Interaction of Nucleotide-Free Hsc70 with Clathrin and Peptide and Effect of ATP Analogues

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ABSTRACT: The functions of the 70 kDa heat-shock proteins (hsp70s) are regulated by their bound nucleotide. We previously observed major differences in the effect of bound ATP and ADP on the interaction of hsc70 (constitutive hsp70) with its protein substrates. In the present study, we investigated the interaction of protein substrates with nucleotide-free hsc70 and with hsc70 with bound ATP analogues. We found, first, that nucleotide-free hsc70 appeared to interact differently with different substrates. Specifically, nucleotide-free hsc70 behaved much more like hsc70−ATP than hsc70−ADP in that clathrin very rapidly bound to and dissociated from nucleotide-free hsc70 in contrast to its very slow binding to and dissociation from hsc70−ADP. On the other hand, nucleotide-free hsc70 behaved more like hsc70−ADP than hsc70−ATP in that cytochrome *c* peptide dissociated very slowly from nucleotide-free hsc70 compared to its rapid dissociation from hsc70−ATP. Second, binding of the ATP analogues AMP-PNP, dATP, and ATPγS to nucleotide-free hsc70 had very little further effect on the properties of the nucleotide-free hsc70. Therefore, previously observed effects of ATP analogues may have been due to removal of the bound ADP rather than to the presence of analogues.

The 70 kDa heat-shock proteins (hsp70s) are among the major heat-shock proteins produced by cells both constitutively and under stress conditions [for reviews, see Craig (1985), Lindquist (1986), and Nover and Scharf (1991)]. Studies show that the hsp70s act as molecular chaperones, disaggregating proteins, and keeping newly synthesized proteins in an unfolded form as they move within the cell. As molecular chaperones, the constitutive hsp70s, or hsc70s, are involved in many fundamental cellular processes such as protein translocation across the membranes of cell organelles (Chirico et al., 1988; Deshaies et al., 1988; Shi & Thomas, 1992; Imamoto et al., 1992; Brodsky et al., 1993), nascent protein folding and multiunit protein assembly (Beckmann et al., 1990; Bole et al., 1986; Flynn et al., 1989), antigen presentation (Vanbuskirk et al., 1989; DeNagel & Pierce, 1993), protein degradation in the lysosome (Chiang et al., 1989; Terlecky et al., 1992), and uncoating of clathrin-coated vesicles (Schlossman et al., 1984; Greene & Eisenberg, 1990; Gao et al., 1991).

Hsc70 has a single binding site for nucleotide (Gao et al., 1993b) and binds both ATP and ADP very tightly (Gao et al., 1994). In addition, ATP hydrolysis apparently plays an important role in almost all of the functions of hsc70. In investigating the effect of ATP hydrolysis on the interaction of hsc70 with its protein substrates, we had found that both clathrin and several peptide substrates of hsc70 dissociated very slowly from hsc70−ADP but rapidly from hsc70−ATP (Prasad et al., 1994; Greene et al., 1995). The ATP-binding portion of hsc70 has been crystallized (Flaherty et al., 1990), and one of the key questions on the mechanism of action of hsc70 is how the structure of hsc70 as described by crystallography is related to the function of hsc70. In particular, it is important to understand how the interaction of the nucleotide with the nucleotide-binding site of hsc70 controls its interaction with protein substrates. One way to approach this question is to remove the bound nucleotide from hsc70 and determine how nucleotide-free hsc70 interacts with protein substrates.

We previously demonstrated that the tightly bound ADP of bovine brain hsc70 could be replaced by AMP-PNP which could then be removed by extensive dialysis (Gao et al., 1994). The nucleotide-free hsc70 retains its full enzymatic functions including binding and hydrolyzing ATP, binding the nucleotide analogues, AMP-PNP and dATP, and uncoating clathrin-coated vesicles (Gao et al., 1994). In the present study, we investigated the interaction of nucleotide-free hsc70 with clathrin, cytochrome *c* peptide, and peptide C. In addition, we bound the ATP analogues, AMP-PNP, dATP, and ATPγS, to nucleotide-free hsc70 to study their effect on the interaction of hsc70 with protein and peptide substrates. We found that clathrin bound to and dissociated from nucleotide-free hsc70 almost as quickly as from hsc70−ATP, but surprisingly cytochrome *c* peptide dissociated from nucleotide-free hsc70 almost as slowly as from hsc70−ADP. In both cases, ATP analogues had little effect on the interaction of protein and peptide substrates with nucleotide-free hsc70. These data show, first, that the nature of the protein substrate may determine the type of interaction it has with nucleotide-free hsc70 and, second, that ATP analogues have very little effect on the properties of nucleotide-free hsc70.

MATERIALS AND METHODS

ATP (catalog no. A5394), AMP-PNP (catalog no. A2647), and dATP (catalog no. D6500) were purchased from Sigma. ADP (catalog no. 01899) was obtained from Fluka. ATPγS (catalog no. 102342) was from Boehringer.

