Trimeric Binding of the 70-kD Uncoating ATPase to the Vertices of Clathrin Triskelia: A Candidate Intermediate in the Vesicle Uncoating Reaction

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Abstract. Clathrin-coated vesicles were uncoated with the 70-kD "uncoating ATPase" from bovine brain, and the molecular products were visualized by freeze-etch electron microscopy. This yielded images of released clathrin triskelia with up to three 70-kD uncoating ATPase molecules bound to their vertices. Likewise, incubation of soluble clathrin triskelia with purified uncoating ATPase also led to trimeric binding of the ATPase to the vertices of clathrin triskelia. However, this occurred only when either EDTA or nonhydrolyzable analogues of ATP were present, in which case the ATPase also appeared to self-associate. When ATP was present instead, no 70-kD ATPases could be found on clathrin triskelia and all ATPases remained monomeric. These observations support the notion that ATP controls an allosteric conversion of the 70-kD uncoating ATPase between two different molecular conformations, an ATP-charged state in which the molecule has relatively low affinity for itself as well as low affinity for clathrin, and an ATP-discharged state in which both of these affinities are high. We presume that in vivo, the latter condition is brought about by ATP hydrolysis and product release, at which point the ATPase will bind tightly to clathrin and/or self-associate. We further propose that these reactions, when occurring in concert within a clathrin lattice, will tend to destabilize it by a mechanism we call "protein polymer competition". We stress the analogies between such a mechanism of uncoating and the ATP-driven events in muscle contraction. Finally, we show that under experimental conditions in which the uncoating ATPase fully removes the coats from brain coated vesicles, identical aliquots of the enzyme do not affect plasmalemmal coated pits in situ. This remarkable selectivity, the mechanism of which remains a complete mystery, is at least consistent with the idea that the 70-kD ATPase indeed plays a role in uncoating coated vesicles after they have formed in vivo.

In the normal cycle of receptor-mediated endocytosis, clathrin-coated "pits" appear in the plasma membrane and, as they invaginate, convert into coated vesicles (2, 25, 26). Inside the cell, such vesicles rapidly release their coats and fuse with other internal membrane compartments to form endosomes, while the released clathrin presumably recycles to the plasma membrane to generate new coated pits and coated vesicles. A possible mediator of this uncoating event is the 70-kD "uncoating ATPase" discovered in brain cytosol (24, 29) and recently shown to be a member of the family of 70-kD ATPases that includes certain heat shock proteins (6, 20, 34, 38). In vitro, this "uncoating ATPase" (UNC)1 removes clathrin from brain coated vesicles in an ATP-dependent reaction (3, 29) and also binds to soluble clathrin and renders it incapable of polymerizing (30). The uncoating event has been described as a two-stage process, first involving an ATP-dependent displacement of certain clathrin-clathrin interactions in the coat, followed by a capture of released portions of the clathrin molecules which prevents subsequent reassembly (28, 30). The final products of the reaction appear to be free clathrin triskelia complexed with up to three UNC molecules (29).

The two stages of the reaction have been further described as occurring at different sites on the clathrin molecule (30, 31). The first event, involving ATP consumption and clathrin bond-breakage, appears to occur at a site that is only expressed in intact coats, not in isolated clathrin triskelions (3, 30). The subsequent "capture" event, on the other hand, apparently occurs at a site that is accessible in isolated triskelia but masked by clathrin interactions in the intact coat (30, 31). The reaction at the "first" site would appear to be a low affinity, rapidly reversible interaction, because intact coats cannot be isolated with UNC attached to them (29). In contrast, binding at the "second" site seems to be high affinity,
because the final triskelion–UNC complex survives prolonged storage (29, 31, 32). Rothman and Schmid (28) have thus proposed that "the uncoating proteins act in concert (perhaps as a transient trimer) to drive a cooperative and symmetric quaternary rearrangement of the vertex, displacing and then capturing the legs of three next-nearest neighbor triskelions" (28). Support for the notion that a "transient trimer" forms during the reaction is that UNC oligomerizes in the absence of ATP but is monomeric in ATP (32).

