Dissociation of Clathrin Coats Coupled to the Hydrolysis of ATP: Role of an Uncoating ATPase

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ABSTRACT   ATP hydrolysis was used to power the enzymatic release of clathrin from coated vesicles. The 70,000-mol-wt protein, purified on the basis of its ATP-dependent ability to disassemble clathrin cages, was found to possess a clathrin-dependent ATPase activity. Hydrolysis was specific for ATP; neither dATP nor other ribonucleotide triphosphates would either substitute for ATP or inhibit the hydrolysis of ATP in the presence of clathrin cages. The ATPase activity is elicited by clathrin in the form of assembled cages, but not by clathrin trimers, the product of cage disassembly. The 70,000-mol-wt polypeptide, but not clathrin, was labeled by ATP in photochemical cross-linking, indicating that the hydrolytic site for ATP resides on the uncoating protein. Conditions of low pH or high magnesium concentration uncouple ATP hydrolysis from clathrin release, as ATP is hydrolyzed although essentially no clathrin is released. This suggests that the recognition event triggering clathrin-dependent ATP hydrolysis occurs in the absence of clathrin release, and presumably precedes such release.

An important step in interorganelle transport is the removal of the clathrin coat from freshly budded coated vesicles. This facilitates the eventual fusion of the contained vesicle with its target membrane and also liberates the clathrin for use in further rounds of coated vesicle budding (1, 5, 15, 17, 24). The accompanying paper (18) described the purification of a protein capable of removing clathrin from coated vesicles. The uncoating protein will also dissociate artificial cages made only of clathrin and its light chains. Disassembly requires micromolar levels of ATP; moreover, nonhydrolyzable analogues of ATP show little ability to support this enzymatic uncoating (14, 18). These results suggest that nucleotide hydrolysis is needed to power disassembly of clathrin.

Any enzyme that efficiently couples ATP hydrolysis to another process will hydrolyze ATP only when all of the substrates needed for the reaction are present. Therefore, we have investigated whether the uncoating protein possesses an ATPase activity that is manifest only in the presence of its other substrate, clathrin cages.

MATERIALS AND METHODS

Materials: The materials used and manufacturers they were obtained from were as follows: [2-^{3}H]ATP (20 Ci/mmol) from Amersham Corp., (Arlington Heights, IL); [α-^{32}P]ATP (400 Ci/mmol), [γ-^{32}P]ATP (3,000 Ci/mmol) from New England Nuclear (Boston, MA); ATP, GTP, CTP, UTP, ADP, and dATP from P-L Biochemical Inc. (Milwaukee, WI); ATP analogues (AMP-

PNP, AMP-PCP, ATP[γ]S)[1] from Boehringer Mannheim Biochemicals (Indianapolis, IN); Elastase from Worthington Enzymes (Freehold, NJ); Polygram CEL300 PEI and thin layer chromatography plates from Machery-Nagel and Co. (Düren, Federal Republic of Germany).

Preparation of Washed Cages for ATPase Assays: Cages were washed with Triton X-100 to remove a contaminating ATPase activity. These washed cages retained their ability to be dissociated by uncoating protein in the presence of ATP. To do this, clathrin, purified as described in the previous paper (18), was dialyzed at a protein concentration of 1 mg/ml into buffer A (20 mM 2-(N-morpholino)ethane sulfonic acid buffer, pH 6.2, 2 mM CaCl$_2$, 1 mM EDTA) for 14–16 hr at 4°C to generate empty clathrin cages (6, 20). These cages were then incubated at 0.5 mg/ml protein in buffer A containing 0.5% Triton X-100 for 30 min at 4°C, and the stable cages remaining were centrifuged for 90 min at 25,000 rpm in an SW50.1 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 4°C. This pellet of washed clathrin cages was resuspended in buffer B (75 mM KCl, 40 mM HEPES buffer, pH 7.0, 4.5 mM Mg-acetate, 0.8 mM dithiothreitol) at 1 mg/ml protein, and then incubated 10 min at 37°C to dissociate poorly assembled aggregates, and then centrifuged for 10 min in a Beckman A 30/100 airfuge rotor (Beckman Instruments, Inc.) at 95,000 g_{av}. This pellet was resuspended in buffer A at the desired concentration and used as substrate in ATPase assays.

Clathrin-dependent ATPase Assay: The method employed was a modification of the method of Kornberg et al. (8). A typical complete incubation (20 μl) contained, in buffer C (25 mM KCl, 20 mM HEPES buffer, pH 7.0, 10 mM [NH$_4$]$_2$SO$_4$, 2 mM Mg-acetate, 0.8 mM dithiothreitol): 0.2 mg/ml of Trison X-100–washed clathrin cages, 0.5 μCi of [2-^{3}H]ATP, 20 μM unlabeled

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ATP, and the uncoating protein fraction to be tested (0.1–0.8 μg of purified protein). Controls consisted of omitting cages or omitting uncoating protein from the incubations. The time course of hydrolysis at 37°C was followed by applying 1 μl samples to polyethyleneimine cellulose thin layer chromatography plates that had been pre-spotted with 0.5 μl of a mixture of carrier nucleotides (10 mM each of ATP, ADP and AMP). Plates were developed in 0.7 M LiCl/ 1 M HCOOH buffer, and dried. The ATP, ADP, and AMP spots were located with UV light, excised, and counted in Aquasol II scintillation fluid (New England Nuclear). The radioactivity in each spot were expressed as a fraction of the total recovered in each lane. The fractions of ³H present as AMP and ADP contaminants in the unincubated [³H]ATP substrate were determined, and subtracted as background. The specific (clathrin-dependent) hydrolysis was defined as hydrolysis in mixtures of cages and uncoating protein greater than the sum of hydrolysis with the two alone. The cage alone and uncoating protein alone values were determined from parallel control incubations in which one or the other component was omitted. This specific hydrolysis generally exceeded the sum of the rates in the two controls by a factor of about 8–10. The fraction of ATP hydrolyzed in a clathrin-dependent fashion was thus calculated, and converted to picomoles. Fig. 1 shows this raw data presenting the complete and both control incubations. For other figures, only the specific hydrolysis (the complete minus the sum of the two controls) is presented.

