reacted the same as bovine brain Hsc70 in regard to other properties we tested. It bound both ATP and ADP strongly (Table 1), and the nucleotide-dependent interactions of Hsp70 with substrate, i.e., clathrin or cytochrome *c* peptide, were the same for the recombinant human and bovine brain enzyme. On this basis, we used recombinant human Hsp70 prepared from *E. coli* grown at 28 °C to study the functional effects of mutations at D10 and K71 residues of Hsp70.

Interaction of D10S and K71E Hsp70 with ATP and ADP. We first examined the effect of the D10S and K71E mutations on the ability of recombinant Hsp70 to bind ATP and ADP and to hydrolyze ATP. After 60 h of dialysis and several changes of buffer, both WT Hsp70 and the K71E mutant retained about 70% of their bound nucleotide in the form of ADP. The D10S mutant retained only about 10% of its bound nucleotide after overnight dialysis, while extensive dialysis reduced this value to 1%. This suggests that both WT Hsp70 and the K71E mutant bind ADP tightly while the D10S mutant binds ADP much more weakly. This was confirmed by equilibrium dialysis experiments. Table 1 shows that the dissociation constants of the D10S mutant for ADP and ATP are 4 and 16 μM, respectively, showing that these nucleotides bind to D10S at least 2 orders of magnitude more weakly than to Hsc70. In contrast, ADP binds as strongly to K71E mutant as to WT Hsp70, while ATP binds about 2 orders of magnitude more weakly to K71E mutant than to WT Hsc70. It should be noted that the binding of ADP and ATP to WT Hsp70 only represents an upper limit and is probably similar to Hsc70. These results demonstrate that, as suggested by the structure of the nucleotide-binding domain (*13, 14*), D10 is involved in the binding of both ATP and ADP to Hsp70 while K71 appears to be involved specifically in the binding of ATP. In agreement with previous studies (*12, 14, 15*), we also find that these mutations have a marked inhibitory effect on the ATPase activity of the mutant Hsp70s. As shown in Table 1, the ATPase activity of the K71 mutant is less than 10% of the WT ATPase activity, while the D10 mutant is about 20% of the WT ATPase activity.

Effect of the D10S and K71E Mutations on the Properties of Nucleotide-Free Hsp70. We next tested whether the D10S and K71E mutations at the nucleotide binding site have a significant effect on the properties of Hsp70 with no bound nucleotide, specifically on the rates of dissociation of cytochrome *c* peptide and clathrin. Our previous work (*9*) suggested that the substrate-binding site of nucleotide-free Hsc70 is flexible because the rates of substrate dissociation depend on the substrate: slow for peptide and rapid for clathrin. The same reaction kinetics also occur with nucleotide-free WT Hsp70. In these experiments, the D10S mutant could be used directly after preparation since it comes essentially free of bound nucleotide, whereas with the WT Hsp70 and the K71 mutant the nucleotide-free enzyme had to be prepared according to Gao et al. (*21*).