In the present study, we use the quick freeze/mica flake technique (11) to demonstrate that the uncoating ATPase does indeed bind strongly to soluble clathrin, and does indeed form trimers on clathrin triskelia. Interestingly, these trimers occur at the vertices of the triskelia, suggesting that these are important sites for normal lattice assembly. We further show that this sort of complex formation is observed when ATP action is inhibited with EDTA or when the nonhydrolyzable ATP analogues AMP-PNP or ATPγS are present. On the other hand, when ATP is present during preparation of the molecules for electron microscopy, the uncoating ATPase remains monomeric and displays no clear signs of binding to triskelia. This ATP-dependence of clathrin–ATPase interaction suggests that the uncoating mechanism may parallel the ATPase cycles of myosin and dynein (7, 8), and we propose an analogous kinetic scheme for the mechanism of uncoating. In related experiments, we find that under conditions in which UNC fully removes the coats from brain coated vesicles, identical aliquots of the protein do not affect the coated pits in broken-open cells. This demonstrates the specificity that would be required if UNC were to play a selective role in vesicle uncoating in vivo.

**Materials and Methods**

**Materials**

DEAE cellulose was purchased from Whatman Inc. (Clifton, NJ); hydroxylapatite (fast flow) from Calbiochem-Behring Corp. (San Diego, CA); adenosine triphosphate-agarose (6 carbon spacer), ATP, and AMP-PNP from Sigma Chemical Co. (St. Louis, MO). Bovine brain clathrin was purchased from Bethesda Research Laboratories (Gaithersburg, MD) and stored at -70°C until used.

**Isolation of Brain Coated Vesicles and Preparation of Pure Clathrin Triskelia**

Coated vesicles were isolated from bovine brain according to the method of Nandi et al. (21). In a typical preparation performed at 4°C, bovine brains, immediately chilled on ice after slaughter, were cleaned of remaining meninges, pia, and white matter. Approximately 1 kg of gray matter was homogenized in an equal volume of buffer B (0.1 M Mes, pH 6.5, 0.5 mM EGTA, 0.5 mM MgCl₂, and 0.02% (wt/vol) NaN₃) in a Waring blender at maximum speed (four 10-s bursts). The homogenate was put through a series of low speed (10,000 g) and high speed (100,000 g) centrifugations to remove contaminating material. 6-ml aliquots of the suspension were layered onto an equal volume of sucrose/D₂O maintained at pH 6.5 with the same contents as buffer B. The single-step gradients were centrifuged for 2 h at 32,000 rpm (125,000 g) in a rotor (model SW-40; Beckman Instruments, Inc.) at 16°C. The supernatants were aspirated and the pellets were washed several times in buffer B. Further purification of the coated vesicles was achieved by Sephacryl S-1,000 (Pharmacia Fine Chemicals, Piscataway, NJ) gel filtration chromatography. Typically, 40 mg of coated vesicles in a total volume of 8 ml was applied to a S-1,000 column (2 × 83 cm) equilibrated in buffer B. The column was eluted at ~50 ml/h in the same buffer and the samples collected in 2-ml fractions. The column-purified coated vesicles eluted in the included volume as a well-defined peak as determined by its absorbance at 280 nm (1). The coated vesicles were pooled and concentrated by centrifugation at 100,000 g for 60 min. The pellet resuspended overnight in 10 ml of buffer B to a concentration of ~2-3 mg/ml.

Soluble clathrin was prepared by extracting these preparations with 0.5 M Tris, pH 7.0, as described (18). After ammonium sulfate precipitation, the clathrin was further purified by chromatography on Separose 6B, also as described (17).

**Conditions of Uncoating Reactions**

Uncoating reactions were performed by mixing various clathrin assemblies with the 70-kD protein to a final concentration of 0.2 mg/ml for each, in the pH 7.0 buffer originally used by Schlossman et al. (29), henceforth termed buffer C (40 mM Hepes buffer, pH 7.0, 4.5 mM Mg acetate, 75 mM KCl, and 0.8 mM DTT). Nucleotides were provided in their Mg²⁺-chelated form at 2 mM initial concentration; no regenerating systems were used. Potato apyrase (0.01 mg/ml) was sometimes included along with the nonhydrolyzable analogues ATPγS or AMP-PNP to hydrolyze any contaminating ATP or ADP, but did not alter the results. Times and temperatures of incubation are described in the relevant figure legends.

