Hsc70 Chaperones Clathrin and Primes It to Interact with Vesicle Membranes*

(Received for publication, October 19, 1999)

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When Hsc70 uncoats clathrin-coated vesicles in an auxilin- and ATP-dependent reaction, a single round of rapid uncoating occurs followed by very slow steady-state uncoating. We now show that this biphasic time course occurs because Hsc70 sequentially forms two types of complex with the dissociated clathrin triskelions. The first round of clathrin uncoating is driven by formation of a pre-steady-state assembly protein (AP)-clathrin-Hsc70-ADP complex. Then, following exchange of ADP with ATP, a steady-state AP-clathrin-Hsc70-ATP complex forms that ties up Hsc70, preventing further uncoating. This steady-state complex forms only during uncoating in the presence of APs; in the absence of APs, Hsc70 rapidly dissociates from the uncoated clathrin and continues to carry out uncoating. Whether it is complexed with ATP or ADP, the steady-state complex has very different properties from the pre-steady-state complex in that it cannot be immunoprecipitated by anti-clathrin antibodies and is readily dissociated by fast protein liquid chromatography. Remarkably, when the steady-state complex is incubated with uncoated vesicle membranes in ATP, the pre-steady-state complex reforms, suggesting that the clathrin triskelions in the steady-state complex rebind to the membranes and are again uncoated by Hsc70. We propose that Hsc70 not only uncoats clathrin but also chaperones it to prevent it from inappropriately polymerizing in the cell cytosol and primes it to reform clathrin-coated pits.

Receptor-mediated endocytosis is an essential cellular process required for the rapid import of membrane-bound receptors into cells (1–3). During receptor-mediated endocytosis, clathrin triskelions polymerize and form clathrin-coated pits on the plasma membrane; similar clathrin-coated pits form on the trans-Golgi membrane (3–5). In addition to clathrin and receptors, these coated pits contain assembly proteins that both catalyze the polymerization of clathrin triskelions and bind the receptors that are localized in the coated pit. Several clathrin assembly proteins (APs) have been described (6–10) including AP1, AP2, AP3, and AP4, which are multimeric subunit complexes, and AP180 and auxilin, which are single subunit, neuronal specific assembly proteins (11, 12). In addition to assembly proteins, a number of other proteins, including synaptojanin, amphyphinis, epsin, Eps15, and the small GTPase protein, Rab5-GDI, are involved in the formation and invagination of clathrin-coated pits (13–19). Phospholipids also play an important role in clathrin coat assembly and receptor recruitment (20, 21) as does dephosphorylation of the various proteins involved in the formation of clathrin-coated pits (22, 23).

Following invagination of the clathrin-coated pits, the GTP-binding protein dynamin plays a key role in the pinching off of the clathrin-coated pits to form clathrin-coated vesicles (4, 5). Then, once in the cytosol, the vesicles are uncoated in an ATP-dependent process by Hsc70 and its partner protein, auxilin, which is both a clathrin assembly protein and a DnaJ homolog (24, 25). It is not yet clear what triggers clathrin uncoating after the clathrin-coated vesicles pinch off. There is evidence that Hsc70 only uncoats clathrin-coated vesicles and not clathrin-coated pits (26), but the mechanism of this selectivity remains a mystery. In addition to carrying out uncoating, it is possible that Hsc70 plays other roles in endocytosis, analogous to the role of Hsc70 in facilitating protein folding and disaggregation, such as keeping clathrin from inappropriately polymerizing in the cytosol and returning clathrin to the membrane to form new clathrin-coated pits. The involvement of Hsc70 in the latter process could explain why ATP appears to be required for formation of clathrin-coated pits and also why free clathrin triskelions added to the cytosol of permeabilized cells do not form normal clathrin-coated pits that can pinch off (27).

If the only role of Hsc70 in endocytosis were to carry out uncoating, it would be expected to act catalytically to remove clathrin from clathrin-coated vesicles. However, there is strong evidence that Hsc70 acts stoichiometrically rather than catalytically. Detailed studies showed that the time course of uncoating of clathrin-coated vesicles in vitro is strikingly nonlinear, consisting of a rapid initial burst of uncoating followed by very slow steady-state uncoating (28). Furthermore, at pH 7, the initial burst of uncoating is nearly stoichiometric with three molecules of Hsc70 uncoating one clathrin triskelion (28). Later studies showed that the same time course and stoichiometry of uncoating occurs with clathrin baskets prepared with mixed assembly proteins, purified AP2, AP180, and even with myelin basic protein, which acts as an assembly protein (29).
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Therefore, the biphasic time course of uncoating is a general feature of the uncoating reaction (28-31). The observation that uncoating occurs stoichiometrically rather than catalytically not only raises questions about the role of Hsc70 in endocytosis but also raises an important question about the general mechanism of action of Hsc70, since it is still unclear whether Hsc70 works stoichiometrically or catalytically in carrying out other functions in the cell.