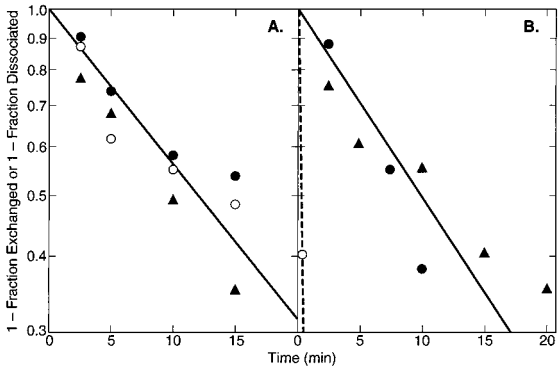

FIGURE 2: Effect of mutations at the nucleotide-binding site of Hsp70 on the rate of dissociation of cytochrome *c* peptide. (A) The dissociation of peptide in the absence of nucleotide was measured using nucleotide-free preparations of Hsp70. Hsp70 (5 μM) was incubated with 80 μM cytochrome *c* peptide at 25 °C for 2 h using WT Hsp70 (○), D10S Hsp70 (▲), and K71E Hsp70 (●). ^{14}C-Labeled cytochrome *c* peptide (10 μM) was then added, and at varying times, the reaction mixture was chromatographed on a Superose 12 column to determine the fraction of exchanged peptide. (B) the dissociation of cytochrome *c* peptide from Hsp70 in ATP. The rates of dissociation of cytochrome *c* peptide from D10S Hsp70 (▲) and K71E Hsp70 (●) were determined as above except that the peptide and enzyme were incubated in the presence of 1 mM ATP and an ATP-regenerating system. The rate of dissociation of peptide from WT Hsp70 (○) in ATP (dashed line) was measured by first mixing nucleotide free Hsp70 (5 μM) with ^{14}C-labeled cytochrome *c* peptide (10 μM) for 2 h at 25 °C. The extent of peptide dissociation was then determined 30 s after the addition of 1 mM ATP.

Figure 2A shows that peptide dissociates with the same slow rates from the nucleotide-free D10S and K71E mutants as from nucleotide-free WT Hsp70, demonstrating that these mutations have little effect on the slow rate of dissociation of peptide from nucleotide-free Hsp70. Likewise, Figure 3A shows that the D10S mutation has almost no effect on the rapid interaction of clathrin with nucleotide-free Hsp70. The K71E mutation does slightly decrease both the binding strength of clathrin to nucleotide-free Hsp70 and the rate of formation of the nucleotide-free Hsp70−clathrin complex. However, the half-life for reaching the equilibrium binding level is still less than 5 min. Therefore, neither the D10S nor the K71E mutant significantly affects the substrate-binding properties of nucleotide-free WT Hsp70.

Interaction of D10S and K71E Hsp70 with Cytochrome c Peptide. If ATP must bind strongly to Hsp70 to affect its interaction with substrate, ATP should have little effect on the interaction of substrates with the D10S and K71E mutants since ATP binds so weakly to both mutants. Figure 2B shows a comparison of the dissociation rate of cytochrome *c* peptide from both WT Hsp70 and the mutant Hsp70s in the presence of ATP. As expected, ATP decreases the half-life of peptide dissociation from WT Hsp70 from about 15 min to less than 30 s (open circle, Figure 2B), but has almost

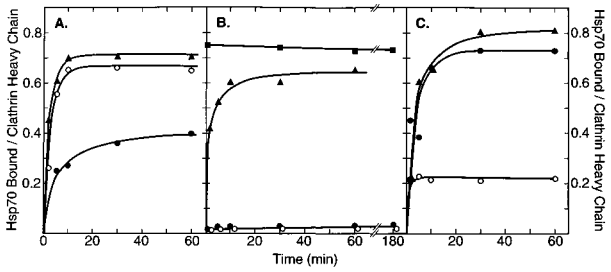

FIGURE 3: Effect of mutations at the nucleotide-binding site of Hsp70 on the interaction of Hsp70 and clathrin. (A) the rate of association of clathrin with Hsp70 in the absence of nucleotide was measured using nucleotide-free enzyme. Hsp70's (5 μM) were incubated with 1 μM free clathrin in buffer A at 25 °C for various times using WT Hsp70 (○), D10S Hsp70 (▲), and K71E Hsp70 (●). At the indicated time point, samples were chromatographed on a FPLC Superose 6 column. The fractions containing Hsp70−clathrin complexes were run on SDS−PAGE, and the Coomassie blue staining of the clathrin and Hsp70 was quantified. (B) The rate of association of clathrin with Hsp70 was measured in ADP using WT Hsp70 (○), D10S Hsp70 (▲), and K71E Hsp70 (●). The experimental conditions were the same as above, except the solution contained 1 mM ADP, 50 units/mL hexokinase, and 5 mM glucose. The rate of dissociation of clathrin from K71E Hsp70 (■) was measured by first forming the complex in the presence of 200 μM ATP, and then hexokinase and glucose were added. At varying times, the reaction mixture was chromatographed, and the extent of clathrin−Hsp70 complex was determined. (C) The rate of association of clathrin with Hsp70 in ATP was measured using WT Hsp70 (○), D10S Hsp70 (▲), and K71E Hsp70 (●). The experimental conditions were the same as in (A) except the solution contained 1 mM ATP and an ATP-regenerating system

