Effect of Constitutive 70-kDa Heat Shock Protein Polymerization on Its Interaction with Protein Substrate

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Constitutive 70-kDa heat shock protein (hsc70) is a mixture of monomers and oligomers in ADP, while in ATP it is monomeric unless certain DnaJ homologs are present which induce hsc70 to form large polymers in an ATP-dependent reaction. A key question regarding polymerized hsc70 is whether it is able to bind protein substrates. Polymerized BiP, the hsc70 present in the endoplasmic reticulum, has been found to bind substrates in vitro although substrates appear to bind only to monomeric BiP in vivo. In this study, we investigated whether substrate binds to polymerized cytoplasmic hsc70 in vitro. Although both stoichiometric ATP and high concentrations of cytochrome c peptide monomerized hsc70, direct binding studies provided no evidence that cytochrome c peptide binds to polymerized hsc70. Furthermore, the time course of cytochrome c peptide and clathrin binding to hsc70 suggested that rather than binding to polymerized hsc70, they monomerized it by reducing free monomer, thereby shifting the monomer-polypeptide equilibrium toward monomer. We conclude that peptide and protein substrates bind at least an order of magnitude more weakly to polymerized hsc70 than to monomer, suggesting that polymerization of hsc70 in vivo, perhaps by DnaJ homologs, may store it in an inactive form.

hsp70s† belong to one of the most prominent classes of proteins produced by the cell under stress. In addition, numerous cognate forms of the hsp70s (hsc70s) are expressed constitutively in the cell. Interactions of the hsc70s with their protein substrates are involved in many important cellular functions including protein translocation across membranes of cell organelles, nascent protein folding and multienzyme protein assembly, antigen presentation, protein degradation in the lysosome, and uncoating of clathrin-coated vesicles (for a review, see Hendrick and Hartl, 1993). In each of these processes, the hsc70s act as molecular chaperones which interact with protein substrates in a nucleotide-dependent manner. In general, hsc70-ATP appears to bind and release protein substrates rapidly and hsc70-ADP appears to bind and release protein substrates very slowly, suggesting that hydrolysis of ATP to ADP traps protein substrates on the hsc70 (Prasad et al., 1994; Greene et al., 1995; McCarty et al., 1995).

ATP and ADP also affect the polymerization of hsc70. Most studies suggest that purified hsc70 occurs as a mixture of monomers, dimers, and higher order polymers in ADP, while in ATP it occurs mostly as monomers (Schlossman et al., 1984; Schmid et al., 1985; Carlino et al., 1992; Toledo et al., 1993; Palleros et al., 1993). There is evidence that polymerization of hsc70 may be important in vivo, in particular, in storing hsc70 in an inactive form. Studying BiP, the hsc70 protein found in the endoplasmic reticulum, Freiden et al. (1992) found that BiP was predominantly monomeric in cells accumulating nontransportable proteins, and only monomeric BiP was complexed with protein substrates, while in control cells BiP appeared to be mostly polymerized and free of protein substrates. As for cytoplasmic hsc70, we recently found that in the presence of catalytic amounts of the yeast DnaJ homolog, YDJ1, or other DnaJ homologs, hsc70 forms large polymers in ATP (King et al., 1995), and at the same time DnaJ homologs inhibit the uncoating of clathrin-coated vesicles by hsc70. It has also been reported that DnaJ homologs inhibit the binding of a protein substrate to hsc70 (Cheetham et al., 1994; Cyr et al., 1992). Since these DnaJ homologs affect the steady-state ATPase cycle of hsc70, it is difficult to determine whether these effects are entirely due to polymerization of hsc70. Nevertheless, it seems possible that under certain conditions, polymerization of both BiP and cytoplasmic hsc70 in vivo prevents them from interacting with their normal substrates. For this reason, we were interested in determining the effect of hsc70 polymerization on its interaction with protein substrates in vitro.