In the present study, we investigated the biphasic time course of uncoating by Hsc70. We found that during the first round of clathrin-coated vesicle uncoating, an AP-clathrin–Hsc70–ADP complex forms. Surprisingly, however, this pre-steady-state complex does not dissociate when ATP exchanges with ADP but rather is transformed into a steady-state AP-clathrin–Hsc70–ATP complex that prevents further uncoating by Hsc70. This steady-state complex is formed only by uncoating in the presence of APs. In the absence of APs, Hsc70 rapidly dissociates from the uncoated clathrin and carries out further uncoating. In contrast to the pre-steady-state AP-clathrin–Hsc70–ADP complex, whether the steady-state AP-clathrin–Hsc70 complex contains bound ATP or ADP, it cannot be immunoprecipitated and readily dissociates during FPLC, suggesting that it has a unique structure. Strikingly, upon incubation with stripped vesicle membranes in the presence of ATP, the steady-state complex is transformed back into the pre-steady-state complex, suggesting that clathrin from the steady-state complex can rebind to the membranes and be uncoated again. We propose that Hsc70 plays several roles in endocytosis: uncoating clathrin-coated vesicles, keeping clathrin from inappropriately polymerizing in the cytosol, and priming the dissociated clathrin to rebind to membranes to form new clathrin-coated pits.

**EXPERIMENTAL PROCEDURES**

**Materials**—ATP, creatine kinase, creatine phosphate, hexokinase, glucose, imidazole, and ATP agarose were from Sigma. ADP was from Fluka. [14C]formaldehyde and [32P]ATP were from NEN Life Science Products. Nickel-agarose resin was from Invitrogen. The Superose 6 preparative gel and Superose 6 quantitative FPLC column were from Amersham Pharmacia Biotech. The pET15b vector and the BL21(DE3) cells were from Novagen. The SDS gels (4–20%) were from Owl Separation Products. Nickel-agarose resin was from Invitrogen. The Superose 6 column and Superose 6 quantitative FPLC column were from Amersham Pharmacia Biotech. The monoclonal antibodies used in this study were AP180-1 form Sigma, X22 from Affinity Bioreagents, TD.1 and CON.1 from鼠抗, and an anti-auxilin antibody (monoclonal antibody 100/4) from Ernst Ungewickell.

**Isolation of Clathrin-coated Vesicles and Purification of Hsc70**—Clathrin-coated vesicles were prepared from fresh calf brains according to the procedure of P. Nandi et al. (32). Isolated clathrin-coated vesicles were stored at 4 °C for a clathrin concentration of 15 μM. Bovine brain clathrin was prepared as described previously (28). Purified Hsc70 was stored as pellets in 70% ammonium sulfate solution at 4 °C and diazotized in buffer A (20 mM imidazole, 25 mM KCl, 10 mM ammonium sulfate, 2 mM magnesium acetate, and 1 mM DTT, pH 7.0) overnight at 4 °C before use. Protein concentrations of clathrin (33) and Hsc70 (28) were determined using extinction coefficients (ε280 = 12 for clathrin and 6.2 for Hsc70).

**Purification of Clathrin and Assembly Proteins and Preparation of Clathrin Baskets**—Clathrin was purified from bovine brain clathrin-coated vesicles according to the procedure described previously (34). Briefly, clathrin was extracted from clathrin-coated vesicles using 0.5 M Tris, pH 7.0, and the high speed supernatant containing clathrin and assembly proteins was concentrated and loaded onto a preparative Superose 6 filtration column (2.5 x 90 cm) equilibrated in 0.5 M Tris, pH 7.0, at 25 °C. Fractions of clathrin peak and assembly protein peak were pooled and stored in 0.5 M Tris at 4 °C.

To prepare mixed-AP-clathrin baskets, isolated assembly protein was mixed with purified clathrin at a 1:3 ratio based on absorbance at A280 and dialyzed in 0.1 M Mes, pH 6.5, overnight. Baskets were collected by centrifugation at 150,000 x g for 1 h. The pelletted clathrin baskets were resuspended in buffer A and clarified with a 5-min spin in a microcentrifuge. The concentration of clathrin in prepared baskets was determined after solubilizing the baskets with 0.5 M Tris, pH 8.0.

In order to make the pure clathrin baskets, clathrin was further purified by repeating the Superose 6 column chromatography step that was used in the original purification procedure. The pure clathrin baskets were polymerized by overnight dialysis of double purified clathrin against buffer B (10 mM Mes, 2 mM calcium chloride, 1 mM DTT, pH 6.5). Polymerized clathrin baskets were sedimented by centrifugation for 1 h at 150,000 x g at 4 °C and suspended in buffer A. The suspension was centrifuged again for 1 h at 150,000 x g at 4 °C. The baskets were resuspended in buffer A and clarified with a 2-min spin in a microcentrifuge. Both the mixed-AP-clathrin baskets and the pure clathrin baskets were stable for at least 2 days.

