Interaction between Hsc70 and DnaJ Homologues: Relationship between Hsc70 Polymerization and ATPase Activity

Camille King, Evan Eisenberg, and Lois E. Greene*

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

Received February 5, 1999; Revised Manuscript Received July 15, 1999

ABSTRACT: We previously found that, in the presence of ATP, DnaJ homologues catalytically induce formation of a metastable Hsc70 polymer and, similarly, the DnaJ homologue auxilin catalytically induces formation of a metastable Hsc70−clathrin basket complex. Since this suggests that the induction of metastable complexes, which form in ATP but dissociate in ADP, may be a general property of DnaJ homologues, in the present study we investigated in more detail the ability of DnaJ homologues to induce polymerization of Hsc70. This study shows that DnaJ homologues induce polymerization of Hsc70 at the same rate as they induce an initial burst of Hsc70 ATPase activity, showing that polymerization is a specific effect of DnaJ homologue binding to Hsc70. However, polymerization does not always accompany the initial burst of ATPase activity. The dependence of the rates of ATPase activity and polymerization on DnaJ homologue concentration shows that DnaJ homologues bind very weakly to Hsc70 in the presence of ATP and do not bind at all in ADP. Surprisingly, however, under certain conditions the rate of polymerization appears to be independent of Hsc70 concentration, suggesting that polymerization is a first-order reaction, perhaps occurring when two Hsc70 molecules bind to a single DnaJ molecule and then shift their binding to each other. We propose that both the polymerization of Hsc70 by DnaJ homologues and the presentation of substrate by DnaJ homologues to Hsc70 involve the bringing of substrate into proximity with Hsc70 and then independently inducing rapid ATP hydrolysis to cause formation of a metastable Hsc70−substrate complex.

It is now clear that cofactors or partner proteins are involved in numerous processes carried out by the Hsc70[1] class of molecular chaperones (*1*). One of the most prominent classes of these partner proteins is the J-domain proteins (*2*−*4*). All J-domain proteins contain at least a portion of a 70 amino acid region known as the J-domain, which was first described in the *E. coli* protein DnaJ (*5*, *6*), a partner protein of the *E. coli* Hsp70 DnaK. J-domain proteins appear to act in vivo by presenting substrates to Hsc70 although how they carry out this function is not clear. In vitro, there are several systems in which DnaJ homologues have been used to study the mechanism of presentation to Hsc70s including P1 plasmid replication (*7*), the folding of nascent polypeptide chains (*8*, *9*), the prevention of rhodanese aggregation (*10*), and the binding of σ^{32} to the DnaK and DnaJ heat shock proteins (*11*−*13*). Perhaps the most extensively studied of these systems is the folding of denatured luciferase by DnaK (*14*−*16*). It has been shown that the denatured luciferase first binds to DnaJ and then the folding occurs in an ATP-dependent reaction by DnaK both in the presence and in the absence of GrpE, a cofactor that catalyzes rapid exchange of the nucleotide bound to DnaK (*6*).

One proposal for explaining how DnaJ homologues induce substrate binding to Hsc70 is that they do so nonspecifically. Detailed studies on the interaction of DnaJ and DnaK have shown that DnaJ homologues induce a rapid initial burst of ATP hydrolysis by DnaK but have little effect on ADP release (*16*−*18*). Since substrates, such as peptide analogues, dissociate much more slowly from DnaK−ADP than from DnaK−ATP (*19*−*23*), it has been suggested that, after substrates transiently bind to DnaK−ATP, DnaJ stabilizes their binding by transforming DnaK−ATP to DnaK−ADP (*17*, *24*−*27*). On the other hand, it has recently been pointed out that this stabilization would be short-lived in vivo, since another partner protein of DnaK, GrpE, causes rapid exchange of ATP with ADP (*26*). In addition, there is evidence that DnaJ homologues inhibit (*28*, *29*) rather than facilitate the binding of peptide substrates to Hsc70 as would be expected if DnaJ homologues nonspecifically stabilize the binding of substrates.

Another possibility is that DnaJ homologues act specifically, only inducing substrates to bind to Hsc70 that are first bound to the DnaJ. There is evidence that DnaJ binds to the *E. coli* σ factor and catalytically induces it to bind to DnaK (*11*, *24*). More detailed evidence comes from studies showing that the DnaJ homologue auxilin is required for Hsc70 to uncoat clathrin-coated vesicles (*30*−*32*). There is strong evidence that auxilin acts by catalytically inducing clathrin baskets to bind to Hsc70 in an ATP-dependent reaction under conditions where clathrin baskets by themselves do not bind

* Address correspondence to this author at the Laboratory of Cell Biology, Building 3, Room B1-22, National Institutes of Health, Bethesda, MD 20892-0301. Telephone: 301/496-1228. Fax: 301/402-1519. E-mail: greenel@helix.nih.gov.

[1] Abbreviations: Hsp70, 70-kDa heat shock protein; Hsc70, constitutive isoform of Hsp70; FPLC, fast protein liquid chromatography; HPLC, high-pressure liquid chromatography.

10.1021/bi9902788
Published on Web 09/03/1999

to Hsc70 (*33*). Other DnaJ homologues not only are unable to substitute for auxilin in this reaction but actually inhibit its effect (*34*, *35*), suggesting that the effect of auxilin specifically depends on its ability to bind to both clathrin baskets and Hsc70.