Almost all of the work on this question has been carried out with BiP, and the various studies are in disagreement. While substrates were reported not to bind to polymerized BiP in vivo (Freiden et al., 1992), in experiments with both BiP prepared from rat liver and recombinant BiP, Bland-Elguindi et al. (1993) reported that certain peptides bound directly to the dimeric form of BiP and monomerized it. Surprisingly, however, in disagreement with previous work on mammalian hsc70 (Schlossman et al., 1984; Schmid et al., 1985), Escherichia coli hsc70 (Dnak) (Palleros et al., 1993), and BiP (Carlino et al., 1992; Toledo et al., 1993), they found that ATP did not monomerize their BiP preparations, and in a related finding they observed that monomeric BiP had a higher ATPase activity than polymerized BiP. On this basis, they proposed that peptide activates the BiP ATPase activity by binding to the polymerized BiP in ATP and then monomerizing it. Since they obtained preliminary data showing that cytoplasmic hsc70 behaved similarly, they argued that this model might be general for all hsc70s. Brot et al. (1994) also studied the interaction of BiP with substrates and ATP, and they too observed direct binding of a substrate (substance P) to BiP dimers in ADP. However, in direct contrast to the findings of Bland-Elguindi et al., they found that although ATP monomerized BiP, the binding of substance P did not.
protein substrates. which polymerize hsc70, inhibit the interaction of hsc70 with polymeric hsc70, which could explain why DnaJ homologs, order of magnitude more strongly to monomeric hsc70 than to suggest that peptides and protein substrates bind at least an monomeric and polymeric hsc70 toward monomer. These data of hsc70, which causes a shift in the equilibrium between hsc70. Instead, substrate binds preferentially to the monomer we found no evidence for binding of substrate to oligomeric binding studies, as well as the time course of substrate binding, neither formation nor dissociation of hsc70 polymers or hsc70-substrate complexes was detectable in this buffer. hsc70 in each fraction quantified by SDS-polyacrylamide gel electrophoresis and densitometric scanning (absorbance was brought to the same scale of FPLC protein profiles for comparison). a–c, profiles of ADP-bound hsc70 (E-ADP) passing through FPLC columns at different flow rates as indicated in each panel. B, SDS gel of cross-linked hsc70 with bound ADP. Fractions of 0.5 ml were collected, cross-linked, and analyzed on SDS gel. Lane 1, cross-linked hsc70 (5 μM) without running through FPLC.

Given these contradictions in the studies on the interaction of BiP with nucleotides and substrates in vitro and the fact that no work had been done on the binding of substrates to polymerized cytoplasmic hsc70, we investigated the interaction of polymerized bovine brain hsc70 with ATP, cytochrome c peptide, and clathrin. First, we found that even stoichiometric ATP completely monomerized the hsc70. Second, based on direct binding studies, as well as the time course of substrate binding, we found no evidence for binding of substrate to oligomeric hsc70. Instead, substrate binds preferentially to the monomer of hsc70, which causes a shift in the equilibrium between monomeric and polymeric hsc70 toward monomer. These data suggest that peptides and protein substrates bind at least an order of magnitude more strongly to monomeric hsc70 than to polymeric hsc70, which could explain why DnaJ homologs, which polymerize hsc70, inhibit the interaction of hsc70 with protein substrates.

MATERIALS AND METHODS

ATP (catalog no. A5394) and AMP-PNP (catalog no. A2647) were purchased from Sigma. ADP (catalog no. 01899) was obtained from Fluka Chemie AG, Buchs, Switzerland.

Purification of Clathrin and hsc70 and Preparation of Nucleotide-free hsc70—Clathrin was purified from bovine brain according to the procedure described previously (Gao et al., 1995). 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 at 4°C as ammonium sulfate pellets, which were resuspended and dialyzed in Buffer A containing 1 mM ADP and 1 mM Pi. Neither formation nor dissociation of hsc70 polymers or hsc70-substrate complexes was detectable in this buffer. hsc70 in each fraction was quantified by SDS-polyacrylamide gel electrophoresis and densitometric scanning (absorbance was brought to the same scale of FPLC protein profiles for comparison). a–c, profiles of ADP-bound hsc70 (E-ADP) passing through FPLC columns at different flow rates as indicated in each panel. B, SDS gel of cross-linked hsc70 with bound ADP. Fractions of 0.5 ml were collected, cross-linked, and analyzed on SDS gel.