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Purification of Clathrin and Hsc70. Clathrin was extracted from isolated clathrin-coated vesicles using 0.5 M Tris, pH 7.0, and precipitated from the high-speed supernatant by adding 1:1 (v/v) saturated ammonium sulfate. Then the ammonium sulfate pellet was dissolved in 0.5 M Tris, pH 7.0, and loaded onto a Superose 6 (Pharmacia, code 17-0489-01) filtration column (2.5 × 90 cm) equilibrated in 0.5 M Tris, pH 7.0 at 25 °C. The clathrin peak was pooled, and clathrin was precipitated by adding 1:1 saturated ammonium sulfate. Finally, the ammonium sulfate pellet was dissolved and dialyzed against buffer A (20 mM imidazole, 25 mM KCl, 10 mM ammonium sulfate, 2 mM magnesium acetate, and 1 mM dithiothreitol, pH 7.0) for 20 h at 4 °C before use. Bovine brain hsc70 was purified by the method of Schlossman et al. (1984) with modifications described by Greene and Eisenberg (1990). Purified hsc70 was stored as ammonium sulfate pellets at 4 °C, which were dissolved and dialyzed in buffer A for 20 h at 4 °C before use. Protein concentrations of clathrin and hsc70 were determined using extinction coefficients reported previously [$\epsilon_{280}^{1\%} = 12$ for clathrin (Unanue et al., 1981) and 6.2 for hsc70 (Greene & Eisenberg, 1990)].

Preparation of Nucleotide-Free Hsc70. Nucleotide-free hsc70 was prepared as described previously (Gao et al., 1994) by replacing bound ADP with AMP-PNP and extensive dialysis to remove AMP-PNP. The nucleotide-free hsc70 prepared by this procedure has less than 5% bound nucleotide and is fully functional in enzymatic activities tested, including binding and hydrolyzing ATP and uncoating of clathrin-coated vesicles.

Analysis of Hsc70 Polymerization by FPLC. Two hundred microliters of hsc70 (10 μM) with various bound nucleotides was loaded onto a Superose 12 analytical column (Pharmacia, code 17-0538-01) equilibrated in buffer A at a flow rate of 0.5 mL/min. The recording speed was set at 0.4 cm/mL and the UV monitor at an OD range of 0.1; 0.5 mL fractions were collected and cross-linked according to the procedure described by Schlossman et al. (1984). The cross-linking was carried out for 1 min at 25 °C with 15 mM glutaraldehyde (Fluka, catalog no. 49632), and the reaction was stopped by adding 80 mM $NaBH_4$ (Fluka, catalog no. 71320). The samples were then analyzed on SDS−PAGE, and the amount of monomeric hsc70 was quantified by desitometric scanning.

Preparation of Hsc70−Nucleotide Complex. Nucleotide-free hsc70 was incubated with different nucleotide or analogue at 25 °C for 10 min before clathrin or peptide was added. Concentrations of nucleotide and analogue used were calculated to ensure near-saturation binding to hsc70 according to the binding strength measured previously (Gao et al., 1994). ATP was used with an ATP-regeneration system containing 50 units/mL phosphocreatine kinase (Sigma, catalog no. C-3755) and 10 mM creatine phosphate (Sigma, catalog no. P-4635). ADP was used with 50 units/mL hexokinase (Sigma, catalog no. H-1131) and 10 mM glucose.

Measurement of Hsc70 Association with Clathrin by FPLC. Free hsc70 was separated from clathrin and bound hsc70 by gel filtration FPLC (Gao et al., 1991) on a Superose 6 column (Pharmacia, code 17-0537-01). Fractions containing clathrin and bound hsc70 were analyzed on SDS gels. The amount of bound hsc70 was quantified by densitometric scanning.

Preparation of Labeled Nucleotide-Free Hsc70 and Measurement of Dissociation of Hsc70 from the Clathrin−Hsc70 Complex. Purified hsc70 was trace-labeled according to the procedure of Jentoft and Dearborn (1983). Generally, 1 mL of hsc70 (30 μM) in 50 mM HEPES, pH 7.0, was incubated with 5 mM $NaBO_3CN$ and 10 μCi of [^{14}C]formaldehyde (NEN, catalog no. NEC039H) at 4 °C for 5 h before 5 mM dithiothreitol was added and the mixture was dialyzed in buffer A. After a brief clarification (Backman TL-100, 100K, 6 min), the labeled hsc70 was further purified with an ATP−agarose column. The labeled hsc70 eluted from the ATP−agarose column was made nucleotide-free as described above. For measurement of clathrin-bound hsc70 exchange, 5 μM nucleotide-free hsc70 was incubated with different nucleotide or analogue, or with no added nucleotide, for 10 min at 25 °C before 1 μM clathrin was added. The mixture was then incubated for 60 min to obtain equilibrium binding, and then 0.3−0.5 μM radioactive nucleotide-free hsc70 or hsc70 with bound nucleotide or analogue was added. Free hsc70 was separated from bound by FPLC (Superose 6 column). The amount of exchange was quantified by liquid scintillation counting of both bound and free hsc70.

