ATPase Activity Associated with the Uncoating of Clathrin Baskets by Hsp70*

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In the presence of ATP, bovine brain hsp70 has been shown to remove clathrin from bovine brain clathrin-coated vesicles in a rapid stoichiometric initial burst followed by slow steady-state uncoating. In addition, it has been found recently that a 100-kDa cofactor is required for hsp70 to uncoat clathrin baskets prepared with the assembly protein AP-2. In this study the ATPase activity associated with uncoating was investigated, with baskets formed from clathrin and assembly proteins. Mixed assembly proteins or assembly protein AP-2 could not be used in ATPase studies because they activated the hsp70 ATPase activity even in the absence of clathrin. However, this was not the case with assembly protein AP-1. A stoichiometric initial burst of ATP hydrolysis was found to accompany the initial burst of uncoating of AP-1-clathrin baskets by hsp70, with 1 mol of hydrolyzed ATP/mol of released clathrin heavy chain. Furthermore, the presence of a 100-kDa cofactor was needed for both processes. These results suggest that an initial burst of uncoating occurs with all clathrin baskets, that an initial burst of ATP hydrolysis accompanies this initial burst of uncoating, and that a 100-kDa cofactor is required for both.

The 70-kDa class of heat shock proteins (hsp70) are present both constitutively and following induction by heat shock in almost all living cells (1, 2). In their role as "molecular chaperones," it has been proposed that these proteins are involved in the folding and unfolding of proteins (3-6), in the translocation of proteins into lysosomes, the endoplasmic reticulum, mitochondria, and the nucleus (7-10), and in the protection of proteins from denaturation during heat shock (11). There is now strong evidence that hsp70 has a single binding site for nucleotide where both ATP and ADP are bound very tightly (Kd ~ 1 x 10^-11 M) (12). However, the role of ATP hydrolysis in the function of the hsp70 proteins remains unclear.

Perhaps the most well defined action of hsp70 in vitro is the ability to remove clathrin from clathrin-coated vesicles (13, 14). In the hope that understanding the mechanism of uncoating will shed light on the general mechanism of action of hsp70, in particular, on the role of ATP hydrolysis in its function, we are carrying out a detailed study of the mechanism of the uncoating reaction. In previous studies, we found that, when hsp70 and ATP are mixed with clathrin-coated vesicles, there is a rapid initial burst of uncoating followed by slow steady-state uncoating (15). The initial burst of uncoating is a stoichiometric reaction in which 1 mol of clathrin triskelions are removed by 3 mol of hsp70, resulting in the formation of a stable complex with 3 mol of hsp70 bound per clathrin triskelion (15). We recently found a similar time course of uncoating with synthetic clathrin baskets prepared with the assembly protein, AP1.2. We also made the interesting observation that a 100-kDa cofactor is required for hsp70 to uncoat these baskets (16), suggesting that uncoating may be like several other reactions involving hsp70 which also require protein cofactors (17-20).

Based on our studies showing that ADP and F inhibit steady-state uncoating, we proposed that a stoichiometric initial burst of ATP hydrolysis accompanies the stoichiometric initial burst of uncoating with 3 mol of ATP hydrolyzed per clathrin triskelion. This model suggests that it is the transition from the ATP form to the ADP form of hsp70 which actually removes the clathrin from the baskets with the concomitant formation of a stable clathrin-hsp70-ADP complex. The model then suggests that clathrin is unable to dissociate from this complex until the bound ADP exchanges with ATP in a reaction assumed to be slow to account for the slow steady-state uncoating which follows the initial burst of uncoating.

This model is supported by our studies on the interaction of monomeric clathrin (21) and peptides with hsp70, which suggest that hsp70 occurs in two major conformations depending on its bound nucleotide. While clathrin binds with nearly the same affinity to both forms of hsp70, its rates of interaction with the two forms are dramatically different with rapid binding and dissociation from the ATP form of hsp70 but extremely slow binding and dissociation from the ADP form. On this basis, the transition from the ATP form to the ADP form of hsp70 would remove the clathrin from the coated vesicles by locking it onto hsp70, while exchange of ADP with ATP would dissociate the clathrin from the ATP form of hsp70 later in the cycle.

