

An Enzyme That Removes Clathrin Coats: Purification of an Uncoating ATPase

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ABSTRACT Uncoating ATPase, an abundant 70,000-mol-wt polypeptide mediating the ATP-dependent dissociation of clathrin from coated vesicles and empty clathrin cages, has been purified to virtual homogeneity from calf brain cytosol. Uncoating protein is present in cells in amounts roughly stoichiometric with clathrin. This enzyme is isolated as a mixture of monomers and dimers, both forms being active. ATP can support protein-facilitated dissociation of clathrin at micromolar levels; all other ribotriphosphates as well as deoxy-ATP are inactive. The clathrin that is released from cages consists of trimers (triskelions) in a stoichiometric complex with uncoating ATPase. These complexes with clathrin have little tendency to self-associate at neutral pH, and at acidic pH they interfere with the assembly of free clathrin. The possible existence and function of these complexes as clathrin carriers in cells would explain why uncoating protein is made in quantities equivalent to clathrin.

Clathrin-coated vesicles transport selected sets of membrane-associated macromolecules between subcellular compartments (6, 10, 13, 25–30). Isolated coated vesicles have offered fruitful opportunities for structural study (3, 7, 8, 11, 12, 14, 24, 39, 41, 42, 46, 47), but as inert objects they can only provide limited insights into the dynamic mechanisms that underlie intracellular transport.

Can purified coated vesicles be activated *in vitro* to function as they do in cells? Coated vesicles can be regarded as intermediates in a transport cycle (30) that happen to be trapped during their isolation. Thus, addition of suitable cellular components to isolated coated vesicles may engage them to pass through subsequent steps in the cycle, offering at once a means to identify the needed components, to elucidate unknown steps in the pathway, and to study the mechanisms involved. In this paper we illustrate this approach and describe the purification of the first enzyme believed to facilitate intracellular protein transport.

Shortly after budding of a coated vesicle, and apparently before fusion of the vesicle with its target, clathrin appears to be removed from the membrane (1, 13, 28, 44). We have reported (23) that the high speed supernatant fraction of brain possesses an ATP-dependent activity that rapidly disassembles clathrin coats. In this and the following paper (5) we describe the purification and characterization of the protein responsible for this activity. Because this 70,000-mol-wt polypeptide uses the hydrolysis of ATP to drive the energetically unfavor-

able release of the clathrin from coats, we have termed this activity “uncoating ATPase.”

MATERIALS AND METHODS

Materials

Proteins used as molecular weight standards and ATP-Agarose were from Sigma Chemical Co. (St. Louis, MO). Yeast hexokinase, AMP-PNP,¹ AMP-PCP, and ATP- γ -S were from Boehringer Mannheim Biochemicals (Indianapolis, IN), other nucleotides from P-L Biochemicals, Inc. (Milwaukee, WI). Sephacryl S-300, Sephadex G-25, and Sepharose 4-B were from Pharmacia Fine Chemicals (Piscataway, NJ), DEAE cellulose from Whatman Laboratory Products Inc. (Clifton, NJ), and hydroxylapatite from Calbiochem-Behring Corp., San Diego, CA). NaB[³H]₄ (5–10 Ci/mmol) was from Amersham Corp. (Arlington Heights, IL).

Buffers

BUFFER A: 20 mM 2-(*N*-morpholino)ethane sulfonic acid (MES)-NaOH (pH 6.2), 1 mM Na₂EDTA, 2 mM CaCl₂.

BUFFER B: 40 mM HEPES-NaOH (pH 7.0), 4.5 mM Mg acetate, 75 mM KCl, and 0.8 mM dithiothreitol.

BUFFER C: 20 mM HEPES-NaOH (pH 7.0), 2 mM Mg acetate, 25 mM KCl, 10 mM (NH₄)₂SO₄, and 0.8 mM dithiothreitol.

BUFFER D: 25 mM Tris-HCl (pH 7.0), 250 mM sucrose.

¹ *Abbreviations used in this paper:* AMP-PNP, 5'-adenylylimidodiphosphate; AMP-PCP, 5'-adenylyl-(β , γ -methylene)-diphosphonate; ATP- γ -S, adenosine-5'-O-(3-thiotriphosphate); MES, 2-(*N*-morpholino)ethane sulfonic acid; TCV, Triton X-100-extracted coated vesicles.

BUFFER E: 0.75 M Tris HCl, 25 mM MES-NaOH (pH 6.2), 0.25 mM Na₂ EGTA, and 0.12 mM MgCl₂.

BUFFER F: 0.5 M Tris HCl, 50 mM MES-NaOH (pH 6.2), 0.5 mM Na₂ EGTA, 0.25 mM MgCl₂.

Purification of Coated Vesicles, Clathrin Triskelions, and Empty Clathrin Cages

Calf brains were frozen on dry ice within 15 min of slaughter and stored at -80°C . Crude membrane vesicle fractions were prepared (16); these were further purified in some cases to obtain coated vesicles using sucrose or Ficoll-D₂O gradients (24, 27). To prepare clathrin, either crude membranes (21) or coated vesicles (16) were extracted in buffer E at 4°C for 60 min, centrifuged at 100,000 g_{av} for 60 min, and the supernatant precipitated with ammonium sulfate (30% of saturation). The precipitate, redissolved in 8 ml of buffer F (but not dialyzed) was chromatographed on a 3-X-115-cm (800-ml) column of Sepharose 4B (33) equilibrated in buffer F (flow rate about 40 ml/min). The clathrin containing fractions (located by SDS PAGE of column fractions) were pooled, and clathrin was precipitated with ammonium sulfate (50% of saturation), redissolved in buffer F at a protein concentration of 10 mg/ml, frozen in liquid nitrogen, and stored as aliquots at -80°C . As reported (16), these preparations consisted mainly of clathrin (180,000 mol wt) and light chains (33,000 and 36,000 mol wt), although contaminants (e.g., the 100,000–110,000-mol-wt family of coated vesicle proteins) were present up to 10% of the total. The procedure yielded ~15 mg of clathrin per kilogram of brain tissue (when coated vesicles were extracted) and ~40 mg of clathrin per kilogram of tissue (when crude membranes were extracted). This "column-purified clathrin" was reassembled into empty cages by dialysis into buffer A for 12–16 h at 4°C as described (16, 23, 32).

Synthesis of ^3H -Clathrin and Preparation of ^3H -Empty Cages

Clathrin was labeled by reductive methylation with formaldehyde and NaB[^3H]₄ according to the procedure of Tack et al. (37). Typically, 0.2 ml of buffer F containing 2 mg of clathrin was dialyzed extensively against 0.2 M Na-borate buffer (pH 8.9), transferred to a microfuge tube and held on ice for 30 min to thoroughly equilibrate the temperature. Then 15 μl of 0.2 M formaldehyde in water and 10 μl of 0.12 M NaB[^3H]₄ in 0.01 N NaOH (8.4 Ci/mmol) were added sequentially, and the tube was held on ice for an additional 10 min. The reaction was then terminated by addition of 10 μl of 0.5 M (NH₄)₂SO₄, and the labeled clathrin was separated from unbound radioactivity by filtration over a 10-ml column of Sephadex G-25 equilibrated in buffer F. Fractions of 0.6 ml were collected and the labeled protein peak was located and pooled. The final product, typically ~1.4 mg of ^3H -clathrin ($1\text{--}2 \times 10^5$ cpm/ μg ; 90% trichloroacetic acid precipitable), was frozen in liquid nitrogen and stored as aliquots at -80°C .

For use in assays, the ^3H -clathrin triskelions were mixed with unlabeled triskelions to achieve a final specific activity of $1\text{--}5 \times 10^3$ cpm/ μg , and then dialyzed against buffer A to form ^3H -empty cages. The cages were pelleted by centrifugation at 95,000 g_{av} for 10 min in an airfuge (Beckman Instruments Inc., Palo Alto, CA) (A 30/100 rotor), and the pellet (containing 80–90% of the starting material was resuspended in buffer C and preincubated at 37°C for 10 min to release a pool of poorly assembled clathrin that would otherwise contribute to the background in the release assay. The remaining cages (30–40% of total) were pelleted in the airfuge, and resuspended in buffer A at 1–2 mg/ml. These "preincubated" ^3H -empty cages, now stable to further incubations in buffer C, were indistinguishable from standard preparations of unlabeled empty cages by electron microscopy, agarose gel electrophoresis (31), polypeptide composition, and limited tryptic hydrolysis (32). Possibly, some of the ^3H -clathrin molecules, damaged by the labeling procedure, are unable to assemble normally but co-polymerize with unlabeled clathrin to render a fraction of the final cages defective and unstable.

