Auxilin-Induced Interaction of the Molecular Chaperone Hsc70 with Clathrin Baskets

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ABSTRACT: We previously reported that a 100-kDa cofactor, recently identified as auxilin, is a DnaJ homolog which is required for Hsc70 to uncoat clathrin baskets. In the present study we investigated the effect of auxilin on the interaction of Hsc70 with pure clathrin baskets at pH 6, where no uncoating occurs. In a reaction which required auxilin, the baskets activated the Hsc70 ATPase activity more than 100-fold with an apparent dissociation constant of about 0.2 μM. Maximal ATPase activity occurred at a 1 to 1 molar ratio of auxilin to clathrin triskelion independent of the Hsc70 concentration suggesting that auxilin is primarily complexed with the clathrin baskets. The binding of Hsc70 to baskets also required auxilin, but less auxilin was needed for maximum binding than for maximum ATPase activity showing that auxilin can catalytically induce binding of Hsc70. The binding also required ATP; Hsc70 dissociated from baskets with a 6 min half-life when ATP was hydrolyzed to ADP. In contrast to auxilin, the assembly proteins, AP-2 and AP_{180}, did not support activation of the Hsc70 ATPase activity by clathrin baskets nor did soluble clathrin triskelions at pH 7 significantly activate the ATPase activity with auxilin present. Therefore, the interaction of auxilin, clathrin baskets, and Hsc70-ATP is highly specific with auxilin first binding to a clathrin triskelion in the baskets and then Hsc70-ATP strongly binding to the auxilin-clathrin complex; the auxilin can then migrate to another clathrin triskelion before the ATPase cycle is complete.

Members of the Hsp70 family of proteins play a crucial role in a number of physiological processes, and in several of these processes, members of the DnaJ family have been shown to act as partner proteins of the Hsp70s and the related constitutive proteins, the Hsc70s.[1] This was first demonstrated for the *Escherichia coli* Hsp70, DnaK, which interacts with *E. coli* DnaJ (Zylicz *et al*., 1985). More recently DnaJ-like partner proteins have been shown to be involved in several important functions of Hsc70s in eukaryotes. Members of the Hsc70 family are the key ATPases involved in the translocation of proteins across mitochondrial and endoplasmic reticulum membranes, and in both cases, partner proteins showing at least partial homology to DnaJ appear to be involved in the interaction of protein substrates with Hsc70 (Rassow *et al*., 1995). Hsc70 also plays an important role in the interaction of Hsp90 with steroid receptors and tyrosine kinases, and here too there is evidence that DnaJ homologs may be involved (Caplan *et al*., 1995; Kimura *et al*., 1995). Finally, Hsc70 appears to be responsible for the uncoating of clathrin-coated vesicles during endocytosis (Schlossman *et al*., 1984; Greene & Eisenberg, 1990), and recent evidence suggests that auxilin, originally identified as a minor assembly protein (Ahle & Ungewickell, 1990), is required for the uncoating reaction (Ungewickell *et al*., 1995).

We previously showed that Hsc70 uncoats clathrin-coated vesicles in a stoichiometric initial burst of uncoating followed by much slower steady-state uncoating (Greene & Eisenberg, 1990). The same time course of uncoating occurs with artificial clathrin baskets prepared with purified clathrin and various assembly proteins such as AP-2 and AP_{180}, and the initial burst of uncoating is accompanied by activation of the Hsc70 ATPase activity (Barouch *et al*., 1994). Both the initial burst of uncoating and the accompanying activation of the ATPase activity require only catalytic amounts of auxilin (Prasad *et al*., 1993; Barouch *et al*., 1994; Ungewickell *et al*., 1995). On the basis of both its activity and the presence of a J-domain at its carboxyl-terminal end, which is homologous to the J-domain of DnaJ, auxilin appears to be a DnaJ homolog (Ungewickell *et al*., 1995). If, indeed, auxilin is acting as a DnaJ homolog, the uncoating reaction becomes an excellent model system for studying the mechanism of interaction of DnaJ homologs and Hsc70 since, in contrast to the mitochondrial and endoplasmic reticulum translocation systems, the interaction of Hsc70 with clathrin baskets and auxilin can be readily studied in a soluble system *in vitro*. Clathrin baskets cannot be uncoated by Hsc70 at pH 6, unlike at pH 7, while at the same time, they still activate the Hsc70 ATPase activity (Braell *et al*., 1984). This enabled us to study the steady-state interaction of Hsc70 with clathrin baskets at pH 6 and quantitatively determine the role of auxilin in this interaction. We found that, in the presence but not the absence of auxilin, clathrin baskets activated the Hsc70 ATPase activity 100-fold with a dissociation constant of 0.2 μM. Maximal ATPase activation occurred at a 1 to 1 molar ratio of auxilin to clathrin, whereas a 3-fold lower ratio was required for maximal binding of Hsc70 to baskets. We conclude that auxilin first forms a binary complex with clathrin, which induces Hsc70 to bind, and then the auxilin

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[1] Abbreviations: Hsc70, 70-kDa heat shock protein; AP, assembly protein; DTT, dithiothreitol; MES, 2-(*N*-morpholino)ethanesulfonic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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can migrate to another clathrin site before the ATPase cycle is complete.

