Organization of Clathrin Coat Structures

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Received July 27, 1988; Revised Manuscript Received December 5, 1988

ABSTRACT: Clathrin (8S), when purified, polymerizes under low-pH conditions (0.1 M MES, pH 6.0–6.2) into a heterogeneous population of baskets with sedimentation coefficients ranging from 150 to 400 S. Several groups of proteins of molecular masses 180, 110, 100, 50, and 47 kDa (based on sodium dodecyl sulfate gel electrophoresis) present in the isolated coated vesicles are involved in polymerizing clathrin under physiological conditions to a homogeneous population of baskets [Zaremba, S., & Keen, J. H. (1983) *J. Cell Biol. 97*, 1339; Ahle, S., & Ungewickell, E. (1986) *EMBO J. 5*, 3143]. We now report that in 0.1 M MES, pH 6.0, where pure clathrin polymerizes by itself, the above proteins (together known as associated proteins or APs) induce polymerization of clathrin into three distinct sizes of baskets with sedimentation coefficients of 150, 220, and 300 S. Low ratios of clathrin to APs give rise to smaller sizes, whereas higher ratios give rise to predominantly the larger sizes. The smaller size baskets (150S) are intermediates in the polymerization of clathrin to larger size baskets (300S) as inferred from the dissociation of larger size baskets into smaller size baskets and the formation of larger size baskets from smaller size baskets upon the addition of pure clathrin.

Coated vesicles are involved in a variety of cellular processes such as the transport across and between membranes (Goldstein et al., 1985; Pearse & Bretscher, 1981). The best understood of these is receptor-mediated endocytosis (Goldstein et al., 1979). The coat of the coated vesicles is made up of a number of proteins, the best characterized among these being clathrin, the protein responsible for the polygonal organization of the coat (Pearse, 1975; Crowther & Pearse, 1981). The clathrin protomer, as isolated from coated vesicles, contains three identical subunits of 180 kDa[1] and three light chains of 33–36 kDa (Kirchhausen & Harrison, 1981). It has a sedimentation coefficient of 8 S and is very asymmetric (Ungewickell & Branton, 1981; Pretorius et al., 1981).

Coated vesicles isolated from bovine brain contain several other proteins aside from clathrin, the principle ones being a group of proteins of 110–100 kDa in association with a 50- or a 47-kDa protein (Keen et al., 1979; Prasad et al., 1985; Pearse & Robinson, 1984;, Manfredi & Bazari, 1987), a 180-kDa protein that has recently been discovered (Ahle & Ungewickell, 1986), and a doublet of 55 kDa that has been identified as tubulin (Pfeffer et al., 1983; Kelley et al., 1983). Several other proteins present in minor amounts have been identified as integral membrane proteins (Weidenman et al., 1985). Clathrin is readily solubilized from CVs by a variety of processes such as treatment with 2 M urea (Schook et al., 1977) or dialysis against 0.01 M Tris, pH 8.5 (Nandi et al., 1982b), or 0.5 M Tris, pH 8.0 (Keen et al., 1979). Depending on the method of solubilization, a certain proportion of 180-, 110-, 100-, 50-, and 47-kDa proteins (together all these proteins are henceforth referred to as associated proteins or APs) are solubilized with clathrin in the supernatant of the pelleted CV extract. Clathrin thus obtained is further purified by gel filtration on either Sepharose 4B-CL (Keen et al., 1979) or Sephacryl S-300 (Prasad et al., 1985) equilibrated with 0.5 M Tris, pH 8.0 buffer so that it no longer contains APs. The purified clathrin polymerizes into a heterogeneous variety of basketlike structures under very low pH conditions (0.1 M Mes, pH 6.0–6.2) (Zaremba & Keen, 1983; Prasad et al., 1985). Sedimentation velocity and equilibrium studies performed on partially purified clathrin indicated two distinct size distributions of sedimentation velocities 150 and 300 S and molecular weights 25×10^6 and 100×10^6, respectively (Nandi et al., 1980). However, recent electron microscopic examination revealed that there are three distinct sizes of baskets in the polymerized products formed from pure clathrin (Heuser & Kirchhausen, 1985). The APs that formed the soluble extract of coated vesicles in the preparation of clathrin have been shown to polymerize pure clathrin to a smaller size basket of uniform distribution (Zaremba & Keen, 1983; Pearse & Robinson, 1984; Prasad et al., 1985; Ahle & Ungewickell, 1986). They have also been implicated in the binding of clathrin to the vesicles stripped of clathrin and assembly proteins (Unanue et al., 1981; Zaremba & Keen, 1985; Prasad et al., 1985) and enhancing the rate of clathrin polymerization (Prasad et al., 1985).

