Assembly Polypeptides from Coated Vesicles Mediate Reassembly of Unique Clathrin Coats

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ABSTRACT A protein activity has been identified in extracts of coated vesicles that enables purified clathrin triskelions to reassemble in vitro into coat structures of uniform size. Coats formed in the presence of this preparation, regardless of the buffer system employed, are uniform in size with a mean diameter of 78 nm (±5 nm SD) and a sedimentation coefficient ($s_{20,w}$) of ~250S. Analysis of the reassembled coats on dodecyl sulfate acrylamide gels reveals that they have specifically incorporated three polypeptides from the preparation: those of $M_r \simeq$ 52,000, 100,000, and 110,000. The 52,000-, 100,000-, and 110,000-mol-wt polypeptides are incorporated in molar ratios of 0.85, 1.11, and 0.26, respectively, per three clathrin monomers (equivalent to one triskelion). We therefore designate these as assembly polypeptides (AP). In contrast, coats formed from clathrin alone, under permissive buffer conditions, are larger (400S), more heterogeneous in size (101 nm ± 15 nm SD), and are composed only of clathrin and its associated light chains. These biochemical and biophysical characteristics distinguish AP-reassembled coats from coats formed by triskelions alone.

AP-reassembled coats can be isolated, dissociated, then reassembled in the absence of any other factors. This recycling indicates that all the information needed for reassembly is present in the coat-incorporated polypeptides themselves. Reassembly is stoichiometric and saturable with respect to both clathrin and AP concentration. In the presence of AP, significant coat reassembly occurs at clathrin concentrations as low as 0.06 mg/ml. AP-mediated reassembly proceeds at 4°, 22°, and 37°C. Coat formation also proceeds efficiently at intracellular pH values (7.2–7.5) in the presence of AP. In its absence, reassembly does not occur at all above pH 6.7. In summary, AP promotes clathrin reassembly into coat structures of uniform size and distinctive composition under physiologically relevant salt, temperature, and pH conditions. In addition, the close similarity in size between AP-reassembled coats in vitro and coated membranes in the Golgi region in vivo raises the possibility that AP in the cell may be associated with this subpopulation of coat structures.

Coated vesicles are unique subcellular organelles found in virtually all eucaryotic cells. Their distinguishing feature, the bristle coat seen on the cytoplasmic surface of the membrane, was first described by Roth and Porter (41), who suggested that these structures play a role in the selective uptake of specific macromolecules. Since then, numerous studies have established the pathways of receptor-mediated endocytosis for a variety of ligands and described the steps involving coated membranes (for reviews, see references 10, 31, 36, 42). In brief, ligand-occupied plasma membrane receptors cluster in localized patches of membrane, on the cytoplasmic side of which are visible the characteristic bristle coat (coated pits and membrane). By a process incompletely understood, these ligands are internalized into the cell and are rapidly observed in smooth-surfaced vesicles. Coated pit endocytosis is clearly a general pathway since different ligands have been detected in the same structure (4, 17, 19, 26, 52).

Coated membranes also participate in other intracellular functions. Coated membranes have been detected on Golgi cisternae (9) and are thought to mediate the transport of products between intracellular compartments, as in the transport of maternal immunoglobulins (1) and the processing of the vesicular stomatitis virus G-glycoprotein (43, 44). Additional roles for coated membranes in membrane recycling are

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indicated in the retrieval of secretory vesicle membranes after exocytosis (8, 11). These observations suggest that the coated membrane is a dynamic intracellular organelle, perhaps undergoing facile assembly-disassembly reactions in vivo.

Electron microscopy of purified coated vesicles or of freeze-etched fibroblasts revealed that the coat appears as a polygonal latticework surrounding the vesicle (12, 16). Pearse (33) obtained purified coated vesicles from bovine brain and showed that the coat structure was comprised of one major polypeptide, which she named clathrin ($M_r \cong 180{,}000$). Subsequent investigators have extracted the coat material into solution and obtained highly purified clathrin (17, 46, 54). Under conditions of low salt and slightly acid pH, purified clathrin can form reassembled coats (17, 20, 29, 40, 46, 51, 53). The coat structure of these enclosed coats resembles that of coated vesicles, but without the underlying vesicle membrane.

We have previously shown (17, 18) that extraction of coated vesicles with 0.5 M Tris-Cl at neutral pH released clathrin and other coated vesicle proteins. Gel filtration of this extract resolved clathrin from a fraction containing partially purified assembly polypeptides (AP).[1] When clathrin and AP were combined and dialyzed to remove dissociating Tris-Cl salts, reassembled coat structure were formed. In this report, we extend these observations by characterizing and quantitating the clathrin-AP reaction. Our data indicate that coats formed in the presence of AP are of a strikingly uniform diameter, are characterized by a single symmetric peak in sedimentation velocity experiments, and are composed of clathrin and stoichiometric amounts of three of the polypeptides from the AP pool. Significantly, the AP-mediated reassembly was found to proceed efficiently under conditions approximating cellular pH and ionic strength. An abstract of this study has been presented in November, 1982 (55).

MATERIALS AND METHODS

Buffers: Vesicle isolation buffer (buffer A) consisted of 0.1 M NaMES (2-[*N*-morpholino]ethane sulfonic acid), 1.0 mM EGTA, 0.5 mM $MgCl_2$, and 0.02% sodium azide, pH 6.5. Coats were extracted from purified coated vesicles in buffer B, a 1:1 (vol/vol) mixture of buffer A and 1.0 M Tris-Cl, pH 7.0. In some reassembly procedures, a buffer containing 5 mM NaMES, 2 mM $CaCl_2$, pH 6.2 (buffer C), was used.