**Preparation of Reaction Mixtures for Electron Microscopy**

In preparation for freeze drying, protein samples were diluted to 10 µg/ml in appropriate stabilization buffers (B or C) and mixed with finely ground mica flakes (ratio of 10 vol protein solution to 1 vol of packed mica). After
5 s of exposure, the mica was separated by centrifugation at 100 g for 10 s and washed in 100 vol of protein-free buffer C, then allowed to settle at 1 g and placed on the surface of a thin slab of fixed rabbit lung (the usual support sponge for quick freezing). After blotting away excess buffer, the mica slurry was quick-frozen by impact against a liquid helium cooled copper block, using a homemade freezing machine as described (11). In a standard Balzer's freeze-etch unit, the frozen slurry was then freeze-fractured and deep-etched for 4 min at -100°C, and finally replicated with 2 nm of platinum rotary-deposited at 10° above the horizontal and backed with 10 nm of carbon rotary-deposited at 75°. The replica was separated from mica by flotation on full-strength hydrofluoric acid for 18 h at room temperature, rinsed several times in H2O and picked up on 400 mesh formvar-coated grids.

**Preparation of Cell Membrane Fragments for Uncoating of Coated Pits**

Cell membrane fragments used to test for in situ uncoating of coated pits were obtained by culturing to near confluence several mammalian cell types on 4 × 4-mm glass coverslips, then rinsing them briefly in buffer C and exposing them to a 1-s burst with an ultrasonic microprobe (Kontes Glass Co., Pittsburgh, PA). This breaks a large proportion of the cells and leaves behind on the glass coverslip a number of attached cell bottoms. Coated pits are abundant on these membrane fragments and are thereafter directly accessible to applied reaction mixtures. To view them in the electron microscope, the coverslips were fixed for 30 min with 2% glutaraldehyde in buffer C, then washed in distilled water, and quick-frozen as described above. Thereafter, they were freeze-dried for 15 min at -80°C in a Balzer's freeze-etch machine and rotary replicated with 2 nm of platinum deposited at 24° above the horizontal. Replicas separated immediately from the coverslip by immersion at an angle into full strength hydrofluoric acid, and did not require further cleaning. They were rinsed and mounted as described for mica replicas above.

**Electron Microscopy**

Replicas were viewed at 100 kV in a standard transmission electron microscope and photographed at ±10° of tilt to obtain stereo images. The micrographs were analyzed and quantified by viewing them with a Wild APT-I stereo map reader connected via a camera lucida to a Zeiss MOP-3 stereology calculator. All images were printed as photographic reversals according to previously published procedures (10, 13, 15).

**Results**

**Characterization of the Uncoating ATPase Preparations**

Fig. 1 shows successive stages in purifying the uncoating ATPase, as monitored by SDS-PAGE and Coomassie blue staining. The final material (lane 8) displayed a major band at ~70 kD and a trace band at 120–130 kD. Silver-stained gels displayed several additional minor bands, but >95% of the staining density was associated with the 70-kD band (not shown). Purity was also assessed by diluting the affinity-purified material to 10 μg/ml and adsorbing it to mica flakes for freeze-drying. In the presence of ATP, this process yielded fields of uniformly globular molecules (Fig. 2). The molecules measure 8 ± 1 nm in diameter (~2 nm of which represents their coat of platinum) and are basically compact and symmetrical. They exhibit essentially the same shape and size as 68-kD BSA prepared under identical conditions (Fig. 2, inset), but they adhere to mica much more avidly than albumin, suggesting they have higher pl (11).

**Uncoating Reactions on In Vitro Clathrin Lattices**

To assess the uncoating potency of this protein, it was reacted with purified preparations of bovine brain coated vesicles or reconstituted cages composed of clathrin plus assembly polypeptides (the latter kindly provided by Dr. J. Keen, Temple University, Philadelphia, PA; cf. reference 17). Uncoating was monitored either as the progressive replacement of coated vesicles by naked vesicles (naked vesicles being extremely rare in the initial brain preparations), or else as the

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*Figure 1. SDS-PAGE of clathrin uncoating ATPase shown as a 10% separating gel with a 4% stacking gel. Proteins were separated by an 8-h run time at 25–30 mA. Molecular mass standards (lane 1) are phosphorylase B, 94 kD; BSA, 68 kD; ovalbumin, 43 kD; and CA1, 30 kD. Lanes 2 and 3 show the flow-through and KCl wash from the DE-52 column; lanes 4 and 5 show the flow-through and salt-wash of the hydroxylapatite column; lanes 6 and 7 show the flow-through and salt-wash of the ATP-agarose column; and lane 8 shows the UNC eluted from the ATP-agarose column with ATP.*

