Polymerization of 70-kDa Heat Shock Protein by Yeast DnaJ in ATP*

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DnaK, the Escherichia coli hsp70 protein, interacts with DnaJ, a protein cofactor that appears to be involved in presenting protein substrates to DnaK. The yeast DnaJ homolog, YDJ1, has also been shown to interact with yeast hsp70, although the function of this interaction is unknown. In the present study, we investigated the interaction of YDJ1 with both yeast and bovine brain hsp70. We found that, in the presence of ATP, where hsp70 is normally monomeric, YDJ1 induced almost all of the yeast and bovine brain hsp70 to form large polymers, which are readily sedimentable. These polymers were much larger than the dimers and trimers of hsp70, which normally form in the presence of ADP. YDJ1 appeared to be acting catalytically since very little YDJ1 copolymerized with the hsp70, and maximum polymerization occurred at low ratios of YDJ1 to hsp70. The polymerization required ATP and was completely reversed when ATP was replaced by ADP. These data suggest that, in the presence of ATP, YDJ1 may present one hsp70 to another just as under other conditions DnaJ is able to present protein substrates to DnaK.

The 70-kDa heat shock proteins act as molecular chaperones in that they fold, unfold, and disaggregate proteins in numerous processes in vivo (Craig, 1993; Hendrick and Hartl, 1993). In addition, the hsp70s are involved in the protection of proteins from denaturation during heat shock. In their role as molecular chaperones, the hsp70s interact with such diverse substrates as clathrin, denatured proteins, nascent polypeptide chains on the ribosome, proteins that are imported into mitochondria and the endoplasmic reticulum, and various synthetic peptides (Lakey et al., 1987; Deshaies et al., 1988; Flynn et al., 1989; Beckmann et al., 1990).

Our laboratory (Greene and Eisenberg, 1990; Prasad et al., 1994b; Greene et al., 1995) has previously characterized the effect of nucleotides on the interaction of brain hsp70 with clathrin and peptide substrates. We found that the rate of dissociation of protein substrates from hsp70 is much slower with ADP than with ATP at the active site; in agreement with this observation, we found that dissociation of protein substrates is caused by ATP binding to hsp70 rather than by hydrolysis of ATP to ADP at the active site. On this basis, we proposed a model of hsp70 action in which protein substrates rapidly bind to and dissociate from the ATP form of the enzyme while, following ATP hydrolysis, these substrates are locked onto the ADP form of the enzyme, unable to dissociate until ADP is released and ATP rebinds (Greene et al., 1995).

Although the hsp70 proteins interact directly with protein substrates and nucleotides in vitro, there is considerable evidence that, in vivo, the interaction of both protein substrates and nucleotides is controlled by protein cofactors. Two such cofactors have been isolated from E. coli. One is GrpE, a protein cofactor that has been reported to greatly increase the rate of exchange of nucleotides bound to DnaK, the hsp70 protein present in E. coli (Liberek et al., 1991). A second is DnaJ, which is apparently involved in presenting substrates to DnaK. Studies of P1 plasmid replication by Wickner and co-workers (1992) found that DnaJ binds to RepA dimers and then targets the complex to DnaK, which monomerizes the RepA, allowing it to participate in the initiation of DNA replication. In an analogous reaction, DnaJ has been shown to bind to heat-denatured luciferase and then target it to DnaK, which with GrpE is able to renature the protein (Hendrick et al., 1993). Finally, Langer et al. (1992) found that the substrate rhodanese simultaneously binds to DnaK and DnaJ to form a stable ternary complex, suggesting that, here too, DnaJ may be presenting rhodanese to DnaK.

Cofactors are also involved in presenting proteins to hsp70 in eukaryotes. There is evidence that hsp40, a eukaryotic DnaJ homolog, binds to nascent polypeptide chains as they emerge from the ribosome, passing them on to hsp70 and TCP-1, the hsp60 chaperonin, for proper folding (Frydman et al., 1994). We have recently found that the 100-kDa protein, auxilin, is required for hsp70 to uncoat clathrin baskets (Prasad et al., 1994a) and have also obtained evidence that auxilin may be required for hsp70 to bind to the clathrin baskets (Barouch et al., 1993). Finally, it recently has been shown that there is a protein in the mitochondrial membrane that is involved in presenting unfolded proteins to hsp70 in the mitochondrial matrix as these proteins are translocated through the membrane (Kronidou et al., 1994; Schneider et al., 1994). Therefore, in addition to DnaJ, there appears to be a number of other cofactors that are involved in presenting protein substrates to hsp70.