Clathrin Purification: Coated vesicles were purified from calf brains, extracted, and chromatographed as previously described (17). A column packed with Sepahrose CL-4B (1.9 × 94.0 cm) was used to resolve clathrin and AP (*vide infra*). Ascending chromatography with buffer B at 12 ml/h and at room temperature was employed. Fractions of 3 ml were collected and clathrin and partially purified AP were obtained by pooling fractions 31–36 and 41–44, respectively (Fig. 1).

Coat Reassembly: Coat reassembly was performed by dialysis of column-purified clathrin with or without AP against buffer A at 4°C and pH 6.5, unless otherwise noted. After overnight dialysis, samples were layered onto linear gradients of 10–30% sucrose (wt/wt) in buffer A. Sedimentation was at 26,000 rpm in the SW 27.1 rotor of a Beckman ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA) for 2 h at 4°C. Approximate sedimentation coefficients ($s_{20,w}$) were determined through the use of standard calibration charts (2). Gradients were fractionated into 1-ml fractions and protein was determined fluorimetrically (λ_{ex} = 278 nm, λ_{em} = 335 nm) using a 650–10S fluorescence spectrophotometer (Perkin-Elmer Corp., Eden Prairie, MN). For each gradient, the extent of reassembly was quantitated by measuring the area under the protein curve for the reassembled and unassembled material (generally fractions No. 5–11 and 1–4, respectively). The results are expressed as the fraction of the total protein sedimenting as 250S coats.

Two other reassembly procedures were employed for comparison. In one, clathrin with or without AP was dialyzed against a calcium-containing, low ionic strength buffer (buffer C) and analyzed on 10–30% sucrose gradients in the same solution. The other procedure was that of Nandi et al. (29). Clathrin with or without AP was dialyzed against 0.1 M ammonium acetate, pH 8.0, for 2–3 h at 4°C. The pH of the samples was then adjusted to 6.25 by addition of predetermined volumes of 1.0 M NaMES, pH 6.2. Samples were incubated overnight and were analyzed on 10–30% sucrose gradients in the same buffer.

In the recycling experiment (*vide infra*) the reassembled coats were sedimented on a discontinuous two-step gradient in buffer A consisting of 1.25 ml 10% sucrose and 0.3 ml 50% sucrose in 1.2 × 5.0 cm nitrocellulose tubes. After centrifugation at 41,000 rpm for 2 h in the Beckman SW 50.1 rotor, the gradients were fractionated manually. The reassembled coats were found cushioned on the 50% sucrose layer and were dissociated and reassembled as described in Results.

Electrophoresis: Electrophoresis in dodecyl sulfate was performed on 5–15% gradient acrylamide gels (21). Sucrose gradient fractions were prepared for electrophoresis by precipitation with cold 10% trichloroacetic acid in the presence of 0.075 mg cytochrome *c* added as a carrier. Gels were stained with 0.25% (wt/vol) Coomassie Blue in 50% trichloroacetic acid or with a sensitive silver staining method (27). Coomassie Blue-stained gels were quantitated by densitometry on a Zeineh Soft Laser Scanning Densitometer.

Electron Microscopy: Samples were applied to carbon-coated Formvar grids (300-mesh) that were glow-discharged immediately before use. After negative staining with 3% sodium phosphotungstate, pH 5.1, or saturated uranyl formate, samples were examined on a Philips 300 electron microscope at 60 kV. Micrographs of random fields were printed at × 126,000 final magnification, and coat diameters were measured on separated, nonoverlapping structures. Edge lengths were measured on polygons whose axis of symmetry appeared perpendicular to the plane of the grid.

Materials: Calf brains were obtained from Venuto Meat Packers, Philadelphia, PA. Uranyl formate was obtained from Polysciences, Inc., Warrington, PA. Viral standards were kindly supplied by Dr. K. S. Kim of the Department of Plant Pathology, University of Arkansas (TMV) and Drs. K. Soprano and R. Baserga of the Department of Pathology, Temple University (SV40). Chemicals for electrophoresis were products of Bio-Rad Laboratories, Richmond, CA. All other chemicals were reagent grade or better and were obtained as described previously (17).

RESULTS

Separation of Clathrin and Assembly Polypeptides

In a previous report (17), we described the fractionation of a Tris-Cl extract of coated vesicles over a column packed with Sepharose CL-4B (Fig. 1). The polypeptides in the major peak (fractions 32–36) have been reported to be associated in a complex, termed triskelions, consisting of three clathrin chains ($M_r \cong 180{,}000$) and approximately three light chains ($M_r \cong 33{,}000$–$36{,}000$) (20, 49).

Clathrin was resolved from a subsequent fraction (Fig. 1, fractions 41–44) that was qualitatively shown by electron microscopy to promote in vitro clathrin reassembly into coat structures resembling the polyhedral surfaces of coated vesicles. This reassembly activity was shown to be heat sensitive and susceptible to proteolysis (17). The inset to Fig. 1 shows that these column fractions contained a heterogeneous group of polypeptides with the major bands at $M_r \cong 100$–$110{,}000$ and at $M_r \cong 50$–$55{,}000$. We therefore attribute the activity in this fraction to the presence of AP.

The AP-mediated reassembly previously identified by electron microscopy has now been quantitatively studied by sucrose gradient sedimention. When Tris-Cl-extracted clathrin was combined with AP and dialyzed against buffer A, a new macromolecular species was formed which sedimented on sucrose gradients at a position corresponding to $s_{20,w} \cong 250$ (Fig. 2). The inset to Fig. 2 confirms that this new species consisted of reassembled coat structures with characteristic hexagonal and pentagonal faces similar to those of coated vesicles (12, 16, 33).

[1] *Abbreviations used in this paper:* AP, assembly polypeptides; MES, 2-(*N*-morpholino)ethane sulfonic acid.

The action of AP was specific. Reassembly showed an absolute dependence on AP since clathrin alone dialyzed against buffer A formed little or no 250S coat structures (Fig. 2). Instead, virtually all of the protein remained at the top of the gradient. A similar result was obtained when AP alone was dialyzed against buffer A (data not shown). Finally, reassembly activity was confined to fractions 41–44 from the Sepharose column. An equivalent amount of protein from fractions 48–50 failed to induce any clathrin reassembly (data not shown).

