Clathrin Assembly Proteins: Affinity Purification and a Model for Coat Assembly

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Abstract. Assembly protein (AP) preparations from bovine brain coated vesicles have been fractionated by clathrin-Sepharose affinity chromatography. Two distinct fractions that possess coat assembly activity were obtained and are termed AP-1 and AP-2. The AP-1, not retained on the resin, has principal components with molecular weights of 108,000, 100,000, 47,000, and 19,000. The AP-2, bound to the resin and eluted by Tris-HCl at a concentration that parallels the latter's effect on coat disassembly, corresponds to the active complex described previously (Zaremba, S., and J. H. Keen, 1983, *J. Cell Biol.*, 97:1339–1347). Its composition is similar to that of the AP-1 in that it contains 100,000-, 50,000-, and 16,000-mol-wt polypeptides in equimolar amounts; minor amounts of 112,000- and 115,000-mol-wt polypeptides are also present. Both are distinct from a recently described assembly protein of larger subunit molecular weight that we term AP-3. These results indicate the existence of a family of assembly proteins within cells.

On incubation with clathrin both AP-1 and AP-2 induce the formation of coat structures, those containing AP-1 slightly smaller (mean diameter = 72 nm) than those formed in the presence of AP-2 (mean diameter = 79 nm); both structures have been detected previously in coated vesicle preparations from brain. Coats formed in the presence of AP-2 consistently contain approximately one molecule each of the 100,000-, 50,000-, and 16,000-mol-wt polypeptides per clathrin trimer. By low angle laser light scattering the molecular weight of native AP-2 was determined to be ~343,000, indicating that it is a dimer of each of the three subunits, and implying that it is functionally bivalent in clathrin binding. A model for AP-mediated coat assembly is proposed in which a bivalent AP-2 molecule bridges the distal legs or terminal domains of two clathrin trimers that are destined to occupy adjacent vertices in the assembled coat. Binding of a second AP-2 molecule locks these two trimers in register for assembly and further addition of AP-2 to free trimer legs promotes completion of the clathrin lattice. Effects of AP binding on the angle and flexibility of the legs at the hub of the trimer (the "pucker") are suggested to account for the characteristic size distributions of coats formed under varied conditions and, more speculatively, to contribute to the transformation of flat clathrin lattices to curved coated vesicles that are thought to occur during endocytosis.

CLATHRIN coated membranes are ubiquitous subcellular structures that have been shown to be involved in numerous membrane transport processes. Among the best characterized of these is receptor-mediated endocytosis, during which ligand–receptor complexes accumulate in coated pits at the plasma membrane surface and are internalized within vesicles into the cell (5, 18). Because of the specificity and efficiency with which these complexes are concentrated in coated pits, the coat structure is thought to intimately participate in the uptake process. Although receptor-mediated endocytosis has been well documented morphologically, its operation is not understood at the biochemical level. That is, the mechanisms by which the coat assembles, what role assembly plays in driving membrane invagination and vesicle formation, and how receptors are actively clustered in coated pits are, at the molecular level, not known. To address these questions we are studying the mechanisms by which coat formation occurs and is modulated.

The major component of the coat is clathrin, a trimeric protein consisting of three heavy and three light chains (14, 27). In addition, a number of other polypeptides are also present, including prominent species with molecular weights of 100,000–120,000 and 45,000–55,000. When partially purified coated vesicle preparations are extracted with high concentrations of Tris-HCl, clathrin and other peripheral proteins are released in a soluble form (13). This extract can be fractionated on sizing columns to yield clathrin trimers and a subsequent fraction, devoid of clathrin, whose predominant components are polypeptides with molecular weights of 100,000–115,000 and 50,000–55,000, as well as other more minor species (13, 27). We initially showed that, under the relatively physiological buffer conditions used for the initial isolation of intact coated vesicles, this second fraction was

required for the reassembly of the extracted and purified clathrin; the responsible activity was therefore attributed to an assembly protein, (AP)[1] (13, 32). This observation has since been confirmed by others (1, 20, 22).

Here we report that purification of the AP has been achieved by affinity chromatography with clathrin-Sepharose. Two distinct, though possibly related, assembly proteins have been identified, which are designated AP-1 and AP-2; compositionally they appear similar to the preparations denoted HA-1 and HA-2 by Pearse and Robinson (20) although there are significant differences. Our results show that both AP-1 and AP-2 are active in promoting clathrin assembly, but they yield coat structures of distinctly different size. A third assembly protein, here termed AP-3, has recently been reported by Ahle and Ungewickell (1). These results suggest that a family of assembly proteins with diverse functions and localizations is likely to exist within the cell. Finally, based on determination of molecular weight, subunit composition, and stoichiometry of AP-2 incorporation into coat structures, a model for AP-2–mediated clathrin assembly is presented.

Materials and Methods

Materials

Clathrin coated vesicle preparations were performed as described previously (13) with the following modifications, which reduced preparation time without materially affecting purity of the product. Three calf brains (~400–500 g) were obtained from Venuto Meat Packers (Philadelphia, PA) and were processed within 1 h of slaughter. The first sucrose gradient was modified to contain 12 ml of 10% sucrose, 14 ml of 40% sucrose, and 2 ml of 50% sucrose, all (wt/vol) solutions in buffer A. The samples were spun at 25,000 rpm for 60 min at 4°C in a rotor (SW27; Beckman Instruments, Inc., Palo Alto, CA) with the brake on. The second gradient was modified to accommodate a larger sample by using 10 ml of 5% sucrose and 4 ml of 30% sucrose in buffer A. After centrifugation at 19,500 rpm for 45 min at 4°C in a rotor (SW27.1; Beckman Instruments, Inc.) the turbid 5% sucrose layer was retained while the upper clear solution and substance at the interface of the 5 and 30% solutions were discarded. This partially purified coated vesicle preparation was immediately extracted with Tris-HCl and subjected to gel filtration as described below.

Clathrin-Sepharose was prepared by mixing a clathrin solution (1.3 mg/ml) in 0.1 M sodium bicarbonate, 0.5 M sodium chloride, pH 8.5, obtained by Superose 6B gel filtration (see below), with an equal volume of CNBr-Sepharose equilibrated in the same buffer and which had been prepared according to the manufacturer's directions (Pharmacia, Inc., Piscataway, NJ). Coupling was essentially complete as determined by protein assay of wash solutions, yielding a preparation containing 1.3 mg clathrin/ml resin. After blocking and washing, the resin was equilibrated in buffer B and stored at 4°C.

Gel filtration media (Superose 6B, Sephacryl S-400, CNBr-Sepharose) were obtained from Pharmacia, Inc. The Bradford reagent was from Pierce Chemical Co. (Rockford, IL), and ultrapure Tris base and ammonium sulfate were purchased from Bethesda Research Laboratories (Gaithersburg, MD). Dithiothreitol, EGTA, and other chemicals were reagent grade or better and were obtained from Sigma Chemical Co. (St. Louis, MO) or Calbiochem (La Jolla, CA).

The following buffers were used. Buffer A: 0.1 M sodium 2-(*N*-morpholino) ethane sulfonic acid, 1.0 mM EGTA, 0.5 mM magnesium chloride, 0.02% sodium azide, pH 6.50; buffer B: buffer A:1.0 M Tris-HCl, pH 7.0 (1:1, vol/vol); buffer C: 0.2 M Tris-HCl, 1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, 0.02% sodium azide, pH 6.5; buffer D: 0.1 M sodium 2-(*N*-morpholino) ethane sulfonic acid, 1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, 0.02% sodium azide, pH 6.5.