EXPERIMENTAL PROCEDURES

Preparation of Proteins. Hsc70 (the uncoating ATPase) was prepared as described previously (Greene & Eisenberg, 1990). Auxilin (100-kDa cofactor), clathrin, AP_{180}, and AP-2 were prepared as described (Barouch *et al.*, 1994). The buffers used were buffer A (20 mM imidazole (pH 7.0), 2 mM magnesium acetate, 25 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM DTT) or buffer B, which is the same as buffer A except the buffer was 20 mM MES (pH 6.0). When APs (stored in 10 mM Tris, pH 8.0) were added to the reaction, the slight increase in pH was compensated by adding 10% (by volume) of 10× concentrated buffer B at a pH adjusted to achieve a final pH of 6.0.

Preparation of Clathrin Baskets. The clathrin peak which was separated from the APs on a Sepharose-6 column was then rechromatographed on the same column to remove any residual APs. The purified clathrin was dialyzed overnight in 0.1 M MES (pH 6.0), 2 mM DTT centrifuged for 1 h at 150000*g*, and the resulting pellet suspended in buffer B (above). The clathrin basket concentration, defined as the concentration of clathrin triskelions in the baskets, was determined from spectrophotometric absorbance at 280 nm of a small aliquot depolymerized in 0.5 M Tris (pH 8.5) and the extinction coefficient $\epsilon_{1\%}$ 280 nm of 12 for clathrin triskelions (Prasad & Lippoldt, 1988).

ATPase and Binding Assays. All assays were performed at 25 °C in buffer B and 20 μM [γ^{32}P]ATP (specific activity 0.4 Ci/mol) for ATPase assays, or nonradioactive ATP for binding, unless otherwise indicated. Hsc70 was preincubated in [γ^{32}P]ATP or ATP for 10 min at 25 °C before addition to the final reaction mixture (Barouch *et al.*, 1994). The binding of Hsc70 to baskets was routinely performed by incubating Hsc70 with clathrin baskets for 15 min at 25 °C. The reaction mixture was then centrifuged in a TL-100 ultracentrifuge at 380000*g* for 6 min. After centrifugation, the Hsc70 in the supernatant was quantified by SDS−gel electrophoresis, followed by gel scanning. The quantification of the released clathrin was determined by SDS−gel electrophoresis on 4−20% SDS gels followed by gel scanning on a LKB Ultroscan-XL laser densitometer.

HPLC Analysis of Nucleotide Content. The bound nucleotide of Hsc70 was converted from ADP to ATP by an ATP-regenerating system to measure the rate of ADP release (Gao *et al.*, 1993). The samples were analyzed using a HP 1090 liquid chromatograph from Hewlett-Packard. The samples were treated with perchloric acid followed by neutralization by KOH according to Gao *et al.* (1993).

Materials. Superose-6 was obtained from Pharmacia; ATP, MES, creatine kinase, creatine phosphate, hexokinase, and imidazole from Sigma; [γ^{32}P]ATP from New England Nuclear; and 4−20% polyacrylamide SDS gels from Integrated Separation Systems.

RESULTS

We investigated the effect of auxilin on the interaction of Hsc70 with pure clathrin baskets at pH 6, where uncoating does not occur (Braell *et al.*, 1984); the baskets were prepared without assembly proteins. We first checked whether the addition of auxilin would promote uncoating of these baskets by Hsc70 at pH 6. At pH 7 maximal uncoating occurs when

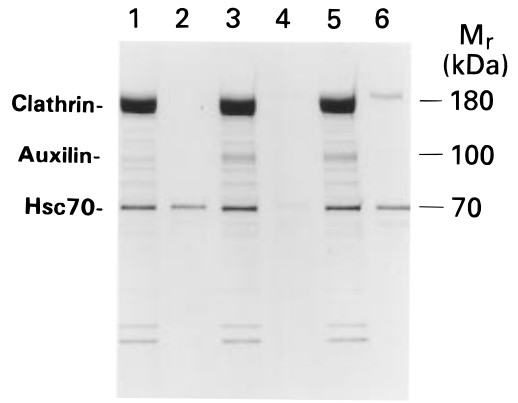

FIGURE 1: Hsc70 does not uncoat but binds clathrin baskets in the presence of auxilin and ATP at pH 6.0 (SDS−PAGE). Lanes 1, 3, 5: total mixture before centrifugation. Lanes 2, 4, 6: supernatant of preceding lane after centrifugation at 25 °C. Lanes 1−4: ATP added. Lanes 5−6: ADP added. Lanes 1, 2: clathrin baskets plus Hsc70. Lanes 3, 4: auxilin plus baskets and Hsc70. Lanes 5, 6: same as lanes 3 and 4, but with ADP instead of ATP. Experimental conditions: 0.3 μM Hsc70, 0.2 μM clathrin baskets, 0.4 μM auxilin, and 20 μM ATP or ADP in buffer B. Centrifugation of Hsc70 alone resulted in 85% recovery of the protein in the supernatant. The higher staining of Hsc70 in lanes 1, 3, and 5 is due to a 70 kDa contaminant present in the clathrin preparation.

auxilin is present at a molar ratio of about 1 to 5 to both clathrin baskets and Hsc70 (Prasad *et al.*, 1993; Barouch *et al.*, 1994). However, at pH 6, both at this ratio and at considerably higher ratios of auxilin to pure clathrin baskets and Hsc70, auxilin induced very little uncoating (Figure 1, lanes 1−4). On the other hand, auxilin has a major effect on the interaction of Hsc70 with pure clathrin baskets; it promotes the binding of Hsc70 to the baskets (Figure 1, lanes 3 and 4 compared to lanes 1 and 2). This binding shows an absolute dependence on nucleotide. In the presence of ATP but not ADP, auxilin promotes the binding of Hsc70 to clathrin baskets, (Figure 1, lanes 4 and 6). We previously obtained similar results with AP_{180}−clathrin baskets at pH 6.5 (Ungewickell *et al.*, 1995). Note also that almost all of the auxilin binds to the clathrin baskets in the presence of both ATP and ADP.