Assay of ATP-dependent Release of ^3H -Clathrin

Incubations (50 μl final volume) contained ATP (2.5 nmol) and the protein fraction to be tested (0.25–1 μg of pure uncoating ATPase; 10–40 μg of crude cytosol) in 45 μl of buffer C. Reactions were initiated by adding 5 μl of buffer A containing either 5 or 10 μg of preincubated ^3H -empty cages. To stop the incubation (usually after 5 min at 37°C), we degraded free ATP by adding 5 μl of a mixture of yeast hexokinase (100 U/ml) and glucose (5 mM) in buffer C. After a further incubation of 45 s at 23°C , samples were chilled on ice, diluted to 120 μl with ice-cold buffer C, and the remaining cages were pelleted in the airfuge at 95,000 g_{av} for 10 min. The top half (60 μl) of each supernatant was

counted for ^3H , and the percent of total clathrin released was calculated as twice this radioactivity divided by the total added to the assay.

The background of spontaneous release of ^3H -clathrin into the supernatant was measured in parallel incubations in which ATP was omitted and the hexokinase-glucose cocktail was added at the start of the incubation. This background was $10 \pm 5\%$ of the total ^3H in the assay. All of the values for ATP-dependent clathrin release reported in this paper have had this "minus ATP" background subtracted. Similar backgrounds were also obtained when cages were incubated in buffer without uncoating protein.

Purification of Uncoating ATPase

All operations were at 4°C unless otherwise noted.

STEP I: CRUDE CYTOSOL. Frozen calf brains (~500 gm) were thawed overnight at 4°C in 1 liter of buffer D, rinsed, and then homogenized in 1 ml/gm of fresh buffer D by three 15-s pulses in a Waring blender. The homogenate was centrifuged for 30 min at 14,000 rpm in a Beckman JA-14 rotor (Beckman Instruments, Inc.), and the supernatant was further centrifuged for 60 min at 45,000 rpm in a Beckman Type 45 Ti rotor. This high speed supernatant (~400 ml) was dialyzed for 8 h against 20 liter of 25 mM Tris-HCl (pH 7.0), centrifuged for 60 min at 45,000 rpm in the 45 Ti rotor, and dialyzed for 12 h against a second 20-liter portion of 25 mM Tris (pH 7). Crude cytosol at this stage could be frozen in liquid nitrogen and stored at -80°C for up to 6 mo without detectable loss of activity.

STEP II: DEAE-CELLULOSE. The dialyzed cytosol was applied at a flow rate of 150 ml/h to a 4-X-18-cm (225-ml) column of Whatman DE-52 equilibrated with 25 mM Tris-HCl (pH 7), and the column was washed (flow rate 250 ml/h) with 200 ml of 25 mM Tris (pH 7) followed by 450 ml of 25 mM Tris (pH 7) containing 50 mM KCl. No activity was found in these fractions. The column was then eluted with 25 mM Tris (pH 7) containing 150 mM KCl at a flow rate of 250 ml/h and 8-ml fractions were collected and assayed for ATP-dependent clathrin releasing activity. Uncoating activity emerged as a broad peak between 120 and 280 ml of this buffer.

STEP III: HYDROXYLAPATITE. The pooled active fractions (160 ml) from step II were applied at a flow rate of 100 ml/h to a 2.5-X-25-cm (125-ml) hydroxylapatite column equilibrated in 25 mM Tris-HCl (pH 7), 150 mM KCl. The column was washed with 150 ml of 20 mM HEPES-NaOH (pH 7) followed by 250 ml of 20 mM HEPES (pH 7) containing 50 mM potassium phosphate (pH 7). The column was then eluted with 20 mM HEPES (pH 7) containing 150 mM potassium phosphate (pH 7) at a flow rate of 150 ml/h, and 6-ml fractions were collected and assayed for ATP-dependent release of clathrin as described. This activity emerged in a broad peak between 114 and 222 ml of elution.

STEP IV: ATP-AGAROSE. The peak fractions from step III (108 ml) were dialyzed for 12 h against 4 liter of buffer C and then applied at a flow rate of 60 ml/h to a 1.5-X-16-cm (28-ml) column of ATP-Agarose equilibrated in buffer C. The column was then washed successively with 60-ml portions of buffer C, buffer C containing 1 M KCl, and buffer C again. These fractions contained most of the protein but did not contain significant activity and were discarded. The column was then eluted with buffer C containing 1 mM ATP at a flow rate of 60 ml/h, and 8-ml fractions were collected and assayed for activity. These fractions were, of course, no longer nucleotide-dependent because of the ATP present in the buffer; therefore, ^3H -clathrin released in an incubation containing cages and ATP but no column fraction was subtracted as background. Activity emerged as a broad peak between 32 and 128 ml of the ATP eluate.

STEP V: (NH₄)₂SO₄ PRECIPITATION. A combined ammonium sulfate precipitation and an EDTA treatment served to concentrate the activity and to restore ATP-dependence in the release assay by removing tightly bound Mg-ATP. The pooled active fractions from step IV (96 ml) were adjusted to 4 mM EDTA (from a 200 mM stock). After about 15 min, solid (NH₄)₂SO₄ was gradually added to achieve 75% of saturation, and the mixture was stirred for 30 min. The precipitate was collected at 100,000 g_{av} for 1 h and redissolved in a minimum volume (~2 ml) of buffer C. This fraction, consisting of essentially pure protein (~2 mg/ml) was frozen in liquid nitrogen and stored at -80°C after dialysis against buffer C. The frozen protein was stable for months at -80°C , and when thawed, aliquots typically retained uncoating activity for up to 2 wk at 4°C .

Other Methods

SDS PAGE was performed as described by Laemmli (18) using 10% polyacrylamide/0.1% SDS gels unless otherwise specified. For quantitation, gels stained with Coomassie R-250 or autoradiographs were scanned with a densitometer (Quickscan [Helena Laboratories, Beaumont, TX]). Electron microscopy was performed as previously described (23). Protein was determined as described by Bradford (4), using bovine serum albumin as the standard.

RESULTS

Assay of Uncoating Activity

Assay of ATP-dependent release of ^3H -clathrin into the supernatant was approximately linear with protein up to 20 $\mu\text{g}/\text{ml}$ of purified uncoating ATPase (or 800 $\mu\text{g}/\text{ml}$ of crude cytosol). Under these conditions, the assay was also linear with time up to 7.5 min of incubation (Fig. 1). Clathrin and light chains were released together (Fig. 2) and seemed to be free of proteolysis, since we could detect no net loss of ^3H , decrease in molecular weights, or change in the molar ratio of clathrin heavy chains to light chains.

Purification of Uncoating ATPase

Uncoating ATPase was purified 38-fold from bovine brain cytosol using a combination of DEAE-cellulose, hydroxylapatite, and ATP-agarose column chromatography (Table I). The product was a single polypeptide chain of 70,000 mol wt that was >95% pure as measured from densitometer tracings of SDS polyacrylamide gels (Fig. 3). Mixing experiments did not reveal activators of purified uncoating ATPase activity present in crude cytosol that might have made us overestimate total activity at the start of purification.

We were surprised that such a modest degree of purification was necessary, implying that the uncoating ATPase was abundant. We were therefore concerned as to whether the major protein we had purified was in fact responsible for the uncoating activity. The evidence in support of this, and a possible explanation for the abundance of this protein based on our findings, is provided in the Discussion.