Gel Filtration

Coated vesicle suspensions, obtained as described in the previous section, were incubated with an equal volume of 1.0 M Tris-HCl pH 7.0 for 10 min at room temperature and then centrifuged at 100,000 *g* for 60 min. The resulting supernatant was diluted with an equal volume of saturated ammonium sulfate and, after incubation for 10 min centrifuged at 10,000 *g* for 30 min. The pelleted protein was resuspended with homogenization in 3–4 ml of buffer B and centrifuged briefly to remove insoluble material. This sample was loaded on a column (2.6 × 59.5 cm) containing Superose 6B equilibrated with buffer B. Elution downward at room temperature was at 1.0 ml/min; 4.5-ml fractions were collected and immediately refrigerated.

Affinity Purification of the AP

In a typical preparation, conducted at 4°C except as noted, 5 mg of AP protein obtained by Superose 6B gel filtration was precipitated by treatment with 50% saturated ammonium sulfate and resuspended in ~12 ml of buffer C. This material was then used to resuspend 7 ml (settled volume) of clathrin-Sepharose resin (prepared as described in Materials) that had been previously equilibrated with buffer C. The suspension was transferred to a Spectrapor 2 dialysis bag (Spectrum Medical Industries, Los Angeles, CA) and dialyzed overnight with vigorous stirring against two changes of buffer D. After measuring the conductivities of the dialysate and buffer D to insure that equilibrium had been attained, the contents of the bag were poured into a column and the resin washed at room temperature with buffer D. Fractions (5 ml) were collected until little or no protein absorbance was detected. A 40-ml linear gradient from 0–0.4 M Tris-HCl in buffer D was then applied at a flow rate of 10 cm/h and 1.0-ml fractions were collected and immediately refrigerated.

The clathrin-Sepharose was washed and stored at 4°C in buffer B supplemented with 10% glycerol after each use; resin has been reused successfully more than five times over a period of 6 mo. Inclusion of at least 10% glycerol in buffers C and D greatly increased protein recovery (from <10 to ~60–80% overall); yield also increased after the first use of the resin, suggesting that nonspecific adsorptive sites had been blocked. Affi-gel 10 (Bio-Rad Laboratories) was also tested as an adsorbent and worked satisfactorily in the purification; underivatized Sepharose 4B did not.

Low Angle Laser Light Scattering

Experiments were performed using a KMX-6 photometer (LDC/Milton Roy, Milton Roy Co., Sunnyvale, CA) equipped with a thermostated flow cell kept at 25°C for these studies. Purified AP-2 was dialyzed at 4°C against buffer C that had been extensively degassed, and was centrifuged immediately before use at 100,000 *g* for 30 min at 4°C. The sample was brought to 25°C and pumped from a syringe through a prewashed 3-mm-diam 0.20 μm pore size nylon filter (Micron Separation, Inc., Honeyoye, NY) and then through the flow cell of the photometer at 0.09 ml/min using an infusion pump (Sage Instruments Div., Orion Research Inc., Cambridge, MA). After stable scattering readings had been obtained, the solution emerging from the flow cell was collected to accurately determine protein concentration in the light path. Measurements were made at three protein concentrations, using 0.2- and 0.3-mm field stops, yielding Rayleigh scatter values of 4.4–13.4 × 10^{-6} cm^{-1}; R_θ of buffer C was 2.86 × 10^{-6} cm^{-1} and that of water alone was 1.1 × 10^{-6} cm^{-1}. The data were analyzed by a linear least squares fit to yield a weight-average molecular weight and are plotted in Fig. 8 as Kc/R_θ vs. AP-2 concentration (the latter expressed as mg/ml for readability).

Other Procedures

Coat reassembly was assayed essentially as described previously (32) except that gradients containing 5–20% (vol/vol) glycerol in buffer A were centrifuged at 26,000 rpm for 90 min at 4°C in a rotor (SW 27.1; Beckman Instruments, Inc.). Quantitative densitometry of Coomassie Blue–stained gel bands was performed on an LKB 2202 Ultrascan equipped with LKB 2190 Gelscan software (LKB Instruments, Inc., Gaithersburg, MD). Multiple scans at varied loadings were performed to insure linearity. Calculations of molar stoichiometries presented in Table I rely upon two assumptions: firstly, equivalent dye binding per unit mass; secondly, that the polypeptide's migration in SDS gels accurately reflects subunit molecular weight. These assumptions appear valid for clathrin and the AP-2 polypeptides (11, 26;

A preliminary account of parts of this work has been published in abstract form (1984. *J. Cell Biol.* 99[4, pt. 2]:366*a*.[Abstr.]).

1. *Abbreviation used in this paper*: AP, assembly polypeptides or assembly protein.

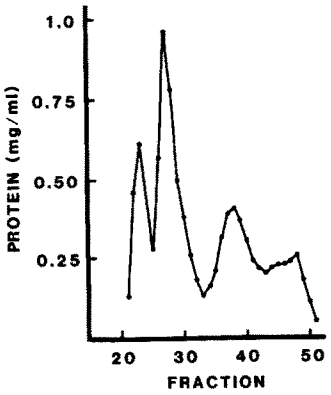

Figure 1. Separation of clathrin and assembly protein fractions by gel filtration. A Tris-HCl extract of a coated vesicle preparation was applied to Superose 6B and eluted with buffer B. Clathrin (fractions *26–29*) was resolved from the peak containing assembly proteins (fractions *35–41*).

Keen, J., manuscript in preparation), but have not been independently verified for the AP-1 and 155,000-mol-wt polypeptides.

Negative-staining electron microscopy and coat diameter measurements were performed as described previously (32); the distributions of coat diameters were statistically evaluated using the Student's *t* test for independent samples. Heuser and Kirchhausen (8) have shown that coats measured by negative staining have larger apparent diameter than those observed by deep etching, apparently due to flattening on the grid. Thus, the baskets of 79 nm diam reported here (formed in the presence of AP-2) correspond to the hexagonal baskets of 60 nm observed on etching; the smaller (71 nm) structures seen here (in the presence of AP-1) correspond to the "B" structures of 50 nm diam by deep etching (using the terminology of Crowther et al. [6]).

One-dimensional and two-dimensional isoelectric focusing gel electrophoresis were performed as described previously (9). Conductivity was measured at room temperature with a Radiometer CDM3 (Radiometer, Inc., Copenhagen, Denmark) equipped with a CDC114 flow cell. AP-2 samples were hydrolyzed in 6 N HCl at 110°C for 25, 48, and 72 h and composition was calculated by extrapolation to zero-time. Protein was assayed either by a Coomassie Blue dye-binding assay (4) or by absorbance at 280 nm using an extinction coefficient of 11.0 for clathrin (31) and 8.7 for AP-2 (based on amino acid analysis).

