**Deep-Etch Visualization of Proteins Involved in Clathrin Assembly**

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**Abstract.** Assembly proteins were extracted from bovine brain clathrin-coated vesicles with 0.5 M Tris and purified by clathrin-Sepharose affinity chromatography, then adsorbed to mica and examined by freeze-etch electron microscopy. The fraction possessing maximal ability to promote clathrin polymerization, termed AP-2, was found to be a tripartite structure composed of a relatively large central mass flanked by two smaller mirror-symmetric appendages. Elastase treatment quantitatively removed the appendages and clipped 35 kD from the molecule's major ~105-kD polypeptides, indicating that the appendages are made from portions of these polypeptides. The remaining central masses no longer promote clathrin polymerization, suggesting that the appendages are somehow involved in the clathrin assembly reaction. The central masses are themselves relatively compact and brick-shaped, and are sufficiently large to contain two copies of the molecule's other major polypeptides (16- and 50-kD), as well as two copies of the ~70-kD protease-resistant portions of the major ~105-kD polypeptides. Thus the native molecule seems to be a dimeric, bilaterally symmetrical entity. Direct visualization of AP-2 binding to clathrin was accomplished by preparing mixtures of the two molecules in buffers that marginally inhibit AP-2 aggregation and cage assembly. This revealed numerous examples of AP-2 molecules binding to the so-called terminal domains of clathrin triskelions, consistent with earlier electron microscopic evidence that in fully assembled cages, the AP's attach centrally to inwardly-directed terminal domains of the clathrin molecule. This would place AP-2s between the clathrin coat and the enclosed membrane in whole coated vesicles. AP-2s linked to the membrane were also visualized by enzymatically removing the clathrin from brain coated vesicles, using purified 70 kD, uncoating ATPase plus ATP. This revealed several brick-shaped molecules attached to the vesicle membrane by short stalks. The exact stoichiometry of APs to clathrin in such vesicles, before and after uncoating, remains to be determined.

**The role played by clathrin polymerization in the events of receptor clustering and membrane vesiculation during endocytosis is a subject of broad current interest (4, 20, 21).** Certain of the polypeptides released from clathrin coated vesicles by 0.5 M Tris have been shown to promote clathrin polymerization in vitro (13, 22, 33). These so-called assembly polypeptides are of interest because they may modulate in vivo coat assembly and clathrin–receptor interactions. We have purified and characterized that fraction of assembly proteins that binds to clathrin affinity columns and displays maximal assembly activity (10); this fraction we term AP-2. It has hydrodynamic properties suggesting a ~340-kD species composed of two copies of the three major polypeptides (16-, 50-, and ~105-kD) seen on SDS gels. Such a complex may thus be bivalent and may promote assembly by bridging adjacent clathrin triskelion during cage formation (10). Additional studies have identified two different functional domains within the AP-2 complex, one associated with the clathrin assembly function (34) and another with phosphorylation of its 50-kD components (11, 12, 19).

Here we determine the overall morphology of AP-2 and demonstrate that it also displays two physical domains: one, a central brick-like structure and the second, a pair of elastase-sensitive appendages. Correlation of these morphological observations with earlier biochemical and sedimentation studies (12, 34) leads us to conclude that the 50-kD phosphorylation reaction must occur exclusively within the brick-like core of the molecule whereas the assembly function includes both the core and the appendages. The latter, being paired, could well be involved in bivalent clathrin crosslinking (10). We also find that AP-2s bind to the terminal domains of isolated clathrin triskelion, confirming previous indications of this relationship from views of whole clathrin cages containing APs (9, 30). Finally, we show that
brain coated vesicles stripped of clathrin with the uncoating ATPase (18, 25) continue to display several surface projections that look like AP-2 molecules.

Materials and Methods

Coated vesicles were prepared from calf brains by sucrose gradient centrifugation, and clathrin and APs were stripped from them with 0.5 M Tris, pH 7, as described (10, 13). After fractionation via ammonium sulfate precipitation and chromatography on Superose 6B, APs were purified by clathrin-Sepharose chromatography as described (10). For comparison, Sepharose 6B fractions of APs were occasionally purified instead by hydroxyapatite column chromatography as described by others (22, 24).