In this report we studied the polymerization of pure clathrin and the reaction products obtained when variable amounts of APs are included in the reaction mixture. We also examined the role of the APs when they are added to the already polymerized baskets. In addition, we examined the question of whether smaller baskets are intermediates in the polymerization of clathrin to larger size baskets.

Materials and Methods

Chemicals. Analytical-grade sucrose and tris(hydroxymethyl)aminomethane were obtained from Bethesda Research Laboratory. Ethylene glycol bis(β-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and 2-(*N*-morphalino)ethane sulfonic acid (Mes) were from Sigma. *N*-(1-Anilinonaphthalenyl)-maleimide (ANM) was from Polysciences. Sodium dodecyl sulfate (SDS) was from BDH Chemicals Ltd. Dithiothreitol (DTT) was from United States Biochemical Corp., Cleveland,

[1] Abbreviations: kDa, kilodalton(s); AP(s), assembly protein(s) or associated protein(s); CV(s), coated vesicle(s); UV(s), uncoated vesicle(s); ANM or AN-maleimide, *N*-(1-anilinonaphthalenyl)maleimide; AN, anilinonaphthalene; Tris, tris(hydroxymethyl)aminomethane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; SDS, sodium dodecyl sulfate; DEAE, diethylaminoethyl; CHC, clathrin heavy chain(s).

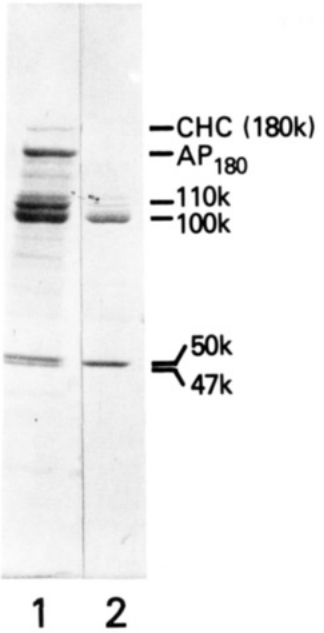

FIGURE 1: SDS gel electrophoretic pattern of the APs: (lane 1) preparation obtained from a 0.01 M Tris, pH 8.5, extract of CVs; (lane 2) preparation obtained from a 0.01 M Tris, pH 8.5, extract of stripped vesicles. In both cases, the solutions were treated with 0.5 M Tris, pH 8.0, and filtered on Sephacryl S-300 columns to obtain the APs.

OH. Sephacryl S-300 was from Pharmacia.

Preparative Procedures. CVs were prepared from bovine brain by a modification of the method of Pearse (1975), as previously described by Nandi et al. (1982a). They were stored in 0.10 Mes, pH 6.5, the buffer used for their isolation.

Clathrin was prepared as described by us earlier (Prasad et al., 1985). Briefly, the method is as follows. CVs were dialyzed for 15 h against 0.01 M Tris, pH 8.5, and the resultant solution was centrifuged at 50000 rpm, 23 °C, in a Ti 70.1 rotor for 1 h. The supernatant is mostly clathrin with 10–15% APs. The supernatant was then dialyzed against 0.5 M Tris, pH 8.0, and 5 mM DTT for 15 h and loaded on a Sephacryl S-300 column equilibrated with the same buffer. Two major peaks were obtained in the elution profile. The first peak is clathrin and the second peak consists mostly of APs with a small contamination of clathrin. Both the peaks were concentrated and dialyzed extensively against 0.01 M Tris, pH 8.5, to remove the high concentration of Tris.

The APs were prepared in two ways. One is the preparation described above and shown in Figure 1 (lane 1). The other is from the uncoated or stripped vesicles obtained as a pellet in the preparation of clathrin after extracting the CVs with 0.01 M Tris, pH 8.5, as described elsewhere (Prasad et al., 1986). This preparation has very little clathrin or AP_{180} contamination as judged from SDS gels (Figure 1, lane 2). In most of the experiments described in this paper, the APs shown in lane 1 of Figure 1 were used. However, in some experiments for reasons given in the text, the pure APs shown in lane 2 of Figure 1 were used.

Methods of Analysis. Unpolymerized clathrin (8S) and polymers of clathrin (150S, 220S, 300S, and 400–500S) can be differentiated by the distribution of their sedimentation coefficients either by analytical ultracentrifugation or by the position, width, and symmetry of the bands on sucrose gradient ultracentrifugation. These two procedures, therefore, served as analytical tools for the studies reported here.

(*a*) *Analytical Ultracentrifugation.* A Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanner was used with 12-mm optical path length, double-sector cells. A detailed analysis of the sedimentation patterns of clathrin baskets has been reported elsewhere (Pretorius et al., 1981).