Preparation of Labeled Peptides and Determination of Peptide Association with Hsc70 and Dissociation from the Hsc70−Peptide Complex. Amino acid sequences of the peptides used were published previously (Greene et al., 1995). The peptides were trace-labeled by alkaline methylation (Jentoft & Dearborn, 1983). Generally, 1 mL of the peptide (100 μM) was incubated with 5 mM $NaBO_3CN$ and 10 μCi of [^{14}C]formaldehyde at 4 °C for 5 h before 5 mM dithiothreitol was added and the reaction mixture was dialyzed in buffer A in a dialysis tubing with a molecular weight cutoff of 500 daltons (Spectrum, catalog no. 131051) to remove free formaldehyde. For measurement of binding, free peptide was separated from bound peptide and hsc70 either by an FPLC filtration column (Superose 12 analytical column) or by Nick columns as described by Flynn et al. (1991). Then the bound and free peptide fractions (when FPLC was used) or bound peptide fraction only (when Nick columns were used) was quantified by liquid scintillation counting. For measurement of bound peptide exchange, 5 μM hsc70 was incubated with 50 μM peptide at 25 °C with the equilibrium was reached (1 h) before 2−5 μM radioactive peptide was added. At designated time points, an aliquot was loaded onto FPLC, and both free and hsc70-bound peptides were quantified by scintillation counting.

RESULTS

Polymerization of Nucleotide-Free Hsc70 and Effect of the ATP Analogues. To determine the properties of nucleotide-free hsc70 and hsc70 with bound ATP analogues, we first studied the polymerization of nucleotide-free hsc70. Schmid et al. (1985) showed that, in the presence of ATP, hsc70 occurs almost completely as monomer while in the presence of ADP it polymerizes to form dimers, trimers, and higher-order polymers. We confirmed this observation by FPLC gel filtration (Figure 1a,b). We then tested the extent of polymerization of nucleotide-free hsc70 and hsc70 with bound AMP-PNP or dATP. Our results show, first, that removing the bound ADP does not significantly change the extent of hsc70 polymerization; nucleotide-free hsc70 contains similar amounts of monomers, dimers, and higher-order

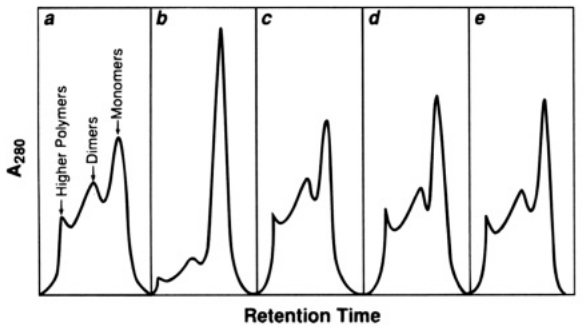

FIGURE 1: FPLC elution profiles showing effects of ADP, ATP, and ATP analogues on hsc70 polymerization. 10 μM of (a) ATP−agarose-purified hsc70 (hsc70−ADP). (b) Hsc70 with bound ATP (25 μM ATP). (c) Nucleotide-free hsc70. (d) Hsc70 with bound AMP-PNP (100 μM AMP-PNP). (e) Hsc70 with bound dATP (200 μM dATP). Peaks were identified by cross-linking and SDS−PAGE of cross-linked samples (see Materials and Methods).

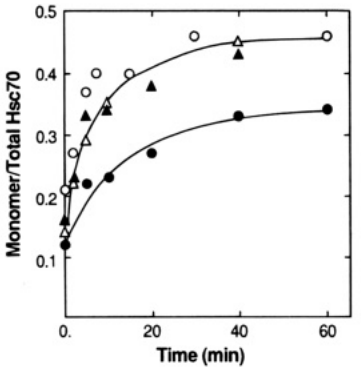

FIGURE 2: Monomer−polymer equilibrium of hsc70 at 25 °C. 50 μM purified hsc70 with bound ADP (●), or nucleotide-free hsc70 (○), or nucleotide-free hsc70 in 100 μM AMP-PNP (▲) or in 200 μM dATP (△) was preincubated at 25 °C for 20 min before being diluted 10 times with buffer A of the same temperature. Samples were then taken at each time point and cross-linked with glutaraldehyde (see Materials and Methods). The amount of monomeric hsc70 was quantified by densitometric scanning of SDS gels of cross-linked samples.

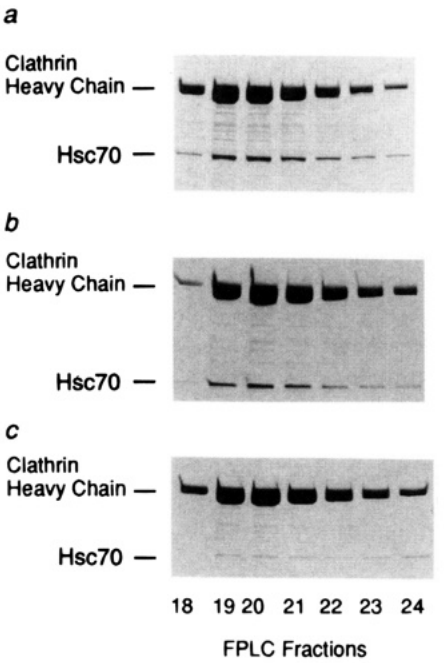

FIGURE 3: Binding of nucleotide-free hsc70 to clathrin and effect of ATP and ADP. 5 μM nucleotide-free hsc70 was preincubated with no nucleotide (a), or with 25 μM ATP (b), or with 25 μM ADP (c) for 10 min at 25 °C before 1 μM clathrin was added and the mixtures were incubated for another 60 min at the same temperature. FPLC fractions of the clathrin peak were analyzed by SDS−PAGE.

polymers as hsc70 with bound ADP (Figure 1c). Second, our results show that the same pattern of hsc70 polymerization was observed for hsc70 with bound AMP-PNP and dATP as for nucleotide-free hsc70 and hsc70 with bound ADP (Figure 1d,e).