Properties of the AP-mediated Clathrin Coats

We and other investigators have shown that purified clathrin can reassemble under certain conditions in the absence of any other proteins (17, 20, 29, 40, 46, 51, 54). These reactions generally required dialysis or dilution of clathrin into buffers of mildly acidic pH and low ionic strength. We found that coats reassembled in the presence of AP differed from those formed from clathrin alone by several significant criteria.

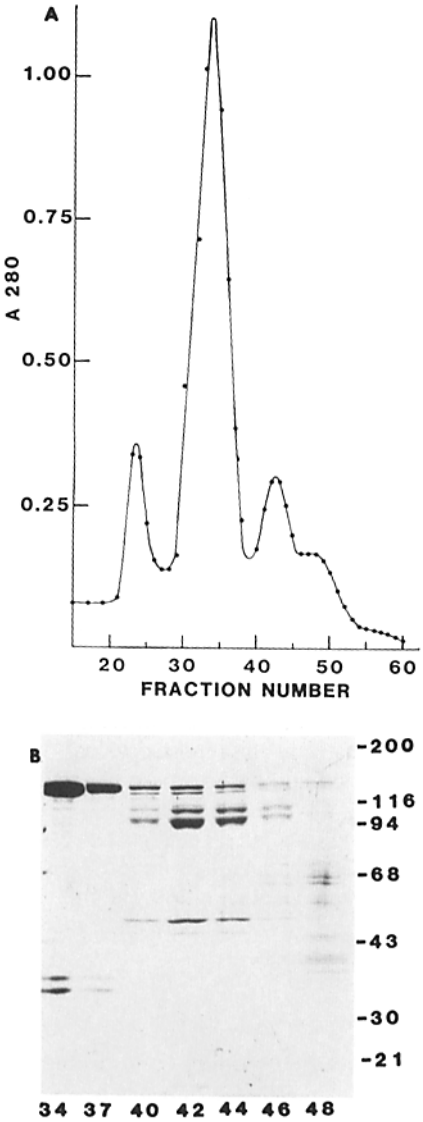

FIGURE 1 Gel filtration of coated vesicle extract. (*A*) The coated vesicle extract in buffer B prepared as described in Materials and Methods was subjected to gel filtration on a 1.9 × 94.0 cm column packed with Sepharose CL-4B. Ascending elution with buffer B was done at room temperature at a flow rate of 12 ml/h, and 3-ml fractions were collected. (*B*) Electrophoretic analysis of the indicated column fractions on a 5–15% gradient acrylamide dodecyl sulfate gel. Markers indicate positions of molecular weight standards. ($\times 10^{-3}$).

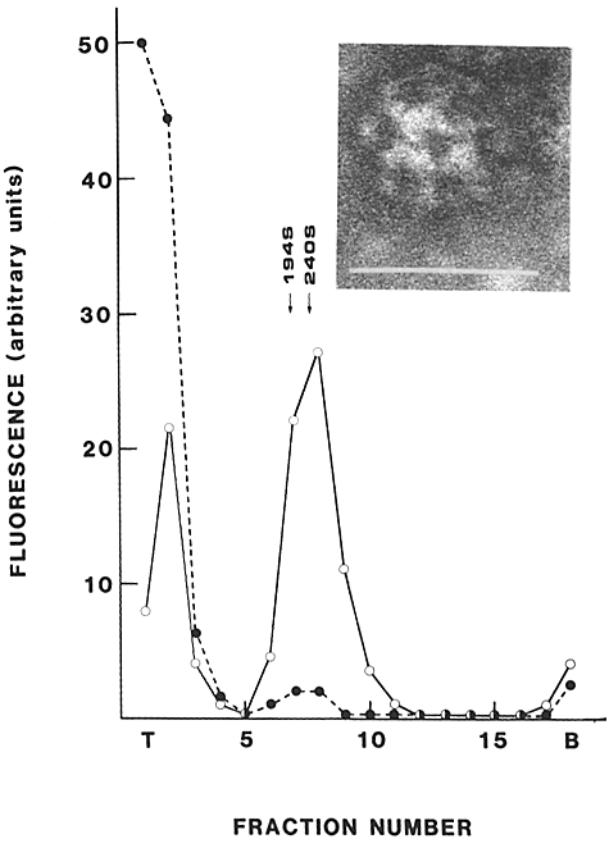

FIGURE 2 Assembly polypeptide-dependent reassembly of 250S clathrin coats. Clathrin (0.38 mg/ml) with (solid line) or without (broken line) AP (0.26 mg/ml) was dialyzed overnight against buffer A at 4°C. Samples were then analyzed on 10–30% sucrose (wt/wt) gradients in buffer A as described in Materials and Methods. Arrows indicate positions of tobacco mosaic virus (194S) and simian virus 40 (240S). *Inset:* Negative staining electron micrograph of an aliquot of gradient fraction 8 (250S). See Fig. 5c. Bar, 0.1 μm. × 252,000.

Coats formed in vitro from clathrin alone by dialysis against buffer C (17, 20), or by titration of clathrin to pH 6.2 in 0.1 M ammonium acetate buffers (29) exhibited broad protein peaks on sucrose gradients (Fig. 3, *a* and *b*). The major peaks had $s_{20,w}$ values of ~400; minor peaks of protein were seen at ~200S and some unpolymerized protein remained at the top of the gradients as reported earlier (29, 40, 51). In contrast, coats formed from clathrin and AP by dialysis against buffer A sedimented in a single, relatively narrow peak at ~250S (Figs. 2 and 3*c*). In addition coats formed from clathrin and AP by dialysis against buffer C or by titration in ammonium acetate buffers, also exhibited narrow 250S peaks on their respective sucrose gradients (data not shown). Furthermore, when unfractionated Tris-Cl extracts of coated vesicles were dialyzed against buffer A, the reassembled coats that formed were exclusively characterized by a sedimentation coefficient of 250S. Thus, regardless of the reassembly procedure or buffer employed, AP-mediated reassembly yields structures with identical sedimentation properties.