(*b*) *Zonal Centrifugation in Sucrose Gradients.* A Beckman Model L8M ultracentrifuge with an SW 28 rotor was used. Linear gradients were formed by mixing equal volumes of 10 and 30% sucrose (w/w) dissolved in the same buffers used in the clathrin polymerization procedure. One-milliliter samples were layered on top of the gradients. Solutions were centrifuged for 100 min at 24000 rpm, 23 °C; 1-mL fractions were collected from the bottom of the tubes. An LKB peristaltic pump set at 2 mL/min was used to collect the fractions.

(*c*) *Fluorescence Measurements.* Fluorescence intensities were measured in a Perkin-Elmer MPF-3 spectrofluorometer. Relative protein concentrations were obtained by exciting at 290 nm and measuring the emission at 340 nm. The ANM-labeled proteins were excited at 350 nm, and their emission was measured at 426 nm.

(*d*) *Labeling of the Proteins.* ANM labeling of the proteins has been described elsewhere (Prasad et al., 1984). The ratio was usually 2 mol of label/mol of protein, by use of an average molecular weight of 600000 for clathrin and 300000 for APs. We have shown elsewhere that the labeling does not affect the reactivity of the proteins with one another (Prasad et al., 1984).

(*e*) *Gel Electrophoresis.* Gradient gels (5–15% acrylamide) were used throughout. The SDS gel electrophoresis was done according to Laemmli (1970).

(*f*) *Protein Concentrations.* Clathrin concentration was measured by its absorption at 280 nm ($E^{280}_{1\%} = 12.1$). The concentration of the APs was estimated by measuring the optical density at 280 nm and at 215 nm according to the method reported elsewhere (Prasad et al., 1986) which is similar to that of Scopes (1974).

(*g*) *Column Chromatography.* The details have been described elsewhere (Prasad et al., 1986).

(*h*) *Electron Microscopy.* Electron microscopic analysis was by negative staining with 1% uranyl acetate (van Bruggen et al., 1960) and was performed by Dr. B. Kramarsky (Electron-Nucleonics, Silver Spring, MD).

RESULTS

The polymerization of pure clathrin with variable amounts of APs was investigated under two conditions: at 0.1 M Mes, pH 6.5, where clathrin by itself does not polymerize, and at 0.1 M Mes, pH 6.0, where clathrin has an intrinsic capability to polymerize. Figure 2 shows the sucrose gradient pattern of pure clathrin (0.4 mg/mL), polymerized at 0.1 M Mes, pH 6.5 or 6.0, by overnight dialysis from 0.01 M Tris, pH 8.0, into the required buffer. The protein concentration was monitored by the intrinsic tryptophan fluorescence of clathrin. There was little, if any, polymerization at 0.1 Mes, pH 6.5 (circles), whereas at 0.1 M Mes, pH 6.0, clathrin polymerized into a heterogeneous population of baskets, predominantly the larger size. Sedimentation velocity analysis gave a value ranging from 400 to 500 S, depending on the particular sample. An electron micrograph of a basket representative of the larger size is shown in the inset of Figure 2.

A different pattern was obtained with solutions consisting of a fixed concentration of clathrin (0.6 mg/mL) and various concentrations of AN-labeled APs after mixing them in 0.01 M Tris, pH 8.0, and dialyzing into either 0.1 M Mes, pH 6.5, or 0.1 M Mes, pH 6.0. Figure 3 shows a typical sucrose gradient pattern after polymerization in 0.1 M Mes, pH 6.5, with 0.15 mg/mL APs. Increasing the concentration of the APs increased the polymerization of clathrin, enhancing the peak at fraction 15 and decreasing the peak at fraction 4 (data

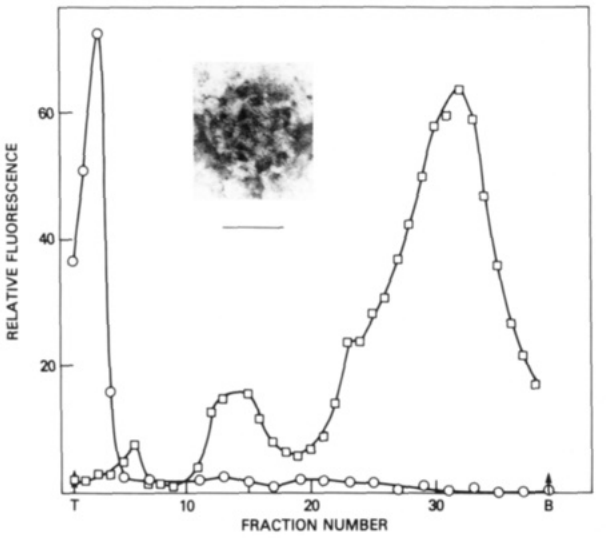

FIGURE 2: Sucrose gradient ultracentrifugation pattern of pure clathrin (0.4 mg/mL) in 0.1 M Mes, pH 6.5 (○), and after polymerization in 0.1 M Mes, pH 6.0 (□). Each sample was dialyzed overnight from 0.01 M Tris, pH 8.5, buffer into the respective buffer. Centrifugation was done in 10–30% sucrose gradients (B = bottom, T = top) for 100 min at 24000 rpm, 23 °C, in an SW 27 rotor. Inset: Electron micrograph of the baskets polymerized at 0.1 M Mes, pH 6.0 (bar = 100 nm).