Of the various cofactors that present proteins to hsp70, DnaJ is particularly interesting because, like hsp70, it consists of a large family of proteins present in many different cell organelles. This has been most clearly demonstrated in yeast. All of the different members of the DnaJ family identified in yeast share a highly conserved 70-amino acid sequence at the N-terminal called the "J" domain, but these different DnaJ s show different sites of localization. SCJ 1 is localized to the mitochondrial (Blumberg and Silver, 1991), Sec63 to the ER membrane (Deshaies et al., 1991), SIS1 to the cytosol and nucleaus (Luke et al., 1991), and YDJ1 to the cytosol and cytoplasmic side of the endoplasmic reticulum and nuclear membranes (Caplan et al.,...
YDJ1 has been extensively studied by Douglas and co-workers (Caplan and Douglas, 1991; Caplan et al., 1992a, 1992b; Cyr et al., 1992; Cyr and Douglas 1994). Physiological studies in vivo showed that YDJ1 deletion causes a translocation defect in yeast with a phenotype similar to that observed for deletion of the SSA class of yeast hsp70 proteins (Caplan et al., 1992a). Biochemical studies in vitro showed that, in the presence of ATP, YDJ1 weakened the binding of the unfolded protein, reduced α-carboxymethylated lactalbumin, to SSA1 (Cyr et al., 1992). Likewise, YDJ1 weakened the binding of the presence peptide, F1(1–15), to SSA1 (Cyr and Douglas, 1994).

Therefore, rather than YDJ1 presenting these substrates to hsp70, it appeared to be interfering with their binding to hsp70. This suggests that the effect of DnaJ and its homologs may be different for different substrates.

Since our laboratory has extensively studied the binding of substrates to brain hsp70, we were interested in determining whether we could detect stable binding of YDJ1 to hsp70 and, if so, whether the YDJ1 bound competitively with these substrates or presented them to hsp70. Therefore, in this study we examine the interaction of YDJ1 with bovine brain hsp70 and yeast SSA1. Surprisingly, we found that YDJ1 induces the formation of large polymers of brain and yeast hsp70 in a reaction that requires the presence of ATP and occurs even at substoichiometric amounts of YDJ1. Formation of these polymers may explain why YDJ1 inhibits the binding of various substrates to hsp70 in vitro.

MATERIALS AND METHODS

Protein Purification—YDJ1 was expressed in E. coli strain BL21(DE3) and purified using a modification of the procedure of Cyr et al. (1992). Cells were disrupted in a French press, clarified, and then loaded on a Q-Sepharose column (1 × 10 cm) in buffer A (20 mM MOPS (pH 7.5), 0.5 mM EDTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). The YDJ1-containing fractions were dialyzed and loaded onto a hydroxylapatite column in buffer B (5 mM potassium Pi, (pH 7.0), 1 mM dithiothreitol, 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride) and eluted with a 5–450 mM potassium Pi gradient. The YDJ1 fractions were precipitated with 75% (NH4)2SO4, dialyzed against buffer C (25 mM KCl, 10 mM (NH4)2SO4, 2 mM magnesium acetate, 20 mM Imidazole (pH 7.0), 1 mM dithiothreitol). YDJ1 was greater than 90% pure. Bovine brain hsp70 was prepared according to Greene and Eisenberg (1990), and SSA1 was prepared according to Gao et al. (1991). Nucleotide-free hsp70, which contained less than 5% bound ADP, was made according to Gao et al. (1994).

FPLC Chromatography—The interaction of YDJ1 with bovine brain hsp70 was routinely measured by mixing these proteins for 30 min in the presence of 1 mM nucleotide in buffer C at 25°C. Unless otherwise noted, the concentration of hsp70 and YDJ1 were both 10 μM. The solution (0.2 ml) was then applied to a Superose 12 column (HR 10/30) at 4°C using a Pharmacia FPLC system. The column was equilibrated in buffer C containing 1 mM ADP and 1 mM Pi, and run at a flow rate of 0.5 ml/min. In the experiment using the Superdex 200 column (HR 10/30), the flow rate was 1.0 ml/min. Fractions were collected and run on a 4–20% SDS gel (from Integrated Separation Systems). The intensity of staining the hsp70 and YDJ1 bands with Coomassie Blue was quantified using the LKB ultroscan XL laser densitometer.