Affinity Labeling with ATP: The procedure used was a modification of the method of Yue and Schimmel (28). A typical incubation contained between 1 and 10 μg protein in 25 μl of buffer C, 10 μCi of [α-³²P]ATP, and 1 μM unlabeled ATP. UV irradiation was performed at 4°C for 20 min with a 1 mW/cm² exposure rate from a General Electric G15T8 15-W germicidal lamp. Samples were then precipitated for SDS-PAGE analysis with 10% trichloroacetic acid, with 20 μg of cytochrome c added as carrier. The pellets were dissolved in sample buffer, neutralized with Tris base, and analyzed by SDS PAGE according to Laemmli (9) using a 10% polyacrylamide gel. Gels were stained for protein with Coomassie R-250 dye, dried, and autoradiographed using Kodak XAR-5 film and Du Pont Cronex intensifying screens. Exposures of ~1 d were typically required.

RESULTS

Clathrin-dependent Hydrolysis of ATP by Uncoating ATPase

To examine whether ATP is hydrolyzed under conditions in which ATP-dependent disassembly of cages occurs, we added [2-³H]ATP to standard incubations containing clathrin cages and the purified uncoating protein (Fig. 1). Samples were analyzed by polyethyleneimine-cellulose thin layer chromatography as described by Kornberg et al. (8), separating ATP, ADP, and AMP. Control incubations containing uncoating protein but not clathrin cages, or vice-versa, showed very little ATP hydrolysis. But, complete incubations containing both uncoating protein and clathrin cages did hydrolyze ATP (Fig. 1, closed circles). Thus, ATP is hydrolyzed only when both cages and uncoating protein are present, i.e., when disassembly is taking place. The products of this ATP hydrolysis appear to be exclusively ADP and free P_i, since all of the ³H released from ATP co-migrated with ADP on thin layer plates. Likewise, all of the ³²P released from ATP when [γ-³²P]ATP was employed as substrate chromatographed with P_i (not shown). As coated vesicles contain a membrane-associated ATPase (27) that obscures the activity of uncoating ATPase, ATP hydrolysis data were not obtainable with coated vesicles as substrate.

The specific or "clathrin-dependent" ATP hydrolysis was calculated by subtracting from the total ATP hydrolyzed the small amounts due to cages and uncoating protein alone. This quantity, pertinent to the uncoating process, was linear with time at early times of incubation, and with added uncoating enzyme up to 10 μg/ml (Fig. 2). The complex kinetics at later times and higher protein concentrations probably reflect changes in the clathrin substrate resulting from disassembly. The data in the previous paper (18) indicate that 80–90% of the clathrin has been released from cage substrate at the times and enzyme levels at which these curves become nonlinear.

The initial rate of cage-dependent ATP hydrolysis followed simple Michaelis-Menton kinetics with respect to ATP and empty cage concentrations (Fig. 3). The K_m values for ATP
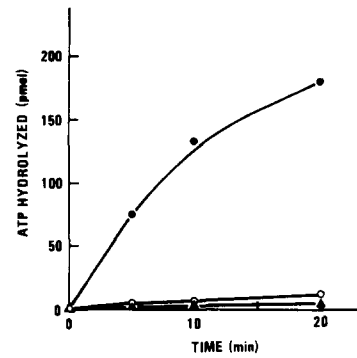

FIGURE 1 Clathrin cage-dependent ATPase assay. Uncoating protein (0.8 μg) was incubated at 37°C in 20 μl volume of buffer D (buffer C, except 20 mM MES buffer at 6.75 was substituted for HEPES) containing also 20 μM ATP, 0.5 μCi of [2³H]ATP, and 0.2 mg/ml of Triton X-100–washed clathrin cages. Samples (1 μl) were removed and analyzed by thin layer chromatography as described in Materials and Methods. For this graph, the raw data for ATP hydrolysis are shown for the complete incubation (●) and also for the two control incubations in which either cages (▲) or uncoating ATPase (○) were omitted. In later figures, the curves shown are obtained by subracting the sum of the two controls (i.e., the expected hydrolysis if the components were additive) from the results for the complete incubation. Plotted is the total amount of ATP (picomoles) hydrolyzed in the 20-μl incubation vs. time.
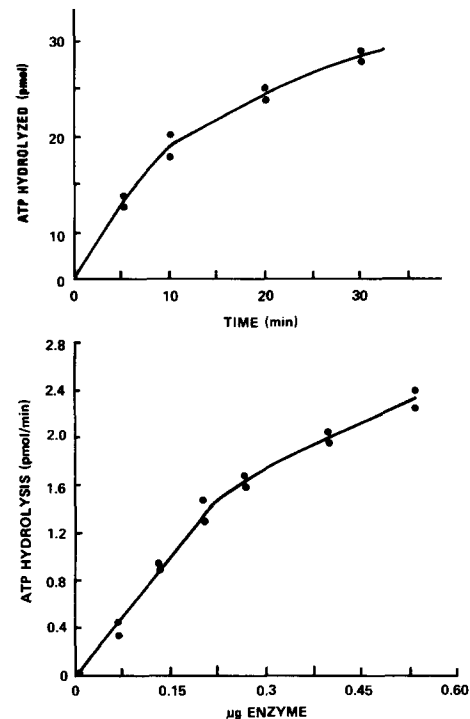