We also determined the rate of the monomer−polymer equilibrium for nucleotide-free hsc70, hsc70−AMP-PNP, and hsc70−dATP. Since the amount of polymer present is dependent on hsc70 concentration, the rate of depolymerization can be determined by diluting concentrated hsc70 and then measuring the rate at which it reaches a new steady-state level of monomer and polymer (Figure 2). The results suggest that, at 25 °C, the half-life for reaching steady-state is about 10 min for hsc70−ADP and about 5 min for nucleotide-free hsc70 and hsc70 with bound AMP-PNP or dATP. Therefore, as we found for the extent of polymerization, nucleotide-free hsc70 and hsc70 with bound ATP analogues closely resemble hsc70−ADP in their rates of depolymerization.

Rate of Binding of Clathrin or Cytochrome c Peptide to Hsc70. We next investigated whether nucleotide-free hsc70 resembles hsc70−ADP in its interaction with clathrin. In our earlier studies, we found that, in the presence of ATP, clathrin bound to hsc70 very rapidly, while in the presence of ADP, the rate of binding of hsc70 to clathrin was too slow to measure (Prasad et al., 1994). Since hsc70, as normally purified, contains a tightly bound ADP, it binds to clathrin so slowly that, over a period of an hour, almost no hsc70−clathrin complex is formed. We therefore began this study by determining whether nucleotide-free hsc70, like hsc70−ADP, is also unable to bind to clathrin over a period of an hour. Surprisingly, we found that after removing the tightly bound ADP, even more nucleotide-free hsc70 bound to clathrin than hsc70−ATP (Figure 3a,b), suggesting that, after removal of bound ADP, nucleotide-free hsc70 acts more like hsc70−ATP than hsc70−ADP in its interaction with clathrin. This effect of removing bound ADP was reversible. While preincubation of nucleotide-free hsc70 with ATP had little effect on its clathrin-binding ability (Figure 3b), adding back ADP caused the hsc70 to again lose its ability to bind to clathrin over a 1-h period (Figure 3c). These data suggest that, with regard to the rate constant for association of clathrin with nucleotide-free hsc70, nucleotide-free hsc70 is much more similar to hsc70−ATP than to hsc70−ADP.

To confirm this result, we measured the rate constant for the binding of nucleotide-free hsc70 to clathrin and found that, indeed, this rate constant was very similar to the rate constant for the binding of hsc70−ATP. In both cases, the half-life for association of hsc70 with clathrin at an hsc70 concentration of 5 μM was less than 2 min (Figure 4a). In contrast, hsc70−ADP associates with clathrin too slowly to measure (Prasad et al., 1994). It should be noted that the rate of association of nucleotide-free hsc70 with clathrin appears to be somewhat biphasic, slowing at the end of the reaction. We think this is due to the fact that, in the absence of ATP, some of the nucleotide-free hsc70 is polymerized into dimers and higher-order polymers, and we have found that the binding of these polymers to clathrin only occurs after their dissociation to monomers, which decreases the rate of binding of the polymerized hsc70 to clathrin (Gao et

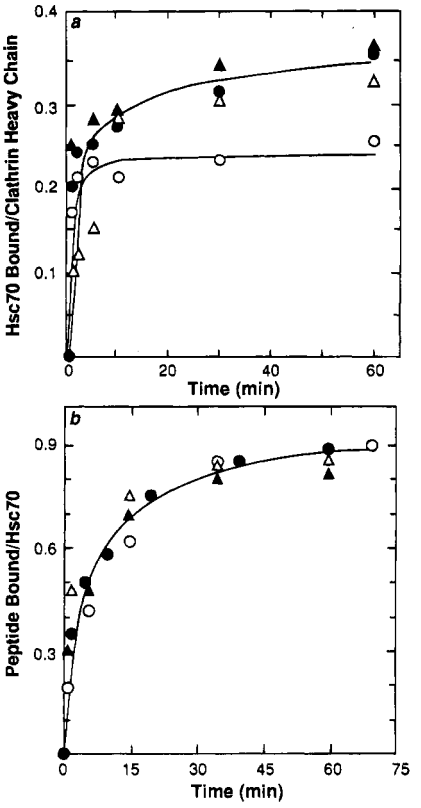

FIGURE 4: Rate of association of clathrin or cytochrome *c* peptide with hsc70. (a) Rate of association of 5 μM nucleotide-free hsc70 (●), or ATP-bound hsc70 (○, in 25 μM ATP), or AMP-PNP-bound hsc70 (▲, in 100 μM AMP-PNP), or dATP-bound hsc70 (△, in 200 μM dATP) to 1 μM clathrin at 25 °C. (b) Rate of association of 30 μM peptide to 5 μM nucleotide-free hsc70 (●), or to AMP-PNP-bound hsc70 (○), or to dATP-bound hsc70 (▲), or to ATPγS-bound hsc70 (△, in 20 μM ATPγS) at 25 °C.