Our observation that auxilin does not induce the binding of Hsc70 to clathrin baskets in ADP could be a kinetic phenomenon in which Hsc70 binds as strongly in ADP as it does in ATP but binds so slowly that essentially no binding occurs over the time course of the experiment. In fact, for the binding of Hsc70 to soluble clathrin triskelions at pH 7, which is not auxilin dependent, Hsc70 was found to bind to and dissociate from clathrin rapidly in ATP but very slowly in ADP (Prasad *et al.*, 1994). To determine whether this is also the case for the binding of Hsc70 to clathrin baskets, Hsc70 was first bound to the baskets in the presence of ATP and stoichiometric auxilin. Hexokinase/glucose was then added to hydrolyze the ATP completely to ADP, and after varying times at 25 °C, the reaction mixture was centrifuged. If the binding of Hsc70 to clathrin baskets is energetically favored in ADP but is kinetically blocked, then the Hsc70 already bound in ATP will remain attached after the ATP is hydrolyzed to ADP. However, Figure 2 shows that the bound Hsc70, in fact, dissociates from clathrin baskets in ADP with a half-life of about 6 min. Therefore, in the presence of auxilin and ADP, Hsc70 does not bind to clathrin baskets because this reaction is energetically unfavorable.

Since the binding of Hsc70 to clathrin baskets in ATP requires auxilin, we examined whether activation of the

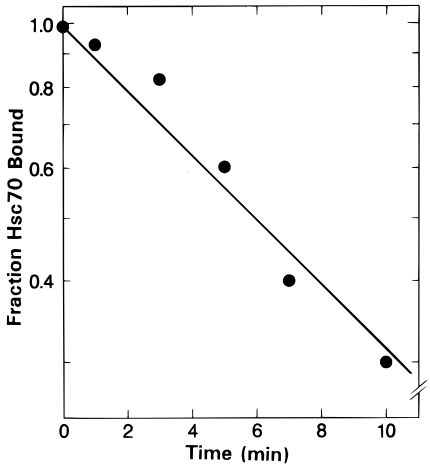

FIGURE 2: Time course of dissociation of Hsc70 from clathrin baskets upon hydrolysis of ATP. A solution of 0.9 μM Hsc70, 0.4 μM clathrin baskets, 0.4 μM auxilin, and 20 μM ATP was incubated for 15 min at 25 °C in buffer B. At varying times after addition of hexokinase (50 units/mL) and glucose (5 mM), the solution was centrifuged to determine the amount of Hsc70 bound to clathrin baskets. The zero time data point of 0.6, representing the fraction of Hsc70 bound to baskets in ATP, was normalized to be 1.0.

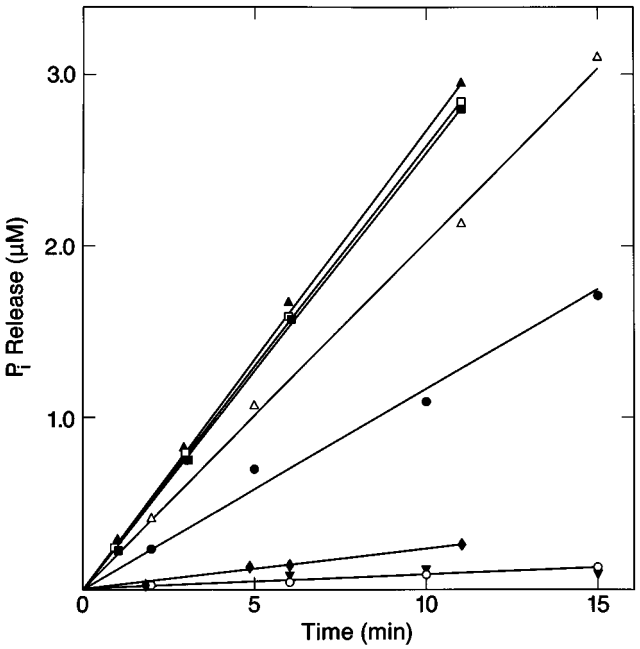

FIGURE 3: Effect of auxilin on activation of Hsc70 ATPase activity by clathrin baskets at pH 6.0. The ATPase activity was measured in buffer B at 25 °C using 0.1 μM Hsc70 alone (○); 0.1 μM Hsc70 with 0.4 μM clathrin baskets (▼); 0.1 μM Hsc70 with 0.4 μM auxilin (♦); 0.1 μM Hsc70, 0.1 μM clathrin baskets, and 0.05 μM (●), 0.1 μM (△), 0.2 μM (▲), 0.3 μM (□) or 0.4 μM (■) auxilin. 20 μM [γ^{32}P]ATP was present in all samples. The rate of ATP hydrolysis with Hsc70 alone was 7×10^{-4} s^{-1}, measured at 4 μM Hsc70.