Elastase treatment of AP's in 0.5 M Tris-HCl was performed as described (34) using a 1:200 (wt/wt) ratio of elastase to AP and incubating for 30 min at 20°C, followed by addition of PMSF (to 1 mM) to stop the reaction. Elastase treatment of AP-containing clathrin coats was accomplished similarly except that a 1:10,000 ratio was employed. In both cases, the inhibitor-treated reaction mixtures were analyzed by SDS-PAGE immediately after incubation and after an additional 24 h on ice, corresponding to the delivery time from one laboratory to the other. These data confirmed that elastase treatment quantitatively cleaved the ~105-kD assembly polypeptides to yield the smaller fragments previously reported (34), but that the 50- and 16-kD assembly polypeptides were not degraded even after 24 h. Clathrin was obtained during the Superose 6B chromatography and mixed with an equal amount (by weight) of AP-2 in 0.5 M Tris, pH 7.0, plus 10% glycerol, and then washed the mica with a solution suitable for deep-etching (i.e., 70 mM KCl plus 30 mM Hepes buffer, pH 7.0; see references 7-9 for details). Replicas of molecules prepared in this manner display relatively squared-off or brick-like masses, many of which possess one or two smaller flanking structures (Fig. 1). Not all molecules display such appendages, however, probably because the molecules adsorb to mica randomly relative to the orientation of their flanking structures. Among those displaying two appendages, the bilaterally symmetrical molecules selected for the gallery in Fig. 2 are representative. These are arranged to show that the two appendages can sometimes be found on op-

![Figure 1](https://example.com/figure1.jpg)
Figure 2. Gallery of selected AP-2 molecules from several different affinity column runs, prepared essentially as in Fig. 1. These were all selected because they display two relatively symmetric small appendages or ears on either side of a relatively squared-off central mass. They are arranged with the widest spans in the left columns and the narrowest spans in the right columns, with intermediates in between. Other molecules in these preparations display only one or no appendages. These are presumably ones that adsorb to mica in different orientations. Note that most of the molecules display bilateral symmetry, in that both appendages are displaced from the central “brick-like” portion of the molecule by roughly the same degree and the same angle. This fact, combined with the biochemical data reviewed in the text, suggests that the observed molecule is a dimer. Window width and height, 75 nm.

posite sides of the molecule, roughly 180° apart, while at other times they appear to cluster together on the same side of the molecule. The latter views give the impression of one larger appendage, probably explaining earlier electron microscopic images which suggested that the AP-2 molecule possesses only one appendage (31). Regardless of this variation in angular separation, the two appendages seen here usually appear to be symmetrically placed relative to the central mass. Furthermore, even though they often appear to be separated from the central mass by 6 nm, their satellite distribution suggests that a thin connection to the central brick does exist. The concentration of other small entities in the vicinity of these larger complexes is too low to suggest random colocalization of the large and small components.

Elastase Treatment of Purified AP Complexes

Insight into the composition of the two appendages on AP-2 was derived from experiments involving controlled elastase proteolysis which, as shown elsewhere (34), cleaves the ~105-kD polypeptides of AP-2 into ~70-kD fragments plus smaller peptides. In this reaction, the smaller peptides are released into the medium but the larger species remain associated with the 50- and 16-kD polypeptide components in the remaining AP-2 complex. The latter are not cleaved (34).

Examining such clipped complexes after adsorption to mica, the appendages are found to be quantitatively removed (Fig. 3). What remains are clean “bricks” of the same dimension as the central masses seen in intact molecules (10 × 12 nm). Only rarely does a hint of an extension remain. However, no isolated appendages are found, possibly because they are further degraded. We conclude that in the intact molecule, the ~105-kD polypeptides not only form much of the brick-like central core of the molecule but also extend out to form the appendages, and that connections to the latter extensions are especially sensitive to proteolysis. The 16- and 50-kD polypeptides, on the other hand, would seem to be packed into the brick-like central mass along with the protease-resistant ~70-kD portions of the original ~105-kD polypeptides. The size of this part of the molecule is large enough to accommodate two copies of each of these polypeptides, which would total ~280 kD. This can be seen by comparing several calibration molecules of roughly the same size, in Fig. 3a (right columns).