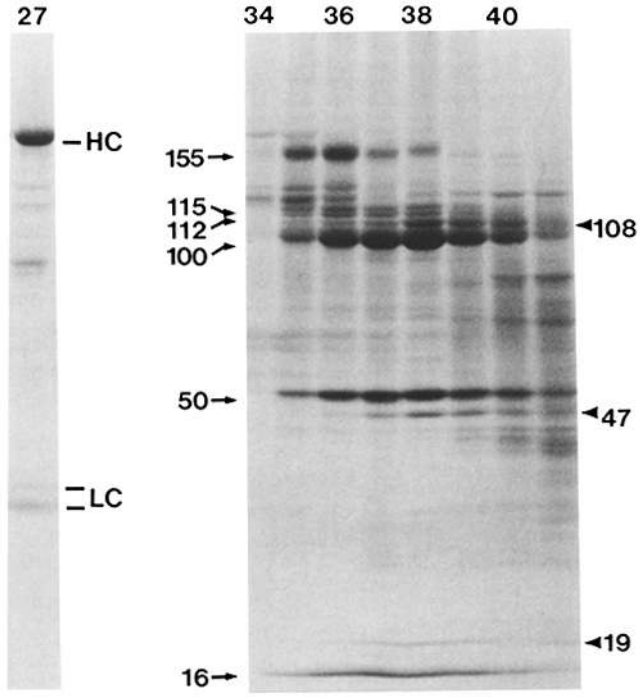

Figure 2. Polypeptide composition of clathrin and assembly protein peaks obtained on Superose 6B gel filtration. Fractions from the Superose elution profile shown in Fig. 1 containing clathrin (*27*) and AP (*34–41*; for clarity only every other lane is numbered) analyzed by SDS gradient (5–15%) gel electrophoresis. The positions of polypeptides in the AP peak are indicated by markers with their apparent molecular weight ($\times 10^{-3}$); in the same gel, clathrin heavy (*HC*) and light (*LC*) chains are shown in the left lane.

*Table I. Molar Ratios of Assembly Polypeptides**

Superose AP		AP-1		AP-2	
		mol wt		*mol wt*	
AP100	1.10	108,000	0.67	99,000–102,000	0.99
AP50	1.00	100,000	0.70	50,000	1.00
AP16	1.20	47,000	1.00	16,000	0.65
155,000-mol-wt	0.11	19,000	0.81		

* Molar ratios are normalized to 1.00 for 50,000- or 47,000-mol-wt polypeptides.

Results

Fractionation of Tris-HCl Extracts of Coated Vesicles

Superose 6B has been used to fractionate Tris-HCl extracts of clathrin coated vesicles with greater resolution than that obtained previously with Sepharose CL-4B (32), permitting a more detailed analysis of polypeptides within the resolved peaks. Thus (Figs. 1 and 2), clathrin (fractions *26–29*) has been completely resolved from the peak (fractions *35–41*) containing assembly polypeptides (AP).

Based on the observation that polypeptides with molecular weights of 100,000–115,000, 50,000, and 16,000 were selectively and stoichiometrically incorporated from this AP fraction into clathrin coats, we hypothesized that these species were present in a discrete complex that was the functional unit responsible for the activity (32). Support for this proposal comes from quantitative analysis of the Superose 6B elution profile by densitometry. These data indicate that the major 100,000-, the 50,000-, and the 16,000-mol-wt bands coelute in fractions *35–41* and on a molar basis are recovered in approximately equal amounts (Table I).

In an earlier study of coated membrane proteins of rat sympathetic neurons maintained in culture (9), we identified a phosphorylated 155,000-mol-wt polypeptide. This polypeptide is of interest because it was incorporated with carrier bovine brain clathrin and AP into coat structures; comigrated on two-dimensional isoelectric focusing with a protein present in coated vesicles derived from cow brain; and on gel filtration with Sepharose CL-4B in buffer B, its elution overlapped that of the AP. With the increased resolution of the Superose 6B resin, the bovine brain 155,000-mol-wt (identified by its acidic position on two-dimensional isoelectric focusing; data not shown) is found to elute sharply on the leading side of the AP peak (Fig. 2, fractions *35–36*). Densitometry of the Superose-6B fractions indicates that there is a substantial amount of the 155,000-mol-wt polypeptide present in the bovine brain extracts (Table I). For the reasons noted in the Discussion, it is likely that this polypeptide is identical to an assembly protein of 180,000 apparent molecular weight (based on SDS gel electrophoresis) recently described by Ahle and Ungewickell (1).

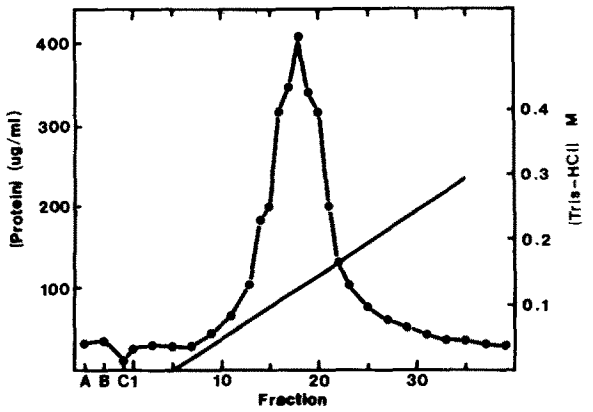

Figure 3. Clathrin-Sepharose affinity chromatography. Fractions *A–C* are flow-through in buffer D (5 ml), containing the polypeptides identified as AP-1. Fractions 1-39 (1-ml each), containing the AP-2 peak, were eluted by a gradient of increasing Tris-HCl, whose concentration is indicated by the solid line and the scale at right.

108,000-, 47,000-, and 19,000-mol-wt polypeptides eluted toward the latter part of the AP peak, appearing predominantly in fractions *38–39*. By densitometry, these polypeptides are present in lesser amounts than the 100,000/50,000/16,000-mol-wt species: the amount of the 47,000-mol-wt is approximately equivalent to that of the 155,000-mol-wt polypeptide (Table I). As reported below, these polypeptides possess clathrin assembly activity and, like the 100,000/50,000/16,000-mol-wt are likely to be associated in a complex.

Affinity Purification of the AP

Initial attempts to isolate the AP from Tris-HCl extracts of coated vesicles using conventional purification techniques did not yield preparations that retained assembly activity. Since the AP polypeptides were incorporated into the reassembled coat structure, we speculated that a stable association of AP with clathrin was likely to occur, and could be exploited as a purification procedure if clathrin were used as a solid adsorbent.

To this end clathrin at alkaline pH, in the form of dissociated trimers, was covalently coupled to Sepharose. An AP preparation was mixed with the clathrin-Sepharose under dissociating conditions (buffer B supplemented as noted in Materials and Methods) and the agitated suspension was dialyzed against assembly buffer (buffer A). After equilibration the suspension was transferred to a column and unbound protein was collected by washing with buffer A. The column was then eluted with a linear gradient of 0–0.4 M Tris-HCl in buffer A. A single major protein peak was obtained (Fig. 3), eluting in approximately 120 mM Tris-HCl.

Analysis of the column fractions by SDS gel electrophoresis (Fig. 4) indicated that there had been fractionation of the starting material (compare with Fig. 2, fraction *38*) into two groups of largely exclusive polypeptides that we designate AP-1 and AP-2. Protein in the first AP-1 fraction, i.e., the unbound flow through, which accounted for ~20% of the applied protein, contained major species of apparent molecular weight of 108,000, 47,000, and 19,000, all of which were largely or entirely absent from the Tris-HCl–eluted peak. It also included a 100,000-mol-wt species whose migration on SDS gels was extremely similar to a more major species eluted by Tris-HCl (see below); other more minor components, amounting to ~15% of the total protein by densitometry, are also detectable. By densitometry it was ascertained that the major polypeptides were present in approximately equivalent molar amounts (Table I). Examination of the Superose 6B elution profile indicates that the 108,000-, 100,000-, 47,000-, and 19,000-mol-wt polypeptides also co-eluted on gel filtration (Fig. 1 *B*), suggesting that they exist as a structural complex.