Differences in the reassembled coats formed in the presence

and absence of AP were also manifest in the respective polypeptide compositions. After reassembly and separation on sucrose gradients, gradient fractions were analyzed by electrophoresis on dodecyl sulfate acrylamide gels. We could not detect any polypeptides smaller than the clathrin light chains because of the presence of the cytochrome *c* carrier. Coats formed from clathrin alone by dialysis against buffer C or by titration to pH 6.2 were comprised only of clathrin and its associated light chains (Fig. 4*a*). However, the coats formed in the presence of AP, in addition to clathrin and the light chains, had incorporated polypeptides that derived from the AP pool (Fig. 4*b*). Examination of all the fractions across the sucrose gradient indicated that these additional bands were found only in the 250S structures. The incorporated polypeptides included a doublet at $M_r \cong$ 100,000–110,000 and a single polypeptide at $M_r \cong$ 52,000. Although Tris-Cl extracts of coated vesicles always contained several protein bands in the $M_r \cong$ 50,000–55,000 range, only one band in this region was consistently incorporated into the reassembled 250S coats.

The relative molar amounts of the different polypeptide bands in the 250S coats were determined by densitometry at different loading levels and are summarized in Table I. The AP-derived polypeptides were incorporated into AP-reassembled coats at similar stoichiometry regardless of the reassembly method employed, i.e., dialysis against buffer A, buffer C, or by pH titration (data not shown).

Morphological differences between coats formed in the presence and absence of AP were detected by electron microscopy. Reassembled coats formed from clathrin alone by dialysis against buffer C or by titration to pH 6.2 showed a wide range of diameters (Fig. 5, *a* and *b*) from 70 to 125 nm (mean = 101 nm ± 15 nm SD, n = 94 coats). In contrast, in the presence of AP (Fig. 5, *c* and *d*) reassembled coats were of a smaller and relatively uniform diameter (mean = 78 nm ± 5 nm SD, n = 150). Similar size coats were formed in each of the reassembly procedures used, i.e., dialysis against buffer A, buffer C, or titration to pH 6.2 in ammonium acetate buffers. The edge length of the polygons in all coats was the same, ~15 nm, for both the large and small coats. This value corresponded closely to the reported values of 16–19 nm of polygon edge length and of the proximal portion of triskelion legs (6, 49, 50).

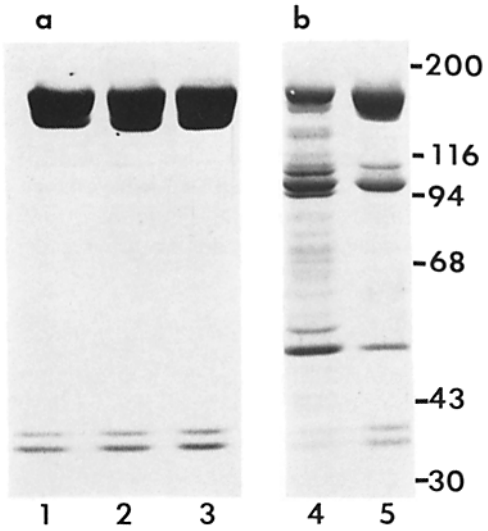

FIGURE 4 Gel electrophoresis of reassembled clathrin coats. Gradient fractions from Fig. 3 were electrophoresed on 5–15% gradient acrylamide dodecyl sulfate gels. Markers indicate position of molecular weight standards (× 10^{-3}). (a) Reassemblies of clathrin alone. Lane *1*: Fig. 3*a*, fraction 11; lane *2*: Fig. 3*b*, fraction 5; lane *3*: Fig. 3*b*, fraction 10. (*b*) Reassembly of clathrin with AP. Lane *4*: Fig. 3*c*, fraction 2; lane 5: Fig. 3*c*, fraction 8.

TABLE I

Molar Ratios of Polypeptide Bands in 250S Coats

Molecular weight	Molar ratio
180,000 Clathrin	3.00 (Triskelion)
110,000	0.26 ± 0.10
100,000	1.11 ± 0.17
52,000	0.85 ± 0.17
36,000	0.83 ± 0.27
33,000	1.62 ± 0.28

Molar ratios are compared with clathrin arbitrarily assigned a value of 3.00, the number of 180,000-mol-wt polypeptides present in one triskelion (20, 49). Means (± SEM) from experiments using nine different preparations are presented.

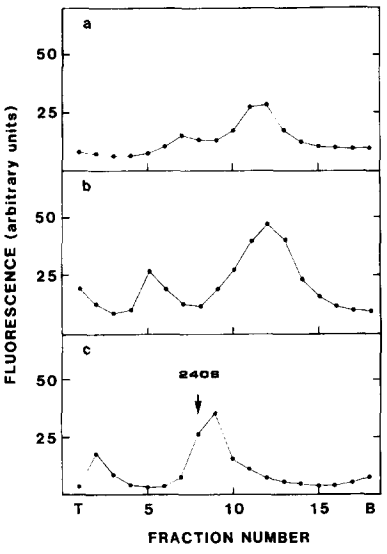

FIGURE 3 Sucrose gradient analysis of reassembled clathrin coat preparations. (*a*) Purified clathrin alone (0.63 mg/ml) dialysed against buffer C. (*b*) Purified clathrin alone (0.49 mg/ml) reassembled by titration to pH 6.25 as described in Materials and Methods (see reference 28). (c) Clathrin (0.16 mg/ml) and AP (0.16 mg/ml) dialysed against buffer A. After dialysis, samples were applied to 10–30% sucrose gradients in the appropriate buffer, centrifuged, and analyzed as described in Materials and Methods. Arrow indicates position of SV40 (240S) on all three gradients.