*Figure 2. Freeze-etch preparation of 70-kD UNC eluted from an ATP-agarose column and adsorbed to mica, still in the presence of 1 mM Mg²⁺-ATP. Under these conditions the protein is monodisperse and measures uniformly 8 ± 1 nm in diameter, commensurate with it being a 70-kD monomer. For comparison, 68-kD BSA is illustrated in the inset. Bar, 50 nm.*
progressive disappearance of reassembled cages and the appearance of triskelia and UNC molecules on the mica. Fig. 3 illustrates typical results. At equal weight ratios (0.1-0.2 mg/ml for both the 70-kD protein and the clathrin in either coated vesicles or reconstituted cages), ATP-induced uncoating was complete by 5 min at 37°C (Fig. 3, columns c and d). Under identical conditions, AMP-PNP did not substitute for ATP (Fig. 3, column a), whereas ATPγS was capable of partial uncoating. In three different experiments, 5 min in 2 mM ATPγS at 37°C reduced 54, 62, and 67% of the brain coated vesicles to the forms shown in Fig. 3, column b. Interestingly, defrocked vesicles often displayed small numbers of brick-shaped bumps on their surfaces (arrows in Fig. 3), regardless of the nucleotide used. These bumps resembled AP-II assembly polypeptides or "adaptins" (14), supporting earlier biochemical data that indicated that when UNC removed ~85% of the clathrin from coated vesicles, it left behind ~80% of their associated assembly proteins (29).

**Uncoating Reactions on In Situ Clathrin Lattices**

When these same preparations of UNC were applied to sonicated fragments of various cultured mammalian cells, very different results were obtained. Invariably, coated pits situated on the plasma membrane fragments of fractured cells were totally unaffected by exposure to UNC (Fig. 4). Regardless of the type of cell that was examined (including various lines of human, rodent, and avian origins), and regardless of the concentration of UNC or of ATP, as well as the time, temperature, or ionic conditions of the exposure, no detectable uncoating of coated pits was ever obtained. Coated pits from bovine MDBK cells were also reacted, since our UNC was of bovine origin; but again, no uncoating was ever detected.

As an extension of these experiments, UNC was applied to MDBK plasma membrane fragments (at 0.2 mg/ml) in the presence of 1 mM AMP-PNP or ATPγS, to test whether the
nonhydrolyzable ATP analogues would promote UNC binding to coated pits in a manner analogous to their promotion of the "rigor" state in various other "motor" ATPases (19, 23, 39). However, no "decoration" of in situ clathrin lattices could be detected in the resultant platinum replicas (Fig. 4, center sections).

In the course of these manipulations, it became obvious that the coated pits on plasma membrane fragments progressively round up during 37°C incubation in vitro, regardless of the incubating medium (Fig. 4), a phenomenon described in detail elsewhere (12). Interestingly, this spontaneous increase in clathrin lattice curvature was not accelerated or retarded by exposure to UNC, regardless of the presence or absence of Mg-ATP or other nucleotides. Thus, we could acquire no evidence that UNC has any effect on coated pit dynamics per se.

**Imaging the Products of In Vitro Uncoating Reactions**

In initial attempts to visualize clathrin–UNC complexes, an ATP-induced uncoating reaction was carried out for 5 min at 37°C and the products were then diluted to 10 µg/ml with ice-cold buffer containing 1 mM Mg-ATP before adsorption to mica. Previous work suggested that in such preparations, we might find UNCs binding to the terminal domains of individual triskelia (28, 31). Instead, the resultant replicas showed nothing but free triskelia mixed with monomeric UNCs (Fig. 5). However, we should stress that this conclusion (e.g., no binding) was complicated by the fact that clathrin terminal domains are themselves globular and approximately the same size as UNC molecules (cf. Fig. 7 of reference 13). Thus we had to consider as possible binding events any triskelia that had terminal domains significantly larger than normal. One such molecule is seen at the circle in Fig. 5, but such images were so infrequent that they could have been coincidental landings of separate UNC and clathrin molecules in close proximity on the mica surface.