FIGURE 2 Clathrin-dependent ATPase as a function of time and of uncoating protein concentration. (*Upper* panel) Time course. Incubation was performed in 20 μl buffer C at 37°C, using 0.2 μg enzyme, 8 μM ATP, 0.5 μCi [2-³H]ATP, and 0.2 mg/ml Triton X-100–treated clathrin cages per assay. The clathrin-dependent ATPase was calculated from raw data as described in Materials and Methods, and is shown here. (*Lower* panel) Concentration dependence. Conditions as above, but for 10 min at 37°C with variable amounts of uncoating protein added to each 20-μl incubation.

and clathrin cages were 0.7 μM and 0.12 mg/ml (~0.8 μM clathrin, corresponding to 7 nM of cages), respectively. These values were in very good agreement with the K_m values for these substrates derived from the ATP-dependent release of clathrin (18). Under conditions in which clathrin and ATP are saturating, the turnover number of the ATPase, calculated from Fig. 3, is about 1 ATP per 70,000-mol-wt polypeptide chain per min at pH 7.0, 2 mM Mg^{++}, 37°C.

The rate of ATP hydrolysis was unaffected when uncoating protein was treated with 5 mM n-ethylmaleimide at 25°C for 60 min. Thus, ATPase and uncoating activity (18) are both n-ethylmaleimide–resistant.

Co-purification of Uncoating and Clathrin-dependent ATPase Activities

The activities of protein fractions were monitored for ATP-dependent release of clathrin from cages and for clathrin cage-
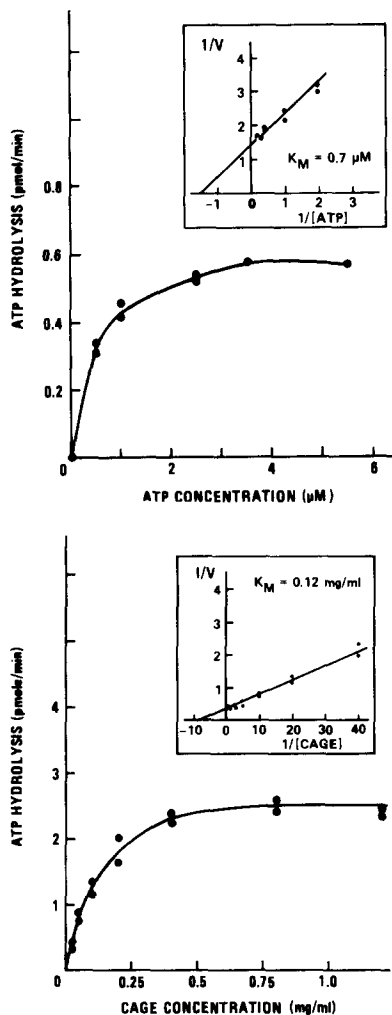

FIGURE 3 Clathrin-dependent ATPase as a function of the concentrations of ATP and of clathrin cages. (*Upper* panel) ATP. The effect of varying ATP concentration on initial rate of ATP hydrolysis is depicted, with the Lineweaver-Burke plot (10) of the data in the inset. Incubations were performed in Buffer C for 10 minutes at 37°, with 2.5 μg/ml uncoating ATPase and 0.5 mg/ml Triton-washed clathrin cages. (*Lower* panel) Clathrin cages. The effect of varying clathrin cage concentration on initial rate of ATP hydrolysis with the Lineweaver-Burke plot of the data in the inset. Incubations were performed in buffer C for 10 min at 37°C, with 4 μg/ml uncoating ATPase.

TABLE I

ATPase Activity Co-purifies with Uncoating Activity

Purification step	Specific uncoating activity	Specific ATPase activity	Purification per step	
			Uncoating	ATPase
	μ/mg	pmol/min/ mg		
Cytosol	352	47	[1]	[1]
DE-52	1,050	152	3.0	3.2
Hydroxylapatite	2,140	323	2.0	2.1
ATP-Sepharose	13,400	2,267	6.3	6.7

Uncoating assays were performed and specific activity calculated as described in the previous paper (18); ATPase assays were performed as described in Materials and Methods. Specific activities for ATPase were calculated as pmole ATP hydrolyzed per minute per mg enzyme protein as measured by the method of Bradford (2). The purification steps shown are described in the previous paper (18).

TABLE II

Inhibition of Uncoating and Clathrin-dependent ATPase Activities by Nucleotides and Nucleotide Analogues

Inhibitor*	Uncoating activity (percent of control)	Cage-dependent ATPase activity (percent of control)
	%	%
None (control)‡	[100]	[100]
300 μM GTP	70	65
300 μM CTP	95	87
300 μM UTP	90	100
300 μM dATP	100	91
300 μM ADP	7	7
300 μM AMP-PCP	100	89
300 μM AMP-PNP	68	58
300 μM ATP(γ)S	50	27

* Uncoating assays, performed as described in the previous paper (18) contained 0.8 μg uncoating ATPase enzyme per reaction. Cage-dependent ATPase assays were performed as described in Materials and Methods for 10 min at 37°C, using 0.1 μg uncoating ATPase per assay. In all cases, reactions were performed using 0.1 mg/ml cage substrate and 7.5 μM ATP, so that all inhibitors were used at 40-fold molar excess over ATP levels.
‡ Contained 7.5 μM ATP and no other nucleotide.

dependent ATPase (Table I) during a purification of the uncoating protein. At each step essentially the same degree of purification of the two different activities was obtained. Their co-purification makes it likely that both activities reside in the 70,000-mol-wt polypeptide brought to near homogeneity by this purification.