In summary, AP-mediated coats possess a set of biochemical and biophysical features that appear constant, regardless of the reassembly protocol we employed, indicating that coats formed in the presence of AP represent a distinct subclass of in vitro reassembled coats.

Coat Recycling

Most of the minor polypeptide bands from the AP fraction as well as some of the major ones in the $M_r \cong$ 50,000 and 100,000 groups did not incorporate into the 250S structures but remained at the top of the sucrose gradient (Fig. 4*b*). It is possible that some of these play a role in coat reassembly without actually being part of the reassembled structures.

To test this possibility, 250S coats, formed by dialysis of clathrin and AP against buffer A, were sedimented through a two-step discontinuous sucrose gradient in buffer A (see Materials and Methods). After centrifugation, the majority of the protein was found cushioned on top of the 50% sucrose layer.

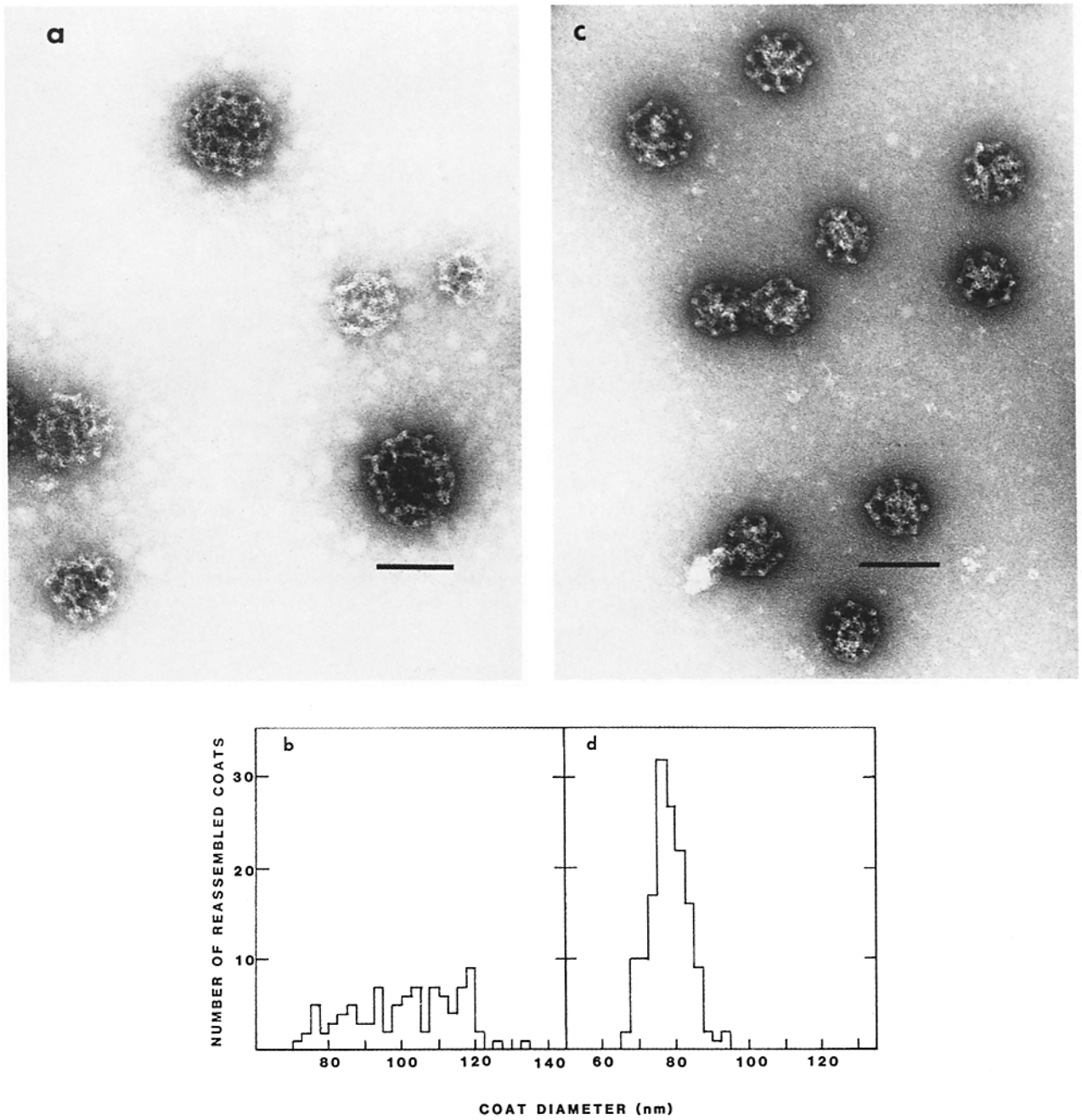

FIGURE 5 Negative staining electron microscopy of clathrin coat preparations. (a) Clathrin alone (0.63 mg/ml) dialysed against buffer C, Bar, 0.1 μm. × 117,000. (*b*) Histogram of diameters of coats from reaction (a). (n = 96). (c) Clathrin (0.16 mg/ml) and AP (0.16 mg/ml) were combined and dialyzed against buffer A, Bar, 0.1 μm. × 117,000. (*d*) Histogram of diameters of coats from reaction (c). (n = 150). All negative staining was carried out with uranyl formate.

An aliquot of this material was analyzed on a 10–30% linear sucrose gradient and found to consist almost entirely (>99%) of reassembled 250S structures (Fig. 6*a*). The balance of this material was dialyzed against buffer B and divided into two equal aliquots. One aliquot was analyzed on a 10–30% linear sucrose gradient in buffer B, the other was dialyzed back against buffer A and analyzed on linear sucrose gradients in buffer A.