FIG. 1. Effect of nucleotide on hsc70 polymerization. A, FPLC profiles of hsc70. 200 μl of purified hsc70 (5 μM) were incubated without (a–c) or with (d) 5 units/ml creatine kinase and 10 mM creatine phosphate at 25°C for 1 h before being loaded onto a Superose 12 FPLC column equilibrated in Buffer A containing 1 mM ADP and 1 mM Pi. Neither formation nor dissociation of hsc70 polymers or hsc70-substrate complexes was detectable in this buffer. hsc70 in each fraction was quantified by SDS-polyacrylamide gel electrophoresis and densitometric scanning (absorbance was brought to the same scale of FPLC protein profiles for comparison). a–c, profiles of ADP-bound hsc70 (E-ADP) passing through FPLC columns at different flow rates as indicated in each panel. B, SDS gel of cross-linked hsc70 with bound ADP. Fractions of 0.5 ml were collected, cross-linked, and analyzed on SDS gel. Lane 1, cross-linked hsc70 (5 μM) without running through FPLC.

RESULTS

It was previously shown that hsc70 occurs as a mixture of monomers, dimers, and higher order polymers in ADP, while in ATP it occurs almost entirely as monomers (Schlossman et al., 1984; Schmid et al., 1985; Palleros et al., 1993). However, recently there have been contradictory reports as to whether BiP occurs as monomers at low ATP concentration (Blondel-Guindi et al., 1993; Brot et al., 1994). Fig. 1 shows that, at 25°C, purified hsc70 that contains equimolar bound ADP (Gao et al., 1993) occurs as a mixture of monomers, dimers, and higher order polymers (Fig. 1A, lane a, and B), while the addition of creatine kinase and creatine phosphate even in the
absence of added free nucleotide converts the hsc70 to almost all monomer (Fig. 1A, panel d). This is consistent with the view that hsc70 with bound ATP is monomeric. Although ATP monomerizes hsc70, the level of polymerization is essentially unaffected by bound ADP; as we reported previously (Gao et al., 1995), nucleotide-free hsc70 shows levels of polymerization almost identical to those of hsc70-ADP.

In the experiments shown in Fig. 1, we quantified the level of monomers, dimers, and polymers by FPLC gel filtration on a Superose 12 column (Fig. 1A) or by cross-linking the protein samples with glutaraldehyde (Fig. 1B). As reported previously, cross-linked monomeric hsc70 occurs as two bands on SDS gels (Schlossman et al., 1984). Since cross-linking of hsc70 (Fig. 1B, lane 1) and FPLC fractions of the hsc70 (Fig. 1B, lanes 2-11) yielded similar amounts of monomers and polymers, there did not appear to be significant dissociation or association of hsc70 during column chromatography at 4 °C. This observation was confirmed by showing that the hsc70 polymerization profile obtained by FPLC was independent of the flow rate of the FPLC column (Fig. 1A, panels a, b, and c). Even when the column was run at one-tenth the normal flow rate, the same profile was obtained showing that hsc70 neither polymerized nor depolymerized during column chromatography at 4 °C.

Although we could not detect the effect of dilution on hsc70 polymerization during column chromatography at 4 °C, we found that the level of polymerization at 25 °C is dependent on protein concentration as has previously been reported for the E. coli hsc70 protein, DnaK (Schönfeld et al., 1995). Fig. 2 shows that the higher the enzyme concentration, the greater is the level of polymerization. Although there was a marked decrease in monomer concentration and a marked increase in oligomer concentration as the hsc70 concentration was increased, the percentage of dimers changed only slightly. To verify that the observation of higher levels of polymerization at higher hsc70 concentration was not an artifact of overloading the FPLC, we loaded one-tenth the volume of 70 μM hsc70 on the column and showed that a similar polymerization profile was obtained (in Fig. 2A, compare f with e).