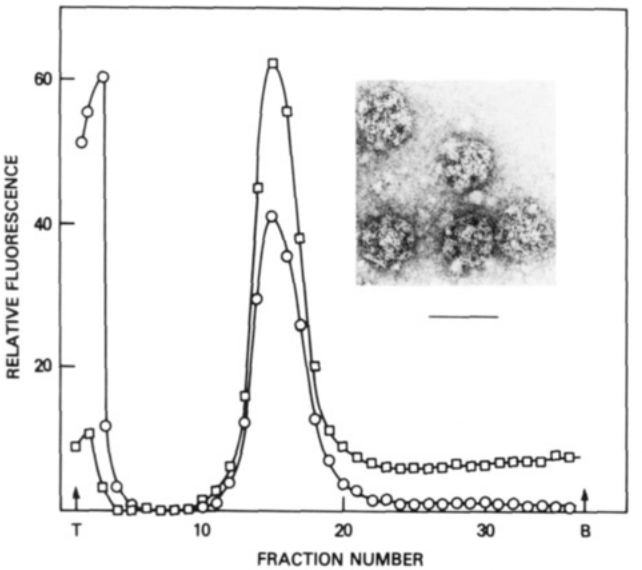

FIGURE 3: Sucrose gradient ultracentrifugation pattern of baskets obtained by polymerization of pure clathrin (0.6 mg/mL) with AN-labeled APs (0.15 mg/mL) in 0.1 M Mes, pH 6.5. Clathrin and APs were mixed in 0.01 M Tris, pH 8.5, and dialyzed overnight against 0.1 M Mes, pH 6.5. Tryptophan fluorescence (○); AN fluorescence (□). The inset shows the baskets polymerized under these conditions (bar = 100 nm).

not shown). The AN fluorescence (squares) was almost exclusively found in the peak at fraction 15. The sedimentation value of the baskets corresponding to fraction 15 has been reported earlier as 150 S (Pretorius et al., 1980). An electron micrograph of the polymerized baskets is shown as an inset in Figure 3. The baskets are of uniform size (60–70 nm) and correspond to the smaller size range.

Panels A, B, and C of Figure 4 show the sucrose gradient patterns of the solutions (clathrin, 0.6 mg/mL, and AN-labeled APs, 0.10, 0.20, and 0.30 mg/mL, respectively) polymerized at 0.1 M Mes, pH 6.0. In contrast to the data at 0.1 M Mes, pH 6.5, in Figure 4A,B, the AN and Trp fluorescence is also found at higher fractions than fraction 15. At low concentrations of APs (Figure 4A) the clathrin as well as the APs are present at fractions 15, 20, and 26. Sedimentation velocity analysis of the peaks at fractions 15 and 26 gave values of 150 and 300 S, respectively, whereas the value of the peak at fraction 20 could not be evaluated because of its low concentration. At a slightly higher concentration of APs (Figure 4B), the peak at fraction 26 disappeared, with only peaks at fractions 15 and 20 becoming more prominent. Once again, the AN fluorescence is present at both the peaks. The sedimentation velocities of the two peaks at fractions 15 and 20 were 150 and 220 S, respectively. At high concentrations of the APs (Figure 4C), only a single peak at fraction 15 containing all the label was obtained, indicating that all the baskets were 150S.