Other laboratories have proposed quite different models of hsp70 action. Several laboratories have suggested that ATP hydrolysis is required for dissociation of substrates from hsp70, because nucleotide analogues are unable to cause this dissociation (3, 5), in contrast to our view that it is ATP binding, not ATP hydrolysis, which is responsible for dissociation of substrates from hsp70. Presumably, these models would predict that ATP hydrolysis would not occur during the initial burst of uncoating but later in the cycle when clathrin dissociates from hsp70. A recently proposed model of hsp70 action suggests that the ATP form of hsp70 is inactive and protein and peptide substrates bind only to the ADP form (22) in contrast to our view that substrates bind to the ATP form and then stimulate the hydrolysis of ATP to ADP. If that model were correct, ATP hydrolysis would presumably occur before the initial burst of uncoating since it would be the hsp70-ADP complex rather

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than the hsp70-ATP complex which would bind to the coated vesicles and remove clathrin. Correlation of the time course of hsp70 ATPase activity with uncoating should allow us to distinguish between these various models of hsp70 action.

This correlation cannot be studied with coated vesicles because of their high level of contaminant ATPase activity. However, clathrin baskets have a much lower level of contaminant ATPase activity. In addition, with clathrin baskets it is possible to determine the effect of the 100-kDa cofactor on the ATPase activity associated with uncoating. In previous ATPase studies with clathrin baskets prepared from partially purified clathrin, Rothman and his collaborators (14, 23) showed that hsp70 ATPase activity is increased during the uncoating of clathrin baskets. However, in contrast to our results with both coated vesicles (15) and clathrin baskets (16), they did not observe an initial burst of uncoating, but a continuous steady-state uncoating, nor did they observe an initial burst of ATPase activity.

In the present study we investigated the ATPase activity and uncoating of clathrin baskets prepared from separately purified clathrin and assembly proteins with and without the 100-kDa cofactor. Due to contaminant ATPase activity, we found that clathrin baskets prepared with either mixed assembly proteins or AP-2 were unsuitable for these studies. However, using clathrin baskets prepared with AP-100 (25), we found that hsp70 caused a stoichiometric initial burst of uncoating which required the presence of the 100-kDa cofactor and that this initial burst of uncoating was accompanied by a stoichiometric initial burst of hsp70 ATPase activity which also required the presence of cofactor.

**EXPERIMENTAL PROCEDURES**

All procedures were carried out at 4°C unless otherwise stated. Preparations of Proteins—Hsp70 (the uncoating ATPase) was prepared as described previously by Greene and Eisenberg (15). Clathrin, mixed APs, AP-100, and AP-2 were prepared essentially by the procedures of Prasad and Keen (24) and Prasad and Lippoldt (26) with minor modifications. Briefly, homogenization and differential centrifugation of Prasad and Keen (24) and Prasad and Lippoldt (26) with minor modifications. Briefly, homogenization and differential centrifugation of calf brains yielded coated vesicles, which were suspended overnight in 10 mM Tris, pH 8.5. Subsequent centrifugation at 180,000 x g for 3.5 h resulted in a supernatant enriched in clathrin and AP-100, which were separated by chromatography on a Superose-6 column (26.7 x 87 cm) at 25°C and 0.7 ml/min in 0.5 M Tris, 2 mM dithiothreitol, 1 mM EDTA, pH 7.0. The eluted major peak consisted of pure clathrin and the minor peak used to obtain AP-100.