Sedimentation Assay—Interaction between YDJ1 and hsp70 was also assayed by a sedimentation assay. In this assay, an ATP regenerating system was added consisting of 30 units/ml creatine kinase and 1.5 mM creatine phosphate. Samples were spun for 20 min at 400,000 × g in the TLA 100 rotor, and the supernatant was then removed.

Chemical Reagents—Q-Sepharose, Superose 12 column, and Superdex 200 column were from Pharmacia Biotech Inc. Imidazole, MOPS, HEPES, ATP, creatine phosphokinase, creatine phosphate, and hexokinase were from Sigma. Hydroxylapatite was from Calbiochem.

RESULTS

In agreement with the observations of Cyr et al. (1992), we found that the yeast DnaJ homolog, YDJ1, activates the ATPase activity of the yeast hsp70, SSA1; in addition, we found that it activates the ATPase activity of bovine brain hsp70 (data not shown). This latter observation establishes that yeast YDJ1 not only interacts with yeast SSA1 but also with brain hsp70. We were, therefore, interested in determining whether we could detect binding of YDJ1 to brain hsp70. We investigated this binding using the same method we used to study the binding of substrates to hsp70. FPLC chromatography on a Superose 12 sizing column. The column was equilibrated and eluted with a buffer containing ADP and Pi, with each profile, the Coomassie Blue absorbance of the protein in all fractions was totaled. This total was used to compute the percent of total hsp70 or YDJ1 in each fraction.
under conditions where hsp70 and YDJ1 are known to interact from ATPase studies. YDJ1 had a profound effect on the elution profile of hsp70, with most of the hsp70 eluting in the void volume (Fig. 1B, solid circles). However, apparently this is not due to complex formation between YDJ1 and hsp70 since the elution profile of the YDJ1 was essentially unaffected by the presence of the hsp70 (Fig. 1B, solid triangles). Therefore, YDJ1 appeared to cause marked polymerization of the hsp70 in ATP without itself copolymerizing with the hsp70.

Schmid et al. (1985) have shown that, in contrast to its monomeric state in ATP, hsp70 polymerizes in ADP. However, as shown by the elution pattern of hsp70 in ADP (Fig. 1A, open circles), this polymerization is much less extensive than the polymerization induced by YDJ1 in ATP. To determine the composition of the hsp70-ADP polymer, the fractions were cross-linked with glutaraldehyde (data not shown). We found that, in addition to monomer (12–12.5-ml elution volume), hsp70 in ADP also forms dimers (11–11.5-ml elution volume), trimers, and higher order aggregates. On the other hand, the polymerized hsp70 induced by YDJ1 in ATP has an apparent molecular weight much greater than the polymerized hsp70 in ADP. Specifically, the Superose 12 column excludes molecules with a molecular mass >300 kDa, showing that the polymers induced by YDJ1 are larger than trimers. Furthermore, when the same mixture of YDJ1 and hsp70 was chromatographed on a Superdex 200 column, which has a void volume corresponding to a molecular mass >600 kDa, the hsp70 polymer was again found exclusively in the void volume (data not shown). Therefore, these results suggest that, in the presence of YDJ1 and ATP, brain hsp70 forms a very large molecular weight polymer.

To confirm that the polymerization of hsp70 induced by YDJ1 in ATP was not due to formation of a copolymer of hsp70 and YDJ1, we attempted to separate polymerized hsp70 from YDJ1 (and monomeric hsp70) by centrifugation. The sedimentation assay was routinely done in the presence of an ATP regeneration system to ensure that the ATP concentration was maintained. Fig. 2 shows that, in ATP, approximately 80% of brain hsp70 sedimented in the presence of YDJ1, whereas only approximately 20% sedimented in the absence of YDJ1. However, the amount of YDJ1 sedimenting (40%) was essentially the same in the presence and absence of hsp70, suggesting that very little YDJ1 was bound to the polymerized hsp70. Therefore, in agreement with the results obtained from column chromatography (Fig. 1B), YDJ1 appears to cause extensive polymerization of hsp70 without itself remaining bound to the polymerized hsp70.