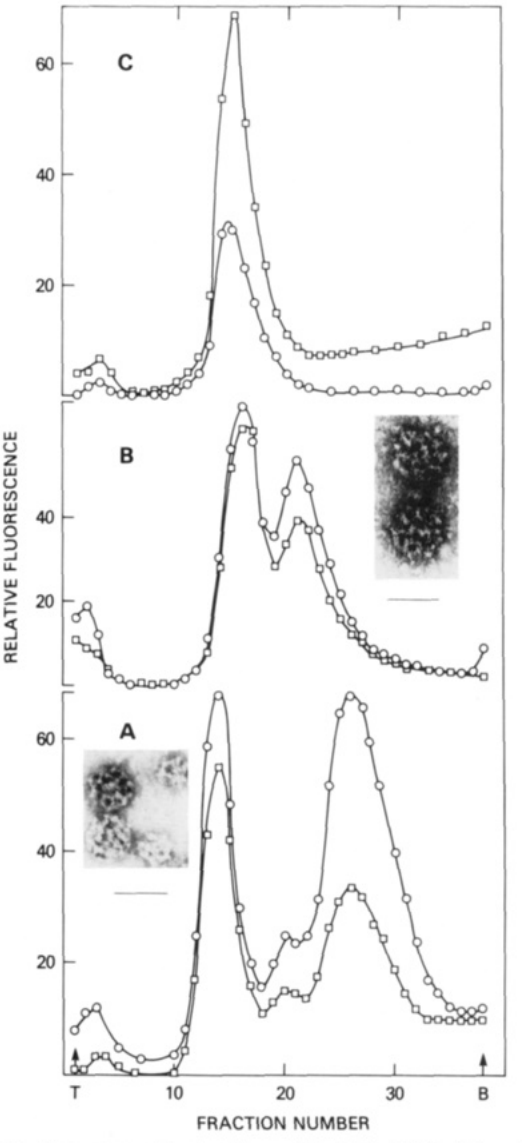

FIGURE 4: Sucrose gradient pattern of baskets obtained by polymerization of pure clathrin (0.6 mg/mL) with varying concentrations of AN-labeled APs at 0.1 M Mes, pH 6.0: (A) 0.10 mg/mL; (B) 0.20 mg/mL; and (C) 0.30 mg/mL. Clathrin and APs were mixed in 0.01 M Tris, pH 8.5, and dialyzed against 0.1 M Mes, pH 6.0. Tryptophan fluorescence (○); AN fluorescence (□). The inset shows the type of baskets formed. The inset in panel A represents the total solution consisting of all sizes of baskets. The baskets in panel B correspond to fraction 21 in the gradient. The baskets in panel C were the same as in Figure 3 (bar = 100 nm).

In addition to the distribution of the labeled APs in Figure 4A,B, it is of interest to note the ratios of tryptophan to AN fluorescence. Most of the tryptophan fluorescence is due to clathrin, and all of the AN fluorescence is due to APs. The

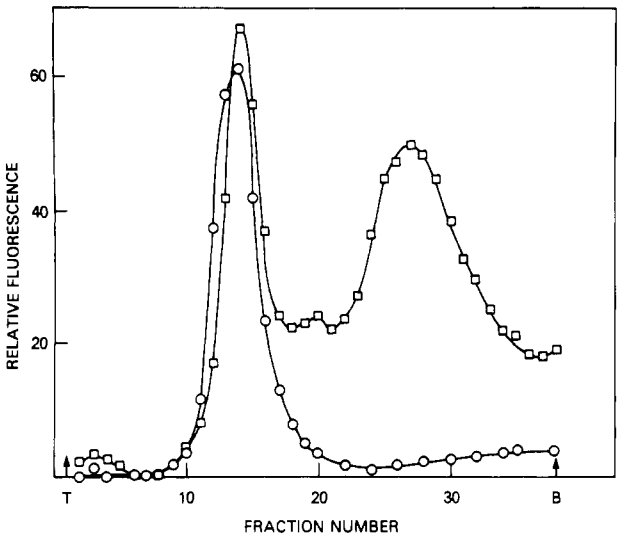

FIGURE 5: Sucrose gradient pattern of baskets (≈0.4 mg/mL) before (□) and after (○) dissociation. Only AN fluorescence is shown. Baskets were formed in 0.1 M Mes, pH 6.0 (as in Figure 4A), and were dialyzed against 0.1 M Mes, pH 6.5, for 15 h.

ratio of tryptophan to AN, 2.0, is highest in the 300S baskets, which decreases to 1.65 and 1.23 in 220S and 150S baskets, respectively.

Electron micrographs of the baskets obtained are shown as insets in the respective figures. In Figure 4A they correspond to both the small (60–70-nm) and large (85–95-nm) sizes. In Figure 4B they correspond to the intermediate size range (70–80 nm), representing the peak at fraction 20. Baskets corresponding to fraction 15 in Figures 4B,C are similar to those shown in Figure 3. A comparison of the larger baskets obtained from pure clathrin alone (Figure 2) and those obtained from a limited incorporation of APS (Figure 4A) indicates that the former are less well-defined and irregular, whereas the latter are clearer with well-defined edges, as is the case with smaller size baskets. A similar conclusion was reached by others (Pearse & Robinson, 1984; Heuser & Kirchhausen, 1985), but the implications of this finding are not clear.