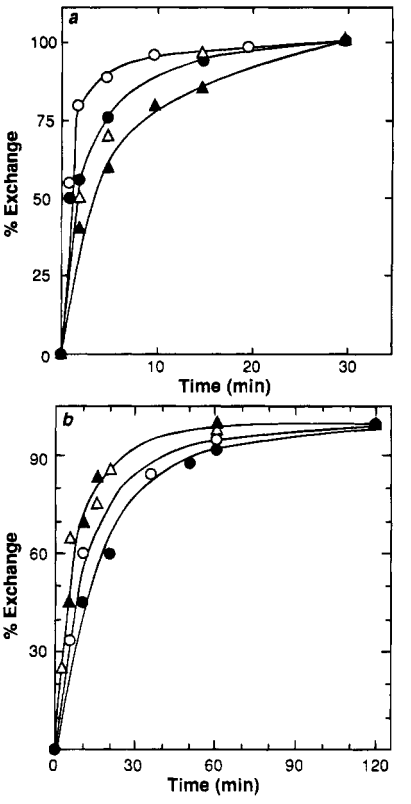

FIGURE 5: Rate of dissociation of clathrin–hsc70 or peptide–hsc70 complex. (a) Rate of dissociation of nucleotide-free hsc70 (●), or ATP-bound hsc70 (○, in 25 μM ATP), or AMP-PNP-bound hsc70 (▲, in 100 μM AMP-PNP), or dATP-bound hsc70 (△, in 200 μM dATP) from clathrin–hsc70 complex. (b) Rate of dissociation of cytochrome *c* peptide from nucleotide-free hsc70 (●), or from AMP-PNP-bound hsc70 (○), or from dATP-bound hsc70 (▲), or from ATPγS-bound hsc70 (△, in 20 μM ATPγS).

al., 1993a). We, therefore, conclude that nucleotide-free hsc70 is very similar to hsc70–ATP in its rate of binding to clathrin.

We next determined whether this was also the case for the rate of binding of cytochrome *c* peptide to hsc70. While clathrin binds to hsc70–ATP much more rapidly than to hsc70–ADP, this effect is not as pronounced for cytochrome *c* peptide. Earlier studies showed that cytochrome *c* peptide bound 7 times faster to hsc70–ATP (k = 140 M^{-1} s^{-1}) than to hsc70–ADP (k = 20 M^{-1} s^{-1}) (Greene et al., 1995). In agreement with our findings for clathrin, we found that the rate constant for binding of cytochrome *c* peptide to nucleotide-free hsc70 was 100–200 M^{-1} s^{-1}, closer to the value for hsc70–ATP than for hsc70–ADP (Figure 4b). It should be noted that, although we have obtained data suggesting that peptides bind only to monomeric hsc70 (Gao et al., 1993a), since, in the present experiments, the half-life for peptide binding to nucleotide-free hsc70 at 25 °C was about 10 min, the shift from polymer to monomer with a 5-min half-life should not significantly affect the observed rate constant or make it appear biphasic as occurred for the binding of clathrin. We therefore conclude that, while nucleotide-free hsc70 is nearly identical to hsc70–ADP in its polymerization properties, it closely resembles hsc70–ATP in its rate constant for binding both clathrin and cytochrome *c* peptide.

Rate of Dissociation of Clathrin or Cytochrome c Peptide from Hsc70. Since clathrin and cytochrome *c* peptide both dissociate very rapidly from hsc70–ATP and very slowly from hsc70–ADP, we previously concluded that the major effect of bound nucleotide on the properties of hsc70 is to control the rate of dissociation of substrates. Therefore, we were particularly interested in studying the rate of dissociation of clathrin and cytochrome *c* peptide from nucleotide-free hsc70. We first determined the rate of clathrin dissociation by measuring the rate of exchange of clathrin-bound nucleotide-free hsc70 with free hsc70. For comparison, we also measured the rate of exchange of clathrin-bound hsc70–ATP. Our results showed that nucleotide-free hsc70 dissociated from clathrin with a half-life of about 2 min or less compared to a half-life of dissociation of hsc70–ATP from clathrin of about 1 min (Figure 5a). In contrast, the dissociation of hsc70–ADP from clathrin is too slow to measure (Prasad et al., 1994). Therefore, nucleotide-free hsc70 appears to be nearly identical to hsc70–ATP in its rate of dissociation from clathrin just as it was in its rate of binding to clathrin.