Specificity of the ATPase for ATP

How selective is the clathrin-dependent ATPase for ATP? The abilities of various nucleotides and their analogues to inhibit the clathrin-dependent hydrolysis of ATP is shown in Table I, and compared with the ability of these same compounds to inhibit the ATP-dependent release of clathrin from cages. The two different assays were performed under identical buffer and substrate conditions, using time and enzyme levels in the linear range of the assays. Only ADP was found to be effective in inhibiting either assay when added in 40-fold excess of ATP (Table II). The other ribonucleoside triphosphates, and dATP had little or no inhibitory effect.

Since CTP, UTP, ATP and dATP did not inhibit ATP hydrolysis, it can also be concluded that they themselves are not hydrolyzed by the uncoating ATPase in a clathrin-de-

pendent manner. This was confirmed directly in the cases of CTP, UTP, GTP, and dATP using radioactive nucleotide as substrate in place of [³H]ATP (data not shown).

The nonhydrolyzable nucleotide analogues had comparable effects upon the two assays: for both clathrin-dependent ATP hydrolysis and clathrin release, AMP-PCP had little effect, AMP-PNP was moderately inhibitory, and ATP-(γ)S inhibited strongly at 40-fold excess levels over ATP.

Uncoating Protein But Not Clathrin Contains a High Affinity Binding Site Specific for ATP

The above results demonstrate that ATP is hydrolyzed only when cages and uncoating protein are present. The active site of ATP hydrolysis interacts with ATP with high (0.7 μM) affinity. Which molecule, clathrin or uncoating protein, is the ATPase? Conceivably, uncoating protein could cause clathrin to bind and hydrolyze ATP, or vice versa. To distinguish these possibilities, incubations containing [α-³²P]ATP were subjected to a photo-cross-linking procedure capable of forming covalent bonds between ATP and proteins (4, 12, 28). The method employed, first described by Yue and Schimmel (28), has two advantages. It is specific for nucleotides bound tightly to a protein, and it does not require the use of a nucleotide analogue to effect cross-linking.

A complete incubation (Fig. 4, lane *J*) or those containing only uncoating protein (lane *C*) or clathrin cages (lane *A*) were irradiated with UV light in the presence of 1 μM [α-³²P]ATP and analyzed by SDS PAGE. No labeling of clathrin or its light chains was observed (Fig. 4, lanes *A* and *J*), but the uncoating protein of 70,000 mol wt was strongly labeled whether or not clathrin was present. The UV-induced labeling of uncoating protein was prevented by the inclusion of 30 or 100 μM unlabeled ATP, or by 100 μM unlabeled ADP, during irradiation (Fig. 4, lanes *D*, *E*, and *F*). However, additions of a 100-fold excess of GTP, CTP, UPT, or dATP had little effect on labeling by [³²P]ATP (Fig. 4, lanes *G*, *H*, *I*, and *K*). These results demonstrate a high affinity binding site specific for ATP in the 70,000-mol-wt polypeptide, but not in clathrin (even during uncoating), at ATP concentrations relevant to the uncoating activity. As observed for the clathrin-dependent ATPase and clathrin releasing activities (Table II), only ADP was an effective inhibitor of the photolabeling of 70,000-mol-wt polypeptide by ATP. The appearance of the 70,000-mol-wt species as an apparent doublet in autoradiography, but not by Coomassie staining, was probably an artifact of the cross-linking procedure, involving an alteration of the electrophoretic mobility of the labeled protein by intramolecular cross-links. This was observed for the bulk protein in the cross-linking experiment of the previous paper (18).

Preparation of an ATP–Uncoating Protein Complex for Active Site Titration in an Initial Burst Experiment

To demonstrate directly that the clathrin-dependent ATPase resides in the 70,000-mol-wt polypeptide chain, we prepared a complex of ATP bound to uncoating protein, and then asked whether this bound ATP was hydrolyzed when clathrin cages were added. This experiment also illustrates the role of this complex as an intermediate in the reaction pathway, and titrates the minimum number of active sites in the uncoating ATPase preparation.

Uncoating protein was incubated with [³H]ATP, and then freed of unbound ATP by gel filtration on Sephadex G-25. The void volume contained ~0.42 mol of nucleotide per mole of 70,000-mol-wt uncoating polypeptide chain. This complex was stable under assay conditions because the bound ATP remained intact during incubations with excess hexokinase and glucose (Fig. 5, upper panel, closed squares). An equivalent amount of free ATP was very rapidly hydrolyzed by hexokinase in a parallel control experiment (Fig. 5, closed circles).

When this ATP-uncoating protein complex was incubated with clathrin cages, the ATP was promptly and nearly quan-
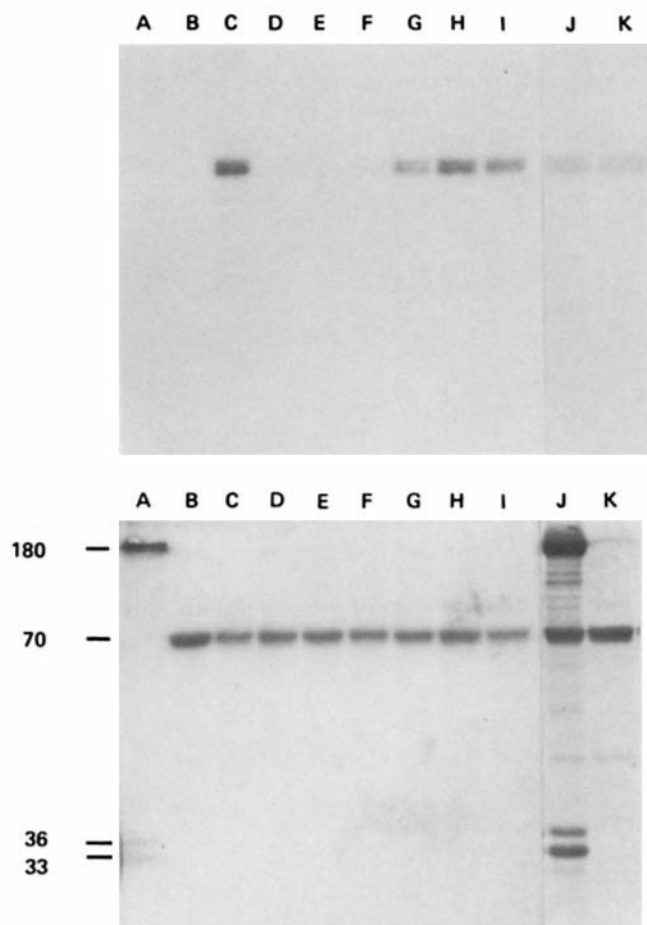