Since the polymer of hsp70 formed in the presence of stoichiometric concentrations of YDJ1 contained only trace amounts of YDJ1, it seemed possible that the YDJ1 was acting catalytically rather than stoichiometrically in inducing polymerization of hsp70 in the presence of ATP. Therefore, we examined whether substoichiometric concentrations of YDJ1 could polymerize hsp70. The extent of polymerization was essentially the same at stoichiometric and substoichiometric YDJ1 concentrations (compare Fig. 3 to Fig. 1B). Therefore, apparently YDJ1 acts catalytically rather than stoichiometrically in inducing polymerization of hsp70.

We next investigated whether the polymerization of hsp70 induced by YDJ1 requires ATP or whether it also occurs in ADP where brain hsp70 already has a tendency to polymerize (see Fig. 1A). Fig. 4 shows that YDJ1 does not induce polymerization of hsp70 in ADP (closed circles), nor does it induce polymerization in AMP-PNP (Fig. 4, open triangles). In addition, YDJ1 did not induce polymerization of nucleotide-free hsp70 (closed triangles) (note that the elution patterns in Fig. 4 lack the detail obtained in Fig. 1A because 1-ml fractions, rather than 0.5-ml fractions, were collected). Therefore, the polymerization of the brain hsp70 appears to require ATP.

Since hsp70 polymerization appears to require ATP, it is possible that this polymerization would be reversed following hydrolysis of ATP to ADP. To test this point, hsp70 polymer was formed in the presence of YDJ1 and ATP; hexokinase/glucose was added to hydrolyze the ATP, and the solution was incubated for varying times at 25°C and was then chromatographed on the Superose 12 column. The polymerized hsp70 (Fig. 5A, closed circles) depolymerized after incubation with hexokinase/glucose for 4 h (Fig. 5A, closed triangles). Fig. 5B shows the time course of this depolymerization. The depolymerization of the polymer upon addition of hexokinase/glucose occurred with a 10-min half-life; nearly maximum depolymerization occurred within about 1 h. This clearly establishes...
that ATP must be present to maintain polymers of hsp70 in the presence of YDJ1.

It should be noted that although depolymerization of hsp70 in ADP is rather slow, some depolymerization may in fact be occurring during FPLC chromatography. In fact, if we markedly reduce the rate of elution, the extent of polymerization decreases (data not shown). Therefore, the size and extent of the polymerized complex may be slightly underestimated by the FPLC chromatography method.

Since YDJ1 is a yeast homolog of DnaJ, we next investigated whether YDJ1 polymerizes SSA1, a yeast isoform of hsp70. Fig. 6 shows that incubating SSA1 with stoichiometric concentrations of YDJ1 in the presence of ATP (closed circles) alters the elution profile so that the SSA1 elutes in earlier fractions compared to SSA1 alone (open circles). The shift in elution profile obtained with SSA1 in the presence of YDJ1 was not as pronounced as that observed with brain hsp70 under the same conditions. Furthermore, even in the absence of YDJ1, SSA1 appeared to be more aggregated in ATP than bovine brain hsp70. Nevertheless, YDJ1 clearly induces polymerization of SSA1, suggesting that the polymerization of hsp70 is a general property of YDJ1. This is further supported by our observation that YDJ1 also induces the polymerization of BiP, although, here too, the extent of polymerization is significantly less than that of bovine brain hsp70 (Fig. 7, open circles). BiP, alone, in ATP gives essentially the same elution pattern as hsp70 in ATP (Fig. 1A). Finally, we found that, like YDJ1, E. coli DnaJ induces polymerization of brain hsp70 in ATP (Fig. 7, closed circles), suggesting that polymerization of hsp70 may be a general property of many "J"-domain-containing proteins and not just a specific property of YDJ1.