The fraction eluted by Tris-HCl, that we now designate AP-2, contained major bands of ~100,000-, 50,000-, and 16,000-mol-wt as well as minor amounts of 112,000- and 115,000-mol wt polypeptides, all species that we have previously identified as components of the assembly protein by virtue of their incorporation into reassembled coat structures (32). Approximately 60% of the applied protein was recovered in these Tris-HCl–containing fractions. The ~100,000-mol-wt band could be resolved into two discrete species with molecular weights of 99,000 and 102,000 present in approximately 3:1 ratio. Considering these two bands together as the AP100, densitometry indicated that the AP100 and AP50 were recovered in approximately equal molar amounts (Table I). Furthermore, the ratio of 100,000:50,000:16,000-mol-wt polypeptides was approximately constant across the elution peak, suggesting that a homogeneous preparation was obtained; electron microscopy studies to be reported elsewhere support this conclusion (Heuser, J. E., and J. H. Keen, manuscript in preparation).

Reexamination of the Superose 6B elution profile (Fig. 2) suggests that the AP-1 and AP-2 can be slightly resolved solely on the basis of size within the broad envelope of the AP peak in fractions *35–41*. In the leading and central part of the peak are the minor AP-2 bands with molecular weights

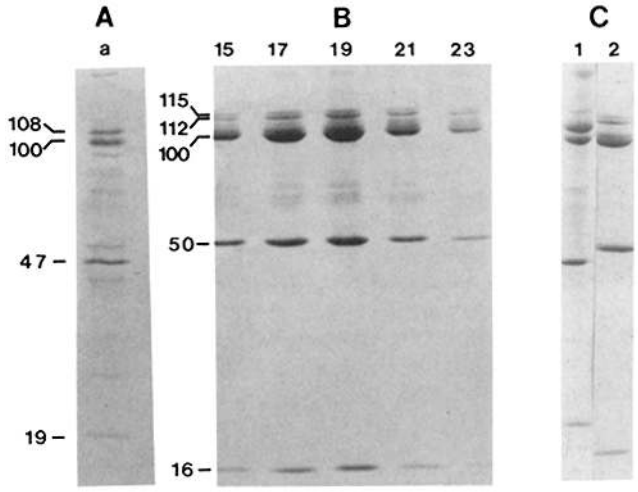

Figure 4. Polypeptide composition of AP-1 and AP-2. Fractions from clathrin-Sepharose affinity chromatography analyzed by SDS gradient gel (5–15%) electrophoresis. (*A*) Fraction A from Fig. 3; AP-1 components indicated by molecular weight ($\times 10^{-3}$) markers at left. (*B*) Indicated fractions across the peak eluted by Tris-HCl in Fig. 3; AP-2 components indicated by molecular weight ($\times 10^{-3}$) markers at left. (*C*) Preparations obtained by hydroxylapatite chromatography, corresponding to HA-1 (lane *1*) and HA-2 (lane *2*) in reference 20.

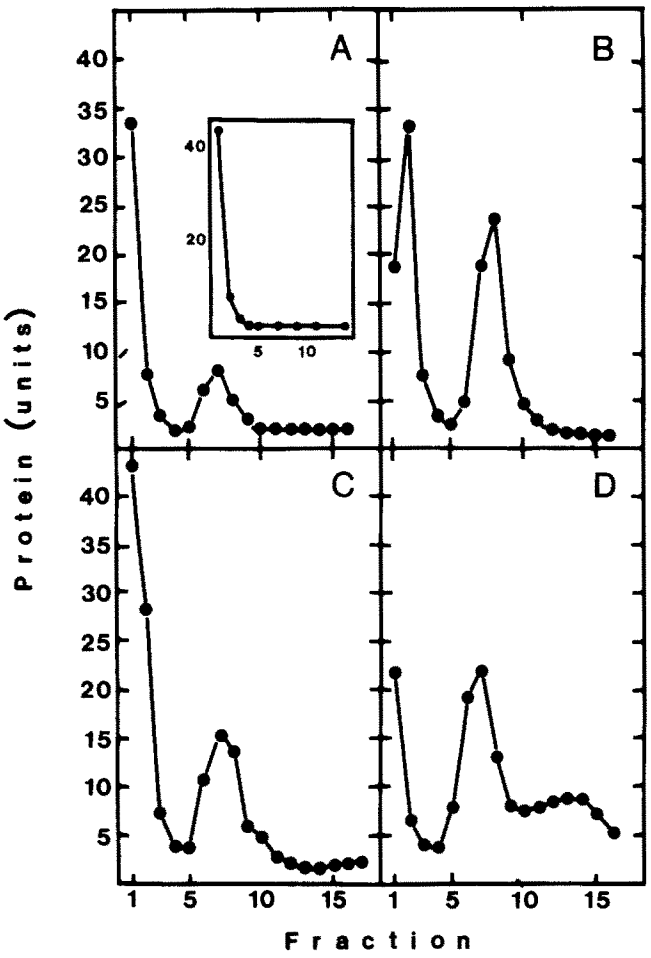

Figure 5. Both AP-1 and AP-2 induce clathrin coat reassembly. Clathrin coat assembly promoted by purified AP preparations was assayed by glycerol gradient ultracentrifugation as described in Materials and Methods. Under these conditions, unassembled material remains at the top of the gradient (fractions *1* and *2*) while coat structures sediment and appear in fractions 6–10 (see text). (*A*, *inset*) 40 μg of clathrin alone (AP/clathrin ratio = 0.00). (*A*) 40 μg clathrin + 12 μg AP-2 (AP/clathrin ratio = 0.30). (*B*) 40 μg clathrin + 46 μg AP-2 (AP/clathrin ratio = 1.15). (*C*) 60 μg clathrin + 15 μg AP-1 (AP/clathrin ratio = 0.25). (*D*) 60 μg clathrin + 60 μg AP-1 (AP/clathrin ratio = 1.00).

of 112,000 and 115,000. These polypeptides share many properties with the major 100,000-mol-wt AP-2 species, including incorporation into coat structures (32), cosedimentation and cleavage by elastase (33), and copurification on clathrin–Sepharose affinity chromatography, suggesting that they may represent a subset of AP-2 with some of the AP100 replaced by 112,000- and/or 115,000-mol-wt components. In contrast, the 108,000, 47,000, and 19,000-mol-wt AP-1 components eluted in the latter part of the peak.

The AP-1 and AP-2 preparations are similar in several respects to two fractions purified from bovine brain coated vesicles by hydroxylapatite chromatography (20), designated HA-1 and HA-2, although there are several prominent differences. Firstly, not reported by Pearse and Robinson (20) are low molecular weight polypeptides in both the AP-1 and AP-2 preparations (19,000 and 16,000, respectively), in amounts approximately stoichiometric with the 100,000-mol-wt polypeptides in each preparation. These data are consistent with our earlier observations using partially purified AP preparations (32, 33) and have been confirmed by others (30). A second difference is that our AP-1 preparation contains equivalent amounts of 100,000- and 108,000-mol-wt polypeptides (Fig. 4 *A*) whereas that obtained by Pearse and Robinson contained only a 108,000-mol-wt species (20). Finally, the assembly products generated by the preparations are different (see below).