Since Hsc70 actually carries out an ATP-dependent reaction on clathrin baskets, converting them to clathrin triskelions, this system allows a distinction to be made between substrates and products of Hsc70. In contrast to clathrin triskelions, which bind strongly to Hsc70 in ADP, the auxilin-induced complex that forms between Hsc70 and clathrin baskets in ATP actually dissociates with a half-life of about 4 min when all of the ATP is hydrolyzed to ADP (*33*). On this basis, we have suggested that the transformation of the metastable Hsc70−ADP−substrate complex to an energetically stable Hsc70−ADP−product complex could drive clathrin uncoating as well as other energy-requiring processes driven by Hsc70 such as protein folding or unfolding and the translocation of unfolded proteins through membranes (*36*).

Interestingly, just as auxilin in the presence of ATP catalytically induces Hsc70 to form a metastable Hsc70−clathrin basket complex, other DnaJ homologues in the presence of ATP catalytically induce Hsc70 to form an Hsc70 polymer that is metastable in ADP (*37*). Since the induction of metastable complexes by DnaJ homologues may be a general phenomenon, in the present study, we investigated polymerization of Hsc70 by DnaJ homologues in more detail. Our results suggest that DnaJ homologues have two independent effects on Hsc70, one to bring substrates into proximity with Hsc70 and the other to cause a rapid burst of ATPase activity. If substrates are not present, DnaJ homologues apparently bring Hsc70s into proximity with each other and therefore cause polymerization of Hsc70 rather than substrate binding.

MATERIALS AND METHODS

Materials. ATP, ATP−agarose, phosphocreatine, creatine kinase, imidazole, and HEPES were purchased from Sigma. Q-Sepharose, monoQ, and Superose 12 columns were from Pharmacia. The Synchropax AX100 HPLC column was from Thomson. [^{14}C]ADP, [^{14}C]formaldehyde, and [γ-^{32}P]ATP were from New England Nuclear. SDS gels (4−20%) were from Integrated Separation Systems.

Methods. Bovine brain Hsc70 was prepared according to Greene and Eisenberg (*38*). Yeast Ssa1p was prepared according to Gao et al. (*39*). Recombinant human Hsp70 was prepared according to Rajapandi et al. (*40*). DnaK was prepared according to Jordan and McMacken (*41*). Ydj1p and Hdj1p were prepared as described in King et al. (*34*, *37*). DnaJ was prepared according to Karzai and McMacken (*18*). The protein concentrations were determined from their extinction coefficients. [^{14}C]ADP−Hsc70 was prepared by exchanging radioactive ADP with the bound ADP for 2 h at 25 °C, followed by extensive dialysis (*42*). Hsc70 was labeled with [^{14}C]formaldehyde by reductive methylation according to Jentroft and Dearborn (*43*).

All reactions were performed in buffer A [20 mM imidazole, pH 7.0, 2 mM magnesium acetate, 25 mM KCl, 10 mM $(NH_4)_2SO_4$, and 1 mM dithiothreitol] at 25 °C. In some experiments, an ATP regenerating system was used composed of 30 units/mL creatine kinase and 15 mM creatine phosphate. To determine the nature of the bound nucleotide during steady-state hydrolysis of Hsc70 in the presence of Ydj1p, 10 μM Hsc70 was incubated with 1 μM Ydj1p in the presence of an ATP regenerating system. At various times, solutions were quenched with perchloric acid, and then the nucleotide content was analyzed on an HP 1090 liquid chromatograph from Hewlett-Packard (*42*).

ATPase was determined from the amount of P_i released from [γ-^{32}P]ATP (*44*). The ATPase activity was performed by preincubating Hsc70 (or its homologues) with a large excess of [γ-^{32}P]ATP for 10 min at 25 °C to exchange the bound ADP on the enzyme with ATP. After this incubation, the reaction was started by addition of the DnaJ homologue followed by measurement of the P_i released. At given times, samples were quenched, and γ-$^{32}P_i$ was determined by liquid scintillation counting using a Beckman LS 3801 counter. The DnaJ homologues, alone, had no ATPase activity.

Polymerization of Hsc70 and Hsp70 by Ydj1p was analyzed by chromatography on a Superose 12 FPLC column equilibrated in buffer A, 1 mM ADP, and 1 mM P_i as described by King et al. (*37*). Fractions (0.5 mL) were collected and analyzed for Hsc70 content by SDS gel electrophoresis. The polymerized Hsc70 eluted in the void volume (fractions 16 and 17), and the free Hsc70 eluted in the included volume (fractions 24−26). In studies of polymerization of Hsc70 at low concentrations ($\leq$1 μM), the fractions were first concentrated by TCA precipitation before running on SDS gels. The Coomassie blue intensity was quantified using an LKB Ultroscan XL laser densitometer.

Radioactive Hsc70 was used in measuring the exchange rate of Hsc70 into the polymer formed in the presence of Ydj1p. At given times, trace amounts of radioactive Hsc70 were added to the polymer, and the reaction mixture was then chromatographed on a Superose 12 column to separate the free and bound Hsc70. The ADP exchange rate was measured using [^{14}C]ADP−Hsc70, and at given times a cold chase of nucleotide was added. The free and bound radioactive ADP was separated on a FPLC Superose 12 column (*42*) equilibrated in buffer A. Radioactivity in fractions from the FPLC column were counted in the above experiments to determine the rate of exchange.

RESULTS

Time Course of the Initial Burst of ATP Hydrolysis. We first investigated the effect of Ydj1p on the time course of ATP hydrolysis by Hsc70 under the condition in which we previously showed Ydj1p polymerizes Hsc70 (*37*). For the ATPase experiments, Hsc70 was first preincubated with ATP to exchange the bound ADP on the enzyme with ATP. Figure 1 shows that, with 10 μM Ydj1p present, Hsc70 shows an initial burst of ATPase activity followed by a slower steady-state ATPase activity. Although the magnitude of the initial burst of ATPase activity is only slightly more than 0.6 mol of P_i/mol of Hsc70, this is probably close to the maximum obtainable value since we previously found that only about 80% of the Hsc70 binds nucleotide (*42*) and only about 80% of this bound nucleotide occurs as ATP (*42*). Consistent with these results, all of the bound nucleotide occurs as ADP after the initial burst of ATPase activity (data not shown). Therefore, Ydj1p markedly accelerates the hydrolysis of the ATP bound to Hsc70.