By taking advantage of the concentration dependence of hsc70 polymerization, we were able to determine the rate of depolymerization of the hsc70 by diluting high concentration hsc70 and determining the rate at which a new monomer-polymerequilibrium was reached. Fig. 3 shows that after dilution, the concentration of higher order polymers decreased and the concentration of monomers increased with a half-life of ~10 min at 25 °C. The true half-life for depolymerization is probably slightly slower than 10 min, because in this experiment depolymerization is not complete; therefore, the rate constant we are measuring is the sum of both a forward and reverse rate constant. The rate of depolymerization that we observe is similar to that of hsc70 polymers formed in the presence of yeast DnaJ and ATP (King et al., 1995a). As we reported previously, nucleotide-free hsc70 showed a similar rate of depolymerization with a half-life of ~5 min (Gao et al., 1995). Again, as we observed at varied concentrations of hsc70 (Fig. 2), the amount of dimeric enzyme appeared to remain constant during depolymerization. The half-life for depolymerization is much longer at 4 °C (data not shown), which explains why we did not observe hsc70 depolymerization during column chromatography at 4 °C even though it was being diluted.
We next investigated the interaction of both hsc70-ADP and nucleotide-free hsc70 with cytochrome c peptide. We first observed, using FPLC, that at 5 μM cytochrome c peptide and 5 μM hsc70, conditions at which the hsc70 is about half-saturated with the peptide, the 14C-labeled peptide bound only to the monomeric fraction of hsc70 (Fig. 4A). To make certain this was not due to dissociation of the peptide bound to dimers and polymers during column chromatography, we used spinning nick columns which separate the free peptide from the bound in 2 min, ~15 times faster than FPLC. If dissociation did occur on the FPLC column, we would see more peptide bound under the same conditions using nick columns. However, the same amount of binding was obtained (data not shown). We also tested the binding of peptide over a wider range of peptide concentration. Our results showed that whether we used FPLC as in Fig. 4A or nick columns, we obtained a linear Scatchard plot that shows the same binding strength and the same stoichiometry of 1:1 binding to the hsc70 at high peptide concentration (Fig. 4B). These data predict that if peptide is binding only to monomeric hsc70, then as the peptide concentration is increased the equilibrium between monomer and polymer hsc70 should be shifted completely to monomer. Fig. 5A shows that this is indeed the case, with both hsc70-ADP and nucleotide-free hsc70, peptide shifts the equilibrium from ~50%
monomeric hsc70 to almost all monomer. In addition, we found that the radioactive peptide bound only to monomeric enzyme and not to polymerized enzyme during the course of monomerization (data not shown).

If peptide is indeed monomerizing hsc70 indirectly by binding to monomer and thus shifting the equilibrium from polymer to monomer, the rate of peptide binding should be biphasic. At high peptide concentration, the peptide should bind rapidly to the monomeric hsc70 followed by a slower rate of binding equal to the rate at which the polymerized hsc70 depolymerizes. Note that this latter rate should be independent of peptide concentration. The results in Fig. 5B show that at high peptide concentrations, the rate of binding of peptide is indeed biphasic. The peptide binds to ~50% of the hsc70 in an initial rapid binding phase followed by a much slower rate of binding. The percentage of hsc70 complexed with peptide during the initial rapid binding is independent of the peptide concentration and is approximately equal to the fraction of hsc70 occurring as monomers under this condition. The rate of the slow binding phase also appears to be independent of peptide concentration with a half-life of ~10 min, which is equal to the rate at which the polymerized hsc70 depolymerizes. These data strongly suggest that the peptide binds to monomeric hsc70 at a rapid rate and that further binding occurs only at the rate at which polymerized hsc70 depolymerizes into monomers. Therefore, it appears that peptide monomerizes hsc70 indirectly by reducing the concentration of free monomeric enzyme, rather than by directly interacting with polymerized enzyme.