Fig. 6*b* shows that the reassembled coats (Fig. 6*a*) were dissociated when dialyzed against buffer B with the resulting protein remaining at the top of the analytical gradient. However, when the dissociated material was dialyzed back against buffer A, 250S coats were reformed (96% of the total protein) with a corresponding decrease in the amount of protein left at the top of the gradient (Fig. 6*c*). These recycled 250S structures possessed the characteristic polypeptide pattern of clathrin, light chains, and $M_r \cong$ 52,000, 100,000, and 110,000 bands. Since the material for the second reassembly was derived exclusively from isolated, reassembled coats from the first cycle, we concluded that all the information necessary for the formation of 250S coats is present in the structural polypeptides themselves.

Stoichiometry of AP-mediated Reassembly

The ability of isolated 250S coats to dissociate and reassemble, and the observation that certain AP polypeptides were

incorporated with clathrin in 250S coats, suggested that AP-mediated reassembly might be a stoichiometric rather than an enzymatic reaction. In examining this hypothesis, a constant amount of clathrin was dialyzed with increasing amounts of AP against buffer A and the extents of reaction were quantitated on individual sucrose gradients. In Fig. 7 the results representative of three such experiments are expressed as the fraction of total protein reassembled into 250S coats. The amount of 250S coats formed from a constant concentration of clathrin was directly proportional to the amount of AP added. At a clathrin/AP weight ratio of 1:1, the formation of coats reached a maximum, in which a near quantitative (≅85%) transfer of protein to the 250S structures occurred. With continued addition of AP the amount of protein in 250S coats remained constant, but unincorporated protein accumulated at the top of the gradient resulting in a decrease in the fraction of total protein in coats.

In converse experiments, increasing amounts of clathrin, with or without a constant amount of AP, were dialyzed against buffer A. In the presence of AP, significant coat formation was evident at clathrin concentrations of 0.06 mg/ml or lower (Fig. 8). As in the previous experiment, increasing amounts of clathrin produced increasing amounts of reassembled coats. Both of these experiments support the hypothesis that AP-mediated coat reassembly is a stoichiometric reaction.

In the absence of added AP, dialysis of increasing amounts of clathrin against buffer A resulted in a low, constant level of coat formation (Fig. 8). We believe this is due to contamination of the clathrin fractions by trace amounts of AP as a result of incomplete separation on the Sepharose column (Fig. 1). This suggestion is supported by several lines of evidence: first, a lack of concentration dependence is observed on dialysis of increasing amounts of clathrin alone (Fig. 8).

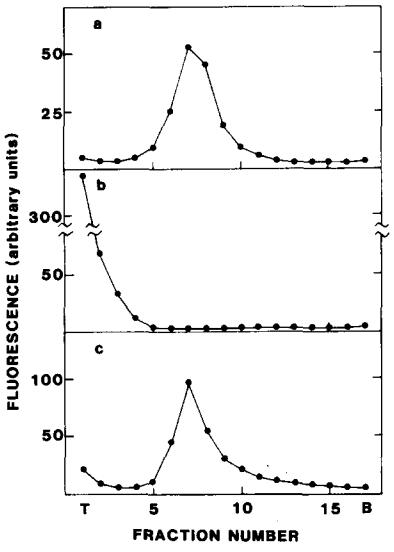

FIGURE 6 Dissociation and recycling of isolated 250S AP-reassembled coats. Clathrin (0.29 mg/ml) and AP (0.28 mg/ml) were combined and dialyzed against buffer A. The sample was then loaded onto a sucrose step gradient in buffer A, centrifuged, and fractionated as described in the text. (*a*) An aliquot of the 50% layer analyzed on a 10–30% sucrose gradient in buffer A. (*b* and *c*) The rest of the 50% layer was dialyzed against buffer B for 4 h at 4°C, then divided into two equal aliquots. (*b*) One aliquot was analyzed on a 10–30% sucrose gradient in buffer B. (*c*) The other aliquot was dialyzed overnight against buffer A then analyzed on a 10–30% sucrose gradient in buffer A.

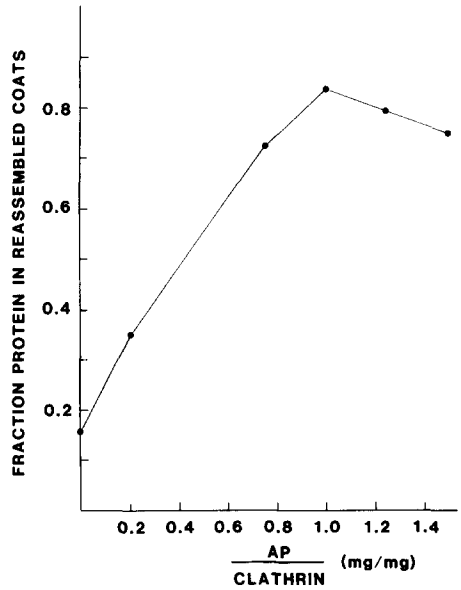

FIGURE 7 Coat formation as a function of AP. Aliquots of clathrin (0.16 mg/ml) were combined with indicated amounts of AP (0.032–0.24 mg/ml) in final volumes of 0.31 ml. Samples were dialyzed overnight against buffer A and reassembly was quantitated as described in Materials and Methods. Results are expressed as the fraction of the total protein sedimenting in 250S coats.