**Labeling Clathrin with [14C]Formaldehyde—**Purified clathrin was trace-labeled according to the procedure of Jentoft and Dearborn (35). Generally, 10 ml of clathrin (~2 μM) in 50 mM HEPES, pH 7.0, was incubated with 5 mM sodium cyanoborohydride and 10 μCi of [14C]formaldehyde for 2 h at 25 °C before 5 mM DTT was added, and the mixture was dialyzed in 10 mM Tris, pH 8.0, containing 1 mM DTT, and subsequently 0.5 M Tris, pH 7.0, containing 1 mM DTT to remove free label. Purification of Recombinant His8-Hsp70—Recombinant His8-Hsp70 was overproduced in E. coli BL21(DE3) cells, grown at 25 °C, containing a pET15b vector with the human Hsp70 gene. The His8-Hsp70 fusion protein was purified using an ATP-agarose column. The human Hsp70 was subcloned from pET11 into the pET15 vector.

**Uncoating Activity Assay—**The uncoating reaction typically was carried out using Hsc70 and clathrin baskets in the presence of ATP in buffer A at 25 °C. The concentrations of Hsc70, clathrin baskets, and ATP are specified for each experiment in the figure legends.14C radioactively labeled clathrin baskets were routinely used in the experiments testing the activity of the Hsc70 in the uncoating supernatant. The clathrin released by Hsc70 from the clathrin baskets was determined by quantification of the clathrin in the supernatant after centrifugation either by densitometric scanning of SDS gels or by scintillation counting when 14C-labeled clathrin was used.

**Measurement of Clathrin-Hsc70–ADP Complex by FPLC Chromatography—**Clathrin–Hsc70–ADP complexes were measured as described previously (34, 36). Briefly, following centrifugation of the uncoating mixture, the free Hsc70 was separated from Hsc70 bound to clathrin by gel filtration FPLC on a Superose 6 column equilibrated in buffer A containing 1 mM ATP and 1 mM Pi. Fractions containing clathrin and clathrin–Hsc70 complexes were analyzed on SDS gels. The amount of Hsc70 associated with clathrin was quantified by densitometric scanning.

**ATPase Assay—**The Hsc70 was incubated for 8 min with [γ-32P]ATP to replace all of the bound nucleotide on the Hsc70 with [γ-32P]ATP (37) before the addition to the final reaction mixture. ATPase activity was determined from the amount of Pi released from [γ-32P]ATP (38). Radioactivity was measured by liquid scintillation count using a Beckman LS 3801 counter.

**His8-Hsp70 Exchange Assay—**Standard His8-Hsp70 exchange assays consisted of the supernatant of the uncoating reaction that was carried out by His8-Hsp70. The solution was incubated for 30 min at 25 °C with or without Hsc70. The nickel-agarose resin was then added to the solution. After incubating for 10 min on a rotator, the resin was spun down in a microcentrifuge, and the uncoating activity of the Hsc70 remaining in the supernatant was tested by adding baskets assembled with [14C]clathrin.

**Hexokinase/Glucose Treatment—**The initial uncoating reaction was carried out in the presence of 0.1 mM ATP, Hsc70, and mixed AP-clathrin baskets at the concentrations specified for each experiment in the figure legend. When the uncoating reached steady state, 50 unit/ml hexokinase and 3 mM glucose were added to the reaction solution at 25 °C and incubated for 1 h. This was followed by the addition of 60 units of glucose-6-phosphate dehydrogenase and 15 mM phosphocreatine, and, after incubating for 10 min at 25 °C, the uncoating activity of Hsc70 was tested. The conversion of nucleotide from ADP to ATP was confirmed by high pressure liquid chromatography analysis.