Having determined that cytochrome c peptide interacts only with monomeric hsc70, we next investigated whether the same effect occurs with a protein substrate of hsc70, clathrin triskelion. Since clathrin is much larger than hsc70 we could not directly measure the binding of clathrin to monomeric and polymeric hsc70 using FPLC as we did with peptide. Only the reverse measurement could be made, that is, we could determine the depletion of free monomeric hsc70 compared to polymeric hsc70. This measurement is valid only if the rate of binding of hsc70 to clathrin is fast compared to the rate of depolymerization of the polymeric hsc70. Therefore, since hsc70 with bound ADP binds very slowly to clathrin while nucleotide-free hsc70 binds rapidly (Prasad et al., 1994; Gao et al., 1995), we used nucleotide-free hsc70 for this experiment. In addition, we carried out this study at 4°C, a condition at which we found great difference between the rate of binding of nucleotide-free hsc70 to clathrin and the rate of depolymerization of the hsc70.

We first investigated whether, like peptide, clathrin binds to nucleotide-free hsc70 in a biphasic reaction. Fig. 6A shows that at 4 °C, there is indeed biphasic binding of hsc70 to clathrin just as occurred with peptide. Furthermore, as with peptide, the magnitude of the initial rapid phase of binding (~60%) is approximately equal to the amount of monomeric hsc70 present under this condition, and the rate of the slow binding phase is similar to the rate of the monomer-polymer equilibrium which has a half-life of ~1 h compared to ~5 min at 25 °C (Gao et al., 1995). Results of hsc70-clathrin binding studies at 25 °C showed similar biphasic binding, although to a lesser extent, since as the temperature is increased, the rate of the monomer-polymer equilibrium increases more than the rate of clathrin binding (data not shown). Therefore, these experiments on the rate of binding of hsc70 to clathrin suggest that only monomeric enzyme binds to clathrin.

We next directly tested whether monomeric hsc70 binds preferentially to clathrin. To avoid depolymerization of the hsc70 during the reaction we incubated the hsc70 with clathrin at 4 °C and the mixture was rapidly applied to FPLC. Fig. 6B shows that the monomeric fraction of the hsc70 was indeed preferentially removed from the free enzyme pool; presumably, the monomeric hsc70 became associated with the clathrin during the initial rapid binding phase before significant depolymerization of the hsc70 could occur. Therefore, as with peptide, clathrin appears to bind only to monomeric hsc70.

**DISCUSSION**

This study confirmed that monomeric hsc70 is in equilibrium with dimeric and higher order polymeric hsc70 in ADP, while the binding of ATP converts almost all of the hsc70 to monomer. Similar observations have been made for DnaK (Schönfeld et al., 1995; Palleros et al., 1993), the hsc70 of *E. coli*. We also found that the half-life of hsc70-ADP depolymerization is ~10 min at 25 °C and 2–3 h at 4 °C. These kinetics are again approximately similar to that observed with DnaK and also with hsc70 polymerized by DnaJ homologs in ATP and then depolymerized in ADP (King et al., 1995a). The concentration of
dimers remained constant during depolymerization of hsc70. This raises the possibility that just as G-actin monomer is in equilibrium with large actin filaments (Korn, 1982), dimeric hsc70 may be in equilibrium with much larger hsc70 polymers with very few intermediate-sized polymers present.