FIGURE 4   Affinity labeling of uncoating protein with [α-³²P]ATP. Proteins were irradiated with ultraviolet light and [α-³²P]ATP as described in Materials and Methods, with the indicated components present. The reactions were analyzed by SDS PAGE and stained for protein (*lower* panel) before autoradiography (*upper* panel). Lanes *B–K* have 4 μg uncoating protein each. Lanes: (A) 20 μg clathrin, with UV illumination. (B) No ultraviolet illumination. (C) With UV illumination. (D) Irradiated, with 30 μM unlabeled ATP added. (E) Irradiated, with 100 μM unlabeled ATP added. (F) Irradiated, with 100 μM unlabeled ADP added. (G) Irradiated, with 100 μM unlabeled GTP added. H, Irradiated, with 100 μM unlabeled CTP added. (I) Irradiated, with 100 μM unlabeled UTP added. (J) 4 μg uncoating protein with 20 μg clathrin cages, with illumination. (K) 4 μg uncoating protein irradiated, with 100 μM unlabeled dATP added. Lanes *J* and *K* are from a different experiment than *A–I* in which exposure time was shorter. Molecular weights are indicated for the 180,000-mol-wt clathrin heavy chain (16, 21) for the 70,000-mol-wt polypeptide, and for the 36,000- and 33,000-mol-wt clathrin light chains (3, 11) on the lower panel.
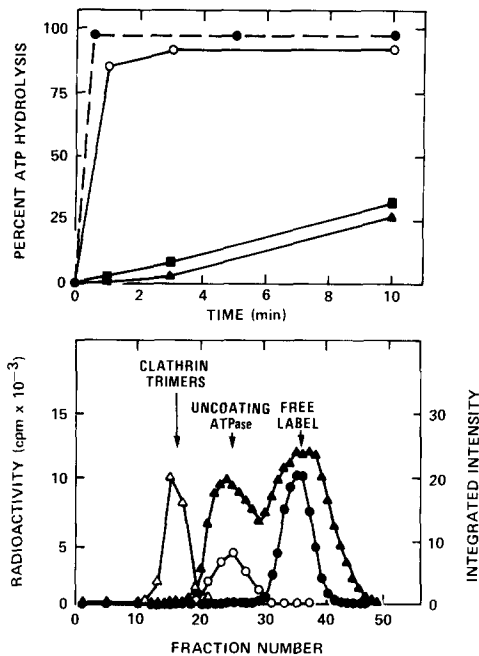
FIGURE 5 Specific hydrolysis of ATP in enzyme·ATP complex. (*Upper* panel) Titration of active sites and products of a single hydrolytic step of uncoating ATPase. Uncoating protein (77 μg) was incubated for 3 min at 37°C, 0.7 mg/ml in buffer C with a mixture of unlabeled ATP, [2-^{3}H]ATP, and [γ-^{32}P]ATP. The total ATP concentration was 6 μM and the final specific radioactivities of ^{3}H and ^{32}P were 16 Ci/mmol and 2.5 Ci/mmol, respectively. Unbound ATP was removed by centrifugation at 4°C through a 0.5-x-6-cm Sephadex G-25 column equilibrated in buffer C. This was accomplished by applying the incubation to the column (after having pre-centrifuged the column at 1,000 g for 5 min to remove the void volume) and then collecting the desalted complexes obtained as the flow-through of a similar centrifugation. These enzyme-ATP complexes were eluted at a protein concentration of 0.22 mg/ml, and contained ^{3}H and ^{32}P corresponding to 0.42 mol nucleotide per mole of 70,000-mol-wt polypeptide of the bound nucleotide, 67% was ATP and the remainder ADP. These uncoating protein-ATP complexes were then incubated at 37°C in buffer C at a concentration of 75 μg/ml protein either alone (▲), with 300 μg/ml yeast hexokinase plus 10 mM glucose (■), or with 0.6 mg/ml Triton-washed clathrin cages (○). In parallel, an equivalent concentration of free [2-^{3}H]ATP, 1.2 μM with a specific activity of 16 Ci/mmol, was incubated at 37°C in buffer C with 300 μg/ml yeast hexokinase and 10 mM glucose (●). 1-μl samples were removed at the indicated times for thin layer chromatographic analysis as described in Materials and Methods. The percent of the ATP isolated in the complexes that had been hydrolyzed during incubation is plotted as a function of time. (*Bottom* panel) An entire incubation of enzyme-ATP complex with Triton-washed clathrin cages (15 mm at 37°C) was loaded onto a 0.7 × 26 cm Biogel A0.5M column and eluted at 2 ml/h with buffer C, collecting 0.25-ml fractions. The elution profile of clathrin heavy chain (△) and the 70,000-mol-wt uncoating polypeptide (○) was determined by SDS PAGE, Coomassie staining, and densitometry. ^{3}H (▲) and ^{32}P (●) radioactivity were also measured, representing the products of hydrolysis, [^{3}H]ADP and ^{32}P$_i$.

titatively hydrolyzed to ADP (Fig. 5, upper panel, open circles). Very little hydrolysis occurred when the complex was incubated by itself (Fig. 5, closed triangles). Adding hexokinase plus glucose along with clathrin cages to the complex during incubation did not change the rate of ATP hydrolysis observed (not shown). This shows that the ATP molecule must be hydrolyzed in the same site at which it was bound in the complex with uncoating protein.