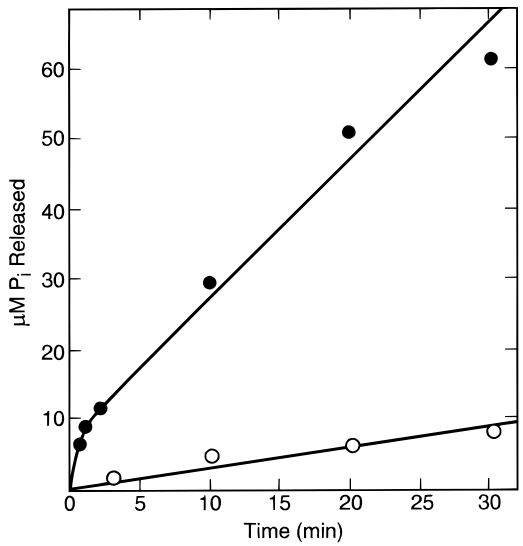

FIGURE 1: Time course of hydrolysis of ATP by Hsc70 is biphasic in the presence of Ydj1p. Hsc70 was incubated with 200 μM ATP for 10 min at 25 °C, at which time Ydj1p was added. The final concentrations of Hsc70 and Ydj1p were both 10 μM. At given times, aliquots were removed for the determination of $^{32}P_i$. The data were plotted by subtracting the amount of ATP hydrolysis prior to addition of Ydj1p. The open and closed circles are the data in the absence and presence of Ydj1p, respectively.

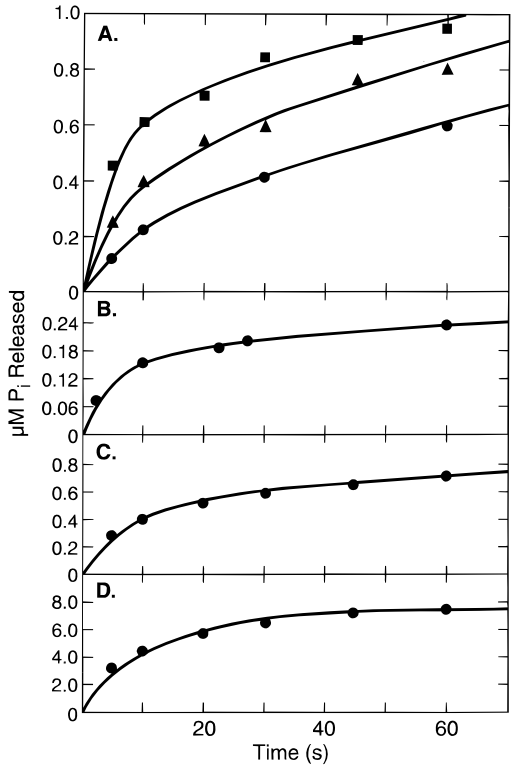

FIGURE 2: Rate of initial burst of ATP hydrolysis is dependent on Ydj1p concentration, but is independent of Hsc70 concentration. In (A), the concentration of Hsc70 was 1 μM, and the concentrations of Ydj1p were 1 μM (closed circles), 3 μM (closed triangles), and 10 μM (closed squares). In (B), the concentration of Ydj1p was 3 μM, and the concentration of Hsc70 was 0.3 μM. In (C), the concentration of Ydj1p was 3 μM, and the concentration of Hsc70 was 1 μM. In (D), the concentration of Ydj1p was 3 μM, and the concentration of Hsc70 was 10 μM. The ATP concentration was 25 μM, except 200 μM ATP was used in the experiment with 10 μM Hsc70. The experiment was performed, and the data were corrected as in Figure 1.

Figure 2A shows that the rate of the initial burst of ATP hydrolysis depends on the Ydj1p concentration. With 1 μM Hsc70 present, the rate constant for the initial burst markedly increases from 2×10^{-2} to 9×10^{-2} s^{-1} as the Ydj1p concentration is increased from 1 to 10 μM. It should be noted that in all cases rate constants were determined by measuring the initial slope of the rate curves which, if anything, leads to an underestimation of the rate constants particularly for the faster rates. If the binding of Ydj1p to Hsc70 were strong, all of the Hsc70 would be complexed with the Ydj1p when the Ydj1p concentration was 1 μM and no further increase in the rate of the initial burst would occur as the Ydj1p concentration was increased to 10 μM. Therefore, the simplest explanation for these data is that Ydj1p binds quite weakly to Hsc70−ATP with a dissociation constant of 10 μM or greater so that as the concentration of free Ydj1p increases, the fraction of the total Hsc70−ATP that is complexed with Ydj1p also increases, thereby increasing the rate of the initial burst of ATP hydrolysis.