Table 2: Effect of Nucleotide on Peptide Binding to WT and Mutant Hsp70s

	K_{DISS} (μM)[a]		
Hsp70	nucleotide-free	ATP	ADP
WT	9	200	5
D10S	4	5	6
K71E	20	5	4

[a] K_{DISS} values were obtained from Scatchard plot analysis of the data in which varying peptide concentrations (at least five different concentrations) were added to a fixed concentration of Hsp70.

no effect on the rate of peptide dissociation from the D10S and K71E mutants (solid symbols, Figure 2B) even though these mutants are saturated with ATP under these conditions. Therefore, ATP that is weakly bound to the D10S and K71E mutants does not induce rapid dissociation of peptide from these mutants. This, in turn, predicts that ATP will also not weaken the binding of cytochrome *c* peptide to the mutants, and the data shown in column 3 of Table 2 confirm this prediction and also show that, because of detailed balance, the binding of peptides does not weaken the binding of ATP to the nucleotide-free mutants. In contrast, strongly bound ATP markedly weakens the binding of cytochrome *c* peptide to nucleotide-free WT Hsp70.

Gao et al. (*9*) previously found that tightly bound ADP only has a relatively small effect on the rate of dissociation of cytochrome *c* peptide from nucleotide-free Hsc70, increasing the half-life of dissociation from about 15 min to about 75 min. We found that tightly bound ADP has a similar effect on the half-life for dissociation of cytochrome *c* peptide from both nucleotide-free WT Hsp70 and the nucleotide-free K71E mutant, but even with the D10S mutant, which binds ADP weakly, the half-life for dissociation of cytochrome *c* peptide increases about 3-fold in the presence of saturating levels of ADP (data not shown). Since peptide already dissociates slowly from nucleotide-free Hsp70, the abilities of both strongly bound and weakly bound ADP to further decrease this rate of dissociation are relatively small. Therefore, with these small effects, it is difficult to determine the effect of strongly bound versus weakly bound ADP on the interaction of peptide with Hsp70.

Interaction of D10S and K71E Hsp70 with Clathrin. Although strongly bound ADP only has a small effect on the interaction of nucleotide-free Hsp70 with peptide, it dramatically decreases both the rate of formation and the rate of dissociation of the complex of nucleotide-free Hsc70 with soluble clathrin triskelions. If ADP must bind strongly to Hsp70 to cause this effect, this effect should not occur with the D10S mutant which binds ADP weakly but it should occur with the K71E mutant which binds ADP strongly. Figure 3B confirms this prediction. Clathrin triskelions reach their equilibrium level of binding to the D10S mutant with a half-life of less than 3 min even though the D10S mutant is saturated with ADP under these conditions. In contrast, as with WT Hsp70, clathrin triskelions bind to and dissociate from the K71E mutant so slowly in ADP that we were unable to obtain these rate constants. In fact, to measure the dissociation rate of clathrin from K71E mutant, it was necessary to first allow the mutant to bind the clathrin in ATP (see below) and then to add hexokinase and glucose to measure the dissociation of the clathrin−Hsp70 complex (solid squares, Figure 3B).

These data suggest that the effect of ADP correlates with its strength of binding to the mutant Hsp70s. ADP binds tightly to the K71E mutant and correspondingly affects the interaction of the K71E mutant with clathrin in the same way that it affects the interaction of WT Hsp70 with clathrin. On the other hand, ADP binds weakly to the D10S mutant and correspondingly has no effect on the rapid binding of clathrin to and dissociation of clathrin from the nucleotide-free D10S mutant.

We next investigated the effect of weakly and strongly bound ATP on the interaction of clathrin with Hsp70. Gao et al. (*9*) previously found that, since clathrin already binds rapidly to nucleotide-free Hsp70, tightly bound ATP has little further effect on the rate of this binding. On this basis, we did not expect to observe significant differences between the effects of weakly and strongly bound ATP on the interaction of clathrin with Hsp70, and Figure 3C confirms this expectation. As was observed for bovine brain Hsc70 (*9*), clathrin binds 3−4-fold more strongly to nucleotide-free Hsp70 than to WT Hsp70−ATP (open circles, Figure 3A,C), but strongly bound ATP has almost no effect on the rate of interaction of clathrin with nucleotide-free WT Hsp70. Weakly bound ATP also has almost no effect on the rapid interaction of clathrin with the nucleotide-free D10S mutant. It does increase the rate and strength of binding of clathrin to the nucleotide-free K71E mutant by about a factor of 2 (solid circles, Figure 3A,C), but, like the small effect of ADP on the rate of dissociation of peptide from the D10S mutant, this small effect does not imply that the binding of ATP causes a physiologically important change in the conformation of the K71E mutant.