This experiment also serves to titrate the minimum number of active sites in the uncoating protein preparation. Over 90% of the bound ATP was hydrolyzed in the burst, corresponding to 0.42 mol of ATP per mole of 70,000 mol wt polypeptide. Therefore, at least 42% of these protein molecules must have been catalyzing clathrin-dependent ATP hydrolysis. Given this high value, it seems certain that the protein active in uncoating and ATP hydrolysis is the major 70,000-mol-wt species.

Clathrin Is Released Free of Nucleotide or Phosphate Derived from ATP

What is the fate of the ADP and P$_i$ generated from ATP hydrolyzed in a round of uncoating? Is any linked to the released clathrin, either covalently or noncovalently? A complex of uncoating ATPase with a mixture of [2-^{3}H]ATP and [γ-^{32}P]ATP was prepared, and incubated with clathrin cages. The whole incubation was then chromatographed on Biogel A0.5M to separate the products of the reaction to enable the fate of the nucleotide to be determined (Fig. 5, lower panel). Tritium, marking the position of the ADP product and the small amount of remaining ATP, was found bound to the uncoating protein as well as free in the included volume. Phosphorous radiolabel, representing trace residual ATP and the P$_i$ product, was exclusively free in the included volume. No ^{3}H or ^{32}P was detected in the fractions that contained clathrin triskelions. We estimate from this data that <0.002 mol of ATP, ADP, or P$_i$ are bound (covalently or noncovalently) per mole of clathrin heavy chain in these fractions. No uncoating enzyme was found associated with triskelions in the gel filtration profile. Evidently, hydrolysis of the stoichiometric levels of ATP present in these reactions is not sufficient to generate the isolable enzyme-triskelion complexes observed after enzymatic release in the previous paper (18).

All of the ^{3}H associated with the peak of uncoating protein was noncovalently bound, as all of the isotope was acid-soluble and also was not associated with the 70,000-mol-wt polypeptide after SDS gel electrophoresis. A total of 0.15 mol of ^{3}H-adenine nucleotide was bound per mole of 70,000-mol-wt polypeptide in these fractions, of which >95% was ADP, as determined by polyethyleneimine-cellulose thin layer chromatography. The remaining [^{3}H]ADP label, like the phosphorous label, was present as free label in the included volume.

Clathrin Packed in a Cage Is the Preferred Substrate for Uncoating ATPase

Does the packing arrangement of clathrin influence its ability to be recognized as substrate by uncoating ATPase? The ATPase assay, unlike the clathrin release assay, affords an opportunity to investigate this issue. Clathrin cages and triskelions can be tested for their ability to elicit the ATPase activity of uncoating protein under the same conditions. This can be accomplished because free triskelions do not assemble into cages to a significant extent under the conditions (pH 7.0) employed in the assay (13, 23). As shown in Fig. 6, cages elicit ATPase activity as expected. But clathrin trimers, prepared from these same cages, were at least 15 times less effective, when incubated at the same protein concentration in the same buffer. Moreover, when these triskelions were reassembled into cages by dialysis into buffer A, their ability to elicit ATP hydrolysis from the enzyme was completely restored (Fig. 6). Thus, the arrangement of clathrin triskelions
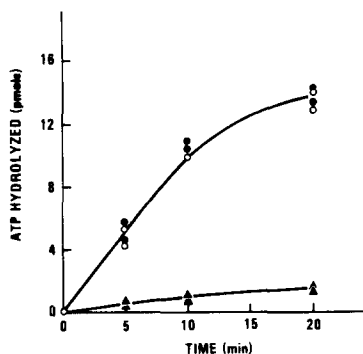
FIGURE 6 Specificity of uncoating ATPase for assembled clathrin trimers. Assays were performed in 20 μl buffer C at 37°C. Each assay contained 10 μg/ml uncoating ATPase and 0.2 mg/ml clathrin protein (added as cages or triskelions). The clathrin cages substrates (●) had been washed with Triton X-100 and preincubated as usual (Materials and Methods). Free triskelions (▲) were prepared from these cages by incubation for 30 minutes at 4°C in 1 M Tris-HCl, pH 6.2, followed by dialysis into 5 mM Tris at pH 8.1. Reassembled cages (O) were prepared from these triskelions by dialysis into buffer A for 16 h at 4°C, followed by a preincubation of 10 min at 37°C in buffer B as described in Materials and Methods.

in a cage structure is clearly an important factor in the recognition of the clathrin substrate by uncoating ATPase. These seem to be appropriate properties for an enzyme whose substrate is assembled clathrin and whose product is disassembled clathrin disassembled clathrin, and would explain why the rate of ATP hydrolysis observed in Fig. 2 declined after most of the clathrin had been released.

Stoichiometry of ATP-dependent Clathrin Release

The ratio of the rate at which clathrin is released to the rate at which ATP is hydrolyzed defines the apparent stoichiometry of ATP-dependent clathrin release. Under assay conditions optimal for ATP-dependent clathrin release (buffer C, pH 7.0), this apparent stoichiometry is about 3 to 4 ATP hydrolyzed per trimer of clathrin released.[2] This may, in fact, represent a minimum estimate since some clathrin may be released in spontaneous events accompanying enzymatic "hits" (see Discussion of previous paper). It is important to recognize that this number may have no absolute significance, apart from the fact that it is small.