Hsc70 ATPase activity at pH 6 by clathrin baskets is also dependent on auxilin. Increasing concentrations of auxilin in the presence of clathrin baskets resulted in increasing activation of the steady-state Hsc70 ATPase activity, so that at high ratios of auxilin to baskets there is marked activation of the Hsc70 ATPase activity while almost no activation occurs in the absence of auxilin (Figure 3). This latter result is contrary to the results of Rothman and collaborators (Braell *et al.*, 1984), who found that pure clathrin baskets by themselves did activate the Hsc70 ATPase activity. However, it is likely that there was auxilin present in the clathrin preparation of Braell *et al.* (1984) since the clathrin used in their experiment was not column purified twice, which is necessary to remove all auxilin. We found there was slight activation of the Hsc70 ATPase by auxilin alone (Figure 3), which is consistent with our results showing direct interaction between Hsc70 and auxilin (Jiang *et al.*, 1997). However, this activation is much less than occurs with both auxilin and clathrin baskets. Furthermore, at pH 7, soluble clathrin triskelions did not significantly activate the Hsc70 ATPase activity in the presence of auxilin (open triangles, Figure 4). This latter experiment was carried out at pH 7 because clathrin spontaneously assembles into baskets at pH 6. Therefore, it appears that there is a specific requirement for both auxilin and clathrin baskets in order for marked activation of the Hsc70 ATPase activity to occur. Note that at pH 6, just as at pH 7 (Gao *et al.*, 1993), essentially all of the nucleotide bound to Hsc70 is ATP in the presence of an ATP-regenerating system. This indicates that ATP hydrolysis as opposed to product release is the rate-limiting step in the Hsc70 ATPase cycle at pH 6. Therefore, activation of the Hsc70 ATPase activity by auxilin and clathrin baskets must involve a marked increase in the rate of the actual ATP hydrolysis step.

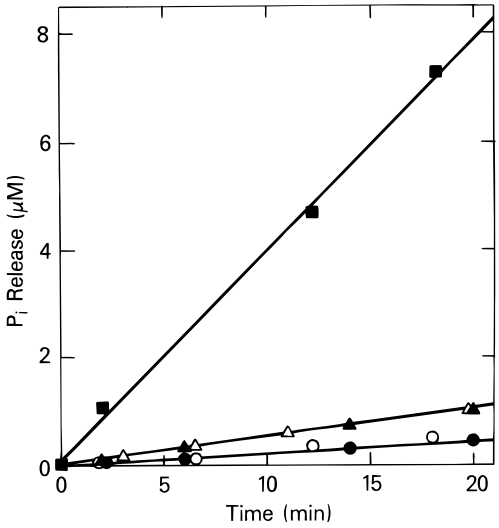

FIGURE 4: Clathrin baskets and auxilin are required for activation of Hsc70 ATPase activity. The ATPase activity was measured in buffer B (pH 6) at 25 °C using 0.6 μM Hsc70 alone (○); 0.6 μM Hsc70, 0.4 μM clathrin baskets, and 0.1 μM of the following proteins; AP-2 (●), AP_{180} (▲), and auxilin (■). The ATPase activity in the presence of soluble clathrin (△) was measured in buffer A (pH 7) using 0.6 μM Hsc70 plus 0.1 μM auxilin and 0.4 μM soluble clathrin triskelions.

Aside from being a cofactor needed in the uncoating reaction, auxilin has also been shown to act as an assembly protein for clathrin baskets (Ahle & Ungewickell, 1990). Therefore, we were interested in determining whether other assembly proteins could substitute for auxilin in inducing clathrin baskets to activate the Hsc70 ATPase activity. As shown in Figure 4, neither AP-2 nor AP_{180} supported activation of the Hsc70 ATPase activity by clathrin baskets. Furthermore, the presence of these assembly proteins did not appear to interfere with the effect of auxilin; in the presence of auxilin, clathrin baskets prepared with assembly proteins activated the Hsc70 ATPase activity only slightly less than clathrin baskets prepared without assembly proteins (data not shown). Finally, whether auxilin was added before or after clathrin basket formation, it had the same effect on the Hsc70 ATPase activity. Therefore, although auxilin acts as an assembly protein, it probably binds to a different site on the

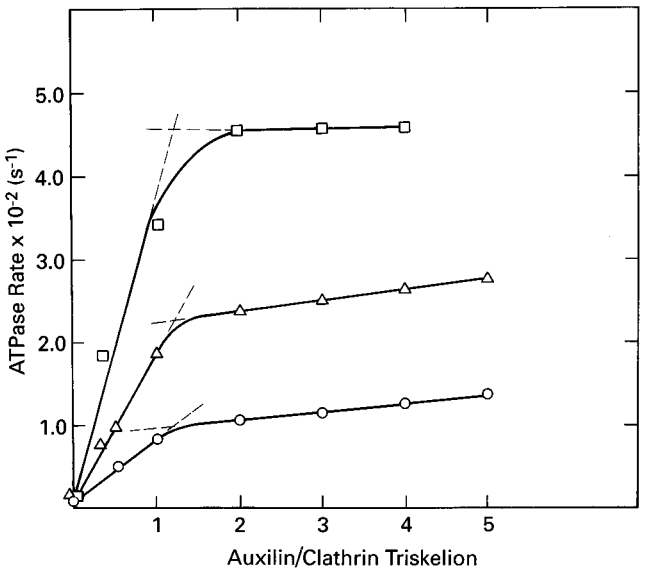

FIGURE 5: ATPase activity of Hsc70 as a function of the ratio of auxilin to clathrin triskelion. The rate of ATPase was calculated from five data points over a 20-min time interval using varying concentrations of auxilin with (○) 0.6 μM Hsc70 plus 0.1 μM clathrin baskets; (△) 0.1 μM Hsc70 plus 0.1 μM clathrin baskets; (□) 0.1 μM Hsc70 plus 0.3 μM clathrin baskets. Note that ATPase rate was calculated per μM Hsc70.

baskets than the other assembly proteins. In addition, since the J-domain of auxilin interacts with Hsc70 (Jiang *et al*., 1997), this interaction is probably crucial in the ability of auxilin to induce the binding of Hsc70 to clathrin baskets.