**Immunoprecipitation—**A mixture of clathrin antibodies consisting of X22, CON.1, and TD.1 was added to the supernatant and then rotated for 2 h at room temperature. Protein A-Sepharose CL-4B beads (Amersham Pharmacia Biotech) were then added, and the mixture was incubated for another 2 h on a rotator. The beads were washed twice prior to boiling in SDS gel loading buffer. Samples were analyzed for clathrin and Hsc70 by SDS gel electrophoresis.
Fig. 1. Properties of the uncoating reaction. A, the uncoating of 0.6 μM mixed AP-clathrin baskets by 0.3 μM Hsc70 was carried out in buffer A with an ATP-regenerating system containing 30 units/ml creatine kinase and 15 mM phosphocreatine (filled circles). As the uncoating reaction reached steady-state (arrow), 0.3 μM additional Hsc70 (open triangles) or 0.6 μM additional mixed AP clathrin baskets (open circles) was added to the reaction solution. The amount of clathrin released in the uncoating supernatant was determined by densitometric scanning of SDS gels. B, 0.6 μM mixed AP-clathrin baskets were uncoated by 0.6 μM Hsc70 for 10 min, followed by centrifugation (filled triangles). The resulting supernatant was tested for uncoating activity by adding fresh mixed-AP-[14C]clathrin baskets to it after the supernatant was treated as follows: incubation at 25 °C for 0 min (open circles), incubation at 25 °C for 60 min (open triangles), and incubation at 25 °C with hexokinase/glucose for 20 min followed by excess creatine kinase/creatine phosphate for 15 min (open squares). In these experiments, only the [14C]clathrin released in the second round of uncoating is shown. The effect of free clathrin and APs on the first round of uncoating was determined by uncoating 0.6 μM clathrin and 0.2 mM APs on the first round of uncoating (filled squares).

**RESULTS**

Following the Initial Burst of Uncoating, Hsc70 Cannot Carry Out Further Uncoating—We showed previously that, during the initial burst of uncoating of clathrin-coated vesicles by Hsc70, a clathrin-Hsc70-ATP complex forms with three molecules of Hsc70 bound per clathrin triskelion (28); the complex can then be isolated by FPLC chromatography on Superose 6 (39). On this basis, we explained the slow steady-state uncoating by proposing that very slow release of ADP from the clathrin-Hsc70-ATP complex limits the rate at which the complex dissociates in ATP and thus limits the rate of further uncoating by Hsc70 (28). However, we later observed that ADP dissociates quite rapidly from the clathrin-Hsc70-ATP complex in ATP with a half-life of less than 5 min at 25 °C. In these experiments, the rate of ADP dissociation was measured by making use of the fact that Hsc70 only dissociates from the clathrin-Hsc70 complex during Superose 6 chromatography if ADP bound to the complex has exchanged with ATP (39). Thus, slow steady-state uncoating cannot be explained by the slow release of ADP from the clathrin-Hsc70-ATP complex.

Another possible explanation for the slow steady-state uncoating is that partially uncoated clathrin baskets become resistant to further uncoating by Hsc70, but we ruled out this possibility by showing that adding fresh Hsc70 during steady-state uncoating caused a second round of uncoating of the residual baskets (Fig. 1A, open triangles), while adding fresh clathrin baskets during steady-state uncoating did not cause a second round of uncoating (Fig. 1A, open circles). Furthermore, when we isolated the supernatant of the uncoating reaction, we found that, even after it was incubated with ATP for 1 h at 25 °C, it still could not carry out a second round of uncoating when mixed with fresh mixed AP-clathrin baskets (Fig. 1B, open triangles). It is therefore clear that, after carrying out the initial burst of uncoating, Hsc70 is unable to carry out further uncoating.

The simplest explanation for this phenomenon is product inhibition; i.e. clathrin released during the initial burst of uncoating rebinds to Hsc70 and prevents further uncoating. However, we found that neither free clathrin nor a mixture of free clathrin and APs had any effect on the uncoating activity of Hsc70 (Fig. 1B, filled squares). Therefore, only newly uncoated clathrin and APs are able to inhibit the uncoating activity of the Hsc70. Furthermore, this effect is stoichiometric with each clathrin triskelion inhibiting the uncoating of the three molecules of Hsc70 involved in its uncoating but, as we noted above, not the uncoating activity of freshly added Hsc70. It therefore seemed possible that when ATP exchanges with ADP on the pre-steady-state clathrin-Hsc70-ATP complex formed during the initial burst of uncoating, the clathrin is transformed into a steady-state clathrin-Hsc70-ATP complex that is maintained over many cycles of ATP hydrolysis and thus prevents further uncoating by Hsc70.

**Isolation of a Steady-state Complex That Prevents Uncoating by Hsc70**—Clearly, such a steady-state clathrin-Hsc70-ATP...
complex cannot be isolated by FPLC chromatography on Superose 6, since we (39) have already shown that, following exchange of ATP with ADP, the clathrin and Hsc70 dissociate on Superose 6. Therefore, to minimize dissociation of the possible steady-state complex, we attempted to isolate it using FPLC on Superose 12 in ADP and also carried out the uncoating reaction at higher concentrations of Hsc70 and clathrin-coated vesicles. Fig. 2A shows the chromatography profile from the Superose 12 column of clathrin and Hsc70 from the supernatant of the uncoating reaction. The clathrin (circles) eluted in a single peak in the void volume of the column, while the Hsc70 (triangles) eluted over three regions, one clearly associated with the clathrin, a second trailing the clathrin peak, and a third consisting of free Hsc70. The ratio of Hsc70 to clathrin in the clathrin peak, obtained from measuring the intensity of Hsc70 and clathrin on SDS gels, showed that the clathrin fractions were about 60% saturated with Hsc70. Furthermore, since a considerable fraction of the Hsc70 trailed behind the clathrin peak in the elution pattern, even more Hsc70 was complexed with the clathrin when the supernatant was initially applied to the column. Therefore, following exchange of ATP with ADP, the pre-steady-state complex released during the initial burst of uncoating appears to be transformed to a steady-state complex that can be isolated from the supernatant of the uncoating reaction.