The major step in the purification was the ATP-agarose affinity column, whose use was suggested by the unusually low K_m for ATP evident from our earlier work with crude cytosol (23). The DEAE cellulose step served principally to concentrate the activity, but also afforded a threefold purification. The hydroxylapatite step removed a major 45,000-

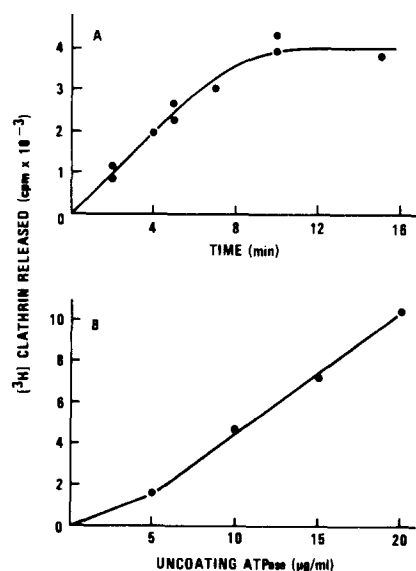


FIGURE 1 Assay for ATP-dependent release of clathrin. (A) Linearity with time. Assays containing 15 $\mu\text{g}/\text{ml}$ purified uncoating ATPase and 10 μg ^3H -empty cages (12,500 cpm) were incubated at 37°C for the indicated time. (B) Linearity with protein. Assays containing the indicated amount of purified uncoating ATPase and 5 μg ^3H -empty cages (25,000 cpm) were incubated at 37°C for 5 min.

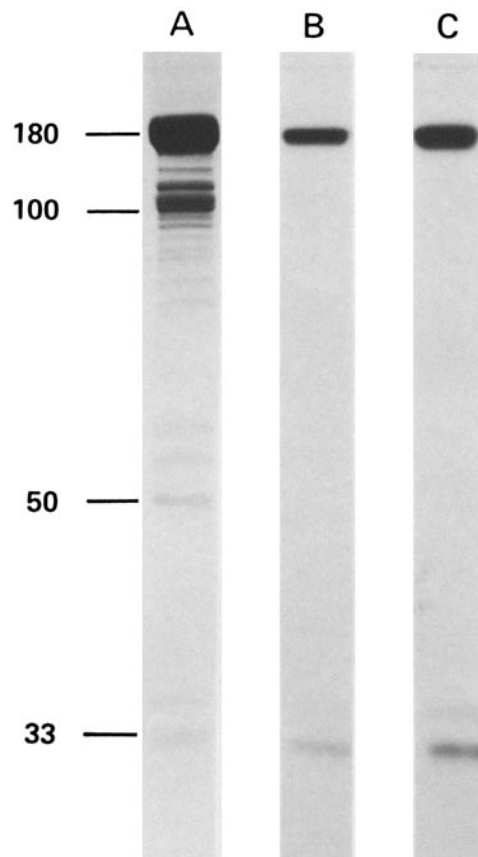


FIGURE 2 SDS PAGE of enzymatically released ^3H -clathrin. A standard assay containing 2 μg of purified uncoating ATPase and 10 μg of ^3H -empty cages (47,700 cpm) was incubated at 37°C for 10 min, stopped, sedimented, and a portion of the supernatant electrophoresed. (Lane A) Purified calf brain coated vesicles (10 μg) run as marker. Molecular weights of known coated vesicle proteins are indicated ($\times 10^{-3}$). (Lane B) ^3H -empty cages (1.9 μg , 9,000 cpm) before incubation. (Lane C) ^3H -Clathrin released to the supernatant (2.8 μg , 14,000 cpm). Lane A was stained with Coomassie R-250 and lanes B and C were autoradiographed after treatment with $\text{En}[^3\text{H}]\text{ance}$ (New England Nuclear, Boston, MA).

mol-wt contaminant (Fig. 3, lane E), believed to be actin, that could also be removed by DNase-I-agarose (2). Trace amounts of an 135,000-mol-wt contaminant were also present.

After the ATP-agarose step, the uncoating protein was found to be ATP-independent in the clathrin-release assay. This is due to tightly bound ATP that resists dialysis (our unpublished observations) acquired during elution from the ATP-agarose column. ATP-dependence could be restored by adding EDTA (4 mM) to remove the tightly bound Mg-ATP, followed by dialysis or gel filtration on Sephadex G-25. Routinely, this was combined with an ammonium sulfate precipitation, as described in Step V of the purification scheme in the Materials and Methods section, but an additional G-25 desalting step was sometimes necessary.

Properties of Uncoating ATPase

The subunit molecular weight of the uncoating ATPase protein, under denaturing conditions, was determined by SDS PAGE (45) to be 70,000 (Fig. 3, lane D). However, native uncoating ATPase consists principally of a mixture of monomers and dimers as judged from its pattern of elution from

TABLE I
Purification of Uncoating ATPase

Purification step	Total protein	Specific activity	Total activity	Purification	Yield
	mg	U/mg	U	(fold)	%
1. Cytosol	2,330	352	8.2×10^5	[1]	[100]
2. DEAE-Cellulose	411	1,050	4.3×10^5	3.0	52
3. Hydroxylapatite	143	2,140	2.4×10^5	6.1	29
4. ATP-Sepharose	6	12,440	0.75×10^5	35	9.1
5. $(\text{NH}_4)_2\text{SO}_4$ ppt.	4.3	13,400	0.58×10^5	38	7.1

Samples of the pooled active fractions from each purification step (see Materials and Methods) were dialyzed exhaustively against buffer C before assay. Assays contained 5 μg ^3H -empty cages, and were all confirmed to be in the linear range. 1 unit of activity is defined as the ATP-dependent release of 1% of the ^3H -clathrin into the supernatant in 1 min.

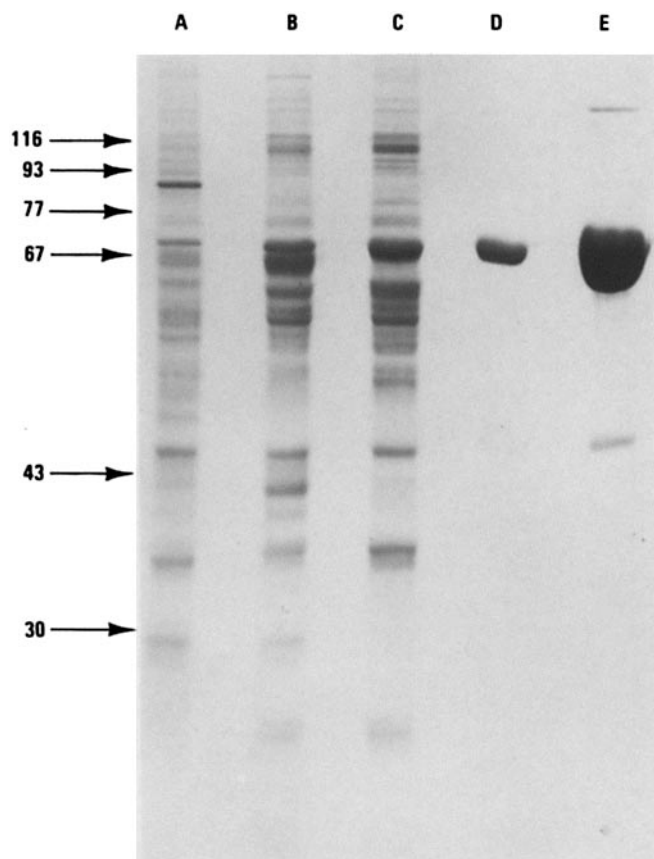


FIGURE 3 SDS polyacrylamide gel of pooled fractions at each step in the purification of uncoating ATPase (Table I). Arrows indicate the migration positions of reference proteins (not shown) with molecular weights as follows ($\times 10^{-3}$): *Escherichia coli* β -galactosidase (116), phosphorylase b (93), transferrin (77), bovine serum albumin (67), ovalbumin (43), and carbonic anhydrase (30). (Lane A) Crude cytosol (30 μg); (lane B) DEAE-cellulose fraction (30 μg); (lane C) hydroxylapatite fraction (30 μg); (lane D), ATP-agarose fraction, concentrated by ammonium sulfate precipitation (4 μg); (lane E) same as lane D, except 20 μg were loaded to emphasize contaminants.

Sephacryl S-300 in assay buffer (Fig. 4A). Protein and activity both eluted in two major peaks corresponding to apparent molecular weights (for spheres) of 100,000 and 220,000 and were recovered in 75% yield.