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Preparation of Mixed Assembly Proteins and AP-2—The pellets which contained uncoated vesicles from the last 180,000 x g spin (see "Preparation of Proteins," above) were suspended in 0.5 M Tris, pH 7.0, overnight, then centrifuged at 180,000 x g for 3 h. The resulting supernatant was chromatographed on Superose-6 under the same conditions as above. The fractions from the eluted AP-2 (minor peak) were pooled and used as mixed assembly proteins, or were further purified into AP-2 on a hydroxylapatite column (1.0 x 9 cm) connected to a Pharmacia FPLC apparatus, and equilibrated in 0.5 M Tris, pH 7.0. AP-2 was eluted with a gradient of 0.5 mM to 25 mM phosphate, 0.5 mM Tris, pH 7.0 at 0.1 ml/min. The extinction coefficient ε280 of 3.8 for AP-2 was used (26).

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Purification of the 100-kDa Co-factor—Pooled fractions from the top of the AP peak (last paragraph) were injected in 0.5 M Tris, pH 7.0, on the same hydroxylapatite FPLC system as for AP-100, washed with 60 mM phosphate, 0.5 M Tris, pH 7.0, and then eluted with 0.5 M phosphate, 0.5 M Tris, pH 7.0. The extinction coefficients ε280 of 5.6 for AP-2, and 6 for the mixed APs were used (27).

Purification of the 100-kDa Co-factor—Pooled fractions from the top of the AP peak (last paragraph) were injected in 0.5 M Tris, pH 7.0, on the same hydroxylapatite FPLC system as for AP-100, washed with 60 mM phosphate, 0.5 M Tris, pH 7.0, and then eluted with 0.5 M phosphate, 0.5 M Tris, pH 7.0, at 0.3 ml/min. The co-factor was further purified by FPLC on a prepacked Pharmacia Mono Q HR 5/5 (1 ml) column equilibrated in 20 mM Tris, pH 8.5. After a brief wash with 0.1 M NaCl, 20 mM Tris, pH 8.5, co-factor was eluted at 0.3 ml/min with a gradient of 0.1 to 0.25 M NaCl, 20 mM Tris, pH 8.5. Co-factor was determined spectrophotometrically and chromatographed on a Superose-6 column (26).

The mixed assembly proteins, co-factor, and clathrin were dialyzed and stored in 10 mM Tris, pH 8.5.

**Preparation of Clathrin Baskets—**Baskets were made by overnight dialysis into 0.1 M MES, pH 6.5, of Superose-6 column purified clathrin together with either mixed APs or AP-100. Before addition to AP-100, the clathrin was further purified as described below. The proteins were mixed in the following ratios (by weight): 1 clathrin:1 mixed APs, and 5 clathrin:1 AP-100. The baskets formed by dialysis were centrifuged for 1 h at 150,000 x g, and suspended in buffer A (20 mM imidazole, pH 7.0, 2 mM magnesium acetate, 25 mM KCl, 10 mM (NH4)2SO4, and 1 mM dithiothreitol). For assays involving unpolymerized basket components, the ratio of clathrin and respective APs used was the same as that for polymerization into baskets.

**Uncoating and ATPase Assays—**Both uncoating and ATPase experiments were done at 25°C in buffer A. When assembly proteins, unpolymerized components, or depolymerized baskets (all in 10 mM Tris, pH 6.0) were included in the reaction solution, the slight increase in pH was compensated by adding 10% (by volume) of 10 x concentrated buffer A at a pH adjusted to achieve pH 7.0 in the final solution. The hsp70 was preincubated for 8 min with ATP or [γ-32P]ATP (specific activity, 0.4 Ci/mmole), to replace all the bound nucleotide on the hsp70 with ATP or [γ-32P]ATP (28) before addition to the final reaction mixture. None of the proteins, including AP-2, aggregated in buffer A under these conditions.

ATPase activity was determined as described previously (16), they did not observe an initial burst of uncoating, but a continuous steady-state uncoating, nor did they observe an initial burst of ATPase activity.