Interaction of D10S and K71E Hsp70 with DnaJ Homologues. We recently found that the DnaJ homolog auxilin

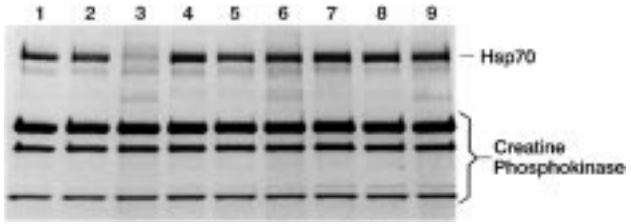

FIGURE 4: Mutant Hsp70s do not bind to clathrin baskets at pH 6.0. Hsp70 (0.6 μM) was incubated in the absence and presence of mixed AP clathrin baskets (0.6 μM) for 15 min at 25 °C in 1 mM ATP and an ATP-regenerating system in a pH 6.0 buffer. The samples were centrifuged, and 20 μL of each sample was loaded on SDS gels. Lanes 1−3 are with WT Hsp70, lanes 4−6 are with D10S Hsp70, and lanes 7−9 are with K71E Hsp70. Lanes 1, 4, and 7 show the total Hsp70; lanes 2, 5, and 8 show the Hsp70 in the supernatant after centrifugation in the absence of mixed AP clathrin baskets; lanes 3, 6, and 9 show the Hsp70 remaining in the supernatant after centrifugation in the presence of mixed AP clathrin baskets.

is required for Hsc70 to uncoat clathrin-coated vesicles or artificial clathrin baskets (*25, 26*). Furthermore, at pH 6.0, under conditions where clathrin baskets cannot be uncoated by Hsc70 (*27*), Hsc70 binds to baskets in an ATP-dependent manner (*22, 26*). Since ATP is required for the binding at pH 6.0, we were interested in whether binding would occur with the D10S or K71E mutants which both bind ATP weakly. The SDS gel in Figure 4 shows the supernatant of WT and mutant Hsp70s after centrifugation of the Hsp70 in the presence and absence of mixed AP clathrin baskets at pH 6.0. We found that, in addition to their inability to uncoat clathrin vesicles at pH 7.0, the D10S and K71E mutants bind considerably more weakly to clathrin baskets in the presence of auxilin at pH 6 (lanes 6 and 9, Figure 4) than does WT Hsp70 (lane 3).

A similar result was obtained for another ATP-dependent interaction of DnaJ homologues with Hsc70, the ability of YDJ1 and HDJ1 to catalytically induce polymerization of Hsc70 (*24*). Figure 5A (solid symbols) shows that WT Hsp70 in the presence of YDJ1 elutes in two peaks, a peak corresponding to Hsp70 polymer, which corresponds to the void volume, and a monomeric peak. Therefore, YDJ1 induces polymerization of WT Hsp70, although to a lesser extent than it induces polymerization of Hsc70; in this regard, WT Hsp70 appears to be similar to SSA1, which is also only partially polymerized by YDJ1 (*24*). However, in contrast to the ability of YDJ1 to induce partial polymerization of WT Hsp70, there was no significant difference in profile of the mutant Hsp70s in the presence and absence of YDJ1 (Figure 5B,C). This shows that neither the D10S nor the K71E mutants were significantly polymerized by YDJ1.

DISCUSSION

Before studying the functional effect of mutations at the ATP-binding site of Hsc70, it was first necessary to test whether WT recombinant Hsp70 has the same activity as Hsc70 prepared directly from bovine brain. In these studies, we used clathrin uncoating as a measure of activity. We found that, provided the recombinant protein was prepared in *E. coli* grown at 28 °C (see Results), the human Hsp70 has the same uncoating activity as bovine brain Hsp70.