We next studied the rate of dissociation of cytochrome *c* peptide from nucleotide-free hsc70. Earlier observations showed that cytochrome *c* peptide dissociated from hsc70–ADP with a half-life of about 75 min while it dissociated form hsc70–ATP with a half-life of about 15 s. We expected that like the association and dissociation of clathrin and the association of cytochrome *c* peptide, cytochrome *c* peptide dissociation from nucleotide-free hsc70 would be rapid just as it is from hsc70–ATP. Surprisingly, however, Figure 5b shows that cytochrome *c* peptide dissociates from

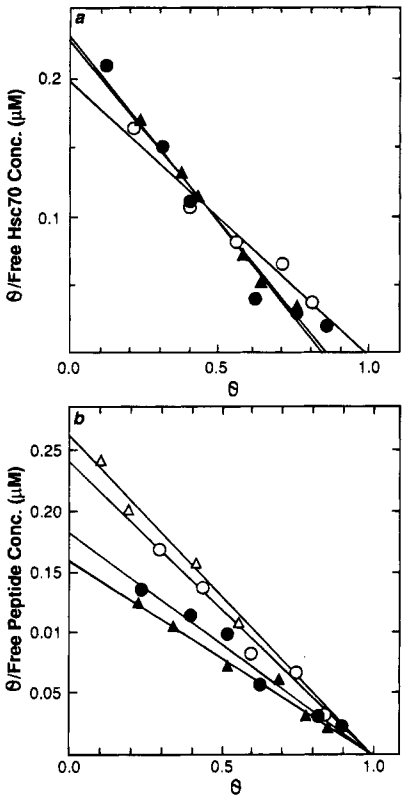

FIGURE 6: Binding strength of clathrin or cytochrome *c* peptide for hsc70. (a) Equilibrium binding of purified clathrin (1−2 μM) to nucleotide-free hsc70 (●), or to AMP-PNP-bound hsc70 (○, in 100 μM AMP-PNP), or to dATP-bound hsc70 (▲, in 200 μM dATP) of varying concentration (0.5−20 μM) at 25 °C. θ represents the percentage of clathrin with bound hsc70. (b) Equilibrium binding of cytochrome *c* peptide of varying concentration (1.5−50 μM) to 5 μM nucleotide-free hsc70 (●), or to AMP-PNP-bound hsc70 (○), or to dATP-bound hsc70 (▲), or to ATPγS-bound hsc70 (△, 20 μM ATPγS) at 25 °C. θ represents the percentage of hsc70 with bound cytochrome *c* peptide.

nucleotide-free hsc70 with a half-life of about 15 min, about 5 times faster than the rate of dissociation of the peptide from hsc70−ADP, but more than 60-fold slower than the rate of dissociation of the peptide from hsc70−ATP. Therefore, nucleotide-free hsc70 appears to be much more similar to hsc70−ADP than to hsc70−ATP in regard to the rate of dissociation of the peptide. This, in turn, suggests that the rate of dissociation of substrate from nucleotide-free hsc70 is not simply an intrinsic property of the nucleotide-free hsc70 but depends on the nature of the substrate as well.

Binding Strength of Clathrin or Cytochrome c Peptide to Hsc70. To confirm our measured rate constants for the interaction of clathrin and cytochrome *c* peptide with nucleotide-free hsc70, we measured their dissociation constants from nucleotide-free hsc70 which should be equal to the ratio of the dissociation and association rate constants. There is relatively little difference in the binding strength of hsc70−ATP and hsc70−ADP to clathrin because the rates of both association and dissociation increase proportionally when ADP is replaced by ATP. Since nucleotide-free hsc70 closely resembles hsc70−ATP, this should also be true for the binding strength of nucleotide-free hsc70 to clathrin, and the Scatchard plot in Figure 6a shows that this is indeed the case; the dissociation constant of nucleotide-free hsc70 from clathrin is 4×10^{-6} M, which is intermediate between the value of 12×10^{-6} M for hsc70−ATP binding and the value of 3×10^{-6} M for hsc70−ADP binding to clathrin.

The dissociation constant of cytochrome *c* peptide from nucleotide-free hsc70 is also consistent with the ratio of the rate constants for dissociation and association. The Scatchard plot in Figure 6b shows that the dissociation constant of cytochrome *c* peptide from nucleotide-free hsc70 is about 6×10^{-6} M, nearly identical to the value predicted by the ratio of the rate constants for dissociation and association (Table 2). Note that this dissociation constant is similar to the dissociation constant of cytochrome *c* peptide from hsc70−ADP rather than hsc70−ATP, as would be expected since the rate of dissociation of peptide from nucleotide-free hsc70 is almost as slow as from hsc70−ADP.

To determine if short peptides in general interact with nucleotide-free hsc70 differently than clathrin does, we tested the binding of another peptide, peptide C, to nucleotide-free hsc70. Earlier results showed that peptide C acted like cytochrome *c* peptide; it dissociated rapidly from hsc70−ATP and slowly from hsc70−ADP (Greene et al., 1995). Results in Table 2 show that peptide C acts likes cytochrome *c* peptide in interaction with nucleotide-free hsc70. It binds nucleotide-free hsc70 strongly and dissociates slowly like it does with hsc70−ADP.