**Generating Complexes from Pure Clathrin and Uncoating ATPase**

Having obtained these images, we went on to show that identical clathrin–UNC complexes could be generated simply by incubating free clathrin triskelia with UNC in the presence of 1 mM ATPyS, no uncoating reaction being necessary (Fig. 8, rows a and b). Likewise, a few such images of complex formation could be generated by adding 1 mM AMP-PNP to a soluble clathrin–UNC mixture (Fig. 8, row c); however, they were never seen when 1 mM Mg-ATP was present. In this light, it was particularly interesting to find that complexes could be generated simply by adding 1 mM EDTA to our standard clathrin–UNC mixtures (Fig. 8, row d). This suggested that EDTA was removing Mg++-chelated ATP that was left in the UNC from the purification, and was thereby creating the same state as did the nonhydrolyzable ATP analogues. This would be analogous to the way in which EDTA creates "rigor" in muscle tissue (4, 5). Table I summarizes the relative degrees of clathrin–UNC binding observed in these various protein-mixing experiments.

**Further Work Indicated**

Further work indicated that it was the inclusion of ATP in our final dilution buffers that was preventing us from finding clathrin–UNC complexes. When instead we used the nonhydrolyzable analogue ATPyS in the initial uncoating reaction and in the final dilution buffer, positive results were consistently obtained (Fig. 6 and Table I). In this case, a significant number of free clathrin triskelia displayed ~15-nm-diam "masses" at their central vertices (though still nothing obvious at their terminal domains). These central masses reminded us of ones seen previously on triskelia under conditions in which a normal ATP-driven uncoating reaction was terminated with hexokinase and glucose to get rid of the ATP (29). However, our freeze-dry images provided sufficiently more resolution to reveal that these central masses were often composed of three relatively distinct globular entities, each about the size of an individual UNC monomer, and were located exactly in between adjacent legs on the ventral (i.e., membrane-apposed) surface of the triskelion. This pattern of binding can be seen in three-dimension at the bottom of Fig. 6, is diagrammed in Fig. 7, and is described more fully in the legend to Fig. 7.

**Figure 5.** Gallery of selected examples of clathrin triskelia released during an uncoating reaction and maintained in 1 mM Mg++-ATP during adsorption to mica and freeze-drying. No obvious binding of UNC molecules to triskelia occurs under these conditions. As in unreacted molecules (Fig. 7 of reference 13), the central vertices of the triskelia are only slightly thicker than the rest of their legs, while the legs themselves terminate in globular or scroll-shaped 7-nm swellings. Rarely, one of these "terminal domains" appears larger than normal (circled), but this was not a consistent finding. Bar, 50 nm.
Uncoating ATPase Self-Associations

An interesting concomitant of the presence or absence of clathrin-UNC complexes in the above experiments was that UNC itself appeared to oligomerize under the same conditions that promoted complex formation. Thus, whenever EDTA, ATPγS or AMP-PNP was added, UNC no longer displayed the uniformly monodisperse distribution on mica seen immediately after preparation (Fig. 2), but instead displayed variable degrees of clumping. This can be seen in the background of Fig. 6 and Fig. 8 and is shown more clearly in Fig. 9, which represents a relatively unsuccessful attempt to "decorate" clathrin triskelia with UNC's that had already been oligomerized by preexposure to AMP-PNP. Unlike the positive results described above, this experiment yielded little or no indication of UNC binding to triskelia. Nevertheless, it clearly produced clumping of UNC into variously sized aggregates, a few of which were distinctly trimeric (circled). These closely matched the classical trimeric image of IgG (Fig. 9, inset), but other UNC aggregates were more irregular and included examples that were larger than would be expected for trimers.

Such irregularity of self-association was in marked contrast to the appearance of UNC molecules attached to clathrin triskelia under the same conditions of ATP depletion or

Figure 6. Triskelia released by incubating clathrin cages with UNC in the presence of 2 mM Mg\textsuperscript{2+}·ATPγS. (Stereo pair in lower portion of plate represents the same field as above, but shown in 3-D.) In contrast to their appearance in ATP, triskelia here generally display the binding of irregular masses at their vertices (though still nothing at their globular terminal domains). The numbered triskelia in this field can be described thusly: (1) displays a "canonical" mass on its vertex (i.e., distinctly triangular and tripartite), even though one of its legs is missing (probably via inadvertent proteolysis); (2) displays three globules scattered a short distance away from its vertex, probably via dissociation during adsorption to mica; (3) displays a smaller mass on its vertex and an immediately adjacent mass, possibly indicating partial dissociation; (4) displays nothing at its vertex, like all the triskelia in ATP (see Fig. 5); (5) displays a large three-part mass on its vertex; (6) looks like molecule No. 3, suggesting partial dissociation of a vertex-mass; (7) a molecule on its side, with a smaller mass at its vertex presumably representing one or two UNC molecules. Bars, 50 nm.
Table 1. Observed Degree of Binding of the Uncoating ATPase to Clathrin Triskelia under Different Conditions