To resolve these discrepancies we subjected our partially purified AP preparations to hydroxylapatite chromatography and obtained two protein peaks corresponding to those designated HA-1 and HA-2. By one-dimensional SDS gel electrophoresis these preparations (Fig. 4 *C*) were indistinguishable from the AP-1 and AP-2 fractions prepared by clathrin-Sepharose affinity chromatography as described above (Fig. 4, *A* and *B*). Thus, the distinctions noted above do not reflect the isolation of different assembly species but, more likely, experimental differences between the laboratories. These may include, for example, the use of Triton X-100 during coated vesicle isolation (19).

The affinity-purification procedure presented here is relatively simple yet yields AP-2 preparations of greater purity than that obtained by hydroxylapatite chromatography (compare Fig. 4 *B* with Fig. 1 in reference 20), and is apparently equivalent to those obtained by repeated hydroxylapatite chromatography in the presence of SDS (Fig. 2 in reference 20). The yield of AP-2 is also improved severalfold, with ~5 mg of AP-2 recovered per kilogram of bovine brain. With much more to be learned about the structure and interrelationships amongst this putative family of assembly proteins, this technique should prove useful.

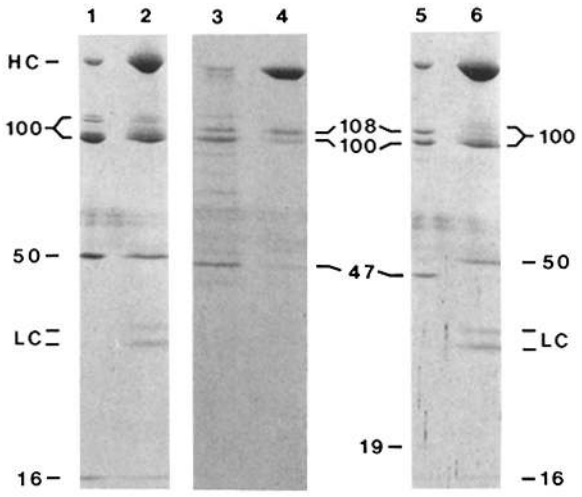

Figure 6. Both AP-1 and AP-2 are incorporated into reassembled coats with clathrin. Glycerol gradient fractions (shown in Fig. 5) containing reassembled coats and unsedimented protein were separately pooled and analyzed by SDS gradient gel electrophoresis (5–15%). Markers denoting the clathrin heavy (*HC*) and light (*LC*) chains and the AP-2 polypeptides are shown on the left and right siges; migration position of the AP-1 polypeptides are indicated beside lanes *4* and *5*. AP-2–mediated assembly: unincorporated protein (lane *1*) and reassembled coat protein (lane *2*). AP-1–mediated assembly: unincorporated protein (lane *3*) and reassembled coat protein (lane *4*). Lanes *5* and *6*, AP-1 and AP-2 mixed with clathrin and unincorporated (lane *5*) and reassembled coat protein (lane *6*) analyzed separately.

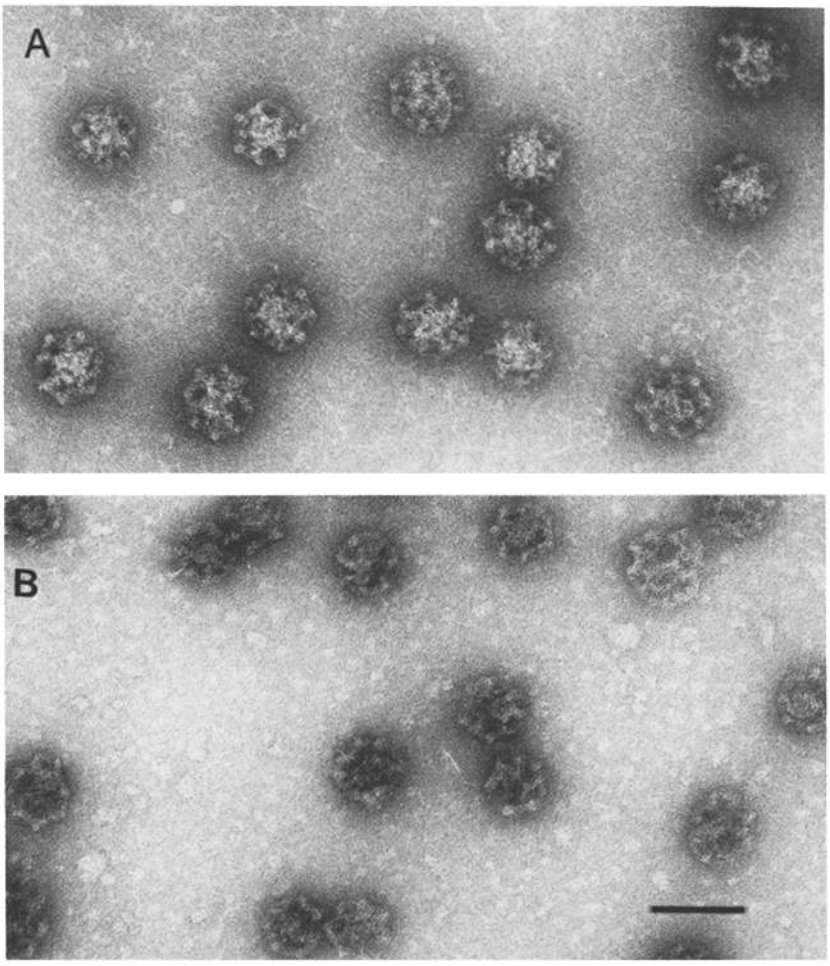

Figure 7. Negative-staining electron microscopy of clathrin coat structures formed in the presence of AP-2 (*A*) or AP-1 (*B*). Bar, 0.1 μm.

Reassembly of AP-1 and AP-2

The ability of the purified AP fractions to promote clathrin assembly into coat structures was evaluated by both glycerol gradient ultracentrifugation and negative staining electron microscopy. As we have noted before (32), little or no assembly is observed with purified clathrin alone (Fig. 5 *A*, *inset*). Purified AP-2, the major species present in coated vesicle extracts, promoted the assembly of structures that sediment with an apparent $s_{20,w}$ of ~250S (Fig. 5, *A* and *B*, fractions 7–9), and negative-staining electron microscopy (Fig. 7 *A*) revealed the formation of intact coats with a mean diameter of 79 nm (SD = 6 nm; $n = 60$) (Fig. 8 *A*). These parameters are similar to those observed in our previous studies with partially purified AP preparations (32).

The AP-1 was also active in promoting clathrin assembly. On gradient centrifugation these coats sedimented slightly slower than those prepared with AP-2 (cf. Fig. 5, *B* and *D*). Electron microscopy (Fig. 7 *B*) provided morphological evidence that coats formed in the presence of AP-1 were indeed smaller (mean diameter 72 nm, 6 nm SD, $n = 54$; Fig. 8 *B*) than those formed with AP-2. Statistical evaluation of the distribution of coat diameters confirmed this difference ($p < 0.05$). While the AP-2 coats correspond to the "hexagonal barrel" and "tennis ball" structures described by Crowther et al. (6), those prepared in the presence of AP-1 correspond in size to the "B" structures noted by these authors, containing only four instead of eight hexagons present in the barrel structures.