We next determined the stoichiometry of auxilin to clathrin baskets and Hsc70 required for maximum ATPase activation. In these experiments, the auxilin concentration was varied at a constant basket concentration and Hsc70 concentration. In Figure 5, the ATPase rate was plotted as a function of the ratio of auxilin to clathrin triskelions at different clathrin basket and Hsc70 concentrations. When the concentration of clathrin baskets was increased, the amount of auxilin required for maximum ATPase activity increased proportionally; the ratio of auxilin to clathrin triskelions required for maximum ATPase activity was about one auxilin per clathrin triskelion. Therefore the auxilin appears to be binding stoichiometrically to the baskets rather than occurring free in solution, confirming the data in Figure 1. In addition, auxilin appears to be interacting primarily with the clathrin triskelions rather than with the Hsc70, since the same ratio of auxilin to clathrin triskelions was required for maximum ATPase activation even at a 6-fold higher Hsc70 concentration (open circles, Figure 5). Note that, in this experiment, the maximum ATPase activity (expressed as mol of ATP hydrolyzed/mol of Hsc70) was lower because of the lower ratio of clathrin baskets to Hsc70. Thus, the amount of auxilin required for maximum ATPase activation depends on the basket concentration rather than the Hsc70 concentration, suggesting that most of the auxilin binds to the clathrin baskets rather than to the Hsc70.

In contrast to the ratio of one auxilin per clathrin triskelion required for maximum activation of the Hsc70 ATPase activity, only one auxilin for every 5−10 clathrin triskelions is required for maximum uncoating of the clathrin coated vesicles (Prasad *et al*., 1993; Barouch *et al*., 1994). Furthermore, substoichiometric auxilin is sufficient to induce maximum binding of Hsc70 to clathrin baskets with AP_{180}−clathrin baskets (Ungewickell *et al.*, 1995). Therefore, we

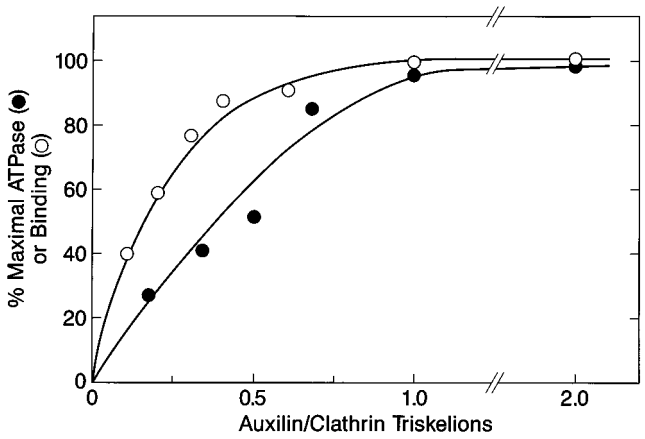

FIGURE 6: Comparison of the amount of auxilin needed for maximal binding and maximal ATPase activity. Both the ATPase activity (●) and binding (○) of Hsc70 to clathrin baskets were measured in buffer B at 25 °C using 0.5 μM Hsc70, 0.3 μM baskets, 20 μM ATP, and a range of auxilin concentrations (0.06−0.6 μM). The data were then normalized by equating to 100% the values measured obtained at high ratios of auxilin to clathrin baskets. Maximal binding and ATPase activity were obtained at auxilin to clathrin triskelion ratios of approximately 0.5 and 1.0, respectively.

were interested in directly comparing the auxilin required for maximum binding of Hsc70 to the pure clathrin baskets with the auxilin required for maximum activation of the Hsc70 ATPase activity by the pure clathrin baskets. Figure 6 shows that considerably less auxilin is required to induce half-maximum binding of Hsc70 than to induce half-maximum ATPase activation by the clathrin baskets.

Finally, we quantitatively investigated the effect of clathrin baskets on the Hsc70 ATPase activity in the presence of saturating auxilin concentration at pH 6. Figure 7A shows the dependence of the rate of ATP hydrolysis on basket concentration using an auxilin concentration always twice the basket concentration. When plotted in double-reciprocal form (Figure 7B), these data fell on a straight line. The *x*-intercept yielded a K_d of 0.2 μM clathrin, which indicates very strong interaction between the clathrin baskets and Hsc70 in the presence of auxilin. This contrasts with the binding constant of Hsc70 to soluble clathrin triskelions of 4 μM in ATP at pH 7 (Prasad *et al.*, 1994). Auxilin is necessary for this effect because in the absence of auxilin no binding of Hsc70 to clathrin baskets could be detected at pH 6, in the presence of either ATP or 90% ADP 10% ATP. This was the case even when the baskets were present at 2 μM, which is a 1 order of magnitude higher concentration than is required for binding in the presence of auxilin (data not shown).