| UNC incubated with | Total yield of triskelia with extra mass at their vertices | Yield of triskelia with three distinct ~5-nm masses at their vertices |
|--------------------|----------------------------------------------------------|------------------------------------------------------------------|
| Whole clathrin cages plus the following nucleotides | | |
| ATP (released large numbers of triskelia) | 4/100 | 0/100 |
| ATPyS (released low numbers of triskelia) | 22/31 | 17/31 |
| AMP-PNP (released no triskelia) | 0/0 | 0/0 |
| EDTA (released no triskelia) | 0/0 | 0/0 |
| Free clathrin triskelia plus the following nucleotides | | |
| ATP | 4/100 | 0/100 |
| ATPyS | 53/91 | 43/91 |
| AMP-PNP | 36/96 | 9/96 |
| EDTA | 56/68 | 31/68 |

* Denominator indicates the total number of triskelia that were included in the tabulation. Generally, some 10–30% of the total number of triskelia in each experiment were excluded as "indeterminant" on purely technical grounds; e.g., not all three of their legs were intact, they lay on their sides so that their vertex was obscured, they overlapped each other or lay under dirt, etc. Numerator indicates how many of the triskelia expressed in the denominator displayed masses directly on their vertices. Near-misses often gave the subjective impression of having fallen apart during adsorption to mica (cf. Fig. 6), but were not included as positives in the numerator.

† This category represents a subset of the same populations of triskelia shown in the adjacent column, chosen according to more rigid criteria: masses not only had to be located directly on triskelial vertices, but had to be triangular in shape with vertices pointing out between each triskelion leg, as indicated in Fig. 7.

replacement, as described above. The complexes on triskelia generally appeared to involve three UNC molecules at each vertex, rarely two or one, but never appeared to be as large as four or more. This would fit with earlier biochemical estimates of the stoichiometry of binding, which also suggested that triskelia possess three binding sites for UNC molecules (29). Three such sites would, of course, allow clathrin triskelia to stabilize the trimeric state of the uncoating ATPase under conditions in which it might otherwise tend to oligomerize more randomly. Indeed, experiments like that shown in Fig. 9 indicated that once formed, UNC oligomers have relatively low affinity for individual triskelia. This observation, taken together with earlier biochemical work showing that micromolar ATP stimulates UNC monomerization and binding to free triskelia (32), suggests that UNC molecules first bind to triskelia individually and only then polymerize into trimers. This behavior will figure prominently in the model for clathrin–UNC interactions proposed below.

Figure 7. Diagram of the characteristic geometry of UNC binding to clathrin triskelia in ATPyS and other nonhydrolyzable ATP analogues. UNC molecules are depicted as circles, three of which bind in close proximity to the vertex of each clathrin triskelion. The triskelion is portrayed with leg-bends or "knees" facing to the left, which is the orientation in which clathrin molecules preferred to adsorb to mica in these experiments. Elsewhere, we have shown that this represents a view of the membraneside of the triskelion, relative to its orientation in an assembled clathrin lattice (cf. reference 14). Thus, the diagram indicates that UNC molecules bind to the same side of the triskelion as do the three terminal domains of the nonadjacent triskelion in the assembled lattice (cf. references 9, 13, 26).

Discussion

This study has shown that in the presence of ATP, the 70-kD clathrin–UNC is monomeric, while in the absence of ATP or in the presence of nonhydrolyzable ATP analogues it is partially oligomerized. Furthermore, in the absence of ATP or in nonhydrolyzable ATP analogues UNC binds strongly to clathrin triskelia, in which case it becomes distinctly trimeric and localizes at the triskelion vertex. Upon presentation of ATP, this vertex-binding disappears and no other locus of binding to triskelion is detectable. Finally, under no conditions does UNC bind to or otherwise alter the clathrin lattices around coated pits on the plasma membranes of broken-open cells, consistent with its having a specific role in vesicle uncoating.
Figure 9. Clathrin triskelia and UNC molecules treated with 1 mM AMP-PNP to oligomerize them before complex formation. UNC aggregation still occurred, but little or no binding of the aggregates to clathrin triskelia was seen when this sequence was followed. A few of the more distinctly trimeric aggregates are circled. For comparison, murine IgG is shown at the same magnification in the inset. Its trimeric 3 × 50-kD configuration is very similar to the circled 70-kD UNC trimers. Bar, 50 nm.