Cross-linking of the column fractions with glutaraldehyde followed by sodium borohydride (Fig. 4B) confirmed that the peak centered at fraction 54 consisted primarily of 70,000-mol-wt monomer while the peak centered at fraction 49 contained a species whose migration on SDS PAGE after

cross-linking was most consistent with a 140,000-mol-wt dimer. The possibility of a trimer could not be completely ruled out, however, because intramolecular cross-linking produces broad bands that migrate somewhat anomalously when compared to the uncross-linked standards. Differences in intramolecular cross-linking were probably also responsible for the doublet seen in cross-linked monomer fractions 51–57. Substantial amounts of a higher molecular weight multimer (probably tetramer) were seen in fractions 45 and 46.

All species show activity, indicating either that they are intrinsically active or that they can interconvert during the uncoating reaction. Incubating a pool of fractions 53–55 with ATP, ADP, or a mixture of the two nucleotides did not induce formation of detectable amounts of dimer (Fig. 4B, lanes A–C). There was no detectable molecular weight difference between the material in fraction 49 and that in fraction 54 when they were run on the gel without exposure to the cross-linking reagents (Fig. 4B, lanes D and E). Our preparation of 70,000-mol-wt polypeptide also focused as a single spot ($\text{pI} = 5.2$) upon two dimensional SDS PAGE (22; data not shown). Therefore, the dimer probably consists of two identical 70,000-mol-wt subunits.

Siegel and Monty (34) showed that the elution volume of a protein in gel filtration correlated better with Stokes radius than with molecular weight. Using their methods, Stokes radii of 39 and 59 Å were calculated for the monomer and dimer species respectively (Fig. 4A, inset).

The ATP-dependent release activity of uncoating ATPase was unaffected by treatment with *N*-ethylmaleimide at concentrations as high as 2 mM for 60 min at 25°C. No loss of activity was observed during incubation of uncoating ATPase at 37°C under assay conditions for up to 1 h.

Coated Vesicles and Clathrin Cages as Substrates for Uncoating ATPase

Uncoating ATPase was purified on the basis of its ATP-dependent ability to dissociate cages that consisted almost exclusively of clathrin heavy and light chains. Such cages are artificial substrates representing only the outermost shell of coated vesicles. Does the pure protein retain the ability of the crude cytosol (23) to uncoat coated vesicles? Pure uncoating ATPase was incubated with calf brain coated vesicles in the presence of ATP, and an ATP regenerating system, the latter needed to overcome ATPases present in coated vesicle fractions (31). The coated vesicles were indeed dissociated, but the rate of release of clathrin from coated vesicles was about $\frac{1}{4}$ of that of clathrin cages tested under the same conditions, in which initial rates are being measured (Fig. 5). Although the initial rate of release of clathrin from coated vesicles is slower than from cages, the extent is the same, given sufficient

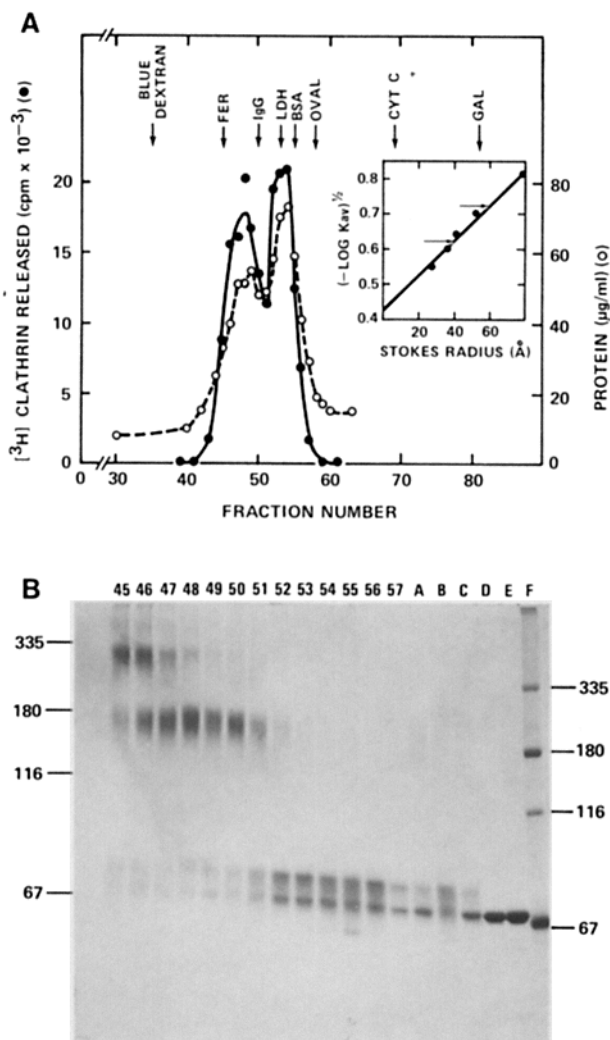


FIGURE 4 Sephacryl S-300 gel filtration of uncoating ATPase. (A) Elution profile of uncoating activity and protein. A total of 0.19 mg of purified uncoating ATPase in 0.1 ml of buffer C was applied to a 25-ml column (0.8 × 50 cm) of Sephacryl S-300 and eluted with buffer C at a flow rate of 2.8 ml/h. Fractions of 0.3 ml were collected. Aliquots of each fraction were assayed for protein (50 μl) and for activity (15 μl). Assays contained 7.5 μg of ³H-empty cages (45,000 cpm). Recovery of both protein and activity was ~75%. The vertical arrows show the peak elution positions of several reference standards which were (from left to right): blue dextran 2000 (V_0), horse spleen ferritin (700,000 mol wt; R_s = 79 Å), rabbit IgG (150,000 mol wt; R_s = 52 Å); rabbit muscle LDH (140,000 mol wt; R_s = 41 Å), bovine serum albumin (67,000 mol wt; R_s = 36 Å); ovalbumin (43,500 mol wt; R_s = 28 Å), cytochrome c (12,000 mol wt; R_s = 17 Å), and [³H]galactose (V_i). The inset shows a plot of $(-\log K_{AV})^{1/2}$ against Stokes radius, allowing an estimate of the Stokes radii of the species in the two peaks by the method of Siegel and Monty (33). (B) Cross-linking of uncoating protein in column fractions. A total of 6 μg of protein from each fraction was diluted to 150 μl with buffer C and incubated successively with 16 mM glutaraldehyde (2 min at 30°C), 80 mM NaBH₄ (20 min at 4°C), and 200 mM Tris-HCl, pH 7.4 (5 min at 4°C). Protein was then precipitated with 10% trichloroacetic acid in the presence of 0.2% Triton X-100 carrier, electrophoresed on a 5–10% polyacrylamide linear gradient gel, and stained with Coomassie R-250. The numbered lanes correspond to the column fractions 45–57. (Lanes A–C) A pool of the monomer form (fractions 53–55) was incubated with 100 μM ATP (A), 100 μM ADP (B), or 100 μM ATP and 100 μM ADP (C) for 15 min at 37°C before cross-linking. (Lane D) A dimer fraction (49), not cross-linked. (Lane E) A monomer fraction (54), not cross-linked.