Evidence that this steady-state complex prevents uncoating by tying up the Hsc70 comes from our observation that, compared with the control (Fig. 2C, bar 1), the uncoating activity of the complex was still inhibited after ATP was restored to the complex eluted in ADP from the Superose 12 column (bar 4). The eluted complex did have a somewhat higher uncoating activity than the supernatant obtained from the uncoating reaction at an Hsc70 concentration comparable with that in the void volume (bar 2), but this is probably due to the 10-fold dilution that occurred during the column chromatography, since the same small increase in activity occurred when the complex that was applied to the Superose column was diluted 10-fold (bar 3).

The Steady-state Complex Remains Intact after ATP Is Hydrolyzed to ADP—Since the complex isolated from the Superose 12 column had bound ADP, our data suggest that, even after the ATP on the steady-state complex is hydrolyzed to ADP, the complex remains intact and is still unable to carry out uncoating when ATP is restored. To directly test whether the steady-state complex remains intact after ATP is hydrolyzed to ADP, we treated the supernatant of the uncoating reaction with hexokinase/glucose and then restored ATP using excess creatine kinase/creatine phosphate. We confirmed that the Hsc70 in the supernatant was still unable to carry out uncoating after this treatment (Fig. 1B, open squares). The fact that the steady-state complex remained intact after ATP was hydrolyzed to ADP is consistent with our observation that the Hsc70 in the supernatant of the uncoating reaction was unable to carry out uncoating for more than 1 h after the supernatant was isolated,
although many cycles of ATP hydrolysis occurred during this period of time. Therefore, our data suggest that very slow steady-state uncoating occurs because the pre-steady-state clathrin-Hsc70-ADP complex formed during the initial burst of uncoating is transformed to a steady-state clathrin-Hsc70-ATP complex that remains intact through many cycles of ATP hydrolysis and therefore ties up the Hsc70, preventing further uncoating.

**Formation of the Steady-state Complex Requires APs**—The fractions eluted from the Superose 12 column that contained the steady-state complex contained not only clathrin and Hsc70 but also APs. Both of the neuronal specific APs, AP180 and auxilin, completely dissociated along with clathrin during the uncoating reaction unlike AP2, which, in agreement with a recent report (40), showed only limited dissociation (data not shown). Fig. 2B shows the elution profile from Superose 12 of auxilin (circles) and AP180 (triangles) compared with that of clathrin (dashed line). Most of the auxilin coeluted with the clathrin, and while the AP180 showed more dissociation from the clathrin during the column chromatography, there was still significant overlap with the clathrin peak. Since mixed AP-clathrin baskets contained about one auxilin and one AP180 per clathrin triskelion, at least one AP per clathrin was present in the steady-state complex eluted from the Superose 12 column.

Since these data suggest that APs may be bound to the steady-state complex, we directly investigated the role of APs in preventing Hsc70 from carrying out multiple rounds of uncoating following the initial burst of uncoating. In these experiments, we used pure clathrin baskets prepared with clathrin that had been twice purified by Superose 6 chromatography to ensure that it was AP-free. Strikingly, the time course of uncoating of these pure clathrin baskets was not biphasic. Rather, a linear time course occurred until the clathrin baskets were completely uncoated (Fig. 3A). Similar to the clathrin baskets prepared with APs (31), the uncoating of the pure clathrin baskets required auxilin, although less than when other APs were also present. The rate of uncoating of pure clathrin baskets was equal to or slower than the rate of the initial burst of uncoating of clathrin baskets prepared with APs (Fig. 1), suggesting that the multiple rounds of uncoating were not caused by disintegration of the pure clathrin baskets. This was confirmed by showing that, as with the initial burst of uncoating (30), the uncoating of the pure clathrin baskets was accompanied by an increase in the Hsc70 ATPase activity, with nearly three ATPs hydrolyzed per clathrin triskelion uncoated (Fig. 3B). Therefore, uncoating of the pure clathrin baskets is due to a direct effect of Hsc70 and not to nonspecific disintegration of the baskets.