The *y*-intercept of the plot in Figure 7B yielded a V_{max} of 9×10^{-2} s^{-1}. Since the rate of Hsc70 alone is 7×10^{-4} s^{-1} under this condition, there is more than 100-fold activation of the Hsc70 ATPase. This is a more than 1 order of magnitude higher activation of the steady-state Hsc70 ATPase activity than occurs with peptide substrates or DnaJ-like proteins at pH 7 (Greene *et al*., 1995; King *et al*., 1995b; Jiang *et al*., 1997). In general, both peptide substrates and DnaJ-like proteins markedly increase the rate of ATP hydrolysis by the Hsc70 proteins but have little effect on the subsequent steps involving product release. Therefore, at pH 7 the rate of the activated steady-state ATPase activity is approximately equal to the rate of product release (Gao *et al.*, 1993; Greene *et al*., 1995; King *et al*., 1995b; Jiang *et al.*, 1997).

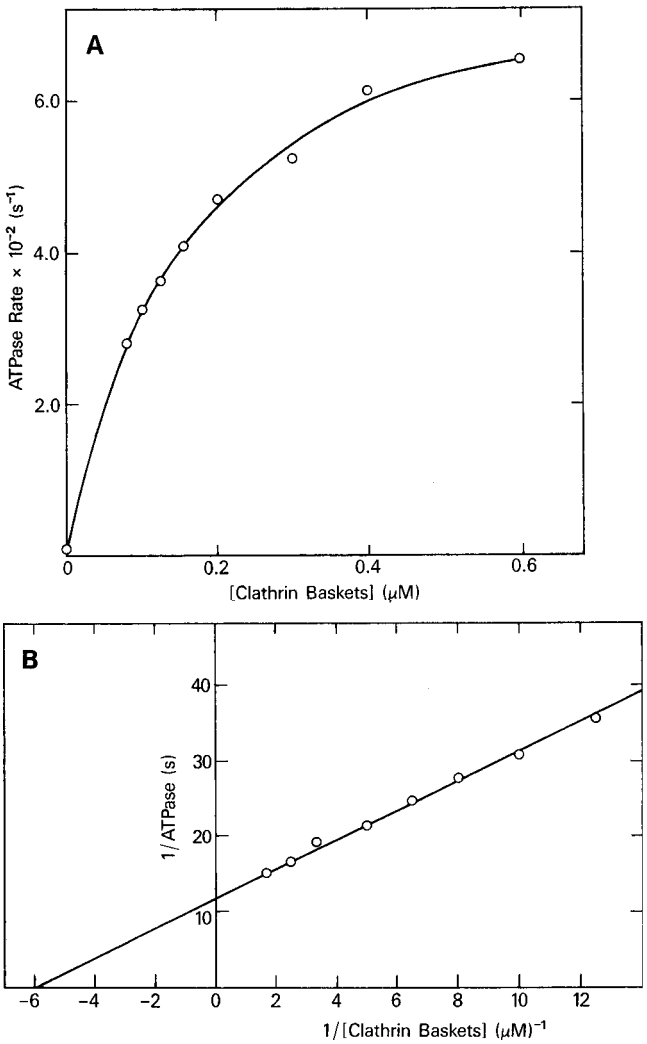

FIGURE 7: Dependence of activation of Hsc70 ATPase activity on varying clathrin basket concentrations at saturating amounts of auxilin. (A) plot of rate vs basket concentration at 0.1 μM Hsc70, 0.08−0.6 μM clathrin baskets; the auxilin concentration was always twice the basket concentration. (B) double-reciprocal plot of data from A. At $x = 0$, $1/v = 1/V_{max}$; $V_{max} = 9 \times 10^{-2}$ s^{-1}. At $y = 0$, 1/[clathrin baskets] $= -1/K_d$; $K_d = 0.2$ μM clathrin.

The high ATPase rate at pH 6 suggested that auxilin and clathrin baskets might not only increase the rate of the ATP hydrolysis step but also increase the rate of ADP release from Hsc70. To test this point, we directly measured the rate of ADP release from Hsc70 alone at pH 6. The rate that ADP bound to Hsc70 is converted to ATP by creatine kinase is a measure of the rate of ADP release (Gao *et al*., 1993). Figure 8 shows that the half-life for ADP release from pure Hsc70 is about 20 s at pH 6, which gives a value of 3×10^{-2} s^{-1} for the rate of ADP release. This value is 1 order of magnitude greater than that measured at pH 7 (Gao *et al*., 1993). Since the maximum steady state rate is 9×10^{-2} s^{-1} at pH 6, the baskets and auxilin must cause at least a 3-fold increase in the rate of ADP release. Therefore, clathrin baskets and auxilin increase both the rate of the ATP hydrolysis step and the rate of product release from Hsc70 at pH 6, although the former effect may be larger than the latter effect.