Structural Comparison of AP-2s with Other’s HA-II APs

Hydroxylapatite chromatography of partially purified APs yields a fraction, termed HA-II, that looks on SDS-PAGE gels and behaves in clathrin assembly experiments like AP-2 (22, 24). Indeed, we find this particular fraction is retained by clathrin affinity columns exactly like AP-2 (10). Preparation of this material for electron microscopy by the procedures used above also yields images of molecules that look identical to AP-2s, in that they are 10 × 12-nm brick-like entities with two bilaterally disposed appendages (data not shown). We conclude that these two fractions are probably identical.

AP-2 in Coat Structures

To probe the organization of AP-2 in coat structures, and to further confirm that these brick-like structures were indeed AP-2, we took advantage of a phenomenon described earlier (9), namely, that clathrin baskets “melt,” or fall apart, when they absorb to mica flakes that have been strongly cationized by pretreatment with polylysine (presumably due to the high local concentration of protonated amino groups; cf. reference 13). In this case, we prepared baskets containing AP-2 by coidialyzing a mixture of equivalent amounts of clathrin triskelions and AP-2s, from 0.5 M Tris, pH 7.0, into 0.1 M MES, pH 6.5 (see Materials and Methods). As noted earlier
Figure 3. Left three columns display several examples of the same AP-2 preparation as in Fig. 2, but subjected to brief elastase digestion before adsorption to mica. Note that both appendages are removed by such treatment. This "clipped" AP is no longer capable of promoting clathrin assembly. For size comparisons, b illustrates β-glucuronidase (280 kD), c illustrates aspartate transcarbamylase (307 kD), and d illustrates leucine aminopeptidase (255 kD), all prepared the same way and printed at the same magnification. Window width and height in left three columns, 75 nm.

(9), APs are not visible on the outside surfaces of such baskets, and cannot be seen down inside them due to technical limitations of metal replication. However, when such copolymers are adsorbed to polylysine-pretreated mica flakes, they melt like pure clathrin baskets, creating clusters of component parts (Figs. 4 and 5). Two components can be recognized: clathrin triskelions, which differ from earlier views (14, 28) because they have assumed their flattened, backward orientation characteristic of high-affinity absorption to mica (9, 16), and large brick-like molecules identical to those observed in purified AP-2 preparations (Fig. 2). The brick-like molecules again measure 12 × 10 nm on a side and display one or two 4-6-nm satellite appendages. Such molecules are never seen when pure clathrin cages are melted on mica (9). Moreover, when AP-2-containing cages are treated with elastase under conditions that cleave the ~105-kD polypeptide into ~70-kD fragments, and the cages are then adsorbed to polylysine-treated mica, the brick-like proteins they release are now devoid of appendages (Fig. 6), just like the species that resulted from elastase-treatment of pure AP-2s (Fig. 3). Thus, we conclude that the released brick-like structures are most likely AP-2 molecules that had been incorporated into these cages. The number of such molecules in individual melted "heaps" of clathrin ranged from 15 to 30 (n = 18). Since the size of our clathrin plus AP-2 cages centers at ~60 nm diam, they should contain an average of 36 triskelions (cf. references 5, 9, 21). Assuming that only a few bricks are lost to the medium during melting, this would suggest that in coassembled cages AP-2 molecules are roughly half as abundant as triskelions.

Direct Visualization of AP-2–Clathrin Interactions
Examination of cages reassembled from purified clathrin, either by direct inspection of deep-etched samples (9) or by image reconstruction of samples embedded in vitreous ice (29), suggests that within the outer polyhedral basket there is an enclosed shell of material composed of inwardly-directed "terminal domains" of clathrin triskelions (cf. reference 15 for definition of terminal domains). In cages assembled from clathrin plus AP-2, additional material is present within this shell of terminal domains. By deep-etch electron microscopy, this additional material appears to fill the cages (9), while in frozen-hydrated material it appears to form another hollow shell inside the terminal domains (30). Earlier negatively stained images suggested, on the other hand, that APs
might reside more peripherally, because they either appeared as eccentric "nuggets" (9, 33) or they appeared to thicken the struts and vertices of the cages generally, thus partially filling in their polygonal openings (22). Clearly, these earlier interpretations do not fit with more recent deep-etched or frozen-hydrated images, both of which indicate that APs are located deep inside the cage, apparently in contact with the shell of terminal domains.