We then asked whether mutations at the nucleotide-binding site of Hsp70 have a significant effect on the interaction of

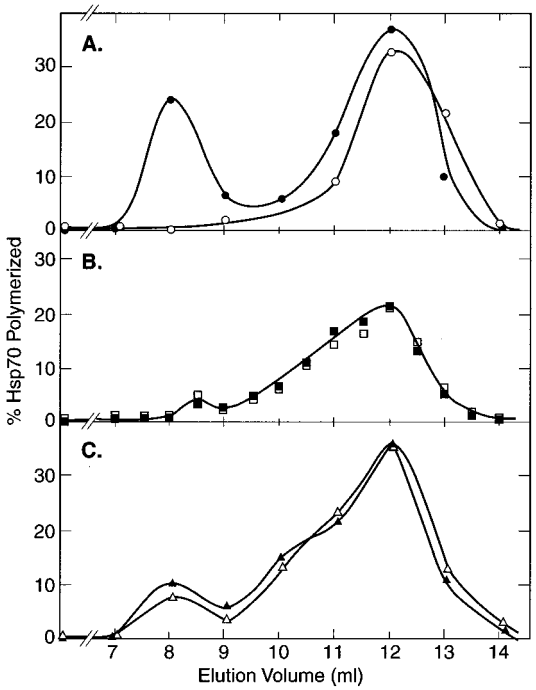

FIGURE 5: Mutant Hsp70s are not polymerized by YDJ1. Hsp70 (10 μM) was incubated in the absence and presence of YDJ1 (10 μM) for 30 min at 25 °C in buffer A and 1 mM ATP and an ATP-regenerating system, followed by FPLC chromatography on Superose 12. In (A), the Hsp70 profiles were obtained with WT Hsp70; in (B), the profiles were obtained with D10S Hsp70; and in (C), the profiles were obtained with K71E Hsp70. In each panel, the Hsp70 profiles in the absence and presence of YDJ1 are given by the open and closed symbols, respectively.

nucleotide-free Hsp70 with substrate. The substrate-binding site of nucleotide-free Hsc70 appears to be flexible in regard to its interaction with peptide and clathrin. Specifically, peptide dissociates slowly from nucleotide-free Hsc70 like it does from Hsc70−ADP while clathrin dissociates rapidly from nucleotide-free Hsc70 like it does from Hsc70-ATP (*9*). Nucleotide-free recombinant WT Hsp70 showed these same specific properties and neither the D10S nor the K71E mutation had a significant effect on these properties; although clathrin binds to the nucleotide-free K71E mutant both about a factor of 2 weaker and a factor of 2 slower than it binds to nucleotide-free WT Hsp70, the effect of this mutation is small compared to the dramatic decrease in the rate of interaction of clathrin with WT Hsp70 caused by the strong binding of ADP. Therefore, although these two mutations have large effects on the binding of nucleotide and the ATPase activity of the mutants, they do not appear to significantly affect the interaction of nucleotide-free Hsp70 with substrate.

We next investigated whether weak binding of ATP and ADP to mutant Hsp70s causes the same conformational changes as the strong binding of ATP and ADP to WT Hsp70. In an earlier study (*9*), we found that when ATP binds to nucleotide-free Hsc70 it causes at least a 30-fold increase in the rate of peptide release, and when ADP binds to nucleotide-free Hsc70, it reduces the rates of clathrin interaction with Hsc70 by at least 2 orders of magnitude; clearly these large effects could not occur unless both ATP and ADP induce physiologically significant conformational changes in Hsc70. Note that, in studying the effect of ADP on nucleotide-free Hsp70, we determined its effect on the

rates of clathrin binding and dissociation while in studying the effect of ATP on nucleotide-free Hsp70 we determined its effect on the rate of peptide dissociation, because ADP has little further effect on the slow rate of peptide dissociation and ATP has little further effect on the rapid rates of clathrin binding and dissociation.