The gradient assay of reassembly demonstrated that increasing amounts of AP-2 generated coordinate increases in coat formation (Fig. 5, *A* and *B*). SDS gel electrophoresis (Fig. 6 *A*) indicated that all of the AP-2 polypeptides had been incorporated into the reassembled coat structures. At the highest ratio of AP-2 (46 μg) to clathrin (40 μg) used,

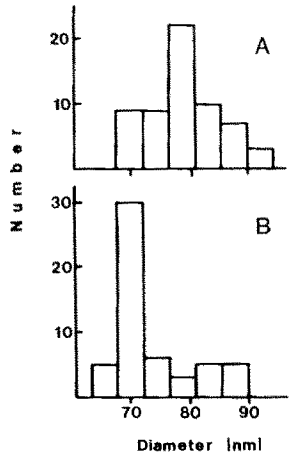

Figure 8. Histogram of coat sizes formed in the presence of AP-2 (*A*, $n = 60$) or AP-1 (*B*, $n = 54$).

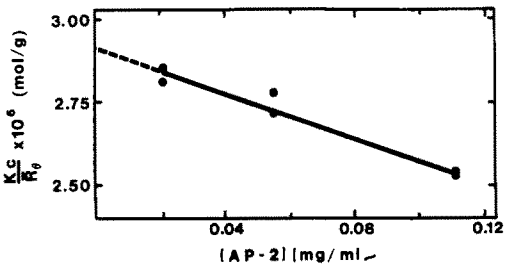

Figure 9. Molecular weight determination of AP-2 by low angle laser light scattering. Plot of Kc/R_θ as a function of AP-2 concentration in the scattering cell yields an ordinate intercept indicating a weight-average molecular weight of ~343,000.

essentially all of the clathrin had been assembled into coat structures and excess unincorporated AP-2 clearly remained at the top of the gradient (Fig. 6, lane *1*). Under these conditions, the molar ratio of AP50/AP100/clathrin trimer (three heavy chains) found in the fractions containing reassembled coat structures (fractions *7–9*, Fig. 5 *B*) was 1.1:1.3:1.0 (mean of determinations on three preparations). This result, similar to that previously obtained with partially purified AP (32), indicates that in reassembled coat structures containing AP-2, approximately one molecule each of the AP50 and AP100 had been incorporated per clathrin trimer, the functional clathrin unit of assembly (14, 27).

AP-1–induced assembly also occurred in a concentration-dependent manner (Fig. 5, *C* and *D*). At higher AP-1 to clathrin ratios, a more rapidly sedimenting minor peak (fractions *12–14*) was observed in addition to the major peak (fractions *6–8*). Electron microscopy of the isolated peaks indicated that the major one contained individual coats while the minor peak contained structures that appeared to be individually similar in size to those in the major peak but in groups of two or three (data not shown). Under conditions where essentially all of the clathrin has been incorporated into coat structures, and excess AP-1 polypeptides remained at the top of the gradient (Fig. 6, lane *3*), gel electrophoresis of the major peak of reassembled coats, fractions *6–7*, revealed incorporation of the major AP-1 bands of 108,000, 100,000, and 47,000 mol wt (Fig. 6 *B*, lane *4*). At the loading level used, the 19,000-mol-wt species was not visible in the fraction containing reassembled coat structures, although it was detectable when a more sensitive silver stain was used (data not shown). The molar amounts of the 100,000- and 47,000-mol-wt AP-1 polypeptides incorporated were somewhat variable, and ranged between 0.5 and 1.0 per 108,000-mol-wt polypeptide; it was not possible to accurately quantitate the amount of 19,000-mol-wt polypeptide incorporated. However, approximately one 108,000-mol-wt polypeptide per 10 clathrin heavy chains was consistently present in reassembled coats, which may reflect the incorporation of one 108,000-mol-wt per three clathrin trimers.

Interestingly, when both AP-1 and AP-2 were incubated with limiting amounts of clathrin, the AP-2 polypeptides were preferentially incorporated to the total exclusion of AP-1 (Fig. 6, lanes *5* and *6*). These results indicate that the AP-1 species is capable of binding to clathrin but that, under the conditions used here for both affinity chromatography and reassembly, it does so with far less effectiveness than the AP-2 complex.

Molecular Weight of Purified AP-2

To determine the molecular weight of AP-2 under native conditions, purified preparations in 0.2 M Tris-HCl and 10% glycerol were subjected to low angle laser light scattering as described in Materials and Methods. The Rayleigh light scattering factor, $\bar{R}_\theta$, was determined using published methods (2) using a 6–7° annulus: with the buffer used this yielded an actual solid scattering angle of 4.796°. Since $\bar{R}_\theta$ is related to molecular weight (M) by Eq. 1:

$$Kc/\bar{R}_\theta = 1/M + 2A_2c, \quad (1)$$

the weight-average molecular weight of a scattering particle is obtained from the ordinate intercept of a plot of $Kc/\bar{R}_\theta$ as a function of c. In this equation c is the concentration of protein (determined by amino acid analysis), of weight-average molecular weight M, A_2 is the second virial coefficient and $\bar{R}_\theta$ is the Rayleigh light scattering factor of the protein solution minus that of the buffer. K is an optical constant (Eq. 2):

$$K = 2\pi^2 n^2 (dn/dc)^2 (1 + \cos^2\theta)/\lambda^4 N, \quad (2)$$

in which λ is the wavelength of the incident light (632.8 nm). N is Avogadro's number, θ is the angle at which scattered light is measured (4.796°), n is the refractive index of the medium (1.3540), and dn/dc is the differential refractive index increment of the protein (assumed to be 0.18 ml/g, see below).

Linear regression analysis ($r = 0.98$) of the data (Fig. 9) yield a straight line with slope -3.33×10^{-3} mol-ml/g^2. This negative value, corresponding to the second virial coefficient, suggests the existence of AP-2–AP-2 interactions (25), and is consistent with the tendency of the AP to aggregate under the relatively low Tris-HCl concentrations used (3). The reciprocal of the ordinate intercept yields a weight-average molecular weight of ~343,000. Given the approximately equal molar stoichiometry of 100,000-, 50,000-, and 16,000-mol-wt species determined by densitometry (Table I), the data are consistent with the existence of a dimeric structure of composition $(100)_2(50)_2(16)_2$, whose molecular weight would be 332,000. The major uncertainty in this measurement involves the value assumed for dn/dc, a parameter that was impractical to directly measure. Fortunately, most proteins have dn/dc of 0.175–0.185 ml/g (21). Even assuming a generous range, and allowing dn/dc to range between 0.16 and 0.20 ml/g, the true complex molecular weight will still be included within 290,000–440,000. Since the subunit molecular weights of the assembly polypeptides estimated by SDS gel electrophoresis are known to be relatively accurate (by comparison with their behavior on gel filtration in 6 M guanidine-HCl; 11; Keen, J., manuscript in preparation), we can still conclude that the AP-2 must be dimeric in structure.

Discussion

AP-1, AP-2, and AP-3

On the basis of in vitro experimentation in this laboratory and others, there is compelling evidence that at least three distinct proteins derived from coated vesicle preparations are independently capable of promoting clathrin assembly into

coat structures (1, 13, 20, 32). Based on common functional attributes and structural similarities that are already apparent, it is likely that these represent a family of assembly proteins: we suggest the designations AP-1, AP-2, and AP-3, which reflect in part their elution order from clathrin-Sepharose (or hydroxylapatite) and the chronology of their description. Data concerning two of these species, the AP-1 and AP-2, are presented here. Ahle and Ungewickell (1) have recently identified another protein that we term AP-3.