It thus became of interest to see if we could demonstrate direct interactions of AP-2 with clathrin terminal domains. We first attempted to do this by searching for residual clathrin-AP-2 contacts around the edges of "melting" cages, but such contacts were found very infrequently. A few examples of APs contacting clathrin terminal domains are shown in Figs. 4 and 5 (arrows), but due to the close positioning of molecules, such images could not be considered defini-
In an effort to stabilize such interactions we prefixed clathrin–AP-2 cages with glutaraldehyde, but the cages then simply failed to melt on polylysine mica (not shown). We also attempted to find such interactions among the background of leftover molecules in reassembly experiments, but we again observed nothing definitive. We were only able to directly visualize clathrin–AP interactions when preparations of HA-II (described above), which elute from hydroxylapatite in relatively high concentrations of phosphate buffer (0.3 M), were mixed with soluble clathrin. Under these conditions, phosphate obviously inhibits the aggregation of AP-2 but apparently permits clathrin–AP interactions to develop without further cage assembly. Adsorbed to the mica under these conditions are many free triskelions and many free AP-2 bricks, but also many clathrin–AP complexes (Figs. 7 and 8). In most cases, these complexes appear to involve binding of APs to the terminal domains of triskelia. This is reminiscent of the occasional observation in melting cages (above) of a single AP molecule appearing to cross-link two adjacent triskelia via their terminal domains (Fig. 5, arrows). Often, a small gap between the central “brick” of the AP-2 molecule and the terminal domain of clathrin suggests that binding may be via one of the APs peripheral appendages (circled in Fig. 8). Only rarely have we observed possible contacts of AP-2s with the proximal parts of triskelia or with their hubs. In control experiments, we have been unable to detect any binding of elastase-treated APs to intact clathrin nor any binding of intact APs to “clipped” clathrin lacking terminal domains.

Visualization of Putative APs on Enzymatically Stripped Brain Coated Vesicles

Finding that APs form readily visible brick-like molecules with characteristic bilateral appendages raised the question of whether similar entities could be found on brain coated vesicles enzymatically stripped of clathrin with the 70-kD uncoating ATPase. This enzyme is reported to remove the clathrin coat without removing APs to any great extent (25). Accordingly, we obtained the 70-kD uncoating ATPase as a...
Figure 8. Gallery of selected examples of AP-2s binding to the terminal domains of clathrin triskelia selected from preparations such as those in Fig. 7. In the circled examples, AP-2 molecules are located immediately adjacent to the terminal domains of the clathrin legs rather than in direct contact, suggesting that they may be bound by one of their smaller appendages. Window width and height, 100 nm.

gift from C. Steer (National Institutes of Health), who prepared and characterized it according to the protocols used earlier (25), and we mixed it with an equal ratio (wt/wt) of bovine brain coated vesicles (100 μg of each) in the presence of 1 mM ATP. After 15 min of incubation at 37°C, mica flakes were added to the reaction mixture, mixed for 30 s, and then washed free of excess protein and vesicles with our usual “intracellular” buffer (70 mM KCl, 30 mM Hepes buffer, pH 7.2, 5 mM MgCl2, 3 mM EGTA). Freeze-etching these flakes revealed numerous examples of ~45-nm-diam membrane vesicles that were not seen in the starting material, thus were most likely to be clathrin-stripped coated vesicles. A few of these displayed an occasional clathrin triskelion clinging to them (not shown) but most instead displayed 3–8 brick-like molecules in their immediate vicinity, often clearly attached by narrow stalks to the vesicle proper (Fig. 9). Often, these brick-like satellites display one or two small appendages or “ears” like those found on isolated AP-2 dimers (Fig. 9, circled). We imagine that these molecules assume their “satellite” distribution during adsorption to mica, by sliding around the vesicle surface until they strike the mica and adhere. Occasionally, similar molecules are found