Effect of ATP Analogues on the Interaction of Hsc70 with Clathrin or Cytochrome c Peptide. Although we observed no significant effect of the ATP analogues, AMP-PNP and dATP, on the polymerization properties of nucleotide-free hsc70, the ATP analogue ATPγS has been reported to greatly affect the binding of peptides to DnaK, the hsc70 protein present in *Escherichia coli*. Therefore, we tested the effect of AMP-PNP, dATP, and ATPγS on the interaction of nucleotide-free hsc70 with cytochrome *c* peptide. Since fresh ATPγS contained 3−5% ADP, we had to use a low ATPγS concentration to keep contaminating ADP substoichiometric. The results in Figures 4b, 5b, and 6b show that, similar to what we observed for polymerization of nucleotide-free hsc70, the interaction of nucleotide-free hsc70 with cytochrome *c* peptide was not greatly affected by the binding of any of the ATP analogues. Neither the rates of dissociation and association of cytochrome *c* peptide (Figures 4b and 5b) nor the dissociation constant of cytochrome *c* peptide (Figure 6b) changed by more than a factor of 3 in the presence of any of these ATP analogues compared to that obtained with nucleotide-free hsc70. It should be noted that when the 3-fold effect caused by binding of ATP analogues is combined with the 3-fold effect caused by removing ADP, the rate constants for association and dissociation of the peptide can be increased about 10-fold for hsc70 with bound ATP analogues compared to hsc70 with bound ADP. Nevertheless, as with their effect on polymerization of hsc70, the ATP analogues, themselves, have relatively little effect on the interaction of nucleotide-free hsc70 with cytochrome *c* peptide.

We also investigated the effect of the ATP analogues, AMP-PNP and dATP, on the rates of formation and dissociation of the hsc70−clathrin complex and on the binding strength of hsc70 to clathrin. The results in Figures 4a, 5a, and 6a show that these ATP analogues have only slight effects on the interaction of nucleotide-free hsc70 with clathrin. AMP-PNP appeared to increase the half-life of dissociation of the nucleotide-free hsc70−clathrin complex from about 2 min to 4 min. However, these analogues had

Table 1: Effect of Nucleotide on Interaction of Hsc70 with Clathrin

	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_d (μM)
ATP− E	≥1200	≥120 × 10^{-4}	12[a]
ADP−E[a]	<30	<1 × 10^{-4}	3
NFE[b]	≥1200	46 × 10^{-4}	4
AMP-PNP−E	≥1200	23 × 10^{-4}	5
dATP−E	≥1200	58 × 10^{-4}	4

[a] Prasad et al. (1994). [b] Nucleotide-free hsc70. E = hsc70. k_{on}, k_{off}, and K_d were calculated from the data in Figures 4, 5, and 6, respectively.

Table 2: Effect of Nucleotide on Interaction of Hsc70 with Cytochrome *c* Peptide

			K_d (μM)	
	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	calculated	measured
ATP−E[a]	140	460 × 10^{-4}	329	300
ADP−E[a]	20	1.5 × 10^{-4}	8	7
NFE[b]	100−200	7 × 10^{-4}	4−7	6
NFE[c]	50−100	7 × 10^{-4}	7−14	15
AMP-PNP−E	100−200	11 × 10^{-4}	5.5−11	5
dATP−E	100−200	17 × 10^{-4}	8.5−17	6
ATPγS−E	100−200	17 × 10^{-4}	8.5−17	4

[a] Greene et al. 1995. [b] Nucleotide-free hsc70. E = hsc70. [c] Peptide C was used as substrate. k_{on}, k_{off}, and K_d were calculated from the data in Figures 4, 5, and 6, repectively.

no measurable effect on either the rate of association or the strength of binding of clathrin to nucleotide-free hsc70. Therefore, as we observed for polymerization of hsc70 and the interaction of hsc70 with cytochrome *c* peptide, the ATP analogues have little effect on the interaction of nucleotide-free hsc70 with clathrin.

Our observation that the ATP analogues have little effect is not due to an inability of these analogues to bind to nucleotide-free hsp70. Even when hsp70−AMP-PNP was prepared by directly replacing bound ADP with bound AMP-PNP, rather than by first preparing nucleotide-free hsp70 and then rebinding the AMP-PNP, its properties with regard to the rate of assocation of clathrin and the rate of dissociation of peptide were the same. Therefore, previous effects observed with these analogues on the interaction of hsc70 with clathrin (Schmid et al., 1985) may have been mainly due to competitive removal of bound ADP from the hsc70 with only a small direct effect of the analogues.

DISCUSSION

Tables 1 and 2 summarize the effects of bound nucleotide on the interaction of the hsc70 with clathrin and cytochrome *c* peptide, respectively. We previously found one consistent effect of bound nucleotide with both peptides and clathrin: dissociation of these substrates was rapid from hsc70−ATP but very slow from hsc70−ADP. On the other hand, although rates of substrate binding to hsc70−ATP were faster than to hsc70−ADP, this effect was much less pronounced with peptides than with clathrin, and, for this reason, while clathrin bound similarly to hsc70−ATP and hsc70−ADP, peptides bound much more weakly to hsc70−ATP than to hsc70−ADP.