If Ydj1p indeed binds weakly to Hsc70, then at a given Ydj1p concentration the fraction of Hsc70 complexed with Ydj1p should be independent of the total Hsc70 present; whether the Hsc70 concentration is more or less than the concentration of Ydj1p, the rate of the initial burst of ATP hydrolysis should be the same. We found that this is indeed the case using 3 μM Ydj1p and varying concentrations of Hsc70. With 0.3 μM Hsc70, the rate constant for the initial burst is 7×10^{-2} s^{-1} (Figure 2B), while with 1.0 μM (Figure 2C) and 10.0 μM Hsc70 (Figure 2D) the rate constant for the initial burst is 5×10^{-2} s^{-1}. Furthermore, at all three enzyme concentrations, the magnitude of the initial burst of ATPase is about 0.7 mol of P_i released per mole of Hsc70. These data show that the fraction of the total Hsc70 complexed with Ydj1p remains essentially constant over a range of Hsc70 concentrations from 0.3 to 10 μM. If Ydj1p bound strongly to Hsc70, all of the Hsc70 would be complexed with Ydj1p at 0.3 and 1 μM Hsc70 but at 10 μM Hsc70 only 30% of the Hsc70 would be complexed with Ydj1p. Therefore these data can only be explained if most of the Ydj1p is not complexed with Hsc70−ATP under these conditions; i.e., the dissociation constant of Ydj1p from Hsc70 is greater than 10 μM. These data further suggest that, if the Hsc70−ATP were fully complexed with Ydj1p, the rate of ATP hydrolysis by the Ydj1p−Hsc70−ATP complex would be about 500-fold higher than the rate of ATP hydrolysis by Hsc70 alone since at 3 μM Ydj1p, the measured rate of the initial burst of ATP hydrolysis is already 100-fold higher than the rate of ATP hydrolysis by Hsc70 alone.

The weak interaction we observed between Ydj1p and Hsc70 is not due to the fact that Ydj1p is a yeast DnaJ homologue while Hsc70 is a mammalian protein. In studies on the interaction of the *E. coli* proteins, DnaJ with DnaK (Figure 3A); the mammalian proteins, Hdj1p with Hsc70 (Figure 3B); and the yeast proteins, Ydj1p with Ssa1p (Figure 3C), we likewise found that neither the rate nor the normalized magnitude of the initial P_i burst changed significantly over a 10-fold range of Hsc70 concentrations when measured at 3 μM DnaJ or DnaJ homologue concentration. In fact, with Ydj1p and Ssa1p (Figure 3C), if anything the normalized burst magnitude increased slightly at the higher Ssa1p concentration although this may be experimental error.

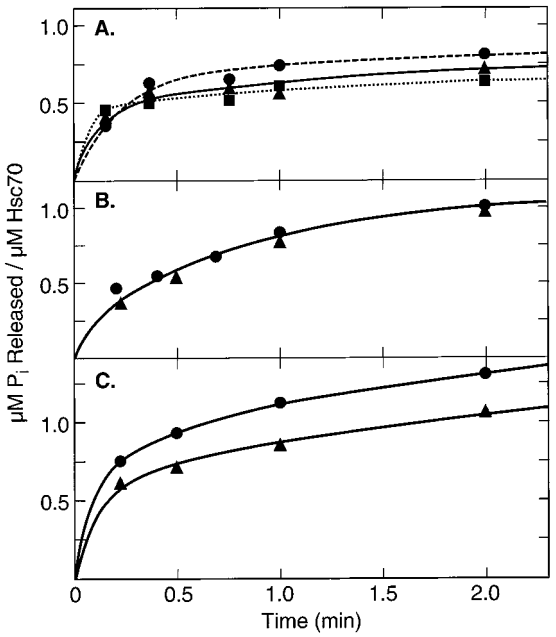

FIGURE 3: Measurement of the magnitude and rate of the initial burst of ATP hydrolysis in the presence of DnaJ or DnaJ homologues at varying concentrations of Hsc70 homologues . In (A), the ATPase determination was made using 3 μM DnaJ and the following concentrations of DnaK: 0.5 μM (closed triangles), 3.0 μM (closed squares), and 10 μM (closed circles). In (B), the ATPase determination was made using 3 μM Hdj1p and either 1 μM (closed triangles) or 10 μM (closed circles) Hsc70. In (C), the ATPase determination was made using 3 μM Ydj1p and either 1 μM (closed triangles) or 10 μM (closed circles) Ssa1p. The data were normalized as P_i released per Hsc70 to enable direct comparison of rates obtained at different concentrations of Hsc70.

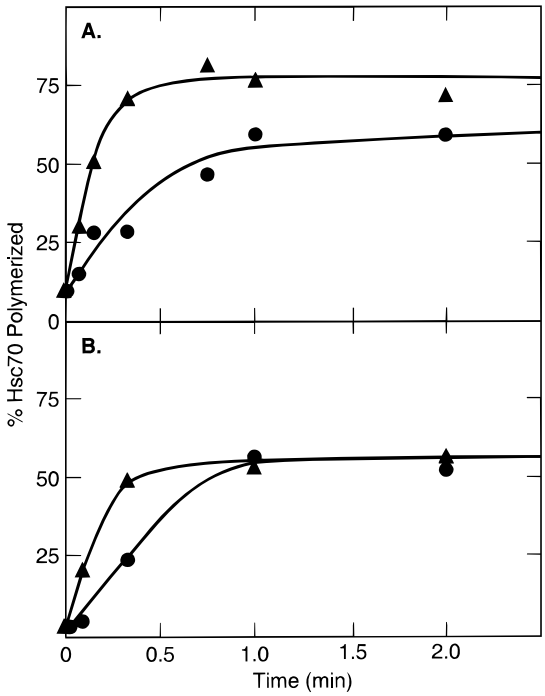

FIGURE 4: Rate of polymerization of Hsc70 is dependent on Ydj1p concentration. In (A), 10 μM Hsc70 was incubated with either 0.5 μM (closed circles) or 3.0 μM (closed triangles) Ydj1p in the presence of 1 mM ATP and an ATP regenerating system at 25 °C. At the indicated times, the reaction mixture was quenched by addition of 0.5 M KCl. The extent of polymer formation was determined by column chromatography using buffer A containing 0.4 M KCl followed by SDS gel electrophoresis of the fractions. In (B), 0.3 μM Hsc70 was incubated with either 0.5 μM (closed circles) or 3.0 μM (closed triangles) Ydj1p. The procedure was the same as in (A). There is a reduction in the extent of polymerization at the lower Hsc70 concentration.