Figure 9. Gallery of brain coated vesicles enzymatically stripped of clathrin before adsorption to mica, illustrating that several AP-sized molecules remain attached to them. Such attachments generally assume a “satellite” distribution during adsorption to mica, though a few remain on top of the vesicles (arrows). Some of the satellites display additional small appendages like those seen on purified AP-2s (circled). Printed at the same magnification as Fig. 2 to facilitate comparison. Window width and height, 125 nm.
on top of the vesicles (Fig. 9, arrows), though often they are hard to discern in this location because the vesicles become slightly wrinkled during freeze-drying. In this position, stalks are of course not visible; but this raises the question of whether a stalk is a natural part of the AP molecule (or of the membrane molecule to which the AP attaches) or whether something gets stretched as the APs slide around the vesicle and adhere to mica. Counting the molecules on top of the vesicles as well as the satellite ones, we obtain a mean of $5 \pm 2$ (SD; $n = 38$) putative AP-2 dimers per stripped vesicle. Further work needs to be done to determine whether this reflects the coated vesicle's original complement of assembly polypeptides, or is a reduced amount due to loss during uncoating.

Discussion

AP-2 Structure

The tripartite structures we find scattered around clathrin plus AP-2 cages melted on mica and brain coated vesicles stripped with uncoating ATPase are identical in morphology to the major species present in our affinity-purified AP-2 preparations and our HA-II preparations. In all cases they display large central masses measuring $12 \times 10$ nm after replication with platinum, and possess two 4-6-nm appendages that lie in variable positions but are usually mirror-symmetric relative to the central mass (Fig. 2). These images differ in one basic respect from earlier images of negatively stained preparations and our HA-II preparations. In all cases they are hard to discern in this location because the vesicles become slightly wrinkled during freeze-drying. In this position, stalks are of course not visible; but this raises the question of whether a stalk is a natural part of the AP molecule (or of the membrane molecule to which the AP attaches) or whether something gets stretched as the APs slide around the vesicle and adhere to mica. Counting the molecules on top of the vesicles as well as the satellite ones, we obtain a mean of $5 \pm 2$ (SD; $n = 38$) putative AP-2 dimers per stripped vesicle. Further work needs to be done to determine whether this reflects the coated vesicle's original complement of assembly polypeptides, or is a reduced amount due to loss during uncoating.

In attempting to determine the polypeptide composition of this part of the molecule, we were greatly aided by the observation that elastase quantitatively clips off the appendages of native molecules and leaves only their central brick-like masses. Elastase is known to clip the ~105-kD components of AP-2 into ~70-kD polypeptides while releasing into solution the fragment(s) that comprised the remaining ~35-kD (34). Thus the appendages must be composed of the ~35-kD portions of the ~105-kD polypeptide. The smaller polypeptides in the complex (50 and 16 kD) are unaffected by elastase and remain associated with ~70-kD cleavage products. Therefore, the central mass must be composed of, at a minimum, one 16-kD polypeptide, one 50-kD polypeptide, and one ~70-kD fragment of the ~105-kD polypeptide (for a total of 130-140 kD). Since its actual size suggests a molecular mass closer to twice that, and since the whole molecule normally displays two appendages, the logical conclusion is that AP-2 is dimeric. However, since this molecule is prone to concentration-dependent aggregation (2), it is worth noting that the morphological observations reported here and the light scattering molecular mass measurements (10) were made on samples prepared identically and at similar protein concentrations.

The image of a central brick-like structure with two projecting globular appendages presented here can be correlated with the available physico-chemical data on the AP-2. From the Stokes radius (6.6 nm; references 10, 13), molecular mass (342 kD; reference 10), and partial molar volume obtained from the amino acid composition (0.74 cc/g; reference 31 and Keen, J., unpublished observations), a frictional ratio ($f/f_o$) of ~1.4 can be calculated (26). The asymmetry component of this ratio (assuming a prolate ellipsoidal shape and hydration of 0.2 g water/g protein) reflects a moderately asymmetric shape with an axial ratio of 5-6. These calculations suggest that in solution the AP-2 molecule adopts a relatively extended configuration in which the appendages reach out from the central brick-like structure, more like the molecules in the left column of Fig. 2. This can also explain the earlier observation that elastase-clipped AP-2 continues to cosediment with native AP-2 on rate zonal ultracentrifugation despite the loss of 20% of its mass (33). Removal of the appendages apparently generates a more symmetrical "core" molecule whose changes in mass and shape are compensatory and result in an unchanged sedimentation rate. We note that the reported (31) value for the sedimentation constant of the AP-2 (8.6 S), in combination with Stokes radius and $v$ (partial specific volume), predict a protein with smaller molecular mass (252 kD). However, a molecule with this smaller mass would have to be considerably more asymmetric with a frictional ratio ($f/f_o$) ~1.6 and axial ratio of 8-9. These values are difficult to reconcile with the images presented here. While this inconsistency cannot be fully resolved at present, one explanation may be that the observed sedimentation constant measured in sucrose gradients is artifactually small, perhaps due to preferential hydration (26) or to a larger value for $v$ than that calculated only from the amino acid composition.