DISCUSSION

Interaction of Auxilin with Clathrin Baskets. Since both auxilin and Hsc70 bind to clathrin at a stoichiometry of 3 mol/mol of clathrin triskelions in the baskets (Ahle & Ungewickell, 1990), it might be expected that maximum activation of the Hsc70 ATPase activity by clathrin baskets would also occur at the same stoichiometry. However, our results show that the binding of one auxilin per clathrin triskelion in the baskets is sufficient to fully activate the ATPase activity of the three Hsc70s bound per triskelion at pH 6. Since the three Hsc70s apparently bind in close proximity to each other at the clathrin vertex (Prasad *et al.*, 1993), it is possible that auxilin also binds to the clathrin vertex, and therefore one auxilin can interact with all three Hsc70s at the vertex. Our data also show that the auxilin binds to a different site on clathrin than AP_{180} and AP-2, since these assembly proteins neither substitute for auxilin nor interfere with the effect of auxilin on the Hsc70 ATPase activity. Finally, our data show that, whether Hsc70 or clathrin baskets are in excess, the same 1 to 1 ratio of auxilin to clathrin triskelions in the clathrin baskets is required for maximum ATPase activity. Since auxilin can interact directly with Hsc70 (Jiang *et al*., 1997) as well as with clathrin baskets (Ahle & Ungewickell, 1990), these data suggest that auxilin binds considerably more tightly to the clathrin baskets than to the Hsc70. Therefore, even in the presence of excess Hsc70, auxilin preferentially binds to the clathrin triskelions in the clathrin baskets at a 1 to 1 stoichiometry.

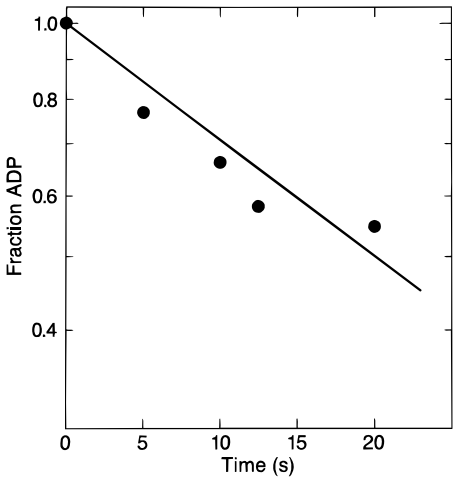

FIGURE 8: Rate of ADP release at pH 6 at 25 °C. ADP bound to Hsc70 was converted to ATP by creatine kinase by incubating 10 μM Hsc70 containing 8 μM bound ADP with 30 units/mL creatine kinase and 15 mM creatine phosphate at 25 °C in buffer B. At varying times the reaction was stopped by mixing with perchloric acid and the nucleotide content was determined by HPLC. After 5 min, 95% of the bound nucleotide was converted to ATP.

Role of Auxilin in Inducing Clathrin Baskets To Bind to Hsc70−ATP. At saturating auxilin, half-maximal Hsc70 ATPase activity occurs at 0.2 μM clathrin baskets (or 0.6 μM clathrin heavy chains), suggesting that auxilin induces very strong binding between clathrin baskets and Hsc70 in ATP. Both auxilin and ATP are required for this binding; even at 2 μM clathrin baskets, no binding could be detected in the absence of auxilin, and in the presence of auxilin, the binding was actually reversed in ADP. We found that, in the absence of clathrin baskets, auxilin binds directly to Hsc70 with a binding constant of about 0.6 μM at pH 7 (Jiang *et al*., 1997). Furthermore, like the binding of Hsc70 to clathrin baskets, this binding occurs in ATP but not in ADP and is reversed when ATP is hydrolyzed to ADP. Therefore, the binding of Hsc70 to clathrin baskets may initially be caused by a direct interaction of Hsc70 with

auxilin which is already bound to the clathrin baskets; that is, auxilin may act as a bridge between Hsc70 and the clathrin baskets. This would be consistent with our previous observation that auxilin has two domains, an N-terminal domain which interacts with the clathrin baskets (Ungewickell *et al*., 1995) and a C-terminal J-domain which interacts with Hsc70 (Jiang *et al.*, 1997).

However, the formation of a ternary complex in ATP cannot completely explain our data because, in contrast to the 1 to 1 molar ratio of auxilin to clathrin triskelions required for maximum ATPase activity, maximal binding of Hsc70 to clathrin baskets occurs at substoichiometric levels of auxilin to clathrin baskets. A similar effect occurs at pH 7 where the maximum rate of uncoating occurs at about 0.1−0.2 mol of auxilin/mol of clathrin (Prasad *et al*., 1993; Barouch *et al*., 1994). One explanation for these observations is that, although most of the auxilin is bound to the clathrin baskets, auxilin rapidly moves from site to site on the baskets, first indirectly linking Hsc70 to the baskets in a ternary complex, then inducing direct binding of Hsc70 to the clathrin baskets, and finally dissociating and moving to another clathrin site before the ATPase cycle is completed, e.g., before ADP is released from the Hsc70. Note that, in such a model, if auxilin accelerates the rate of ADP release, maximum binding of Hsc70 but not maximum ATPase activity can occur at substoichiometric concentrations of auxilin just as we observed.

In inducing direct binding between Hsc70 and clathrin baskets, auxilin may act like other DnaJ homologs which present substrates to Hsc70s. Two explanations of this phenomenon have been suggested. One is that DnaJ homologs induce rapid hydrolysis of ATP to ADP so that the transient complex formed between substrate and Hsc70−ATP is stabilized upon ATP hydrolysis (McCarty *et al*., 1995). However, ADP exchanges with ATP so rapidly at pH 6 that stabilization by ADP is unlikely to be significant in our experiments. A second possible explanation is that DnaJ homologs somehow activate Hsc70−ATP to bind substrates which would otherwise bind more weakly (Wawrzynow *et al.*, 1995). The mechanism of this activation is unknown; in the case of auxilin it might involve the J-domain altering the substrate binding site of Hsc70 or it might involve the J-domain inducing polymerization of Hsc70 at the clathrin vertex. In this regard, it is interesting that other DnaJ homologs induce Hsc70 to polymerize in ATP (King *et al*., 1995a), as does auxilin in the absence of clathrin baskets (Jiang *et al*., 1997). However, these other DnaJ homologs cannot substitute for auxilin in inducing uncoating at pH 7 or the binding of clathrin baskets to Hsc70 at pH 6 (King *et al*., 1997). Therefore, since both the clathrin-binding domain and the J-domain of auxilin must be present for auxilin to function properly (Ungewickell *et al*., 1995; Jiang *et al*., 1997), the clathrin-binding domain may juxtapose the clathrin baskets and the Hsc70 while the J-domain activates the Hsc70 to bind to the clathrin baskets.