Proposed Mechanism of Clathrin Uncoating

Fig. 10 presents a hypothetical cycle for in vivo clathrin uncoating that is consistent with the data presented here and with many of the observations in earlier biochemical reports. UNC is portrayed as cycling back and forth (via adenosine nucleotide binding and release) between one conformation that binds only loosely to itself or to clathrin, and a second conformation that tends to self-associate and to bind tightly to clathrin, so tightly we presume that it distorts vital interactions in the underlying lattice. The cycle is closely analogous to current schemes for other ATPases (7, 8) including other 70-kD heat shock proteins (20, 27). The hypothetical cycle is presented in six steps. Step 1: In an initial "placement" reaction, monomeric UNCs with ATP bound to them would attach individually and sequentially to the three terminal domains of nonadjacent triskelia that converge beneath each vertex in an intact lattice. Weak interactions between UNCs at this point could promote some cooperative binding. (ATP hydrolysis by individual UNCs could occur at any point during this "placement" reaction, assuming that UNC\textsubscript{ADP + P}\textsubscript{i} is physically similar to UNC\textsubscript{ATP}, as is the case for myosin [7, 8].) Step 2: Juxtaposition of three UNCs via such sequential binding would somehow trigger phosphate and/or ADP release and a concomitant allosteric conversion of the UNCs to a high self-affinity state, leading to their polymerization into a stable trimer. Step 3: Trimeric UNC would now shift its position to bind tightly to clathrin vertices in the lattice, and this binding would be so tight that it would displace the three terminal domains from the vertex. At this point, the lattice would fall apart if similar disruptions occurred at all its vertices. However, regardless of whether it did fall apart or not, the cycle would continue in the following manner. Step 4: In the presence of normal in vivo levels of ATP, completion of the displacement reaction and release of ADP would prepare UNC-trimers for receipt of fresh ATP. Step 5: ATP re-binding would induce the UNCs to resume their former low-affinity configuration, to dissociate from each other, and thus to fall off the triskelion vertex. If the lattice had not fallen completely apart by then, the stage would be set for the original terminal domains to rebind to the original vertex

Figure 10. Proposed cycle for clathrin coat dissolution via nucleotide-induced changes in the polymeric state of 70-kD uncoating ATPase molecules. The critical step in dissolution is No. 3 (distortion reaction) and is envisioned as being due to competition between a newly formed trimer of uncoating ATPases (U-U-U) and the three terminal domains of nonadjacent triskelia (TDs). These are depicted as starting off bound to the underside of the triskelion at each vertex or "hub" in the lattice, and being displaced as a result of this "protein-polymer competition."
(Step 6) and for the cycle to repeat. Thereby, clathrin lattices would stimulate UNCs ATPase activity regardless of whether or not they became completely dissociated in the process.

**Evaluation of the Model**

Several aspects of previous and present studies support the proposed kinetic cycle. Stopping an uncoating reaction by an abrupt removal of ATP with hexokinase and glucose yields "capture complexes" with three UNCs bound to each triskelion vertex, as predicted in the lower right corner of Fig. 10 (data shown here and in Fig. 11 of reference 29). This could be described as blocking the cycle just before step 4. Likewise, re-addition of ATP dissociates these complexes (observations described here and in Fig. 2 of reference 31), which could be described as allowing step 4 to proceed. Finally, carrying out an uncoating reaction at pH ∼6.5 allows ATP hydrolysis to proceed but prevents lattice disruption, creating so-called "futile" ATP hydrolysis (30). This could be described as stimulating step 6 via stabilization of the rest of the clathrin lattice.