In the present study, we investigated the interaction of clathrin and cytochrome *c* peptide with nucleotide-free hsc70 and hsc70 with bound ATP analogues. The data presented in this paper illustrate two major findings. First, nucleotide-free hsc70 acts like hsc70−ATP in regard to association and dissociation of clathrin and association of cytochrome *c* peptide, but like hsc70−ADP in regard to its polymerization properties and dissociation of cytochrome *c* peptide. This is not only the case with cytochrome *c* petide, but as shown in Table 2 it is also true for peptide C; therefore, this result may apply to most peptide substrates. Second, our data strongly suggest that the properties of hsc70 with bound ATP analogues are very similar to those of nucleotide-free hsc70. Using AMP-PNP, dATP, and ATPγS, we have found, at most, a 3-fold effect of the nucleotide analogues on the rates of binding or dissociation of protein and peptide substrates from nucleotide-free hsc70. Therefore, our data strongly suggest that the major effect of ATP analogues on the properties of hsc70 is to displace bound ATP or ADP from the hsc70, thus, in effect, creating nucleotide-free hsc70.

To check the validity of our results with cytochrome *c* peptide, we used the measured rate constants for association and dissociation of the peptide to calculate theoretical equilibrium constants which could be compared to our measured equilibrium constants, and we find that these values are in good agreement (Table 2), suggesting that our measurements are internally consistent. However, it remains a key question as to whether other hsc70s, e.g., DnaK from *E. coli*, behave in a similar manner. Recently, a study on the binding of a peptide to DnaK suggested that, when ATPγS binds to nucleotide-free hsc70, it increases the rates of association and dissociation of the peptide 10-fold while the binding of ATP increases the rate of association about 50-fold and the rate of dissociation more than 500-fold (Schmid et al., 1994). This study presented a model in which the binding of ATP or ATP analogues increased the rates of association and dissociation of the peptide 10-fold, while hydrolysis of ATP to ADP further increased the rate of association 5-fold and the rate of dissociation 50-fold.

If this model is correct, the effect of nucleotide on DnaK would be very different from its effect on bovine brain hsc70. However, an alternative explanation of these data is that the DnaK used in these experiments was not nucleotide-free, but initially contained bound ADP. In this case, much of the effect of ATPγS might be due to removal of bound ADP in addition to a small direct effect of the ATPγS. Furthermore, since ATP binds much more strongly than ATP analogues to hsc70, if the same occurs with DnaK, the binding of ATP and ATPγS may have very different effects on DnaK. Therefore, it seems possible that it was ATP binding, not ATP hydrolysis, which increased the rates of association and dissociation of the peptide, 50- and 500-fold, respectively, and, in fact, hydrolysis of bound ATP to ADP would markedly reduce the rates of formation and dissociation of the peptide−DnaK complex, just as we observed with bovine brain hsc70.

One of the central questions about the mechanism of action of hsc70 is how the nucleotide-binding region of the molecule communicates with the protein-binding region. The data presented in this paper show that in regard to dissociation of protein or peptide substrate, nucleotide-free hsc70 can act either like hsc70−ATP or like hsc70−ADP. These data open up the possibility that when nucleotide is absent from the nucleotide-binding site of hsc70, the substrate-binding region may react flexibly with a protein substrate; that is, an induced-fit phenomenon may occur in which the hsc70 folds around the substrate (or in the case of polymerization interacts with another hsc70 molecule) in a manner which

depends on the nature of the substrate. On the other hand, with ATP or ADP at the nucleotide-binding site, the protein-binding region of the hsc70 may be much more rigid in its interaction with substrate.

Of course, this analysis of our data is based on our previous conclusion that clathrin and the peptide bind to the same site on hsc70 (Greene et al., 1995). Alternatively, it is possible that peptide binding really mimics some other type of interaction which occurs with hsc70, e.g., the interaction of a cofactor with hsc70 or the hsc70–hsc70 interaction. In this case, the differences in the way that clathrin and peptides interact with nucleotide-free hsc70 may reflect real functional differences in the nature of the interaction rather than flexibility in the binding site for protein substrates.

The data presented in this paper also suggest that there is a major difference in the interaction of hsc70 with ATP analogues compared to its interaction with ATP and ADP. We have already shown that ATP analogues bind orders of magnitude more weakly to hsc70 than ATP and ADP (Gao et al., 1994), and the current data suggest that, although they bind to the nucleotide-binding site of hsc70, they have very little effect on the protein-binding site. These data suggest that there might be major differences in the structure of the nucleotide-binding site with ATP analogues bound compared to that with ATP or ADP bound. In fact, the 44 kDa proteolytic fragment of hsc70 which contains the nucleotide-binding site has been crystallized, but, surprisingly, analysis of the binding of ATP and AMP-PNP to wild-type and mutant species showed only very subtle differences in the effect of the two nucleotides (Flaherty et al., 1994). There were also only small differences in the structure of the active site with ATP or ADP bound. Therefore, the X-ray crystallography studies do not seem to reflect the major differences in the effect of ATP, ADP, and ATP analogues which we observe experimentally. It is possible that the differences in the way these nucleotides interact with hsc70 are too subtle to detect in the X-ray analysis although these subtle changes have major effects on the protein-binding region of the molecule. Alternatively, it is possible that the structure of the nucleotide-binding site in the 44 kDa fragment does not reflect its structure in the intact hsc70. Further work will be required to correlate the major differences in the effects of ATP, ADP, and ATP analogues on the interaction of hsc70 with protein and peptide substrates with the structure of the nucleotide at the nucleotide-binding site of hsc70.

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