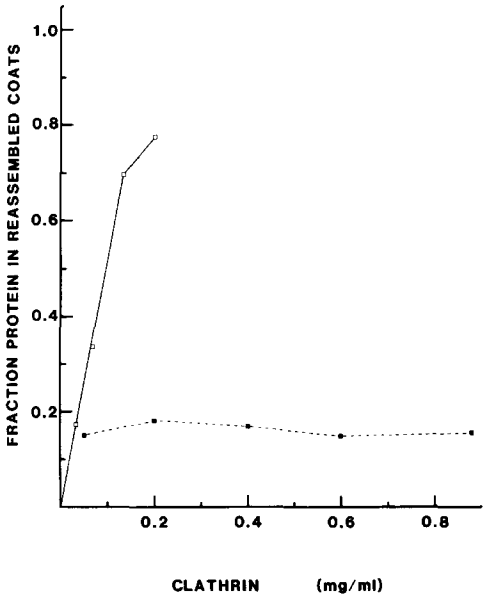

FIGURE 8 Coat formation as a function of clathrin concentration in the presence or absence of AP. Indicated amounts of clathrin (0.035–0.88 mg/ml) were combined with buffer B (broken line) or AP (0.24 mg/ml, solid line) in final volumes of 0.15 ml and were then dialyzed overnight against buffer A, and reassembly was quantitated as described in Materials and Methods. Results are expressed as the fraction of the total protein sedimenting as 250S coats.

Second, the small amounts of reassembled coats appeared only at the 250S position, characteristic of the AP-mediated process, rather than at 400S (compare Fig. 3, *a* and *c*). Finally, examination of these 250S species by dodecyl sulfate gel electrophoresis using a sensitive silver stain revealed the presence of the AP even though none had been added (data not shown).

Reassembly as a Function of Temperature and pH

The dialyses and sedimentations described above were all performed at 4°C. Reassembly at 22°C in the presence of AP was only slightly less efficient than at 4°C (Fig. 9). The concentration of AP for which a given amount of clathrin became maximally reassembled was approximately the same at the two temperatures. In addition, when clathrin and AP were dialyzed against buffer A at 37°C, reassembled coats were detected by negative staining electron microscopy.

Previous investigators have reported that coat reassembly from purified clathrin was extremely pH dependent (3, 17, 20, 24, 40, 46, 51, 54). Reassembly in low salt buffers occurred only at pH 6.7 or below and could be reversed by raising the pH to 7.8 (51). Nandi et al. (30) reported increased rates of clathrin polymerization at more physiological pH, but only in the presence of high concentrations of Mn^{2+} (2–4 mM), Ca^{2+} (10 mM), polybasic amines, or exogenous basic proteins (e.g., lysozyme).

In contrast, the AP-dependent reassembly of clathrin proceeded readily at a pH as high as 7.5, in the absence of high concentrations of cations or exogenous proteins. Clathrin and AP were combined and dialyzed against buffer A adjusted to different pH values. As examined on sucrose gradients, incorporation of protein into coats was optimal at pH 6.6 and was still efficient at pH 7.4 (Fig. 10). In the absence of AP, coat formation at pH 6.6 was low, and barely discernible at pH 7.4.

The ability of AP to promote clathrin reassembly at cellular pH was more striking when reassembly was performed against buffer C adjusted to pH 6.8. After dialysis against such a buffer, clathrin alone does not reassemble, as reported by others and confirmed by sucrose gradient centrifugation in our laboratory. In the presence of AP, however, reassembled coats were readily formed (>80% of the total protein) at this pH (data not shown). Once again, these structures had the characteristic polypeptide composition of AP-mediated coats.

DISCUSSION

Reports over the past several years have shown coated membranes to be present in a wide range of eukaryotic cell types (for reviews, see references 10, 36) and to be implicated in a number of cellular processes (viz., receptor-mediated endocytosis, secretory pathways, intracellular protein traffic, and

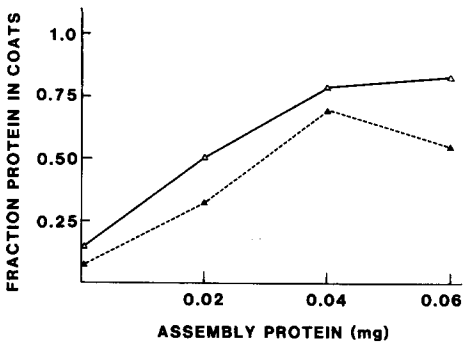

FIGURE 9 Coat formation as a function of AP at 4° and 22°C. Aliquots of clathrin (0.19 mg/ml) were combined with buffer B or indicated amounts of AP in final volumes of 0.26 ml. Overnight dialyses and subsequent sedimentations on 10–30% sucrose gradients were done at 4° (solid line) or 22°C (broken line).

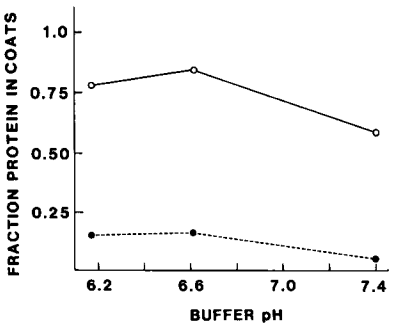

FIGURE 10 Coat formation as a function of pH. Aliquots of clathrin (0.22 mg/ml) in the presence (solid line) or absence (broken line) of AP (0.22 mg/ml) were dialyzed overnight against buffer A adjusted to the indicated pH. Reassembly was quantitated as described in Materials and Methods. Results are expressed as the fraction of the total protein sedimenting as 250S coats.

membrane recycling after neurotransmitter release).

Most investigators agree as to the identity of the major protein of these extrinsic coats. However, there is considerable uncertainty regarding the role of certain minor polypeptides in coated membrane dynamics since it is well established that empty coats can reassemble under certain in vitro conditions from purified clathrin preparations. We previously reported that Sepharose CL-4B gel filtration of coated vesicle extracts resolved clathrin from a partially purified pool of proteins possessing in vitro reassembly activity. We report here that the activity resides in three polypeptides that are among the minor components of coated vesicles in vivo and that are essential structural components of a unique class of in vitro reassembled coats. As will be discussed below, several features of AP-mediated reassembly are consistent with a role for AP in the in vivo coat dynamics.