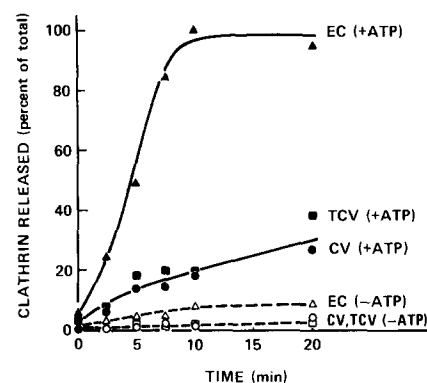


FIGURE 5 Release of clathrin from coated vesicles by uncoating ATPase. Preincubated calf brain coated vesicles (CV), 140 μg; Triton X-100 extracted coated vesicles (TCVs; see reference 26, 140 μg); and empty clathrin cages (EC), 105 μg, were each incubated with uncoating ATPase (3.5 μg) in 350 μl of buffer C either in the absence of ATP (–ATP) or in the presence of 0.7 mM ATP and an ATP regenerating system (+ATP) (22). Assays with TCVs additionally contained 0.5% Triton X-100. At the indicated times, 50 μl samples were removed from the reactions, stopped, and processed as described for the standard release assay. A 60-μl sample of each supernatant was electrophoresed on a 10% polyacrylamide gel. Densitometer tracings of the Coomassie-stained gels allowed comparison of the amount of clathrin in each supernatant with the amount added to the incubation (electrophoresed in parallel). Control lanes containing known amounts of column-purified clathrin confirmed that densitometer response was linear with clathrin. Clathrin release in a 20-min incubation containing coated vesicles and ATP but no uncoating enzyme was 4.7% of the amount added. (▲) EC (+ATP); (●) CV (+ATP); (■) TCV (+ATP); (△) EC (–ATP); (○) CV (–ATP); (□) TCV (–ATP).

time and uncoating protein. For example, the incubations used in Fig. 5 contained uncoating enzyme at a level of 0.025 μg/μg coated vesicle protein. In parallel incubations containing 0.15 μg enzyme/μg coated vesicles, clathrin release was 86% in 15 min, consistent with our earlier results for crude cytosol (23). Also, as observed previously with crude cytosol, release via the pure uncoating protein was highly selective for clathrin and its light chains. For example, when 80% of the clathrin had been released from coated vesicles, only ~15% of the 100,000- and 50,000-mol-wt family of polypeptides had been released (not shown).

To test the possibility that ATP-generated ion gradients across the vesicle membrane might in some way be needed for uncoating from coated vesicles, we employed Triton X-100-extracted coated vesicles (TCVs) as substrate. These lose most of their membrane lipid but retain all of the major proteins (27). TCVs were equivalent to intact coated vesicles in the release assay, even when assayed in the presence of Triton X-100 (Fig. 5). Duramycin, an inhibitor of the ATP-dependent proton pump of coated vesicles (9, 35, 36) also had no effect on uncoating when used at levels up to 50 μg/100 μg of coated vesicles, an amount sufficient to inhibit ATP-dependent proton translocation ~80% (36).

The initial rate of release of clathrin increased with cage concentration until a saturation was achieved (Fig. 6A); half-saturation required 0.14 mg/ml of cages. For cages containing

(Lane F) Markers, which were bovine serum albumin (67), *E. coli* β-galactosidase (116), clathrin (180), and thyroglobulin (335). Molecular weights, × 10³.

36 clathrin trimers (7) this corresponds to 7.2 nM cages. Clathrin is likely to be present at this or higher concentrations in cells.

Nucleotide Requirements for Clathrin Release from Cages

The initial rate of clathrin release via the pure uncoating protein was measured as a function of the concentration of ATP (Fig. 6B). A double reciprocal plot of the data (Fig. 6B, inset) yielded a K_m of 0.9 μ M (19).

Of all the nucleotides tested (Table II), ATP was uniquely effective. Other ribonucleotides and deoxy-ATP did not substitute for ATP, even at millimolar levels, at which point traces of contaminating ATP become significant. Substantial clathrin release was observed with 1 mM of two nonhydrolyzable analogues AMP-PNP and ATP- γ -S (but not AMP-PCP). The ability of these two analogues to release clathrin was largely resistant to hexokinase and glucose treatment (Table II) under conditions in which added radioactive ATP is quantitatively converted to ADP (data not shown). This suggests that the observed release is a direct effect of the analogues and not traces of contaminating ATP. ADP and AMP were inactive.

pH Dependence of Clathrin Release

Because both coated vesicles and clathrin cages are known to dissociate spontaneously on exposure to pH levels 7.5 and above (15, 16, 40, 43, 46, 47), the pH dependence of spontaneous and ATP-dependent clathrin release from empty cages and coated vesicles was carefully investigated. Spontaneous release of clathrin from empty cages incubated in the absence of ATP was quite substantial above pH 7.0 (Fig. 7A, open circles). Release in the presence of ATP increased rapidly above pH 6.6 (Fig. 7A, closed circles). The ATP-dependent component of release (Fig. 7A, inset) was maximal between

TABLE II
Nucleotide Specificity of Clathrin Release from Cages

Nucleotide	Concentration			
	10 μ M	100 μ M	1,000 μ M	1,000 μ M plus hexokinase and glucose*
ATP	0.95	[1]	1.05	<0.05
dATP	0.12	0.05	ND*	ND
GTP	<0.05	0.16	0.12	<0.05
CTP	<0.05	0.23	0.16	<0.05
UTP	<0.05	0.07	0.06	<0.05
ADP	<0.05	<0.05	0.12	<0.05
AMP	<0.05	<0.05	<0.05	<0.05
AMP-PNP	<0.05	<0.05	0.59	0.32 [§]
AMP-PCP	<0.05	<0.05	<0.05	<0.05
ATP- γ -S	<0.05	<0.05	0.32	0.20

Each result is the average of two independent assays containing 0.5 μ g of purified uncoating ATPase and 10 μ g of 3 H-empty cages. Clathrin released under various conditions is expressed as a fraction of the amount released in a parallel control incubation containing 100 μ M ATP.

* Incubations contained 10 U/ml hexokinase and 5 mM glucose in addition to 1,000 μ M of the indicated nucleotide or analogue.

† Not determined.

§ Control experiments showed that 1 mM ATP-PNP did not inhibit the ability of the hexokinase/glucose cocktail to degrade as little as 1 μ M ATP in 30 s at 37°C.

pH 6.8 and 7.2. The pH dependence of clathrin release from coated vesicles was similar to that observed with empty cages, except that for coated vesicles release in the absence of ATP did not become substantial until pH 7.4 and above (Fig. 7B). The data in Fig. 7B reflect initial rates of clathrin release and thus differ somewhat from our previous data on the pH dependence of clathrin release (reference 23, Fig. 2) where the extent of release was measured at the various pH values.

Dependence of Clathrin Release upon Monovalent and Divalent Cations

A broad optimum was observed for KCl (Fig. 8A), and release was stimulated by including up to 10 mM $(\text{NH}_4)_2\text{SO}_4$ (Fig. 8B, filled circles). This was an effect of NH_4^+ and not SO_4^{2-} as NH_4Cl gave similar results (Fig. 8B, open circles). Since release assays contained no membranes, the effect of NH_4^+ did not involve any change in pH gradients. Rather, stimulation was direct and probably independent of the well known mechanisms (6) by which NH_4^+ acts *in vivo*.

Mg^{++} was inhibitory >1 mM (Fig. 9). However, Mg^{++} was only required in amounts about equivalent with ATP (ATP was 50 μ M in Fig. 9), suggesting that the major role played by Mg^{++} lies in its chelation to ATP. Manganese was similar although inhibition began at 0.5 mM and was more precipitous. Calcium ion did not substitute for Mg^{++} at any concentration.

Properties of Clathrin Released from Cages via Uncoating ATPase

What is the oligomeric state of the clathrin released from cages via the uncoating protein and ATP? At one extreme, clathrin could be released as fragments of cages that simply do not sediment under the rather arbitrary conditions employed in the assay. At the other extreme, an ATP-dependent process could release clathrin as monomers rather than as the trimers ("triskelions") produced when physical-chemical conditions favor spontaneous cage disassembly.

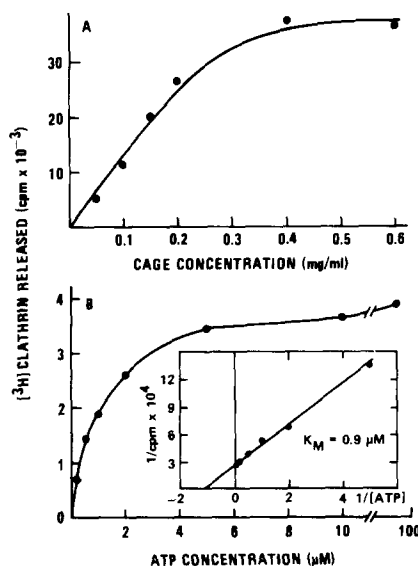


FIGURE 6 Dependence of clathrin release on substrate concentrations. (A) Concentration of cages. Assays contained 0.75 μ g of uncoating ATPase and the indicated level of 3 H-empty cages (5780 cpm/ μ g). (B) Concentration of ATP. Assays contained 0.5 μ g of uncoating ATPase, 10 μ g 3 H-empty cages (17,500 cpm) and the indicated level of ATP. The inset shows a replot of the data according to the method of Lineweaver and Burk (18).