Two aspects of the proposed cycle differ fundamentally from prevailing views, however. First, the current view is that the final "capture complex" should have three UNCs bound, not to the vertex, but to the three terminal domains of each clathrin triskelion (28). Evidence for this alternative has been twofold: first, proteolytic removal of terminal domains appears to abolish the ability of cages to stimulate UNC-mediated ATP hydrolysis and prevents cage dissociation (31); and second, addition of isolated terminal domains appears to competitively inhibit clathrin–UNC reactions (31). However, both of these observations could reflect effects of terminal domains on the initial phases of UNC–cage interaction but not necessarily the final location of UNC on released triskelain. Since in fact we have not observed UNC molecules bound to the terminal domains of released triskelain, and none were reported in previous studies of released triskelain (29), we here proposed as an alternative that terminal domains are involved instead during an initial, transient reaction of UNCs with clathrin lattices (Step 1 in Fig 10). The second major problem in correlating the uncoating cycle proposed in Fig. 10 with previous work is that it does not explain the apparent requirement for light chains in clathrin uncoating (33). Current evidence suggests that light chains exist near the outside of each vertex (2, 9, 25, 26, 35), i.e., physically separated from the underside of the vertex where we find that UNC binding occurs (Fig. 7) and where terminal domain binding would normally occur in intact clathrin lattices (13, 36, 37). Thus we would have to imagine that the presence of light chains on the top side of a vertex somehow alters the conformation of the terminal domains underneath it, such that their affinity for UNCs or their ability to nucleate UNC trimerization is enhanced. This seems rather far-fetched, however, and it may turn out that the proteolysis or chaperone action that was used to remove light chains from clathrin in earlier experiments may have simply damaged the topology of the underlying terminal domains. Further work with genetically clipped clathrin should resolve this issue.

**The Effects of UNC on In Situ Clathrin Coated Pits**

The cycle proposed in Fig. 10 would offer a plausible explanation for the inability of UNC to remove the lattices from coated pits, in spite of its effectiveness on isolated coated vesicles, if we further suppose that clathrin terminal domains are anchored to the membrane (or to receptor-tails or "adaptin" molecules) differently in the two situations, such that UNC can only gain access to them once a coated pit has pinched off to become a vesicle. In fact, this could be a vital constraint on UNC's normal function. If the molecule does play a vesicle-uncoating role in vivo, it should not be able to remove the coats from coated pits before they pinch off, or else it would be in danger of constantly disrupting receptor-mediated endocytosis.

Still, it would have been interesting to learn that UNC had other effects on coated pits. For example, one might have imagined that UNC could transiently free up triskelion vertices within preexisting lattices and thereby provide an opportunity for insertion of new triskelain during lattice growth. Alternatively, UNC activity could permit shifting of adjacent terminal domains within a lattice, thereby facilitating the remodeling of lattice hexagons into pentagons which is thought to accompany changes in lattice curvature during in situ coated vesicle formation (10, 12, 16). Thus, we would have been pleased to observe any effect of UNC on the rate or extent of clathrin lattice curvature, yet we found none. Exposed lattices rounded up spontaneously at such a rapid rate that we could not demonstrate any acceleration by providing exogenous UNC or ATP, nor could we retard their rate of curvature by strictly eliminating ATP with hexokinase and glucose or with EDTA. The latter experiments might have worked if, for example, UNC became bound to lattices as soon as they formed and thus was already on them when we broke the cells open. In fact, however, no one has yet seen any signs of UNC binding to intact clathrin lattices of any sort (cf. Fig. 4). That will ultimately have to be accomplished before we can be certain whether or not UNC is also involved in clathrin lattice dynamics at coated pits.

Thanks to Dr. J. Keen (Temple University, Philadelphia, PA) for providing the reconstructed clathrin cages and Dr. W. J. Nelson (Fox Chase Cancer Center, Philadelphia, PA) for providing TX-100 extracts of MDCK cells containing clathrin–ATPase complexes. Thanks also to Lois Greene (National Institutes of Health) and Tomas Kirchhausen (Harvard Medical School) for sharing their observations on the uncoating ATPase with us before publication. Finally, heartiest thanks to Robyn Roth and Melissa Reichman for preparing all the freeze-etch replicas, Comfree Colman for preparing the figures, and Jan Jones for preparing the manuscript.

Supported by United States Public Health Service grant GM29647 to J. Heuser.

Received for publication 22 December 1988 and in revised form 14 June 1989.

*Note added in proof:* Very recently, Flynn, Chapell, and Rothman reported in *Science* (Wash. DC.) (1989. 245:385–390) that the 70-kD uncoating ATPase binds also to certain short, unfolded peptides attached to Affigel columns, and that ATP, but not ATPyS, dissociates it from the columns, suggesting that nucleotide hydrolysis is required for its release. These data fit well with the nucleotide effects on uncoating ATPase/clathrin interactions described in this report.

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