On this basis, we found that the effect of Hsp70 mutations on the strength of nucleotide binding at the active site appears to be directly related to their effect on substrate binding. Both ATP and ADP bind about 2 orders of magnitude more weakly to the D10S mutant than to WT Hsp70, and ATP has very little effect on the rate of peptide dissociation from nucleotide-free Hsp70 while ADP has very little effect on the rates of interaction of clathrin with Hsp70; with either ATP or ADP bound, the D10S mutant continues to behave like nucleotide-free Hsc70. A similar correlation between the strength of nucleotide binding and the effect of bound nucleotide is observed with the K71E mutant that binds ATP weakly but binds ADP as strongly as wild-type Hsp70. With weakly bound ATP, the K71E mutant essentially behaves like nucleotide-free Hsp70 in regard to the slow rate of peptide dissociation while with strongly bound ADP it essentially behaves like WT Hsp70 with bound ADP in regard to the rates of both binding and dissociation of clathrin. The weak binding of ATP did increase the rate of binding and strength of binding of clathrin to the nucleotide-free K71E mutant by about a factor of 2, but as we discussed above, the rate of binding of clathrin to the nucleotide-free K71E mutant is already quite fast so we do not consider this effect physiologically significant.

The inability of weakly bound ATP to significantly affect the rate of dissociation of peptide from nucleotide-free mutant Hsp70 and the inability of weakly bound ADP to decrease the rates of interaction of clathrin with nucleotide-free mutant Hsp70 are very similar to the inability of weakly binding nucleotide analogues such as AMP-PNP to affect substrate binding to nucleotide-free WT Hsc70 (*9*). These data suggest that ATP and ADP must bind strongly to Hsp70 in order to cause the physiologically important conformational changes that affect substrate binding. Either the use of nucleotide analogues or the perturbation of the active site by mutations prevents this tight binding, thus abolishing the major physiological effects of the bound nucleotide. A similar effect apparently occurs with BiP the Hsc70 homologue present in the endoplasmic reticulum. Mutations at G226 and G227 of BiP, (equivalent to G201 and G202 of bovine brain Hsc70) not only make ATP binding so weak that it cannot be detected in equilibrium dialysis experiments but also qualitatively prevent ATP from dissociating the mutant BiP from a peptide affinity column (*28*). However, it is not clear whether this phenomenon also occurs with DnaK, the Hsp70 present in *E. coli*, since AMP-PNP has been reported to have an effect on the interaction of nucleotide-free DnaK with substrate (*29*). On the other hand, Buchberger et al. (*30*) observed that when DnaK was mutated at E171 (equivalent to E175 of bovine brain Hsc70), ATP binding was weakened 10−100-fold, while at the same time ATP no longer weakened the binding of peptide to the mutant *E. coli* DnaK just as we observed with our mutations of Hsp70. However, the results with DnaK were interpreted as mutation of E171 interrupting the communication between the ATP-binding site and the substrate-binding region of DnaK rather than simply maintaining the nucleotide-free conformation of DnaK. It will be interesting to determine whether mutating the equivalent residue in mammalian Hsp70 (E175) causes similar global effects or, as we found for mutation of D10 and K71, all of the effects of the E175 mutation can be simply explained by a weakened binding of ATP. In this regard, deletion of the carboxyl-terminal four amino acids of Hsp70 has been reported to globally interfere with the interaction of nucleotide- and substrate-binding sites of Hsp70 (*31*).

The D10S and K71E mutants have not only lost their response to ATP in regard to substrate binding, but also no longer carry out reactions which require DnaJ homologues. In contrast to WT Hsp70, neither the D10S nor the K71E mutants polymerize in the presence of YDJ1, carry out the uncoating reaction at pH 7.0, or bind strongly to clathrin baskets at pH 6.0 in the presence of the DnaJ homolog auxilin. It is possible that all of these effects are lost because the ATPase activity of the D10S and K71E mutants is lost in addition to their ability to bind ATP strongly. It is particularly interesting that even the auxilin-induced binding of the D10S and K71E mutants to clathrin baskets at pH 6.0 is greatly reduced. These data raise the possibility that a tightly bound ATP is required for Hsp70 to interact with DnaJ homologues. However, much more work will be required to differentiate the effects of ATP binding and ATP hydrolysis on the interaction of Hsp70s with DnaJ homologues.

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