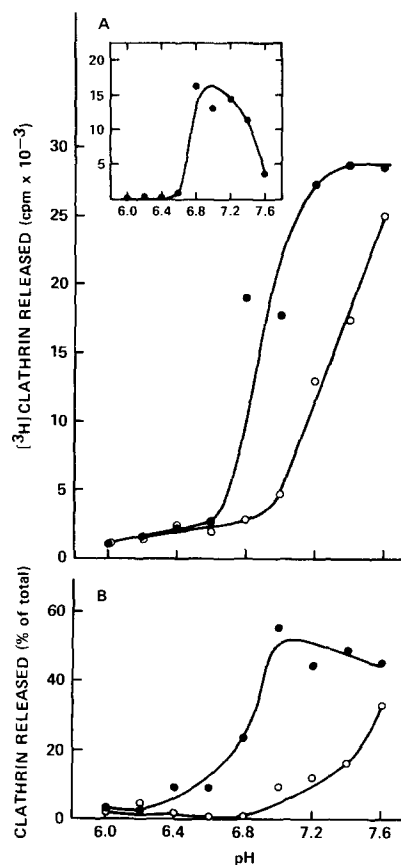


FIGURE 7 pH Dependence of the initial rate of uncoating. (A) Empty cages. Assays in which the buffer C was adjusted to the indicated pH value contained 0.7 μg of uncoating ATPase and 10 μg of ^3H -empty cages (45,000 cpm). For pH values below pH 6.8, the HEPES in buffer C was replaced by 20 mM PIPES titrated to the appropriate pH. Control experiments showed that PIPES itself did not affect the assay. Incubations at 37°C for 5 min were carried out in the presence of 50 μM ATP (●) or the presence of 10 U/ml hexokinase and 5 mM glucose (○). The inset shows the net, ATP-dependent portion of the clathrin release (the difference between the two curves). (B) Coated vesicles. Assays in which the buffer C was adjusted to the indicated pH value contained 1.2 μg of uncoating ATPase and 10 μg of coated vesicles. For pH values below pH 6.8, the HEPES in buffer C was replaced by 20 mM MES titrated to the appropriate pH. Control experiments showed that MES itself did not affect the assay. Incubations at 37°C for 10 min were carried out in the presence of 50 μM ATP and an ATP regenerating system (●) or the presence of 10 U/ml hexokinase and 5 mM glucose (○). Release of clathrin into the supernatant was quantitated by SDS PAGE and densitometry as described in the legend to Fig. 5. Clathrin release in the presence of ATP but no uncoating enzyme did not differ significantly from that observed in the presence of uncoating enzyme but no ATP.

Two lines of evidence demonstrate that the end product of ATP-dependent release is a trimer of clathrin. First, the released clathrin eluted from Sepharose 4B as a symmetrical peak in the position of clathrin triskelions (Fig. 10, filled circles). Second, when material in this peak was examined in the electron microscope after rotary shadowing (38) only trimeric structures were observed (Fig. 11).

To our surprise, these ATP-released trimers contained stoichiometric amounts of bound uncoating ATPase. About half of the uncoating protein (Fig. 10, open circles) co-eluted with the clathrin trimers, evidently bound to them. The ratio of

uncoating ATPase monomer to clathrin heavy chain determined by scanning gels of these fractions was constant across the clathrin peak (Fig. 10, inset). A stoichiometry of about 1.4 70,000-mol-wt uncoating protein for every 180,000-mol-wt clathrin heavy chain can be calculated from these data, assuming that the two species stain equally per unit of mass

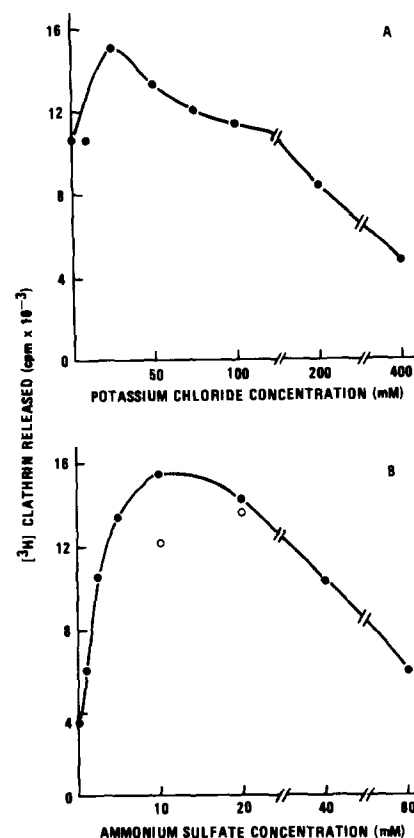


FIGURE 8 Effects of monovalent cations. (A) Effect of KCl. Assays in which the KCl concentration of the buffer C was adjusted to the indicated level contained 0.75 μg of uncoating ATPase and 10 μg ^3H -empty cages (40,900 cpm). (B) Effect of ammonium. Assays in which the $(\text{NH}_4)_2\text{SO}_4$ concentration of the buffer C was adjusted to the indicated level contained 0.75 μg uncoating ATPase and 10 μg ^3H -empty cages (40,900 cpm). The open circles show the ^3H -clathrin release observed when NH_4Cl was substituted for $(\text{NH}_4)_2\text{SO}_4$ at the 10- and 20-mM levels.

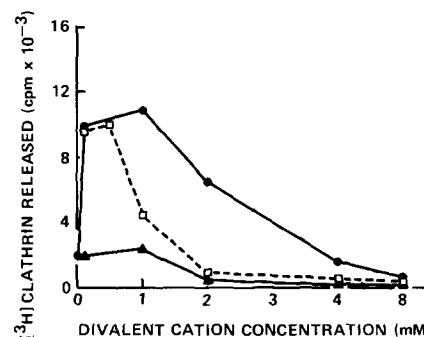


FIGURE 9 Requirement for divalent cations. Assays in which the standard 2 mM $\text{Mg}(\text{CH}_3\text{COO})_2$ in the buffer C was replaced with the indicated level of $\text{Mg}(\text{CH}_3\text{COO})_2$ (●), MnCl_2 (□), or CaCl_2 (▲) contained 0.7 μg of uncoating ATPase and 10 μg ^3H -empty cages (39,300 cpm). Assays labeled "0 mM" divalent cation contained 2 mM EDTA.

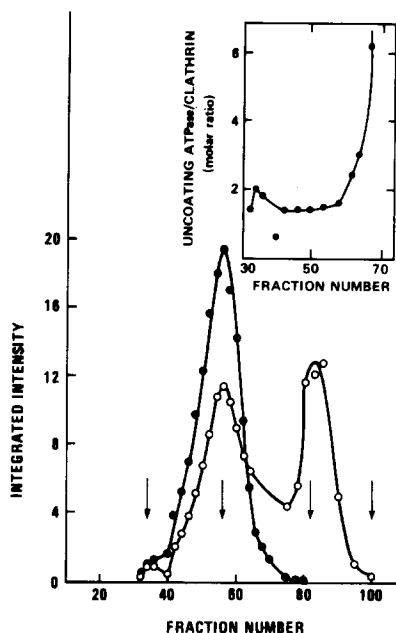


FIGURE 10 Separation of the products of an uncoating reaction on Sepharose 4B. Unlabeled empty cages (4.3 mg/ml) were incubated with uncoating ATPase (1.3 mg/ml) and 0.7 mM ATP and an ATP regenerating system (22) in a total volume of 0.35 ml of buffer B for 30 min at 37°C. The reaction was stopped by addition of hexokinase and glucose and centrifuged at 95,000 g_{av} for 10 min in the airfuge. The supernatant (containing 90% of the added protein) was applied to a 1-X-30-cm column of Sepharose 4B equilibrated in buffer B and eluted at 2.1 ml/h at 4°C while collecting 0.26-ml fractions. A 100- μ l aliquot of each fraction was precipitated with 10% trichloroacetic acid in the presence of 0.2% Triton X-100 as carrier. The redissolved, neutralized precipitates were electrophoresed on 10% polyacrylamide gels and the content of clathrin (180,000-mol-wt band) and uncoating ATPase (70,000-mol-wt band) in each fraction was quantitated from densitometer tracings of the Coomassie-stained gels. The vertical arrows indicate the peak elution positions of several reference standards which were (from left to right): purified calf brain coated vesicles chromatographed in buffer A (V_0), authentic clathrin triskelions chromatographed in buffer F, uncoating ATPase chromatographed in buffer B, and ATP chromatographed in buffer B (V_1). The inset shows the ratio of stain in the uncoating ATPase band to stain in the clathrin heavy chain band in the column fractions, expressed as a molar ratio (i.e., normalized to molecular weights).

with Coomassie Blue. The clathrin light chains also co-eluted with the heavy chains, and were recovered in the expected amounts (1 mol light chain per mole heavy chain). Although the incubation analyzed in Fig. 10 contained 0.3 μ g of uncoating ATPase/ μ g clathrin before gel filtration, the input ratio of uncoating protein to clathrin could be varied over the range of 0.05 to 0.4 without substantially changing the result (data not shown).