Therefore, in all cases, the DnaJ homologues not only induce a rapid initial burst of ATP hydrolysis by their Hsc70 partners but also bind to their Hsc70 partners quite weakly with a dissociation constant of 10 μM or greater.

Time Course of Polymerization. Having determined the time course of the initial burst of ATP hydrolysis, we next determined the time course of Hsc70 polymerization in the presence of Ydj1p to determine if the two time courses were related. It should be noted that Hdj1p has a tendency to aggregate which makes it much more difficult to work with than Ydj1p. So although qualitatively similar results were obtained with Hdj1p in the ATPase and polymerization experiments, we routinely used Ydj1p for the more quantitative studies. To measure the time course of polymerization, we developed a method of rapidly stopping polymerization by markedly increasing the ionic strength; at 0.5 M ionic strength, no new polymer is able to form, but the polymer that has already formed remains stable during FPLC chromatography (data not shown). Using this method, we found that, in the presence of Ydj1p, the rate of Hsc70 polymerization is similar to the rate of the initial burst of ATP hydrolysis; in both cases, the half-life of the reaction is less than 1 min and markedly increases as the Ydj1p concentration is increased from 0.5 to 3 μM. Specifically with 10 μM Hsc70 present, the rate constant for polymerization increased from about 1×10^{-2} s^{-1} at 0.5 μM Ydj1p to about 5×10^{-2} s^{-1} at 3 μM Ydj1p while the rate of the initial burst of ATP hydrolysis increased from about 8×10^{-3} s^{-1} at 0.5 μM Ydj1p to 6×10^{-2} s^{-1} at 3 μM Ydj1p (data not shown). Furthermore, comparison of Figure 4A and Figure 4B shows that, although as previously shown (*34*) the extent of polymerization is somewhat reduced at the lower Hsc70 concentration, at a given Ydj1p concentration the rate of polymerization is nearly the same at 0.3 and 10 μM Hsc70 just as we found for the rate of the initial burst of ATP hydrolysis. Specifically at 0.5 μM Ydj1p, the rate constant for polymerization is about 1×10^{-2} s^{-1} at both Hsc70 concentrations while at 3 μM Ydj1p the rate constant for polymerization is about 5×10^{-2} s^{-1} at both Hsc70 concentrations. These data not only confirm that Ydj1p binds quite weakly to Hsc70 but, in addition, clearly show that Hsc70 polymerization is caused by the same transient interaction with Ydj1p that causes the initial burst of ATP hydrolysis.

This, in turn, raises the possibility that polymerization of Hsc70 is actually tied to acceleration of the ATP hydrolysis step by Ydj1p. On the other hand, we could not detect any polymerization of DnaK by DnaJ (data not shown) even though DnaJ causes a rapid initial burst of ATP hydrolysis by DnaK. However, this may reflect qualitative differences between DnaK and eukaryotic Hsc70s; such qualitative differences in regard to dissociation of ATP have been shown by Craig and her colleagues (*45*). Therefore, we tested whether an initial burst of ATPase activity occurs in the presence of 3 μM Ydj1p at 0.1 μM Hsc70, a concentration where only about 25% of the Hsc70 is polymerized. We found that, similar to results obtained at higher concentrations of Hsc70, the initial burst of ATPase activity was nearly

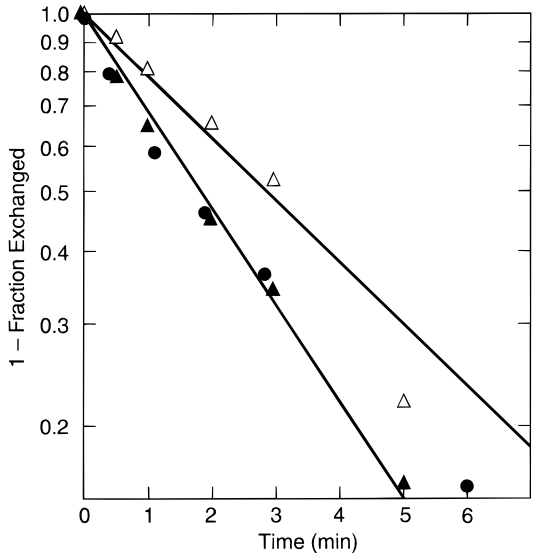

FIGURE 5: Rate of exchange of monomeric Hsc70 into polymer equals the rate of ADP release which is not significantly affected by Ydj1p. The rate of ADP release was measured using 10 μM Hsc70 with bound [^{14}C]ADP either in the presence (closed triangles) or in the absence (open triangles) of 1 μM Ydj1p. In the presence of Ydj1p, the experiment was done in the presence of an ATP regenerating system to form polymeric Hsc70 with bound ADP. At given times, 1 mM ATP was added, followed by column chromatography to determine the amount of [^{14}C]ADP bound to Hsc70. The rate of exchange of monomeric Hsc70 into the polymer (closed circles) was measured by first forming the Hsc70 polymer by incubating 5 μM Hsc70 with 0.2 μM Ydj1p in the presence of 1 mM ATP for 30 min at 25 °C. These are conditions in which about 50% of the Hsc70 polymerizes. Trace concentrations (0.5 μM) of ^{14}C-labeled Hsc70 with bound ADP were then added to the polymer. The exchange of the radioactive Hsc70 into the polymer was measured at the given times by FPLC column chromatography.