**RESULTS**

**Uncoating and ATPase with Mixed AP-Clathrin Baskets—**Although Rothman and co-workers were able to prepare synthetic clathrin baskets from relatively impure clathrin (14, 23), we found that more than 80–90% of the clathrin baskets prepared from pure clathrin by dialysis against low pH buffer (30), spontaneously dissociated in the uncoating assays. Therefore, assembly proteins had to be present to prepare stable clathrin baskets for uncoating assays from pure clathrin. We first used a mixture of assembly proteins isolated by Superose-6 column chromatography of Tris buffer extracts of coated vesicles. Lanes 1 and 2 in Fig. 1 show the clathrin and mixed assembly protein preparations, respectively. The resulting baskets were stable in Buffer A; 90–95% sedimentation occurred upon centrifugation at 400,000 x g for 6 min (data not shown).

When hsp70 and ATP were added to these clathrin baskets, the time course of uncoating was nonlinear (Fig. 2, solid squares) as previously observed with clathrin coated vesicles (15). The initial burst of uncoating, shown by extrapolation of the slower, linear part of the graph to zero time, appeared to be stoichiometric: 0.6 µm added hsp70 dissociated 0.6 µm clathrin heavy chain, i.e., 0.2 µm clathrin triskelions. An initial burst of P, release (Fig. 2, open squares) accompanied this initial burst of uncoating. However, it was difficult to determine if this ATPase activity was associated with the uncoating reaction because it remained high even after the initial burst of uncoating was complete. Furthermore, considerable ATPase activation occurred (Fig. 2, open triangles) when the clathrin and
mixed assembly proteins were combined but not given enough time to polymerize into baskets (indicated by the lack of sedimentation on centrifugation). Treatment of the baskets with X-100 did not affect this result. Therefore, using baskets prepared from mixed assembly proteins and clathrin, we did observe an initial burst of uncoating but we were unable to determine if it was accompanied by an initial burst of ATPase activity.

The high ATPase activity which occurred in the absence of uncoating was not caused by the clathrin but by the mixed assembly protein preparation which showed a significant ATPase activity by itself and, in addition, caused significant activation of the hsp70 ATPase activity. Therefore, we further purified the major assembly protein, AP-2, in hopes of removing the contaminations which caused this ATPase activity. Lane 3 in

Hsp70 and Clathrin Baskets

Fig. 2. ATPase activity associated with the uncoating of mixed AP-clathrin baskets. Solid lines show ATPase activity. Dashed line shows uncoating quantified as release of clathrin HC. 10 μM [γ32P]ATP or ATP was used. ATPase activity of 0.6 μM hsp70 alone (○), with clathrin baskets consisting of 0.4 μM clathrin and 0.8 μM mixed assembly proteins (□), or with a mixture of 0.4 μM unpolymerized clathrin and 0.8 μM mixed assembly proteins (△). Clathrin heavy chain release from 0.4 μM clathrin baskets by 0.6 μM hsp70 (■).

Fig. 3. Different effects of AP-2 and AP180 on the ATPase activity of hsp70. Activity of 0.6 μM hsp70 alone (○), or with the addition of either 0.5 μM AP180 (▼) or AP-2 at 1.3 μM (□), 2.6 μM (△), or 5.2 μM (●), 1.3 μM AP-2 alone (■). All samples contained 10 μM [γ32P]ATP.

Fig. 1 shows the purified AP-2 obtained from hydroxyapatite column chromatography. Surprisingly, although the protein appeared to be pure and had very little intrinsic ATPase activity, it caused a marked initial burst of ATPase activity when it was mixed with hsp70 (Fig. 3). Approximately 1 mol of ATP per mol of hsp70 was hydrolyzed in this initial burst whereas, following the initial burst, the steady-state rate was only about 2-fold higher than the rate of the hsp70 alone. The rate of the initial burst appeared to be proportional to the amount of AP-2 present; the highest concentration of AP-2 caused more than 25-fold activation of the initial ATPase activity. Preliminary data involving further purification of AP-2 suggests that another factor may be involved but more work will be necessary to establish this point.