It is probably significant that the clathrin trimers with stoichiometric amounts of bound uncoating protein eluted from Sepharose 4B in the same place as free triskelions. Evidently, the uncoating protein is positioned in the complex so as to minimally perturb its shape. Though the appearance of the complex in the electron microscope (Fig. 11) was similar to that already published for free triskelions (16, 39), released trimers not infrequently had a bulge at their vertex, possibly representing bound uncoating ATPase (Fig. 11, arrows). This position at the vertex is the same region already documented

as the location of the clathrin light chains (17, 42). However, triskelions prepared by standard chemical methods did not show such a bulge, even though their full complement of light chains was present (data not shown).

Properties of Released Clathrin Complexes

Can uncoating ATPase-clathrin complexes spontaneously assemble into closed cages, as free triskelions do? Complexes were dialyzed against buffer A under our standard conditions for the reconstitution of empty cages and then sedimented in the airfuge. About half the protein assembled into structures that could be pelleted (Table III). These structures appeared as large amorphous extended aggregates rather than closed cages when viewed in the electron microscope (not shown) as in earlier experiments (23).

The aggregates were very unstable under assay conditions. More than 85% dissociated upon incubation in assay buffer (buffer C) for 10 min at 37°C. By contrast, only 37% of empty cages dissociated under the same conditions (Table III). More than half of the authentic clathrin cages survived two successive incubations; only 10% of the aggregates of uncoating protein-clathrin complexes were this stable. Indeed, the small fraction of aggregates that were stable to repeated incubations contained essentially no uncoating ATPase (Table III) and thus represented a subpopulation perhaps equivalent to pure clathrin.

These experiments show that the complexes lack the ability of clathrin to form regular, closed cages at pH 6.2, and that at neutral pH the complexes have little or no tendency to self-associate. In fact, uncoating ATPase-clathrin complexes interfere with the ability of free triskelions to assemble properly. Cages and aggregates electrophorese differently in agarose gels (Fig. 12, compare lanes D and E), as reported earlier (23). When mixtures of complexes and triskelions were dialyzed into pH 6.2 assembly buffer, the two species co-polymerized into aggregates of intermediate electrophoretic mobility whose value was determined by their content of enzyme-clathrin complexes (Fig. 12, compare lanes B, C, and D). Evidently the complexes can interact with triskelions at acidic pH, but do so in a defective manner.

These data with purified uncoating ATPase confirm the behavior documented in our earlier report (23) for clathrin released from coated vesicles by ATP and crude brain cytosol and discussed in terms of an ATP-dependent "modification" of clathrin's assembly properties. It now appears likely that this "modification" of clathrin consists of the noncovalent binding of stoichiometric amounts of the uncoating protein during ATP-dependent release. However, we have not yet established whether dissociation of the bound uncoating protein restores native assembly properties to clathrin. It is not inconceivable that additional ATP-dependent changes in the clathrin trimer may be present as well and account for the modified assembly properties.

DISCUSSION

This paper describes the purification from bovine brain of a 70,000-mol-wt polypeptide that can dissociate clathrin coats in an ATP-dependent fashion. As shown in the next paper, this uncoating protein hydrolyzes ATP in the process of uncoating. To connote these properties, we have named this protein "uncoating ATPase." Uncoating ATPase presumably facilitates intracellular transport in cells through its action

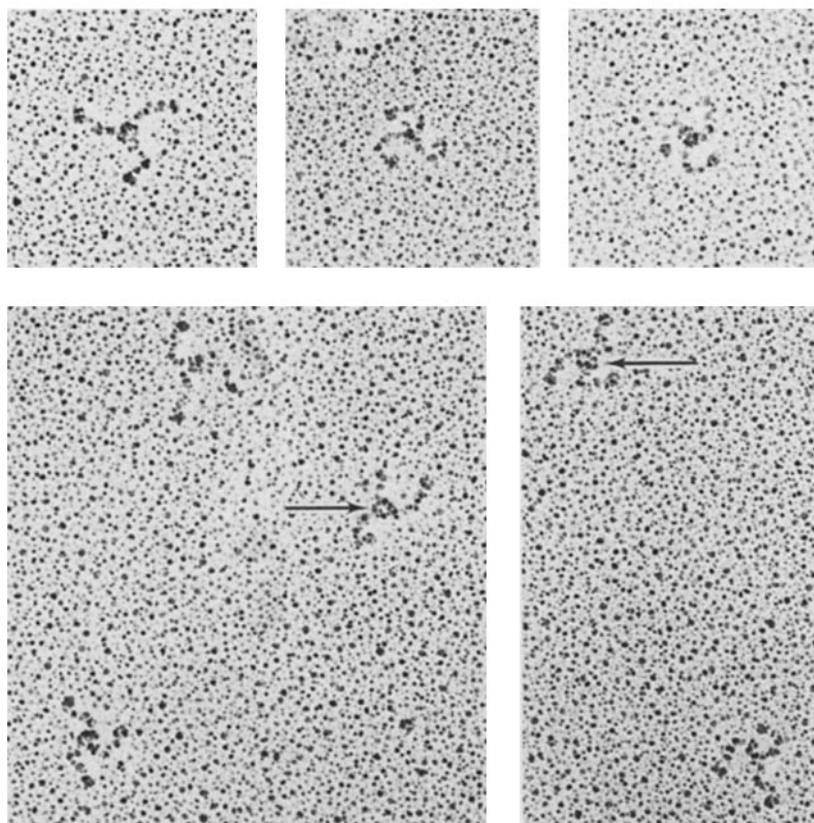


FIGURE 11 Electron micrographs of uncoating ATPase-clathrin complexes. The complexes were prepared by Sepharose 4B chromatography as in Fig. 10. The fractions containing complexes were pooled, concentrated by precipitation with $(\text{NH}_4)_2\text{SO}_4$ (50% of saturation), and dissolved in buffer B. The molar ratio of 70,000-mol-wt uncoating protein to 180,000-mol-wt clathrin was 1.1 in this sample, as determined by densitometry of a Coomassie-stained SDS polyacrylamide gel. Samples were dried from glycerol, rotary shadowed with platinum as described by Tyler and Branton (37), viewed in the electron microscope, and photographed at a magnification of 146,000. Arrows indicate a bulge seen at the vertex of some of the triskelions, possibly representing bound uncoating ATPase.