**DISCUSSION**

Since our laboratory has extensively studied the interaction of brain hsp70 with clathrin and various peptides, we were interested in investigating the interaction of hsp70 with YDJ1.
induces polymerization of hsp70, there is considerable evidence that members of the DnaJ and hsp70 families interact. For the E. coli proteins DnaJ and DnaK, this interaction is demonstrated by the ability of DnaJ to activate the DnaK ATPase activity (Liberek et al., 1993), although there is little evidence for physical interaction between the two proteins. Physical interaction does occur in a hexameric complex between DnaK and DnaJ isolated from the bacteria Thermus thermophilus (Motohashi et al., 1994). This complex consists of three molecules of each protein but is completely different from the polymerized hsp70 we observe since it contains stoichiometric amounts of DnaJ, contains no endogenously bound nucleotide, and is stable in the presence of both ADP and ATP. Brodsky et al. (1993) also observed binding between the yeast endoplasmic reticulum protein BiP, a hsp70 homolog located in the lumen of the endoplasmic reticulum (Silver and Way, 1993), and Sec63, a yeast DnaJ homolog localized in the endoplasmic reticulum membrane (Feldheim et al., 1992). Finally, mammalian hsp40, a DnaJ homolog, recently has been shown to immunoprecipitate with hsp70 (Sugito et al., 1995). Therefore, it is clear that the DnaJ and hsp70 proteins physically interact, although we do not yet understand how this interaction can cause hsp70 polymerization in ATP.

One possible mechanism may relate to the ability of DnaJ to present substrates to hsp70; in the absence of substrate, DnaJ may induce hsp70 polymerization by repeatedly presenting hsp70 molecules to other hsp70 molecules in a process that results in hsp70 polymerization in vitro. It may be that this process does not occur in vivo but simply reflects the ability of DnaJ to present substrates to hsp70. On the other hand, polymerization of hsp70 by DnaJ may be related to the function of hsp70 in vivo. For instance, it is possible that the formation of polymerized hsp70 by YDJ1 facilitates the migration of large amounts of hsp70 to the nucleus, which occurs when a cell is subjected to heat shock conditions (Milarski and Morimoto, 1989). Or perhaps, it is related to the observation of Lindquist and coworkers (Feder et al., 1992) that expressing hsp70 in Drosophila cells at normal temperature led to the localization of hsp70 in granules in which the protein appeared to be irreversibly inactivated.

The ability of DnaJ-like proteins to induce polymerization of hsp70 may also play a role in protein translocation and membrane fusion in yeast. Brodsky et al. (1993) observed that BiP and Sec63 are necessary to obtain both protein translocation across the endoplasmic reticulum membrane and membrane fusion. The DnaJ domain of Sec63 is thought to bind BiP and orient it to facilitate the translocation of proteins across the membrane of the endoplasmic reticulum (Brodsky et al., 1993; Brodsky and Schekman, 1993). It seems possible that as part of this process it might also cause polymerization of the BiP. In addition, the converse of this polymerization process may also occur so that BiP causes aggregation or capping of Sec63 on the membrane of the endoplasmic reticulum in a process that might be required in membrane fusion.

Alternatively, polymerization of hsp70 might inhibit its activity in vivo. There is evidence that only monomeric BiP is able to bind its normal substrates (Freiden et al., 1992), and we have observed that under conditions where clathrin and peptides bind strongly to monomeric hsp70, very little, if any, binding occurs to dimers, trimers, and higher order aggregates of hsp70 (Gao et al., 1993). Therefore, it is possible that the observation of Cyr et al. (1992) that carboxymethylated lactalbumin dissociates from SSA1 in the presence of YDJ1 and ATP may be due to the formation of polymerized SSA1. It is also possible that the arrest of luciferase folding by E. coli DnaJ observed by Hartl and coworkers (Hendrick et al., 1993) may be compli-
YD1 Polymerizes hsp70

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Although the physiological role for hsp70 polymerization by YD1 in ATP is not known, it is clear that this polymerization occurs in vitro. It is interesting that x-ray crystallography studies have shown that the 44-kDa fragment of hsp70 has a tertiary structure that resembles actin (Flaherty et al., 1991), since, like actin, hsp70 is able to polymerize. Polymerization of actin is correlated with hydrolysis of actin-bound ATP (Korn et al., 1987). In future studies on the mechanism of hsp70 polymerization by YD1, it will be important to correlate the ability of YDJ1 to activate the hsp70 ATPase activity with its ability to induce polymerization of hsp70.

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