Stability of 150S, 220S, and 300S Baskets. The stability of the three varieties of baskets was studied by increasing the pH from 6.0 to 6.5 in 0.1 M Mes. A suspension of baskets (0.4 mg/mL), corresponding to that shown in Figure 4A, containing all three varieties was dialyzed against 0.1 M Mes, pH 6.5, for 24 h, and the sucrose gradient patterns are shown in Figure 5. After dialysis, all the label is centered at fraction 14, characteristic of 150S baskets. In addition, a large amount of tryptophan fluorescence was found at fraction 4, corresponding to free clathrin (8S) (data not shown). Similar data were obtained when a solution consisting of 150S and 220S baskets was dialyzed against 0.1 M Mes, pH 6.5, for 24 h (data not shown).

The formation of 150S baskets from 300S baskets raises the question of whether the large baskets dissociate to small baskets and free clathrin or whether they dissociate into clathrin and APs and then reassociate rapidly to form smaller baskets. The results in the following section support the first possibility.

Copolymerization of 150S Baskets and Pure Clathrin. It was reported by Irace et al. (1982) that 150S baskets cannot be converted into larger baskets either by reducing the pH from 6.5 to 6.0 or by raising the concentration of clathrin. To examine the origin of the larger structures in our experiments, we polymerized a solution of clathrin and labeled APs by

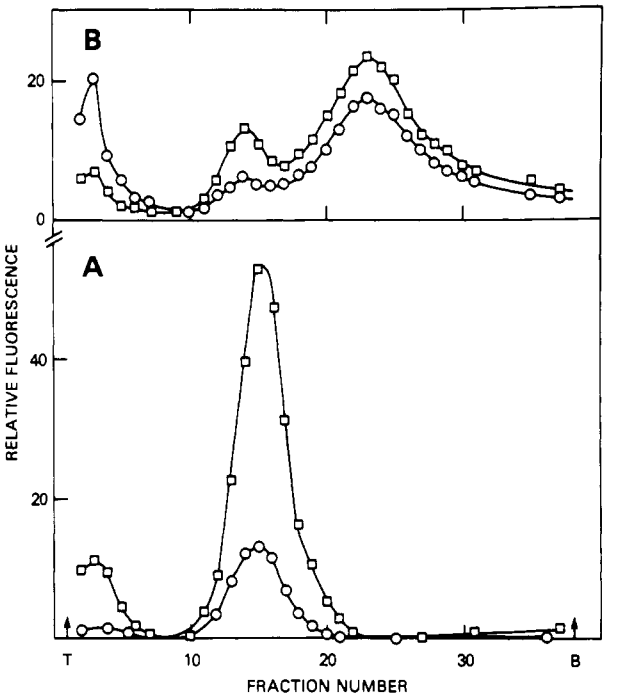

FIGURE 6: (A) Sucrose gradient pattern of baskets formed from clathrin (0.2 mg/mL) and labeled APs (0.1 mg/mL) at 0.1 M Mes, pH 6.5, and after dialysis to 0.1 M Mes, pH 6.0. Both patterns were identical, so only one is shown. Clathrin and APs were mixed in 0.01 M Tris, pH 8.0, and dialyzed against 0.1 M Mes, pH 6.5 or 6.0. (B) Sucrose gradient pattern of baskets formed from the addition of pure clathrin (0.4 mg/mL) to the above baskets (0.3 mg/mL) at 0.1 M Mes, pH 6.0. Solutions were mixed at 0.1 M Mes, pH 6.5, and dialyzed to 0.1 M Mes, pH 6.0, for 15 h. Tryptophan fluorescence (○); AN fluorescence (□). Baskets in panel A resemble the baskets shown in Figure 3, and baskets in panel B resemble the baskets shown in Figure 4A.

dialysis to 0.1 M Mes, pH 6.5, and isolated the 150S baskets from sucrose gradients. To an aliquot of these baskets (0.3 mg/mL) in 0.1 M Mes, pH 6.5, was added pure clathrin (0.4 mg/mL) in 0.1 M Mes, pH 6.5, and the combined solution was dialyzed against 0.1 M Mes, pH 6.0, for 15 h. As a control, another aliquot of the 150S baskets was dialyzed against the same buffer. As seen in Figure 6A, the control 150S baskets remained at fraction 15, whereas the baskets to which clathrin had been added formed larger structures (Figure 6B). Electron microscopy confirmed that these were larger size baskets rather than small baskets with clathrin attached to them. Since clathrin itself tends to polymerize under these conditions, one might infer that what one sees in the electron microscope are those baskets. However, this cannot explain three facts: (1) the labeled baskets at fraction 15 were decreased and were replaced by a peak at fractions 23 and 24, where the larger size baskets appear; (2) the label was superimposable on the tryptophan fluorescence, which has its major contribution from the tryptophan fluorescence of clathrin; and (3) pure clathrin under these conditions would have had its major peak at fractions 30–32, not at fractions 23 and 24. The pattern shown here resembles that shown in Figure 4A where clathrin is polymerized in the presence of a limited amount of APs.