TABLE III
Relative Stability of Clathrin Cages and Aggregates of
Clathrin-uncoating Protein Complexes

Step	Percent of sedimentable protein		Molar ratio of 70,000-mol-wt uncoating protein to 180,000-mol-wt clathrin heavy chain	
	Cages	Aggregates	Cages	Aggregates
	%			
Before dialysis	0	0	0	1.4
After dialysis	70	53	0	1.2
After first incubation	44.5	8.9	0	0.6
After second incubation	38.6	5.7	0	<0.1

Complexes of uncoating protein and released clathrin were isolated, pooled, and precipitated as described under Figs. 10 and 11, except that the $(\text{NH}_4)_2\text{SO}_4$ pellet was redissolved in buffer F. Free triskelions were prepared in parallel by dissociation of standard empty cages in buffer F. Each sample was diluted to 1 mg/ml protein and dialyzed against buffer A for 16 h at 4°C to form either cages (from free triskelions) or aggregates (from uncoating protein-clathrin complexes). Sedimentation in the airfuge and the first incubation in buffer C were as described under Materials and Methods for preincubation of cages. The second incubation was for 5 min at 37°C, and had 0.1 mg/ml of cages or aggregates in buffer C.

upon clathrin. We have also detected this activity in cytosol from rat and bovine liver, primary chick embryo fibroblasts, Chinese hamster ovary cells, *Drosophila* Kc cells, and yeast (data not shown). We have attempted to demonstrate uncoating activity in membrane fractions, but have not detected any (data not shown).

Several lines of evidence imply that the major 70,000-mol-wt polypeptide chain in our preparations is active in uncoat-

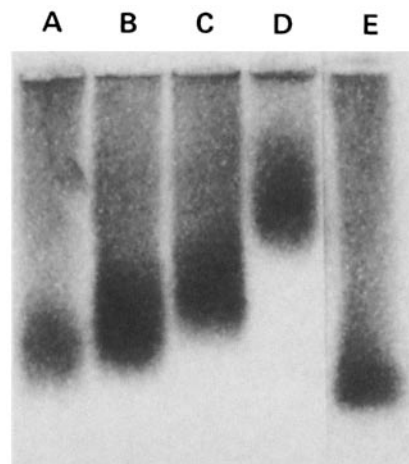


FIGURE 12 Agarose gel electrophoresis of cages, aggregates of uncoating protein-clathrin complexes, and mixtures. Complexes were isolated, pooled, and precipitated as described in Figs. 10 and 11, except that the $(\text{NH}_4)_2\text{SO}_4$ pellet was dissolved in 10 mM Tris-HCl (pH 8.0). The ratio of 70,000-mol-wt uncoating polypeptide to 180,000-mol-wt clathrin was 1.5 in this preparation. Standard triskelions in 10 mM Tris-HCl (pH 8) were mixed in varying proportions with uncoating protein-clathrin complexes to achieve a final concentration of 0.5 mg/ml, and then dialyzed against buffer A for 16 h at 4°C. Assembled structures were pelleted by centrifugation at 95,000 g_{av} for 10 min in the airfuge, resuspended in buffer A, and 15- μ g samples were loaded onto a 0.15% agarose gel and electrophoresed at 4°C for 40 h at 0.75 V/cm as described by Rubenstein et al. (30). The gel was air dried onto Whatman 3 MM paper and then stained with Coomassie R-250. (Lanes B, C, D, and E) Structures assembled from mixtures containing 25%, 50%, 100%, and 0% uncoating protein-clathrin complexes, respectively. (Lane A) A control in which standard triskelions were dialyzed in the presence of 1 mg/ml bovine serum albumin.

ing. First, this polypeptide enriches with activity during the purification (Fig. 3) and coincides with activity on Sephacryl S-300 gel filtration (Fig. 4); although whether the monomer or dimer or both represents the primary active form remains to be determined. Second, this same 70,000-mol-wt protein is bound in molar amounts to the released clathrin (Fig. 10). This same polypeptide is also selectively bound in stoichiometric amounts from crude cytosol to coated vesicles during their uncoating (see Fig. 1, reference 23). Third, as illustrated in the accompanying paper, this 70,000-mol-wt polypeptide has an ATP binding site whose affinity and specificity is the same as that of the ATP-dependent activity that releases clathrin. Fourth, stable complexes of ATP with this protein can be prepared; the ATP in this complex is promptly hydrolyzed when clathrin cages are added (5).

It was because of its surprising abundance that we were especially concerned to establish that we had indeed purified the right protein. About 4 mg of uncoating ATPase were obtained from ~500 gm of calf brain with an apparent yield of 7%. It can be calculated that this corresponds to ~115 mg of uncoating protein per kilogram of brain tissue, roughly 0.1% of total protein. By way of comparison, Pearse (24, 25) estimates that clathrin also comprises ~0.1% of bovine brain protein. One possible explanation for the approximate equivalence of uncoating ATPase and clathrin is that much of clathrin in cells is sequestered in the form of uncoating ATPase-clathrin complexes (as identified in Fig. 10) rather than in coated pits or coated vesicles. This "soluble" form of clathrin may be the pool used in assembling coated vesicles. Louvard et al. (20) have recently presented immunocytochemical evidence for just such a pool. Experiments are in progress to determine whether clathrin in a soluble pool is associated with uncoating ATPase.

The uncoating protein was purified on the basis of its ability to dissociate clathrin cages in an ATP-dependent fashion. The pure protein retains the capacity of crude cytosol (23) to uncoat coated vesicles. However, further data will be required to determine whether this represents the protein's major activity in intact cells. Conceivably, however, this protein may function in cells in other capacities. For example, extensive rearrangement of clathrin lattices (such as transitions from hexamers to pentamers) may need to occur during coated vesicle budding (12). The protein we have purified may catalyze such rearrangements in addition to (or even instead of) its postulated role in uncoating.

The endproduct of ATP-dependent uncoating, mediated by the uncoating ATPase ("enzymatic" uncoating), is a clathrin trimer; clathrin trimers are also released by perturbations in physical-chemical conditions, such as changes in pH ("spontaneous" uncoating). The products of enzymatic and spontaneous uncoating differ in that the former contains bound uncoating ATPase and does not self-assemble into regular cages, whereas the latter lacks accessory protein and assembles correctly.

The conditions in the clathrin release assay were selected to maximize the ATP-dependent release of clathrin from an empty cage substrate by any given amount of uncoating ATPase. The relative efficiencies of coated vesicles and clathrin cages as substrates in the assay may partly reflect this selection. In addition, we do not yet know whether all of the events involved in the uncoating protein-facilitated disassembly of a cage are enzymatic. For example, it may be that only a few ATP-dependent enzymatic "hits" are needed to break

the cage into inherently unstable fragments that then dissociate spontaneously into triskelions. The rates of these spontaneous, secondary dissociations would depend upon prevailing conditions such as pH and divalent cation concentration (14–16, 42, 45, 46) so that the number of enzymatic "hits" needed to disassemble a cage might be greater the more inherently stable the cage. Alternatively, or in addition, clathrin triskelions dissociated at only one or two of the three legs may snap back into the cage structure, increasing the number of hits needed to effect a complete release. Conditions increasing cage stability could slow release due to both of these effects, perhaps explaining why clathrin is released more slowly from coated vesicles than from empty clathrin cages (Fig. 5), and why lower pH and higher Mg^{++} slow the rate of release (Figs. 7 and 9). The empty cages used contain only clathrin and light chains and will be less stable than coated vesicles whose clathrin is additionally held in place by interactions with other coat proteins (such as the 100,000-mol-wt polypeptide family) and the vesicle membrane. Clearly, however, intact membrane permeability barriers are not required for uncoating, since treatment of coated vesicles with Triton X-100 has little effect on this process (Fig. 5).

Is the uncoating "ATPase" in fact an enzyme? As illustrated in the next paper, the uncoating protein is literally an enzyme in the sense that a single uncoating protein can hydrolyze hundreds of molecules of ATP, especially under conditions that stabilize cages against spontaneous dissociation. But it would also appear, at least superficially, that the uncoating protein is consumed during uncoating, as it can be recovered stoichiometrically bound to clathrin (Fig. 10). It seems only reasonable to assume that the uncoating protein acts in repeated rounds of dissociation in cells. We do not yet know if, under the conditions we have employed in vitro, each uncoating ATPase can only participate in only one round or whether it is recycled.

Elucidation of the properties of the complex of clathrin and uncoating ATPase that can now be isolated in milligram quantities (Fig. 10) should help clarify this issue. This complex may be the carrier of clathrin in the cytoplasm. Additional components may be needed to recycle uncoating ATPase from complexes, freeing the clathrin for assembly. If located on membranes, these components might serve to ensure an efficient coupling of clathrin assembly to vesicle budding. Using the complexes as substrates, it may now be possible to construct functional assays to search such for the molecules involved in later steps in the clathrin-coated vesicle cycle.

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