Both the observation that BiP can occur in polymerized form in vivo and our previous observation that DnaJ homologs polymerize hsc70 in ATP suggest that polymerization of hsc70 proteins may be a physiological phenomenon. It is possible that the role of this polymerization is to provide an inactive storage form of hsc70. There is evidence that protein substrates do not bind to polymerized BiP in vivo, and in vitro, YDJ1 not only polymerizes hsc70 in ATP but also inhibits both protein-substrate binding to hsc70 and uncoating of clathrin-coated vesicles by hsc70 (Cheetham et al., 1994; Cyr et al., 1992; King et al., 1995b). Although these latter effects could be due to polymerization of the hsc70, they might also be related to the major effect of YDJ1 on the steady-state ATPase cycle of hsc70. However, our current results, obtained in the absence of DnaJ homologs, confirm the view that substrates probably do not bind to polymerized hsc70. Our results from the time course of binding and from direct binding studies show that high concentrations of substrate depolymerize hsc70. This monomerization is apparently caused by substrate binding preferentially to monomer, resulting in a shift in the monomer-polymer equilibrium toward monomer. Therefore our data strongly suggest that both peptide and clathrin bind at least an order of magnitude more weakly to polymeric hsc70 than to monomeric hsc70, which supports the view that polymerized hsc70 may act as an inactive storage form of hsc70.

The other major studies on the binding of substrates to polymerized hsc70 in vitro have all been carried out with BiP. In contrast to the in vivo study by Freiden et al. (1992), two of these studies found evidence for peptide binding to polymerized BiP in vitro (Blond-Elguindi et al., 1993; Brot et al., 1994). Unfortunately, these two studies disagree with each other in major respects. Specifically, they disagreed on both the effect of ATP on polymerized BiP and the ability of peptide to depolymerize BiP. Blond-Elguindi et al. (1993) found that neither recombinant BiP nor BiP prepared from liver were depolymerized at low concentrations of ATP, although peptide depolymerized BiP. On the other hand, Brot et al. (1994) found that low concentrations of ATP depolymerizes BiP almost completely, although peptide binding had no effect on the amount of polymer.

It is possible that the differences between the results of Blond-Elguindi et al. (1993) and Brot et al. (1994) are due to problems with in vitro preparations of BiP. Recombinant BiP may have folding problems which greatly affect its properties. In this regard, Blond-Elguindi et al. (1993) noticed differences in the effect of ATP on their recombinant BiP and preparations of BiP from liver. In addition, there may be a problem of partial denaturation since the BiP prepared from liver by Brot et al. (1994) did not contain a bound nucleotide, while Blond-Elguindi et al. (1993) stated that they removed bound ATP from BiP with ammonium sulfate precipitation. Since both hsc70 and BiP contain a tightly bound nucleotide (Gao et al., 1993; Wei and Hendershot, 1995), it is surprising that these BiP preparations were nucleotide-free.

In addition to their work on BiP, Blond-Elguindi et al. (1993) also reported, in preliminary experiments, that cytoplasmic hsc70 behaved in the same manner as BiP. On the basis of their observations with BiP and hsc70, Blond-Elguindi et al. (1993) proposed a general model suggesting that ATPase activation by peptide was due to monomerization of the hsc70 by peptide. Our results are not consistent with this model of ATPase activation. We find that stoichiometric ATP almost completely monomerizes hsc70; therefore, the activation of hsc70 ATPase by peptide cannot be due to peptide monomerizing hsc70.

Although it is not clear whether there are differences in the properties of polymerized hsc70 and BiP in ADP, there are major differences in their mechanism of polymerization in ATP. YDJ1, a yeast DnaJ homolog, catalytically induces polymerization of hsc70 in ATP but has a much smaller polymerizing effect on BiP (King et al., 1995a). On the other hand, the polymeric, but not the monomeric, forms of BiP isolated from Ag8 cells are ribosylated and phosphorylated (Freiden et al., 1992), whereas similar modifications have not been observed for cytoplasmic hsc70s. Therefore, in vivo, with ATP present, the process of polymerization and depolymerization may be controlled by ribosylation and phosphorylation with BiP and by DnaJ homologs with hsc70. In both cases, polymerization may be a way of obtaining a storage form of hsc70 which does not bind substrate until monomerization occurs. However, further work with BiP will be required to determine whether there are indeed differences in the polymerization and depolymerization properties of BiP and hsc70 in vitro and, if so, whether these differences are related to differences in the mechanism of polymerization and depolymerization of BiP and hsc70 in vivo.

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