Uncoating and ATPase with AP180—Clathrin Baskets and Cofactor—Since AP-2 caused a rapid initial burst of ATPase activity, clathrin baskets made with AP-2 could not be used to determine if there was ATPase activity associated with the uncoating process. Another possibility was to use AP-1. However, AP-1 is very difficult to purify and also is present in small amounts. Therefore, we decided to try another assembly protein, AP180, a brain specific assembly protein (25) (Fig. 1, lane 4). In contrast to AP-2, purified AP180 not only had very little intrinsic ATPase activity but, in addition, caused almost no activation of the hsp70 ATPase activity (Fig. 3, solid triangles). Furthermore, to prepare clathrin baskets, only 1 mol of AP180 per mol of clathrin triskelion is needed compared to 3 mol of AP-2 per mol of clathrin triskelion so contaminant ATPase activity is less of a problem with AP180. We, therefore, prepared clathrin baskets, using purified AP180 and clathrin, and found they were stable in buffer A with 90% sedimentation following centrifugation at 400,000 x g for 6 min.

When the AP180-clathrin baskets were mixed with hsp70 and ATP, almost no uncoating occurred (Fig. 4A, solid circles). However, as we previously observed with AP-2-clathrin baskets (16), addition of increasing amounts of 100 kDa cofactor caused a corresponding increase in the uncoating of AP180-clathrin baskets (Fig. 4A). Complete uncoating was restored at a molar ratio of cofactor to clathrin of 1 to 5 (Fig. 4B), slightly higher than the 1 to 10 ratio observed with AP-2 clathrin baskets (16).
These data suggest that the requirement of a 100-kDa cofactor for uncoating may be a general one, since the cofactor is already present in preparations of mixed AP-clathrin baskets and must be added to both AP₁₈₀ and AP-2-clathrin baskets.

Neither clathrin baskets at low pH nor artificially cross-linked clathrin baskets can be uncoated by hsp70, but in both cases the clathrin baskets markedly activate the hsp70 ATPase activity (31); this raises the possibility that AP₁₈₀-clathrin baskets, which cannot be uncoated in the absence of cofactor, might also activate the hsp70 ATPase activity. However, we found that, in the absence of cofactor, the AP₁₈₀-clathrin baskets alone only slightly activated the hsp70 ATPase activity, and there was no initial burst of ATPase activity (Fig. 5A, solid circles). Cofactor alone also only slightly activated the hsp70 ATPase activity (Fig. 5A, solid triangles). However, with both cofactor and baskets together there was a significant burst of ATPase activity which was correlated to the amount of cofactor present, as seen with the uncoating process. Furthermore, it was not sufficient to have just a mixture of cofactor, AP₁₈₀, and depolymerized clathrin. Fig. 5B demonstrates a large initial burst of ATPase activity with the polymerized baskets plus cofactor, indicated by the extrapolated dotted line to zero time, but only a slight burst of ATPase activity with depolymerized clathrin baskets under identical conditions. This slight burst was probably due to incomplete depolymerization of the baskets. Therefore, both 100-kDa cofactor and polymerized clathrin baskets appear to be required for the initial burst of ATPase activation which accompanies uncoating.

We next investigated the quantitative relationship between the initial burst of ATPase activity and the initial burst of uncoating at two different concentrations of hsp70. First, the correlation between uncoating and hsp70 concentration was examined quantitatively. When the hsp70 concentration was doubled the amount of uncoating also doubled (Fig. 6, solid symbols, dashed lines), which demonstrates that the initial burst of uncoating is not due to the dissociation of a relatively unstable fraction of AP₁₈₀-clathrin baskets, but is a specific reaction with hsp70. Furthermore, the baskets which were not uncoated at these sub-stoichiometric levels of hsp70 were completely uncoated at higher concentrations (data not shown). As we found with coated vesicles (15), the stoichiometry of uncoating of these baskets is 1 mol of clathrin triskelion per 3 mol of hsp70.