Indeed, ATP hydrolysis can be readily uncoupled from clathrin release. For example, the pH dependence of the

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[2] To calculate the number of ATP molecules hydrolyzed per clathrin triskelion released, parallel reactions were performed as described in Materials and Methods in buffer C, pH 7.0 (optimal conditions for clathrin release). Reactions contained 0.5 mg/ml Triton X-100–washed ^{3}H-clathrin cages, 20 μg/ml uncoating ATPase, and 7.5 μM ATP, either unlabeled or containing 25 μCi/ml [^{3}H]ATP. The rate of ATP hydrolysis was determined by thin layer chromatographic analysis of the reaction containing [^{3}H]ATP, as described in Materials and Methods. Radioactive clathrin, which remained at the origin, was resolved from the nucleotides by the developing solvent. Triskelion release was measured in the reaction containing unlabeled ATP, as described in the accompanying paper (18), and converted to moles using the known clathrin specific radioactivity. The ratio of ATP hydrolysis rate (pmol/min) to triskelion release rate (pmol/min) under these conditions was 3 to 4, i.e., about three to four ATP molecules are hydrolyzed for every clathrin trimer released.

clathrin-dependent ATPase (Fig. 7, top) is strikingly different from that reported for ATP-dependent clathrin release in the previous paper. Whereas, as shown in Fig. 7, top panel, the
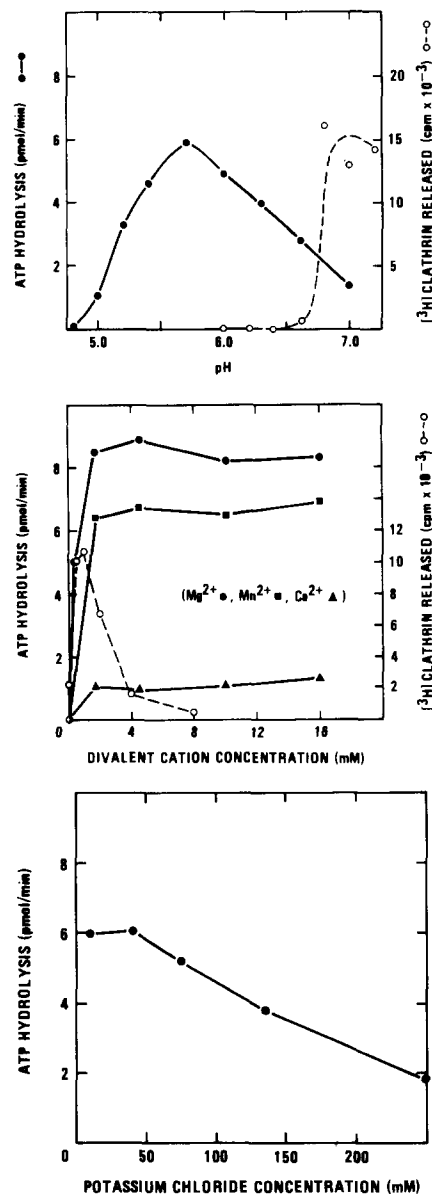

FIGURE 7 Effect of pH, divalent cations, and KCl concentration on the clathrin-dependent ATPase activity. Assays were performed for 10 min at 37°C in 20 μl containing 2.5 μg/ml uncoating protein and 0.25 mg/ml Triton X-100–washed clathrin cages as described in Materials and Methods. (Top panel) pH was varied in a buffer containing 40 mM KCl, 4.5 mM MgCl$_2$, and 20 mM 2-(N-morpholino)ethane sulfonic acid titrated to the appropriate pH. At any given pH, ATPase activity in 2-(N-morpholino)ethane sulfonic acid buffer was equivalent to that using HEPES buffer. The solid line indicates cage-dependent ATPase activity. The dashed line indicates the pH profile for ATP-dependent clathrin release determined earlier under the same conditions (18). (Middle panel) Divalent cation concentration was varied in incubations containing 40 mM KCl, 20 mM HEPES (pH 7.0), and 0.8 mM dithiothreitol, with clathrin and uncoating protein concentrations as in the top panel. The solid lines indicate cage-dependent ATPase activities for the cation shown. The dashed line indicates the clathrin release profile for Mg^{++} under similar conditions (18). (Bottom panel) KCl concentration was varied in a buffer containing 20 mM HEPES (pH 7.0), 4.5 mM MgCl$_2$, and 0.8 mM dithiothreitol, with uncoating and clathrin proteins as in the top panel.

release activity (open circles) declines rapidly below its optimum at pH 6.8, the ATPase (closed circles) actually increases an order of magnitude as pH falls, reaching an optimum near pH 5.7. Below pH 5.7, ATPase activity precipitously declines, probably reflecting denaturation of clathrin cage substrate. This interpretation is indicated because preincubation of cages of pH values below 5.7 reduces their effectiveness as substrate when assayed at pH 6.2, while preincubation of uncoating ATPase at these acid pHs was without effect on assays at pH 6.2.

A similar uncoupling of ATPase from release activity could be induced by adding high concentrations of divalent cations (Fig. 7, middle). The ATPase activity showed a requirement for divalent cation, with a rank order of $Mg^{++} > Mn^{++} > Ca^{++}$, as observed for the release activity in the previous paper (18). However, unlike the sharp optimum seen at low divalent cation concentrations for the uncoating activity (Fig. 7, middle, open circles) the cage-dependent ATPase activity level was essentially constant over a range of 1 to 16 mM divalent cation concentration. At these higher concentrations, ATP is maximally hydrolyzed unaccompanied by the release of clathrin.