The model presented in Fig. 10 portrays the AP-2 molecule as being composed of three discrete globular domains connected by thinner protease-sensitive strands. This model is similar to that for fibrinogen (6, 17, 32), although the relative sizes of the three globular domains are clearly different. The AP-2 is also considerably less asymmetric than fibrinogen.
AP-2 Organization within the Clathrin Cage

It has been known for some time that when clathrin is polymerized in the presence of assembly polypeptides, the latter become incorporated into the newly formed cages (22, 33); however, the location of APs in these cages has been variously described. Knowing now that AP-2 is a relatively large brick-like complex (Figs. 1–3), we can state with certainty that these structures are not present on the outside of such cages (cf. reference 9), nor do they lie along the struts forming the external polygons of such cages, as was proposed earlier (22). Instead, deep etching agrees with a more recent electron microscopic study of frozen hydrated clathrin–AP cages (30), which indicated that APs form a discrete shell of density within that of the clathrin terminal domains. Elsewhere, one of us has proposed that AP-2s promote coat assembly by bridging the terminal domains of clathrin triskelia so that they come to occupy adjacent positions in the assembling coat structure (10). The vertex-to-vertex separation in a normal clathrin lattice is 15–16 nm (9), while the widest dimension of the AP-2 dimer we find to be between 13 and 20 nm (depending on the separation of its ears; cf. Fig. 2). Thus, the AP-2 molecule is big enough to bridge two terminal domains, particularly since the inwardly directed distal arms of clathrin triskelia are oriented radially, bringing neighboring terminal domains closer together within the coat structure than are the vertices on the surface of the coat (29). However, we have rarely visualized such cross-linking to date (see only Fig. 5, arrows).

There is good reason to believe that clathrin assembly involves the appendages on the AP-2 molecule. Elastase treatment of AP-2 removes these structures and quantitatively abolishes its ability to promote clathrin assembly (34). There are also indications that not all of the brick-like head may be necessary, since active ~105-kD species without any 50- and 16-kD polypeptides have also been found to be capable of promoting clathrin assembly (12, 25). However, it is not clear if these particular ~105-kD polypeptides are actually components of the AP-2 and HA-II complexes visualized here, or are components of other sorts of AP complexes that also exist (1, 23).

AP Binding to Coated Vesicle Membranes

Assuming that clathrin terminal domains point inward in bona fide coated vesicles and APs also interact with them in that situation, then APs would certainly be large enough to span the gap between the coat and the enclosed vesicle (21) and would be in a proper position to interact with the vesicle as well as the coat (27). It was thus of interest to observe “halos” of several brick-like molecules around the vesicles that emerge upon enzymatic removal of clathrin from brain coated vesicles (18, 25). Smaller numbers of brick-like molecules are also seen on coated vesicles stripped with 10 mM Tris, pH 8, rather than with the uncoating ATPase (Heuser, J. E., and D. Branton, unpublished observations), but such chemical stripping leaves behind fewer APs (21, 23, 27, 33). Although we cannot be sure of the extent of loss of AP from the stripped vesicles, our images would suggest that brain coated vesicles typically possess at least 10 or so molecules large enough to be AP-2 dimers. This is a low number in comparison to their original content of clathrin triskelions (~60; cf. references 5, 9, 21) and is very different from the situation in reconstituted clathrin–AP cages where the proportions are 2:1 (reference 10 and the data presented here). Our data thus raise the possibility that the AP–clathrin ratio seen in whole coated vesicle preparations from brain (21, 33) may be unduly influenced by the large number of “empty cages” that abound in such preparations and that the AP–clathrin ratio in normal coats and vesicles in situ may be considerably lower. If this were so, it might indicate that the AP functions by nucleating cage assembly (as originally proposed in reference 27), rather than being uniformly incorporated in the organization of the coat. Further work will be necessary to resolve this issue.

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