To determine the ATPase activity specifically associated with uncoating, the background ATPase activation caused by the baskets alone, the cofactor alone, and the ATPase activity of the hsp70 alone were subtracted from the total ATPase activity. At both concentrations of hsp70, 1 mol of ATP was hydrolyzed per mol of clathrin heavy chain released during the initial burst of uncoating, i.e. a stoichiometric initial burst of ATPase activity accompanies the initial burst of uncoating (Fig. 6, extrapolated dotted lines). However, this simple relationship between ATPase activity and uncoating does not appear to hold for the steady-state uncoating and steady-state ATPase activity. After the initial burst of uncoating was complete, the relatively low level of steady-state ATPase activity was still considerably faster than the very slow steady-state rate of uncoating.
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curs when hsp70 is unable to uncoat clathrin baskets in the absence of cofactor. Furthermore, preliminary experiments have shown that, even at pH 6 where no uncoating occurs, cofactor is required for clathrin baskets to activate the hsp70 ATPase activity (34). This provides further evidence that uncoating may involve a specific interaction between hsp70, cofactor, and clathrin baskets.

Although we observed a one to one relationship between the initial burst of ATPase activity and the initial burst of uncoating, there did not appear to be a simple relationship between steady-state uncoating and ATPase activity. We previously suggested that the low rate of steady-state uncoating was due to the slow rate of ADP and P, release from the hsp70-clathrin complex (15), in which case we would expect the steady-state ATPase rate to be as slow as the steady-state uncoating rate. However, even after correcting for nonspecific ATPase activity, we observed ATPase activity which was about twice the ATPase activity of hsp70 alone, much faster than the rate of steady-
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Our results also strongly suggest that this initial burst of uncoating requires the presence of a 100-kDa cofactor. We pre-
viously demonstrated this for AP-2-clathrin baskets (16), and
we have now shown it for AP,^-clathrin baskets. It also ap-
pears to occur with clathrin baskets prepared with the newly
discovered 20-kDa assembly protein (32). Therefore, all of our data are consistent with the view that the 100-kDa cofactor is
necessary for hsp70 to interact with clathrin baskets. Preliminary data obtained in collaboration with Dr. E. Ungewickell (Washington University, St. Louis) suggest that the 100-kDa cofactor is auxilin (33).

In studying the ATPase activity associated with the initial burst of uncoating, we found that clathrin baskets prepared
with mixed APs caused considerable activation of the hsp70 ATPase activity even in the absence of uncoating, and AP-2 caused an initial burst of hsp70 ATPase activity in the absence of clathrin. However, with AP,^-clathrin baskets we were able to demonstrate that, at two different hsp70 concentrations, uncoating was accompanied by a stoichiometric initial burst of ATP hydrolysis with 3 molecules of ATP hydrolyzed per mole-
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active form to which substrates bind, while the ATP form of hsp70 is inactive (22). This model proposed that ATP hydrolysis occurs before substrates bind to the ADP form, while our observation that an initial burst of ATP hydrolysis occurs after the hsp70-ATP complex binds to clathrin baskets strongly suggests that it is the ATP form of hsp70 which binds to the baskets and following this binding, the baskets activate the hydrolysis of ATP bound to hsp70.

Our observation that, with ATP at the active site, clathrin attaches to and detaches from hsp70 very rapidly whereas with ADP and P$_i$ at the active site, clathrin neither binds to nor dissociates from hsp70 (21), may explain how hydrolysis of ATP causes dissociation of clathrin from coated vesicles. It seems possible that it is not the hydrolysis of ATP per se which causes the dissociation of clathrin from baskets but rather the major change in conformation of hsp70 from the ATP form to the ADP form to which clathrin is locked and unable to dissociate. We have suggested that this same conformational change may be responsible for locking other substrates onto hsp70 so that they can be transported from one point in the cell to another (35). It is possible that clathrin may also be passed onto another protein in vivo, perhaps VCP (valosin-containing protein) (36), before coated pits are reformed; but much more work will be required to completely understand the cycling of clathrin between coated vesicles, the hsp70-clathrin complex, and newly formed coated pits.

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