To examine further whether the pure clathrin is reacting with the 150S baskets rather than with the labeled APs that may be in excess, we repeated the above experiment using labeled 150S baskets instead of labeling the APs. When baskets are labeled, the label distributes into all the proteins in the baskets including clathrin heavy chains, clathrin light chains, and APs in proportion to their mass. Gel electro-

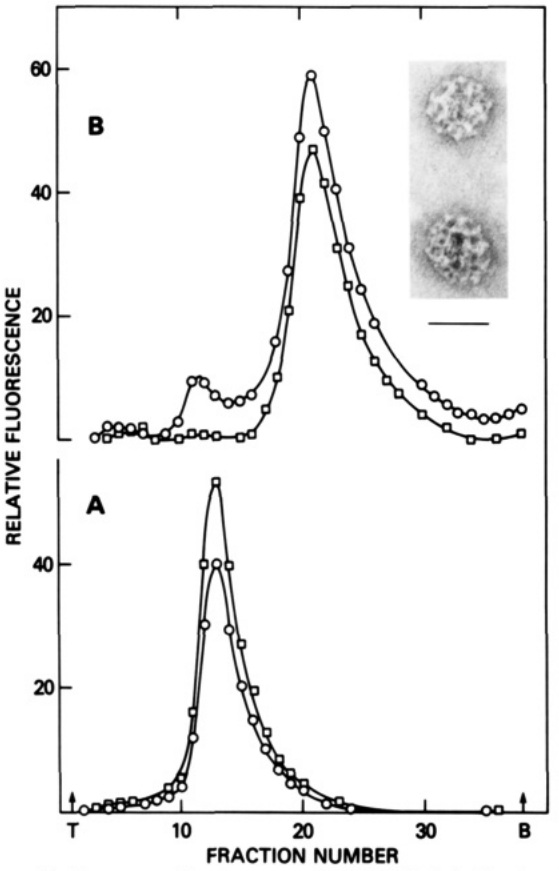

FIGURE 7: Sucrose gradient pattern of (A) AN-labeled baskets (0.2 mg/mL) and (B) AN-labeled baskets (0.2 mg/mL) plus pure clathrin (0.3 mg/mL). Baskets and a mixture of baskets and clathrin at 0.1 M Mes, pH 6.5, were separately dialyzed into 0.1 M Mes, pH 6.0. Tryptophan fluorescence (○); AN fluorescence (□). Inset shows the baskets formed. Baskets corresponding to panel A are similar to those shown in Figure 3 (bar = 100 nm).

phoresis of AN-labeled baskets examined by fluorescence and Coomassie-stained baskets appears virtually identical (Prasad et al., 1985). The results are shown in Figure 7. As observed earlier, the baskets (0.2 mg/mL) themselves did not aggregate with reduction of pH from 6.5 to 6.0 but remained at 150 S (Figure 7A). However, when pure clathrin (0.3 mg/mL) was added (Figure 7B), most of the clathrin reacted, giving the heavier sedimenting baskets at fraction 21. Furthermore, all the AN label moved to fraction 21 and was superimposable on the peak of tryptophan fluorescence. Electron microscopy confirmed the presence of large discrete structures (Figure 7B, inset). By varying the ratio of pure clathrin to baskets, patterns similar to that shown in Figure 6B were obtained (data not shown). Taken together, these data confirm that the smaller baskets are intermediates in the polymerization of the larger clathrin baskets.

Interaction of APs with Polymerized Baskets. We showed earlier that the APs react with clathrin protomers and determine the size distribution of the baskets. To determine whether APs react with already assembled larger baskets, we polymerized clathrin with enough APs to give 150S and 220S baskets of 0.35 mg/mL, at pH 6.0, 0.1 M Mes, and then added AN-labeled APs at 0.07 mg/mL. Figure 8 shows the sucrose gradient pattern of the reaction products as well as the control baskets. As seen by tryptophan fluorescence, the added APs do not change the size distribution; instead, they react with the baskets and probably remain bound to them. The ratio of AN to tryptophan fluorescence shows that a larger amount of the APs bind to 220S baskets compared to 150S baskets.