If APs are indeed involved in inhibition of the steady-state uncoating, it might be expected that the addition of APs to the pure clathrin baskets would change the time course of uncoating from linear to biphasic. Fig. 3C shows that this is indeed the case. The addition of APs to the pure clathrin baskets had little effect on the first round of uncoating but caused a marked decrease in the rate of subsequent rounds of uncoating. Furthermore, this effect was dependent on the AP concentration; as the concentration of APs was increased, the rate of the steady-state uncoating decreased, suggesting that about a 1:1 ratio of APs to clathrin is necessary for formation of the steady-state complex that prevents Hsc70 from carrying out uncoating.

This view was confirmed by adding recombinant auxilin, which functions not only as a cofactor for uncoating but also as an assembly protein, to the pure clathrin baskets. As shown in Fig. 3D, while very low auxilin concentrations were sufficient to support linear steady-state uncoating, only at a ratio of one auxilin to five clathrin triskelions did the uncoating activity show a loss of linearity, similar to that observed when low concentrations of APs were added.

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concentrations of mixed APs were added. Furthermore, when three auxilins per clathrin triskelion were added, the baskets showed an initial burst of uncoating with a negligible steady-state rate (Fig. 3D, triangles) as also occurred when one auxilin was added per clathrin triskelion (data not shown). These data, in combination with our observation that auxilin and AP-180 eluted from the Superose 12 column in the steady-state complex at an approximately 1:1 molar ratio to clathrin, strongly suggest that APs as well as clathrin and Hsc70 are key elements of the steady-state complex that prevents uncoating by Hsc70.

The Steady-state Complex Irreversibly Dissociates When Hsc70 Is Removed—A mixture of clathrin and APs does not inhibit the uncoating activity of Hsc70. However, given the key role that APs play in the formation of the steady-state complex it seemed possible that, if we could isolate the AP-clathrin portion of the steady-state complex, it would inhibit the uncoating activity of fresh Hsc70-ATP. To isolate the AP-clathrin portion of the steady-state complex, we carried out uncoating with recombinant His6-Hsp70, anticipating that it could be easily removed from the supernatant of the uncoating reaction. Control experiments showed that His6-Hsp70 carries out normal uncoating; there was an initial burst of uncoating followed by slow steady-state uncoating with inhibition of the His6-Hsp70 uncoating activity occurring in the supernatant of the uncoating reaction (Fig. 4, bars 1 and 2). We could therefore test whether the His6-Hsp70 in the steady-state complex is removed by passing the supernatant through nickel-agarose resin. We found that although only His6-Hsp70 was removed by the nickel-agarose column and almost all of the clathrin and APs in the supernatant of the uncoating reaction were still present in the resin eluant (data not shown), the eluted clathrin and APs no longer inhibited uncoating when they were mixed with fresh Hsc70 (Fig. 4, bar 3). On the other hand, when the same experiment was carried out using Hsc70 instead of His6-Hsp70, the Hsc70 present in the resin eluant was still inhibited; it could not carry out an initial burst of uncoating (Fig. 4, bar 4), confirming that, other than removing His6-Hsp70, the nickel-agarose did not affect the steady-state complex. Therefore, the AP-clathrin portion of the steady-state complex is only stable while it is actually bound to Hsp70 and breaks down almost immediately after the Hsp70 is removed.

On the other hand, free Hsc70-ATP can exchange with the His6-Hsp70-ATP in the steady-state complex. When Hsc70-ATP was added to the steady-state complex before the complex was passed through the nickel-agarose column, this added Hsc70-ATP partially bound to the AP-clathrin portion of the complex and was thus prevented from carrying out uncoating (Fig. 4, bar 5). Therefore, even Hsc70 that has not carried out an initial burst of uncoating can become part of the steady-state complex that prevents uncoating. However, because the AP-clathrin portion of the steady-state complex breaks down so rapidly, the fresh Hsc70 can only do so by exchanging with the His6-Hsp70 initially present in the steady-state complex.