The complex that we designate AP-1, since it is eluted first on both hydroxylapatite and clathrin-Sepharose affinity chromatography, contains approximately equivalent molar amounts of 108,000-, 100,000-, 47,000-, and 19,000-mol-wt polypeptides. There is good reason to believe that these components are present in a discrete complex, like those in AP-2, since they co-elute on Superose gel filtration and are co-assembled into coat structures with clathrin with qualitatively similar stoichiometry.

The AP-2, eluted by Tris-HCl after being bound to clathrin-Sepharose, is the major species isolated from brain coated vesicles and contains equimolar amounts of 16,000-, 50,000-, and 100,000-mol-wt components, the last of which can be resolved as a doublet of 99,000-, and 102,000-mol-wt polypeptides. In addition it contains minor amounts of 112,000-, and 115-000-mol-wt polypeptides; these appear structurally related to the major 99,000/102,000-mol-wt polypeptides in their sensitivity to elastase (33) as well as in their co-assembly properties (32). There is some data to indicate that the 100,000-mol-wt polypeptides present in AP-1 and AP-2 are at least partially distinct since they differ in their binding to clathrin-Sepharose and have slight differences in electrophoretic migration.

Ahle and Ungewickell (1) have recently reported the identification of another protein derived from bovine brain coated vesicles, distinct from those above, that promoted the assembly of clathrin into coat structures. We suggest the designation of AP-3 for this third assembly protein. The properties of this AP-3 are similar to those of a recently described rat neuronal phosphoprotein (9) that is also found in bovine brain (this report): both are incorporated with clathrin into coat structures, have similar elution properties on gel filtration, and have acidic isoelectric points. Ahle and Ungewickell (1) also showed that the AP-3 migrated anomalously on SDS gel electrophoresis, with an apparent subunit molecular weight of ~180,000; using gel filtration in guanidine-HCl they found its true molecular weight to be closer to 120,000. The phosphoprotein we refer to as the 155,000-mol-wt polypeptide (based on its migration on SDS gels) behaves similarly in that it elutes with, not ahead of, the 100,000-mol-wt components of the AP on gel filtration in guanidine-HCl (Keen, J., manuscript in preparation). These observations indicate that the phosphorylated 155,000-mol-wt protein described earlier is very likely to be identical to the protein identified by Ahle and Ungewickell.

These three assembly proteins appear to be compositionally related in several important respects. All contain major species with molecular weights of approximately 100,000–120,000 and these domains are responsible for directing clathrin assembly (11, 12; Keen, J., manuscript in preparation); all are stoichiometrically incorporated into the coat structure (see below); if, as suggested, the 155,000 mol wt is identical to AP-3, then all are also phosphorylated in the intact cell (9). Phosphorylation may also be responsible for some of the apparent heterogeneity of the 100,000-mol-wt polypeptides. It is known that this posttranslational modification can slightly retard migration on SDS gel electrophoresis (12, 16 and references therein). In a previous study of assembly polypeptide phosphorylation in intact cells (9) within each closely spaced pair of polypeptides (99,000/102,000 and 112,000/115,000 using the molecular weight assignments reported here) only the bands of higher apparent molecular weight, i.e., 102,000 and 115,000, respectively, were detectably phosphorylated. These observations suggest that within each pair of polypeptides the upper band may represent a more highly phosphorylated variant of the lower, parent band and that the heterogeneity of the 100,000-mol-wt assembly polypeptides may be less complex than at first surmised.

In addition to 100,000–120,000-mol-wt polypeptides, the AP-1 and AP-2 contain species with molecular weights of 47,000 and 19,000, and 50,000 and 16,000, respectively. It is apparent that the lower molecular weight species in each complex could arise from a common precursor of subunit molecular weight 66,000. However, it is extremely unlikely that this represents an artifactual cleavage during preparation, since the AP50 has been directly detected in biosynthetically labeled cells (9).

The assembly proteins differ considerably in the nature of their interaction with clathrin. Thus, under the conditions used here, AP-2 binds stably to clathrin trimers immobilized on Sepharose, while the AP-1 (and the 155,000-mol-wt polypeptide; data not shown) do not. Furthermore, when both AP-1 and AP-2 are incubated with limiting clathrin the AP-2 is preferentially incorporated into coat structures, providing evidence for different mechanisms of interaction of the two assembly proteins with clathrin. Finally, AP-1 and AP-2 direct the assembly of morphologically unique coat structures, distinct both in terms of size and in the amount of assembly protein incorporated relative to clathrin. Studies of bovine brain coated vesicle preparations (6, 8) have identified structures that correspond in size to the coats formed by AP-1, termed "B" structures, and those formed by AP-2, described as "hexagonal barrel" (A) and "tennis ball" (C) structures by Crowther et al. (6). Two conclusions may be drawn from these observations: firstly, that structures corresponding to the reassembled coats described here are present in bovine brain, or at least in preparations of coated vesicles derived from this tissue; secondly, that these assembly proteins may be associated with distinct populations of coated vesicles within the cell. Support for the latter possibility comes from the recent work of Robinson (23) who reported a differential distribution of AP-containing coated membrane structures in cells studied by immunofluorescence. Thus, it is reasonable to speculate that assembly may be independently regulated or localized within the cell to modulate clathrin coat formation in response to specialized stimuli.

AP-2 and the Organization of the Coat Structure

The AP-2 is incorporated into reassembled coat structures in discrete stoichiometry. Our measurements have been based exclusively on analysis of reassembled coats isolated by gradient ultracentrifugation. These data, using both partially purified AP preparations (32) and affinity purified AP-2 (this