Comparison of Hsc70 Binding to Clathrin Baskets and Clathrin Triskelions. The data in this paper show that the auxilin-induced binding of clathrin baskets to Hsc70 is qualitatively different than the binding of soluble clathrin triskelions to Hsc70. The soluble clathrin triskelions bind 1 order of magnitude more weakly than the clathrin baskets in ATP and, in contrast to the baskets, still bind in ADP (Prasad *et al*., 1994). Since clathrin baskets are the substrate of the uncoating reaction while soluble clathrin triskelions are the product, these data suggest that studies on the binding to Hsc70 of peptides or denatured proteins which are not presented by DnaJ homologs may be misleading and may, in fact, more accurately reflect the interaction of products with Hsc70 than substrates.

Interestingly, ATP appears to be required for both substrate binding and product dissociation from Hsc70. This may be explained by recent structural studies of the substrate binding domain of DnaK which suggest that the substrate binding site of DnaK must be opened up in order for substrates to bind or products to dissociate (Zhu *et al.*, 1996). However, in reactions such as uncoating, unfolding, or translocation where the energy of ATP hydrolysis is used to perform work, it may be crucial that the rebinding of ATP during the dissociation phase of the cycle not return the Hsc70 to the exact conformation it had in the substrate binding phase, thereby reversing the conformational change which occurred during ATP hydrolysis. The requirement for DnaJ homologs such as auxilin in the binding but not the dissociation phase of the cycle may not only provide specificity in the binding of substrates to Hsc70 but also provide the asymmetry which is required for reactions such as uncoating which use the energy of ATP hydrolysis to perform work.

REFERENCES

Ahle, S., & Ungewickell, E. (1990) *J. Cell Biol. 111*, 19−29.
Barouch, W., Prasad, K., Greene, L. E., & Eisenberg, E. (1994) *J. Biol. Chem. 269*, 28563−28568.
Braell, W. A., Schlossman, D. M., Schmid, S. L., & Rothman, J. E. (1984) *J. Biol. Chem. 99*, 734−741.
Caplan, A. J., Langley, E., Wilson, E., & Vidal, J. (1995) *J. Biol. Chem. 270*, 5251−5257.
Gao, B., Emoto, Y., Greene, L., & Eisenberg, E. (1993) *J. Biol. Chem. 268*, 8507−8513.
Greene, L., & Eisenberg, E. (1990) *J. Biol. Chem. 265*, 6682−6687.
Greene, L., Zinner, R., Naficy, S., & Eisenberg, E. (1995) *J. Biol. Chem. 270*, 2967−2973.
Jiang, R. F., Barouch, W., Eisenberg, E., & Greene, L. E. (1997) *J. Biol. Chem.* (in press).
Kimura, Y., Yahara, I., & Lindqust, S. (1995) *Science 268*, 1362−1365.
King, C., Eisenberg, E., & Greene, L. (1995a) *J. Biol. Chem. 270*, 22535−22540.
King, C., Eisenberg, E., & Greene, L. (1995b) *Mol. Biol. Cell. 6*, 69a.
King, C., Eisenberg, E., & Greene, L. (1997) *Biochemistry* (in press).
McCarty, J., Buchburger, A., Reinstein, J., & Bukau, B. (1995) *J. Mol. Biol. 249*, 126−137.
Prasad, K., & Lippoldt, R. (1988) *Biochemistry 27*, 6098−6104.
Prasad, K., Barouch, W., Greene L., & Eisenberg, E. (1993) *J. Biol. Chem. 268*, 23758−23761.
Prasad, K., Heuser, J., Eisenberg, E., & Greene, L. (1994) *J. Biol. Chem. 269*, 6931−6939.
Rassow, J., Voos, W., & Pfanner, N. (1995) *Trends Cell Biol. 5*, 207−212.
Schlossman, D. M., Schmid, S. L., Braell, W. A., & Rothman, J. E. (1984) *J. Cell Biol. 99*, 723−733.
Ungewickell, E., Ungewickell, H., Holstein, S., Lindner, R., Prasad, K., Barouch, W., Martin, B., Greene, L., & Eisenberg, E. (1995) *Nature 378*, 632−635.
Wawrzynow, A., Banecki, B., Wall, D., Liberek, K., Georgopoulos, C., & Zylicz, M. (1995) *J. Biol. Chem. 270*, 19307−19311.
Zhu, X., Zhao, X., Burkholder, W., Gragerov, A., Ogata, C., Gottesman, M., & Hendrickson, W. (1996) *Science 272*, 1606−1614.
Zylicz, M., Yamamoto, T., McKittrick, N., Sell, S., & Georgopoulos, C. (1985) *J. Biol. Chem. 12*, 7591−7598.

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