When uncoupled by either method, several hundred ATPs are hydrolyzed per 70,000-mol-wt polypeptide in the course of a typical incubation of 10–20 min. This demonstrates that the uncoating protein ATPase can act catalytically with regard to ATP hydrolysis.

The effect of varying KCl on the ATPase activity (Fig. 7, bottom) was similar to that reported in the previous paper for the release activity.

DISCUSSION

The data reported in this paper suggest that ATP hydrolysis is used to power the removal of clathrin from coats. We have shown that ATP is hydrolyzed to ADP and P_i in the process of enzymatic uncoating, and that clathrin is free of the products of this hydrolysis. Several lines of evidence demonstrate that the 70,000-mol-wt uncoating protein, purified on the basis of its ability to dissociate clathrin cages, is responsible for this clathrin-dependent ATPase activity. First, the clathrin-dependent ATPase activity co-purifies at each step with the uncoating activity. Second, the intrinsic properties of the ATPase and uncoating activities are identical, making it quite probable that they represent alternative measures of a single catalytic sequence: the release of clathrin from coats coupled to the hydrolysis of ATP. Thus, the K_m values for ATP and clathrin cage substrates are, within experimental error, the same for both activities. The specificity for ATP is equally exacting in both cases. Of special note is the inability of dATP to promote clathrin release or to be hydrolyzed. Third, ATP but not dATP can be cross-linked to the 70,000-mol-wt uncoating protein, and the specificity of this binding site for nucleotide is the same as that for the uncoating and ATPase activities. Fourth, a stable complex of the 70,000-mol-wt uncoating protein with stoichiometric amounts of ATP can be prepared. This ATP is promptly hydrolyzed when clathrin cages are added.

We currently envision that the uncoating ATPase functions in cells by removing clathrin coats from coated vesicles (but see the discussion in accompanying paper [18] for other possibilities), thereby facilitating the eventual recycling of clathrin and baring the membrane surface to permit fusion of the transport vesicle. This scheme would work best if uncoat-

ing ATPase could recognize coated vesicles and ignore coated pits. In this regard, it is noteworthy that uncoating ATPase greatly prefers closed cages to free clathrin triskelions as substrates. It seems appropriate for a protein designed for uncoating to invest energy in a substrate that needs disassembly (cages) and not in products that are already disassembled (triskelions). The small amount of hydrolysis elicited by triskelions could result from transient associations between triskelions that are recognized by the enzyme.

Uncoating ATPase, as purified, consists of a mixture of monomers and dimers. It is not yet known which species interacts with cages or whether they interconvert during uncoating. A dimeric structure could help uncoating ATPase distinguish assembled from unassembled clathrin. When clathrin is packed into cages, sites of local dyad symmetry are created on the edges (15) that would represent natural binding sites for a symmetric protein dimer—sites that are missing from unassembled clathrin.

ATP hydrolysis by uncoating ATPase always requires the presence of clathrin cages, and is most efficiently used to release clathrin under approximately physiological conditions (1 mM Mg^{++}, pH 7). Clathrin release is never observed in the absence of ATP hydrolysis, even when ADP or the nonhydrolyzable ATP(γ)S are present in sufficient concentration to inhibit ATP-dependent release of clathrin by uncoating ATPase. But hydrolysis is readily uncoupled from release when the pH is lowered or the divalent cation concentration is raised. Indeed, conditions have been found (Fig. 7) wherein ATP is hydrolyzed maximally and essentially no clathrin is released, the ATP hydrolysis occurring in an apparently futile manner. Thus, ATP hydrolysis cannot solely be occurring subsequent to the actual release of a triskelion from a cage. Additionally, the decline of the rate of ATP hydrolysis seen as cage disassembly nears completion (Fig. 2) and the inability of free triskelions to elicit ATP hydrolysis (Fig. 6) suggest that clathrin, once released, loses the ability to elicit ATP hydrolysis from the enzyme. This occurs despite the fact that after clathrin release, enzyme can be found associated with the triskelions (18).

Preliminary evidence suggests that more than one ATP is used, in more than one step, in forming the isolable enzyme-clathrin complexes during the uncoating of clathrin cages. This explains why no such complexes were observed in the experiment of Fig. 4, where the stoichiometric levels of ATP present were completely hydrolyzed to ADP during clathrin release.

As noted in the previous paper (18), the conditions most conducive to enzymatic clathrin release are also those that tend to destabilize clathrin-clathrin interactions within the cage structure (23, 25, 26). We suggested that when physical-chemical conditions favor spontaneous disassembly of cages, fewer enzymatic "hits" will be necessary to promote net disassembly. Likewise, when conditions stabilize clathrin cages, more hits may be needed. Presumably, each enzymatic hit requires the hydrolysis of ATP, so the number of ATPs hydrolyzed per clathrin released would be expected to increase as conditions stabilize cages, as we observe when pH is lowered or Mg^{++} is raised (Fig. 7). When cages are stable, most hits will be futile as the clathrin can "snap-back" readily into position in the cage.

Indeed, it is probable that the simultaneous efforts of several enzyme molecules or successive catalytic events by a single enzyme molecule are needed to release a clathrin trimer. The

inner segments of triskelions in cages interact with those of three nearest-neighbor triskelions (7), suggesting that all three associations might have to be disrupted simultaneously to effect release. Interactions with the outer segments of legs from three next-nearest neighbor triskelions are not needed to maintain stable cages (19, 22) but may need to be broken for release. These considerations would certainly account for the requirement for at least three ATP molecules to be hydrolyzed for every clathrin trimer released, even under optimal conditions.

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