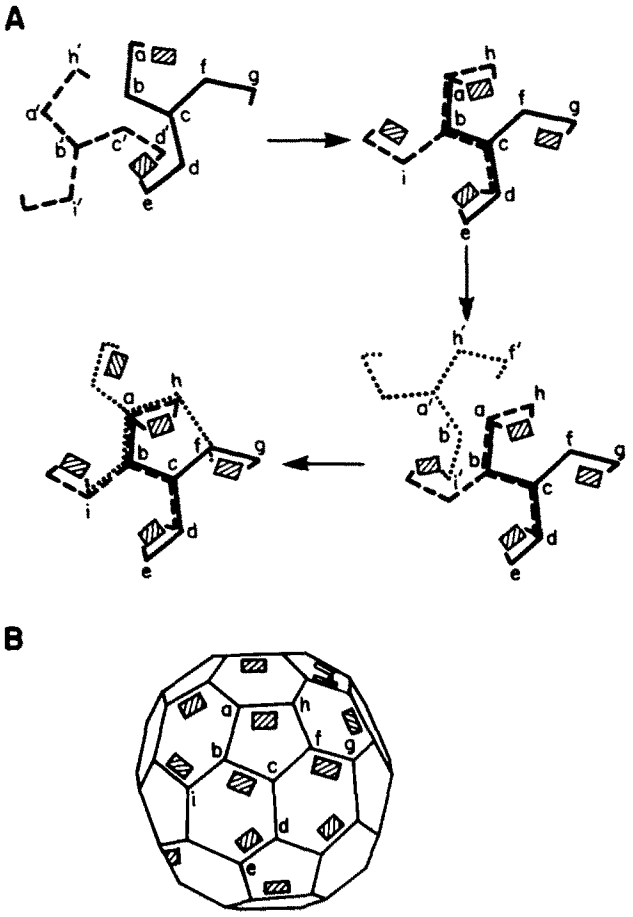

Figure 10. A model for AP-2–mediated clathrin coat assembly. (*A*) Role of AP-2 in initiating assembly. (*Upper left*) AP-2 (*shaded rectangle*) bridges the terminal domains or distal leg regions of two clathrin trimers (positions *d'* and *e*) while a second AP-2 molecule is bound at position *a*. (*Upper right*) Movement of one of the trimers (*broken lines*) into overlap with the other (along *a–b–c–d*) facilitates bridging of a second AP-2 molecule, at *a–h*, and rigidification of the complex. Additional AP-2 molecules are bound near position *i* and at *g*. (*Lower right*) A third clathrin trimer (*dotted lines*) binds to the available AP-2 site at position *i*. (*Lower left*) The third trimer (*dotted lines*) has now become aligned with the first two, stabilized by the action of a second AP-2 molecule bridging terminal domains at positions *f–g*. The three trimers have generated a closed pentagon (*a–b–c–f–h*) and there is multiple leg overlap along *a–b–c–d*. A "free" AP-2 molecule (near *a*) is available for propagation of the assembly reaction. (*B*) At completion, the clathrin domains at each vertex of the assembled coat will interact with one end of a bivalent AP-2 molecule, consistent with biochemical measurements of AP-2 molecular weight and AP-2/clathrin stoichiometry.

report), indicate that approximately one molecule each of the AP50 and AP100 (considering the 99,000- and 102,000-mol-wt species together) are incorporated per clathrin trimer (the latter containing three heavy chains and approximately three light chains). Similar ratios have been reported in studies with isolated 100,000-mol-wt polypeptides (22) or with the apparent 180,000-mol-wt AP-3 (1). Higher, and more variable, proportions of AP to clathrin were found by Pearse and Robinson (20). However their measurements were based on assay of protein pellets obtained after ultracentrifugation, rather than on isolated coat structures, and may have been spuriously influenced by the tendency of AP to aggregate (3) and to form larger AP/clathrin aggregates in the absence of Tris-HCl.

Results presented here indicate that AP-2 is capable of binding to clathrin trimers that have been covalently immobilized in the dissociated trimeric state upon a support resin. That the clathrin-AP-binding interaction is Tris-HCl sensitive, paralleling quantitatively the effect of Tris-HCl on reassembly (13), lends further support to the view that AP-2 binding to clathrin-Sepharose reflects a primary step in the clathrin assembly pathway. The 2:2 stoichiometry of AP100/AP50 in the AP-2 molecule deduced from light scatter measurements, the 1:1 stoichiometry of each of these species to clathrin trimers in reassembled coat structures, and the observation that clathrin assembly is a function of the 100,000-mol-wt domain (12, 33; Keen, J., manuscript in preparation), lead to the inference that the AP-2 is bivalent, i.e., that each AP-2 can bind to two clathrin trimers.

One possible orientation of AP-2 molecules within the coat structure, consistent with the available data, is presented in Fig. 10 *B*. Here the distal leg or terminal domain regions of clathrin heavy chains present under each vertex of the coat structure interact with one of the two clathrin binding domains on each AP-2 molecule. Recent morphological studies suggest that the terminal domains of the clathrin trimers protrude ~6 nm inward toward the vesicle surface from the main shell of clathrin density (8, 28). Although conventional transmission electron microscopy fails to discretely visualize the AP within reassembled coats or intact coated vesicles, studies of purified AP-2 by deep etch electron microscopy, to be reported elsewhere, indicate that it is a bricklike structure ~9 × 7 nm on edge; coats dissociated upon a mica surface confirm that the AP brick structures are indeed contained within the intact coat (Heuser, J., and J. Keen, manuscript in preparation). Finally, image reconstruction studies of coats in vitreous ice (29) indicate a discrete shell of AP density, beneath the clathrin lattice and ~8-10 nm in thickness; these observations are all consistent with the model presented here.

This model suggests a specific pathway for how AP-2 may drive clathrin assembly, schematically depicted in Fig. 10 *A*. Here coat assembly is initiated by a bivalent AP-2 molecule bridging the terminal domains or distal legs of two clathrin trimers that are destined to occupy adjacent vertices in the assembled coat (positions *d'* and *e* on *dashed* and *solid line* trimers, respectively, Fig. 10 *A*, *upper left*). This initial interaction, mediated by the 100,000-mol-wt domains of the AP complex, draws the clathrin trimers into proximity and promotes overlap of other regions of the trimer legs. Binding of a second AP-2 molecule, to the two other terminal domains of these two trimers that are adjacent one another (Fig. 10 *A*, positions *a* and *h'*, *upper left*), locks the two trimers in register (*upper right*). Binding of another AP-2 molecule at the unoccupied leg of one of the trimers, Fig. 10 *A*, position *i*, upper right, facilitates the binding of a third trimer (*broken line*) at *i'*, lower right. Interaction of the latter's distal leg (Fig. 10 *A*, position *f'*) with an AP-2 bound at *g* (*lower right*) locks the third trimer into position for assembly (*lower left*). This yields a closed polygon along Fig. 10, *h–f* (*lower left*) with a free AP-2 available to propagate assembly. Geometrically,

it is apparent that at completion each vertex of the assembled coat will interact with one binding domain on a bivalent AP-2 molecule (Fig. 10 *B*), consistent with the observed stoichiometry.

Importantly, the model is supported by the stoichiometry of AP-2 incorporation relative to clathrin (results presented here), and with measurements of AP-2 size (Heuser, J., and J. Keen, manuscript in preparation). It may also explain observations that coats of different sizes are formed depending on whether or not AP is present (1, 20, 32). Coats formed from clathrin alone, under permissive conditions, are relatively large and heterogeneous in size (101 ± 5 nm SD) while AP-2 containing coats are smaller and more uniform (79 ± 6 nm SD). We suggest that formation of large coats reflects the "resting" solid angle between the trimer legs at the hub, i.e., the "pucker" of the trimer (15), and that the large size variability of coat diameter observed is a consequence of the variability of this angle due to flexibility of the joint during assembly. Coats in which AP-2 is incorporated are proposed to be smaller and more uniform in size because adjacent terminal domains in the assembling coat are bridged by AP-2 molecules, increasing the pucker of the trimers to a defined value and rigidifying them, yielding structures with increased radii of curvature and thus smaller diameter. According to this reasoning, AP-1 may yield yet smaller coats because it draws the legs together even more sharply. More speculatively, it seems possible that AP binding (or activation) in this manner can provide a mechanism to transform relatively flat clathrin lattices into the curved structures that are observed during endocytosis (7, 17).

Further studies are needed to delineate exactly how the AP-2 molecule interacts with clathrin and whether, as we have previously proposed, the 50,000-mol-wt component of the complex is independently active as a protein kinase that is oriented toward the enclosed vesicle surface (12). The available data indicate that the AP isolated from coated vesicles is primed for assembly, and that its activity in vitro is reversibly inhibited by high concentrations of protonated amines, or elevated pH. The clathrin uncoating ATPase has been shown, again in in vitro experiments, to promote a reverse reaction (reviewed in reference 24). Thus, a major unresolved question concerns the spatial and temporal expression of these activities within the intact cell and the extent to which they are independently, or reciprocally, regulated. The findings presented here, and experiments to test the resultant model, should help to advance elucidation of coated membrane function in cells.

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