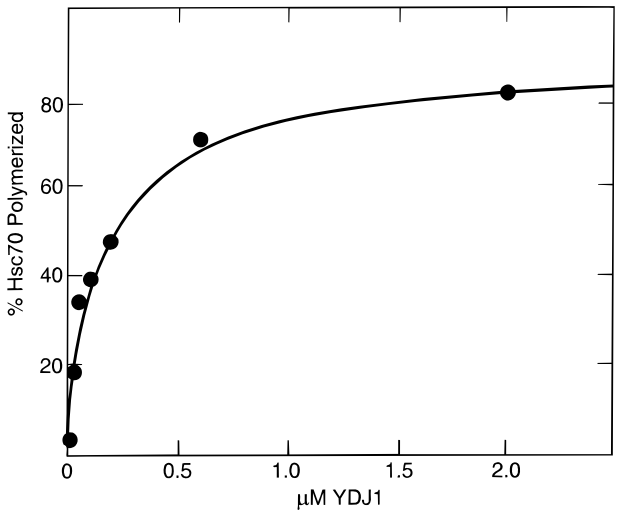

FIGURE 6: Ydj1p dependence of the polymerization of Hsc70. Hsc70 (10 μM) was incubated with varying concentrations of Ydj1p (0.03−4.0 μM) for 30 min at 25 °C in the presence of ATP and an ATP regenerating system. The reaction mixture was then chromatographed to determine the extent of polymerization.

stoichiometric despite the low level of polymerization (data not shown). Therefore, it is clear that DnaJ homologues can induce rapid ATP hydrolysis without inducing polymerization of Hsc70.

Effect of Ydj1p on Steady-State ATP Hydrolysis. In the absence of Ydj1p, we have shown that, during steady-state ATP hydrolysis, about 80% of the nucleotide bound to Hsc70 occurs as ATP (*42*); i.e., the rate-limiting step in the Hsc70 ATPase activity is ATP hydrolysis rather than ADP release. However, since Ydj1p causes a rapid initial burst of ATP hydrolysis, we expected that, in the presence of Ydj1p, most of the nucleotide bound to Hsc70 during steady-state ATPase activity would be ADP rather than ATP. HPLC analysis of the bound nucleotide showed that greater than 95% of the nucleotide bound to Hsc70 is ADP during steady-state hydrolysis of ATP by Hsc70 in the presence of Ydj1p (data not shown). Since the rate of ADP release from Hsc70 is about 6-fold higher than the rate of ATP hydrolysis (*42*), if Ydj1p has no effect on the rate of ADP release it will maximally activate the steady-state Hsc70 ATPase activity about 6-fold. Figure 1 shows that, in fact, Ydj1p activates the steady-state Hsc70 ATPase activity about 8-fold. This suggests that Ydj1p only modestly increases the rate of ADP release. In Figure 5, we directly measured the rate of ADP release from Hsc70 both in the presence (closed triangles) and in the absence of Ydj1p (open triangles). The rates of ADP release were 6×10^{-3} and 4×10^{-3} s^{-1} in the presence and absence of Ydj1p, respectively. Therefore, as predicted, Ydj1p caused only a slight increase in the rate of ADP release from Hsc70.

Dependence of Polymerization on Ydj1p and Hsc70 Concentrations. Figure 6 shows that, in the presence of ATP with 10 μM Hsc70 present, half-maximal polymerization occurs at about 0.3 μM Ydj1p, suggesting that, at this Ydj1p concentration, the rate of Hsc70 polymerization equals the rate of Hsc70 depolymerization. Interestingly, at about 0.3 μM Ydj1p, the rate of Hsc70 polymerization is also about equal to the rate of ADP release from the Hsc70 polymer, suggesting that, in the presence of ATP, the rate of depolymerization of the Hsc70 polymer may be governed by the rate of ADP release from Hsc70. This could occur if, once ADP was released from an Hsc70 present in the polymer, ATP rapidly rebound and caused the monomer to dissociate from the polymer. To test this possibility, we measured the rate at which labeled monomeric Hsc70−ADP exchanges into the Hsc70 polymer in the presence of ATP; since no net polymer is formed under these conditions, the rate of exchange must be equal to the rate of depolymerization of the polymer. The closed circles in Figure 5 confirm that exchange occurs between Hsc70 monomer and polymer and, furthermore, show that the exchange rate is indeed equal to the rate of ADP release from polymerized Hsc70. Note that this rate is much faster than the 10 min half-life we had previously measured for the rate of depolymerization of polymer upon complete hydrolysis of ATP (*37*).

Since polymerization of Hsc70 must involve the interaction of Hsc70s with each other, it might be expected that the rate of Hsc70 polymerization would increase as the Hsc70 concentration was increased just at it increases as the Ydj1p concentration is increased. On the other hand, there is no reason for the rate of depolymerization to be affected by an increase in Hsc70 concentration. Therefore, under conditions where the extent of polymerization is not limited by the Ydj1p concentration, it seemed likely that the level of Hsc70 polymerization would increase with increasing Hsc70 concentration. It has already been demonstrated that, at saturating Ydj1p concentration, different types of Hsc70 homologues show different levels of polymerization. Ssa1p (*34*) and