The Steady-state Complex Has a Unique Structure—The fact that a mixture of clathrin and APs does not inhibit the uncoating activity of Hsc70 and that even the AP-clathrin portion of the steady-state complex breaks down rapidly when Hsc70 is removed strongly suggests that the steady-state AP-clathrin-Hsc70 complex has a unique structure. This was confirmed when we investigated the properties of the steady-state complex with ADP rather than ATP bound to the Hsc70. We expected that the steady-state complex treated with hexokinase/glucose would be converted back into the pre-steady-state AP-clathrin-Hsc70-ADP complex that formed during the initial burst of uncoating when ATP was first hydrolyzed to ADP. Surprisingly, however, we found that this was not the case. Table I compares the properties of the pre-steady-state AP-clathrin-Hsc70-ADP complex formed during the initial burst of uncoating with the steady-state AP-clathrin-Hsc70-ADP complex formed by treating the steady-state AP-clathrin-Hsc70-ATP complex with hexokinase/glucose. We found that the pre-steady-state complex can be immunoprecipitated using a mixture of monoclonal antibodies against clathrin and remains essentially intact following Superose 6 chromatography. In contrast, neither the steady-state ATP nor steady-state ADP
complexes could be immunoprecipitated, and both complexes almost completely dissociated when chromatographed on Superose 6. Similarly, when the pre-steady-state complex was passed through a nickel-agarose column, the whole complex bound to the column, while, when the steady-state ADP complex was similarly treated, only the His6-Hsp70-ADP bound to the column, and the clathrin was eluted (data not shown), just as we observed previously with the steady-state ATP complex. Therefore, when ATP exchanges with ADP, the pre-steady-state complex is permanently transformed into a steady-state complex with quite different properties and presumably a quite different structure from the pre-steady-state complex, and this structure is not altered when bound ATP is replaced by ADP.

The Steady-state Complex Interacts with Vesicle Membranes—When the supernatant of the uncoating reaction is separated from the uncoated vesicles by centrifugation, the pre-steady-state complex is rapidly transformed into the steady-state complex as shown by the fact that it dissociated during Superose 6 chromatography. However, we noticed that this transformation occurred only if the uncoated vesicle membranes were removed by centrifugation. Surprisingly, during steady-state ATP hydrolysis in the presence of uncoated vesicle membranes, the dominant complex present in the supernatant of the uncoating reaction was the pre-steady-state complex, which does not dissociate during Superose 6 chromatography, rather than the steady-state complex, which does. This is just the opposite of what occurs in the absence of membranes (Fig. 5).

This result raised the possibility that if uncoated vesicle membranes were added to the supernatant of the uncoating reaction they would transform the steady-state complex back into the pre-steady-state complex. Fig. 6 (bar 3) shows that this is indeed the case as shown by the observation that, like the pre-steady-state complex (bar 1) but in contrast to the steady-state complex (bar 2) the transformed complex no longer dissociated during Superose 6 chromatography. Furthermore, reformation of the pre-steady-state complex did not occur in ADP (bar 4); nor did it occur when the vesicle membranes were incubated with a mixture of free clathrin, APs, and Hsc70 (bar 5). Both ATP and the supernatant of the uncoating reaction containing the steady-state complex must be present. The simplest explanation for these observations is that the steady-state complex present in the supernatant of the uncoating reaction reacts with the vesicle membranes in such a way that clathrin rebinds to the membranes, and then, when this clathrin is again uncoated by Hsc70, the pre-steady-state complex reforms.

### Discussion

**Inhibition of Uncoating by a Unique Steady-state Complex**—Our results show that the slow steady-state uncoating of clathrin baskets and coated vesicles by Hsc70 occurs because the Hsc70 is prevented from carrying out further uncoating by the transformation of the pre-steady-state AP-clathrin-Hsc70-ADP complex formed during the initial burst of uncoating into a steady-state AP-clathrin-Hsc70-ATP complex that ties up the Hsc70. On the other hand, in the absence of APs, the pre-steady-state complex rapidly dissociates when ATP exchanges with ADP, allowing further uncoating to take place.

The major APs present in the steady-state complex were auxilin and AP180. Hannan et al. (40) recently found that AP2 was not released from clathrin-coated vesicles during uncoating by Hsc70, but they did not determine whether auxilin or AP180 were released. Although we observed only partial release of AP2 during the initial burst of uncoating, we found that both auxilin and AP180 were almost completely released, and it was these APs, particularly auxilin, that were present in the steady-state complex eluted from the Superose 12 column. Moreover, our data clearly show that APs must be present in the steady-state complex at concentrations close to stoichiometric to clathrin for slow steady-state uncoating to occur.

The steady-state AP-clathrin-Hsc70-ATP complex has several unique properties, suggesting that it has a highly specialized structure. First, it cannot be formed by mixing Hsc70-ATP with clathrin and APs; it can only be formed by uncoating. Second, Hsc70 is required to keep it intact. Third, while the pre-steady-state AP-clathrin-Hsc70-ADP complex remains intact when subjected to immunoprecipitation, FPLC on Superose 6, or chromatography on nickel-agarose, the steady-state complex, whether it has bound ATP or bound ADP, dissociates when subjected to these treatments. Therefore, once ATP exchanges with ADP on the pre-steady-state complex, the structure of the complex is permanently altered. Even hydrolysis of ATP to ADP does not change the properties of the steady-state complex, confirming that it has a unique structure quite distinct from the structure of the pre-steady-state complex.