Our experiments showed that coats formed from clathrin and AP possessed biochemical and biophysical properties that distinguish them from coats formed from clathrin alone. Coats formed in the absence of AP sedimented in broad peaks on sucrose gradients with a major peak at 400S. In addition, these coats were composed exclusively of clathrin and the light chain polypeptides and possessed a wide range of diameters (70–125 nm). On the other hand, coats formed in the presence of AP, regardless of the reassembly buffer, sedimented more slowly in sucrose gradients (250S). These AP-reassembled coats contained clathrin, the light chains, and stoichiometric amounts of several AP polypeptides, and were smaller and relatively uniform in diameter (78 nm).

Densitometric examination of the stained gels of AP-mediated coats revealed a constant ratio between clathrin and the other polypeptides in the reassembled coat, suggesting the possibility that these polypeptides are structural components of the reassembled coat and bind at characteristic sites on the triskelion. We speculate that this binding may increase and define the nonplanarity of triskelion subunits, a process that would generate smaller coats of uniform size.

The data in this report, indicating that AP assist in the formation of small coats in vitro, seem to be supported by a recent report on the removal of a 110,000-dalton polypeptide from coated vesicle extracts using lysine-Sepharose (13). When the remaining extracts were reassembled, relatively large coats were formed. When the $M_r \cong 110{,}000$ polypeptide was added back to the reassembly reaction, smaller coats were formed.

The formation of 250S coat structures in vitro exhibited

behavior consistent with the stoichiometric addition of assembly polypeptides to clathrin triskelions. Reassembly increased linearly with the addition of one component and then reached a maximum when the other became limiting. All the information necessary for reassembly of 250S coats was present in the polypeptides that were ultimately incorporated into the coats. Coats formed in the presence of AP could be separated from unincorporated proteins, dissociated, and then reassembled at high efficiency. These observations argue against the operation of a dissociative enzymatic agent and indicate that AP act stoichiometrically to promote reassembly.

Several features of AP-mediated reassembly suggest that AP may play a role in coat formation in vivo. AP that are incorporated into reassembled coats (i.e., $M_r \cong 52{,}000$, 100,000, and 110,000) are ubiquitous components of coated vesicle preparations obtained from many sources (34, 35, 37, 45, 47, 54). They appear to be true coated vesicle components since they are observed in the most highly purified coated vesicles obtained by means other than sucrose gradient sedimentation (37, 38, 45).

The presence of AP also permits in vitro coat reassembly to occur under more physiological salt conditions than previously reported. Reassembly in the absence of AP was found to occur in low ionic strength buffers but was inhibited by biologically common anions (SO_4^{2-}, Cl^-) and cations (K^+, Na^+) (51). The rate of coat reassembly could be increased by the presence of some divalent cations but only at high concentrations, e.g., 2–4 mM Mn^{2+} or 10 mM Ca^{2+} (30). Reassembly of purified clathrin alone also does not occur in buffer A (Fig. 2), the medium in which the coated vesicles are originally isolated. In contrast, the AP-mediated reassembly proceeded extensively in all the buffer systems employed, including buffer A (0.1 M NaMES). In addition, formation of coats by dialysis against buffer A or buffer C also showed that AP-mediated reassembly occurred efficiently in the absence or presence of Ca^{2+} chelators (1 mM EGTA).

Equally important, the AP-mediated coats are unique in that they formed extensively at physiological pH. Previous descriptions of in vitro clathrin coat reassembly have found formation to be inhibited at pH 6.7 and above. Nandi, et al. (30) found that reassembly of clathrin alone could occur at pH 7.95, but only in the presence of high concentrations of free, divalent cations, certain amines such as dansylcadaverine, or lysozyme. On the other hand, AP-mediated reassembly occurred at physiological pH (7.4) in the absence of any other effectors. Reassembly in the pH range 6.8–7.2 was consistently high in the presence of AP in two different buffer systems.

Finally coat reassembly in the presence of AP occurred in high yields at clathrin concentrations as low as 0.06 mg/ml. This value corresponds closely to estimates of the total intracellular clathrin concentration (6).

Polypeptides of similar electrophoretic mobility to those that we have shown to be incorporated into 250S coats have also been noted in other studies of coated vesicles. Polypeptides of $M_r \cong 100{,}000$ and 50,000 are among those reported to specifically interact with Ca^{2+}-calmodulin (22, 23, 28). Indirect evidence has also been presented suggesting that an $M_r \cong 100{,}000$ polypeptide is involved in the binding of clathrin coats to vesicle membranes (48). In addition, coated vesicle preparations have been shown to possess a protein kinase activity with the primary substrate being an $M_r \cong 50{,}000$ polypeptide (15, 32) that is immunologically related to microtubule associated τ proteins (39). Recent experiments in this laboratory indicate that the 52,000-mol-wt AP identified here is also the major substrate phosphorylated in intact coated vesicles and that the kinase activity is present in the partially purified AP preparation (Keen, J. H., and S. Zaremba, manuscript in preparation).

The size and uniformity of in vitro AP-mediated coats strikingly parallels a discrete subset of coated membranes in vivo. Electron microscopic studies of various cell types have established that coated vesicles appear in a range of sizes from 60 to 200 nm diam (5, 9, 12, 16, 36). In general, smaller coated vesicles and membrane segments of relatively uniform size (60–80 nm) are associated with the Golgi region of cells while larger coated profiles (>100 nm) and coated expanses of membrane appear in the plasma membrane region (9, 14, 25, 53). It is therefore possible that the small AP-reassembled coats formed in vitro correspond specifically to those coated membranes involved in Golgi related functions in vivo. Antibodies to AP are being produced in this laboratory and should prove to be useful tools in testing this hypothesis and in further elucidation of the exact role of AP in coated membrane function.

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Note added in proof: We have recently detected a 16,500-mol-wt polypeptide in 250S coats. This polypeptide, derived from the AP fraction, appears distinct from bovine brain calmodulin since it does not exhibit a Ca^{++}-EGTA electrophoretic shift.

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