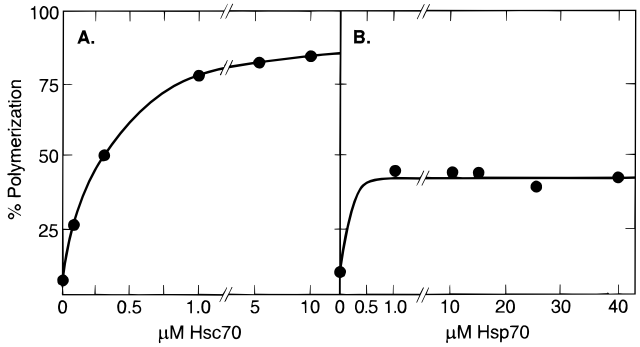

FIGURE 7: Hsp70 does not polymerize to the same extent as Hsc70 at saturating concentration of Ydj1p. In these polymerization experiments, varying concentrations of either Hsc70 or Hsp70 were incubated with 10 μM Ydj1p for 30 min at 25 °C in the presence of ATP and an ATP regenerating system. In (A), Hsc70 concentration ranged from 0.1 to 10 μM. In (B), Hsp70 concentration ranged from 1 to 40 μM. The Ydj1p concentration was saturating in these experiments. The extent of polymerization was determined by FPLC chromatography followed by SDS gel electrophoresis of the eluted fractions.

recombinant human Hsp70 (*40*) show partial polymerization while bovine brain Hsc70 shows marked polymerization, but it is not clear whether in all of these cases the level of polymerization increases as the Hsc70 concentration is increased. We found that this indeed occurs with Hsc70, where at a saturating concentration of Ydj1p the level of Hsc70 polymerization reaches 80% at 1 μM Hsc70 (Figure 7A). Surprisingly, however, this does not occur with recombinant human Hsp70; as the concentration of Hsp70 is increased by more than an order of magnitude (Figure 7B), rather than the level of polymerization increasing, it remains at 50%. This was observed using both 1 and 10 μM Ydj1p, thereby ensuring that Ydj1p was not limiting in the polymerization reaction. Therefore, the level of polymerization cannot be driven to 100% simply by increasing the Hsp70 concentration. A similar phenomenon occurs with Ssa1p, which shows 50% polymerization at both 3 and 10 μM Ydj1p (*34*, *37*). It seems unlikely that this phenomenon occurs because only half of the Hsp70 or Ssa1p is able to polymerize; Hsp70 shows normal uncoating activity, and in addition the levels of both the bound nucleotide and the initial burst of ATPase activity suggest that Hsp70 and Ssa1p are fully active. Therefore, the observation that only partial polymerization occurs at high concentrations of Hsp70 and Ssa1p strongly suggests that the rate of polymerization is not simply controlled by the rate of interaction of Hsc70s with each other during the polymerization process.

DISCUSSION

In the present study we compared the effect of DnaJ homologues on the polymerization of Hsc70 with their effect on the Hsc70 ATPase activity. We found that the polymerization of Hsc70 occurs with the same rapid time course as the initial burst of ATP hydrolysis. The rates of both processes show the same dependence on DnaJ homologue concentration, and in addition the Hsc70 polymer is a dynamic structure with monomeric Hsc70 exchanging with Hsc70 in the polymer at the same rate that ADP is released from Hsc70. Therefore, polymerization of Hsc70 is not a nonspecific effect of DnaJ homologues but results from the same specific interaction of DnaJ homologues with Hsc70 that activates the ATP hydrolysis step.

We also found that several different DnaJ homologues bind very weakly to Hsc70, a result in agreement with a recent observation of Pierpaoli et al. (*27*) that DnaJ binds weakly to DnaK but in disagreement with a previous study by Karzai and McMacken (*18*), who found that the rate of the initial burst of Hsc70 ATP hydrolysis appeared to level off at a DnaJ concentration of 1 μM. Conflicting results have also been obtained by directly measuring the binding of DnaK to the J-domain of DnaJ by NMR analysis and by the Biacore sensor chip method. In the NMR study of Landry and coworkers (*47*), a dissociation constant of 10 μM was obtained, whereas in the Biacore study (*48*) the measured dissociation constant was 0.1 μM.

Our observation that the rate of the initial burst of ATP hydrolysis is activated about 100-fold under conditions where very little Hsc70 is complexed with the DnaJ homologues implies that, if all of the Hsc70 were bound, the rate of ATP hydrolysis would be increased by at least 500-fold and probably much more. Furthermore, since polymerization and ATP hydrolysis occur with the same time course, the rate of polymerization would be extremely rapid as well. In contrast, our data confirm that DnaJ homologues have almost no effect on the rate of ADP release from Hsc70 which accounts for DnaJ homologues activating the steady-state Hsc70 ATPase activity only about 10-fold. It is important to note that not all DnaJ homologues bind weakly to Hsc70. Auxilin also induces polymerization of Hsc70, but in contrast to other DnaJ homologues it binds quite strongly to Hsc70 in the presence of ATP (*49*). It is possible that DnaJ homologues which carry out specific functions in the cell bind more tightly to Hsc70 than DnaJ homologues that act more generally.

In developing a model to explain our data, we attempted to account for several observations. First, we observed a remarkable similarity between the induction of clathrin basket binding to Hsc70 by auxilin at pH 6 where uncoating does not occur (*33*) and the induction of Hsc70 polymerization by DnaJ homologues in the absence of substrate. In both cases, while ATP is required for the complexes to form, the resulting complexes are metastable in that they dissociate in ADP. In the case of the metastable binding of Hsc70 to clathrin baskets, we have suggested that the transition from the metastable Hsc70−clathrin basket complex to the stable dissociated clathrin−Hsc70 complex drives the uncoating process. Second, we observed that DnaJ homlogues induce an initial burst of ATP hydrolysis even under conditions where polymerization does not occur. Finally we obtained the surprising result that, although Hsc70 almost completely polymerizes at high Hsc70 concentration, polymerization of both Hsp70 and Ssa1p levels off at about 50% and over a 10-fold range of Hsp70 or Ssa1p concentration no further increase in polymerization occurs.