### Table I

| Type of Complex | IP (bar 3) | FPLC (bar 4) |
|----------------|-----------|--------------|
| Pre-steady state | 70 ± 10 | 60 ± 5 |
| Steady-state ATP | 15 ± 10 | 25 ± 5 |
| Steady-state ADP | 25 ± 10 | 35 ± 5 |

**Fig. 5.** The pre-steady-state complex is maintained in the presence of clathrin-coated vesicles. 1 μM clathrin-coated vesicles were uncoated by 3 μM Hsc70 in the presence of 5 mM MgATP and an ATP-regenerating system for 15 min at 25°C. The reaction solution was then either immediately centrifuged and the resulting supernatant incubated for the indicated time periods before it was loaded onto the FPLC Superose 6 column (filled circles), or the reaction solution was incubated for the indicated times and only then centrifuged, after which the resulting supernatant was loaded onto the FPLC (open circles). The clathrin-Hsc70-ADP complexes in the supernatant were quantified by densitometric scanning of the clathrin and Hsc70 in the fractions eluted from the Superose 6 column. 100% complex indicates one Hsc70 bound per clathrin heavy chain in the reaction mixture.
Possible Functions of the Steady-state Complex in Vivo—Why does Hsc70 form two types of complex with clathrin and APs? A possible answer is presented in the model shown in Fig. 7. We have suggested that formation of the pre-steady-state AP-clathrin-Hsc70-ADP complex is the driving force for uncoating. We previously showed that, at pH 6, auxilin induces Hsc70-ADP to form a metastable complex with clathrin baskets following ATP hydrolysis (41). On this basis, we suggested that it is the transition from the metastable clathrin basket-Hsc70-ADP complex to the energetically stable pre-steady-state AP-clathrin-Hsc70-ADP complex that drives the uncoating process (42) as illustrated in the model shown in Fig. 7. However, if the pre-steady-state AP-clathrin-Hsc70-ADP complex simply dissociated when ATP exchanged with ADP, the released clathrin triskelions might inappropriately bind to both the membrane-bound and cytosolic proteins involved in endocytosis, in particular various APs.

That such an effect can occur in vivo is shown by our observation that overexpression of auxilin or AP180 in various cells leads to inappropriate sequestration of clathrin away from the normal clathrin-coated pits that form on the plasma membrane and trans-Golgi network (43). Therefore, formation of a steady-state complex dissociates very slowly, thus explaining why Hsc70 only carries out one round of uncoating. However, if stripped vesicles are added to this steady-state complex, the clathrin rebinds to the stripped vesicles (arrow with dotted line) and is again uncoated by Hsc70 to reform the pre-steady-state clathrin-Hsc70-ADP complex.
steady-state complex to rebind to the membrane to form new clathrin-coated pits. Surprisingly, we found that, in the presence of stripped vesicle membrane, the dominant complex present during steady-state uncoating is the pre-steady-state complex that forms during the initial burst of uncoating. In agreement with this observation, we found that when stripped vesicle membranes were added to the steady-state complex they converted this complex back to the pre-steady-state complex. As shown in the model in Fig. 7, one way this could occur is if the steady-state complex returned clathrin to the membrane, where it could then be uncoated again, causing reformation of the pre-steady-state complex. Thus, these data raise the possibility that the steady-state complex may not only function to keep clathrin and APs depolymerized in the cytosol but may also be primed to interact with the plasma membrane to rapidly return clathrin and possibly APs as well to the membrane to form new clathrin-coated pits. Such a rapid return of clathrin to the membrane could be particularly crucial in nerve cells, where endocytosis must be very rapid to ensure rapid reformation of synaptic vesicles.

Our observation that the steady-state complex easily dissociates during chromatography or immunoprecipitation may be related to its involvement in returning clathrin to the plasma membrane. In the cell, the return of clathrin and APs to the membrane must be highly regulated, since, for reasons that are not yet clear, clathrin-coated pits cannot be uncoupled by Hsc70 (26), in contrast to our in vitro system, where the clathrin returned to the membrane appears to be re-uncoupled almost immediately. A number of proteins have already been described that play a key role in endocytosis in nerve cells, and dephosphorylation of these proteins apparently favors formation of clathrin-coated pits (22, 23). Future work will be required to determine the role of auxilin in the formation and maintenance of the AP-clathrin-Hsc70-ATP complex present during steady-state uncoating and to understand how this complex interacts with the numerous other proteins and regulatory factors that are involved in the return of clathrin and APs to the membrane to form clathrin-coated pits.

Acknowledgments—We thank Drs. Brian Freeman and Richard Morimoto for the pET11-Hsc70 vector, Dr. Ernst Ungewickell for the monoclonal 100/4 anti-auxilin antibody, and Dr. Rajapandi Thavamani for helpful discussion of this research.

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J. Biol. Chem. 2000, 275:8439-8447.
doi: 10.1074/jbc.275.12.8439

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