The model of Hsc70 polymerization in Figure 8 provides a possible explanation for these observations. In this model we suggest, in agreement with many other models of DnaJ action, that DnaJ homologues have both a substrate binding site and an Hsc70 binding site; recently, the Hsc70 binding site for DnaJ has been elucidated by several groups (*47*, *48*, *50*). To explain polymerization with this model, we propose, first, that, when substrate is not present, Hsc70 not only binds to the Hsc70 binding site of the DnaJ homologue (step 2) but also binds to its substrate binding site as well (step 1).

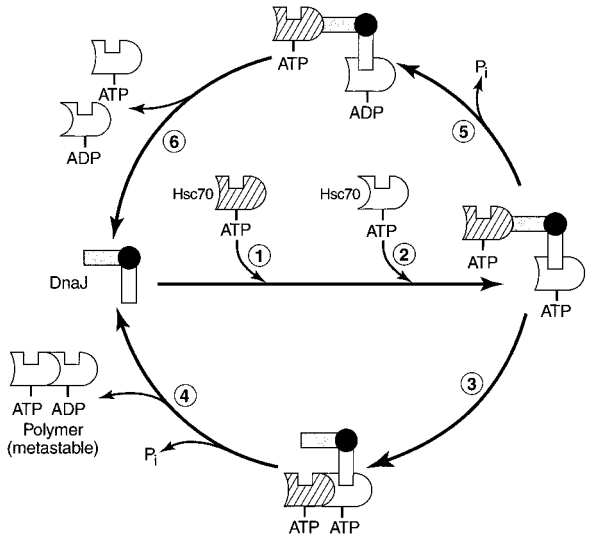

FIGURE 8: Model of the polymerization of Hsc70 by DnaJ homologues. The hatched Hsc70 molecule is presented to Hsc70 by DnaJ homologues in the presence of ATP. Following presentation, there is a burst of ATP hydrolysis whether the hatched Hsc70 detaches (upper pathway) or else remains bound to form a dimer (lower pathway). Repeated addition of Hsc70 to the dimer gives rise to the polymer of Hsc70. Instead of the hatched Hsc70, other substrates of DnaJ can also be presented to Hsc70 using this same model. The steps are described in detail under Discussion.

Therefore, like substrate, it can be transferred to the substrate binding site of the other bound Hsc70 (step 3), resulting in formation of a metastable Hsc70 polymer that is analogous to the metastable complex between Hsc70 and clathrin baskets induced by auxilin. In addition, we suggest that the transfer of Hsc70 from the substrate binding site of the DnaJ homologue to the other bound Hsc70 (step 3) and induction of ATP hydrolysis (step 4 or step 5) are separate reactions, so that ATP hydrolysis can occur before the transfer of Hsc70, in which case the Hsc70−ATP acting as substrate will not transfer to the substrate binding site of Hsc70−ADP and polymerization will not occur (step 6).

At low Hsc70 concentration, statistically only one Hsc70 will bind per DnaJ homologue, and, therefore, the initial burst of ATP hydrolysis will occur without polymerization. At higher Hsc70 concentration, Hsc70 will bind to both sites on the DnaJ homologue, but the level of polymerization will still depend on the relative rates of Hsc70 transfer and ATP hydrolysis (steps 3 and 5) so that, at saturating Ydj1p concentration, as the Hsc70 concentration is increased the level of polymerization will become independent of the Hsc70 concentration as we observed experimentally. Whether a similar effect occurs with the usual substrates presented to Hsc70 by DnaJ homologues is not known at the present time.

The polymerization of Hsc70 and Hsp70 by DnaJ homologues may have several physiological roles in the cell. First, the cell tightly regulates the expression of Hsp70 at normal temperature; Feder et al. (*51*) showed that overexpression of Hsp70 at normal temperature has a deleterious effect on growth of *Drosophila* cells. Furthermore, they (*51*) found that in the cells that overproduce Hsp70 at normal temperature, the distribution of Hsp70 changed from diffuse to granular, indicating sequestration of of Hsp70, perhaps mediated by DnaJ homologues . Second, since polymerized Hsc70 does not bind substrate (*46*), the ability of DnaJ homologues to transiently polymerize Hsc70 when the DnaJ homologue is not carrying a substrate may protect the cell from Hsc70 inappropriately binding nonspecific substrates. In this regard, there is evidence that polymerization of BiP (*52*) is involved in regulation of its activity. On the other hand, DnaJ does not polymerize DnaK. Based on our model, this could be explained if, when DnaK is at the substrate binding site of DnaJ, its rate of transfer to the other DnaK is much less than the rate of ATP hydrolysis. Such a difference between DnaK and other Hsc70s would not be surprising since other differences also occur. For example, DnaJ does not increase the rate of ATP release from DnaK, in contrast to the effect of Ydj1p on ATP release from Ssa1p (*45*).

In summary, our data show that DnaJ homologues specifically induce Hsc70 polymerization at the same rate that they induce an initial burst of ATP hydrolysis. Furthermore, the resulting Hsc70 polymer is metastable in ADP just like the complex that auxilin induces between Hsc70 and clathrin baskets. On this basis, we have proposed that, in the absence of substrate, Hsc70 binds to both the substrate binding site and the Hsc70 binding site on DnaJ homologues. This facilitates the transfer of one Hsc70 to the substrate binding site of the other Hsc70 followed by rapid ATP hydrolysis which our data suggest occurs in a reaction that is independent of the transfer reaction. Thus, a metastable Hsc70 polymer is formed just as in the presence of substrate a metastable complex forms between substrate and Hsc70. Polymerization of Hsc70 may prevent it from nonspecifically interacting with substrates that are not presented by DnaJ homologues in vivo.

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BI9902788