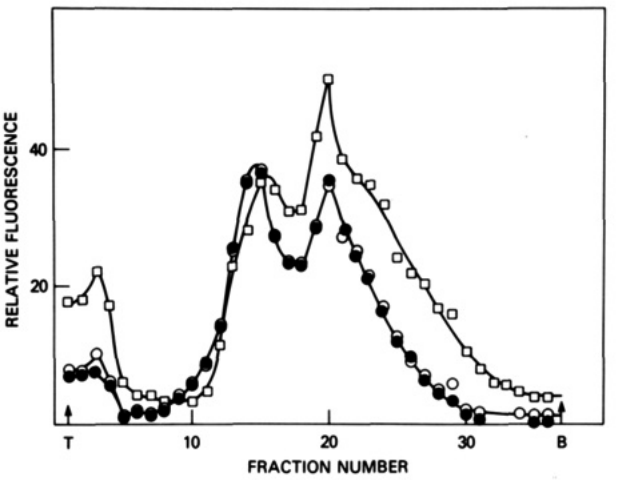

FIGURE 8: Sucrose gradient pattern of baskets to which AN-labeled APs were added at 0.08 M Mes, pH 6.0. Baskets (0.35 mg/mL) were prepared at 0.1 M Mes, pH 6.0, and a small aliquot of a concentrated solution of APs (giving a final concentration of 0.07 mg/mL) in 0.01 M Tris, pH 8.0, was added. Final buffer conditions were 0.002 M Tris and 0.08 M Mes, pH 6.0. Tryptophan fluorescence of baskets (control) (○); tryptophan fluorescence of reacted baskets (●); AN fluorescence of reacted baskets (□).

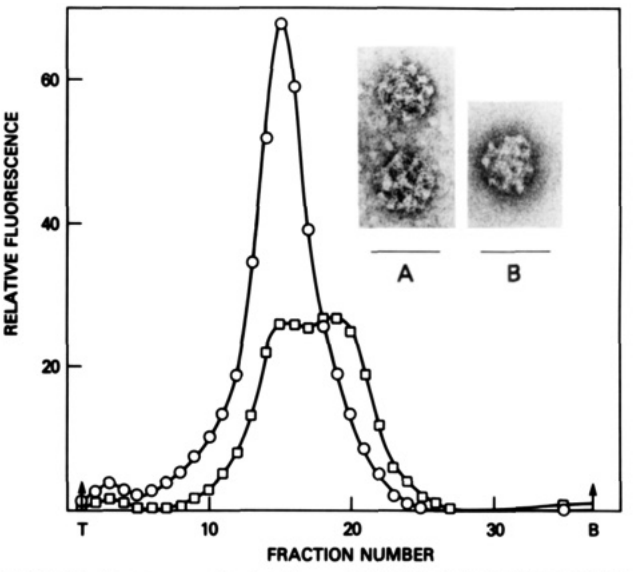

FIGURE 9: Sucrose gradient pattern of 150S baskets (0.15 mg/mL) to which additional APs (0.20 mg/mL) were added at 0.1 M Mes, pH 6.0. Baskets were prepared by dialysis of clathrin and APs in 0.01 M Tris, pH 8.0, to 0.1 M Mes, pH 6.0. To this solution was added a concentrated solution of APs in 0.01 M Tris, pH 8.0, to give final buffer conditions of 0.002 M Tris and 0.08 M Mes, pH 6.0. Tryptophan fluorescence of baskets (control) (○); tryptophan fluorescence of reacted baskets (□). The inset shows the baskets corresponding to the reacted products: control baskets (A); reacted products (B) (bar = 100 nm).

Presumably, their binding to baskets depends on the surface area of the basket. The tryptophan fluorescence distribution does not change significantly since most is due to clathrin. A similar study carried out with 300S baskets showed that the added APs bind to them as well, again without significantly altering the size distribution (data not shown).

To clarify further the nature of the interaction of APs with baskets, we studied their interaction with 150S baskets. The 150S baskets were isolated from gradients, and to an aliquot (0.15 mg/mL) was added a comparable amount of APs (0.20 mg/mL). Figure 9 shows the sucrose gradient pattern of the reacted products along with the control baskets. In the control, only 150S baskets corresponding to the peak at fraction 15

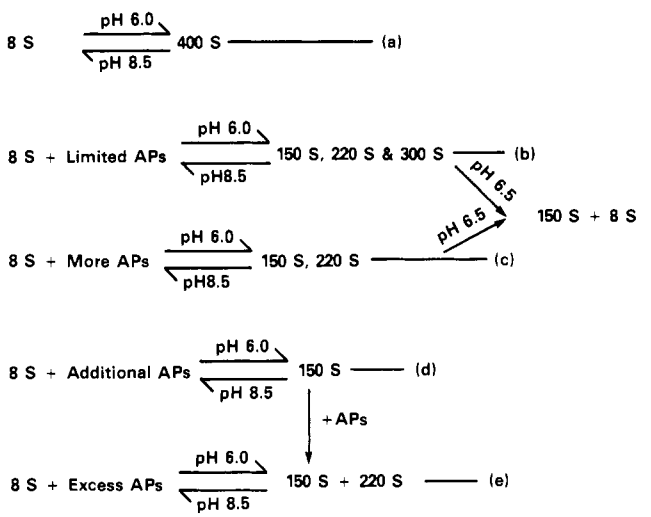

FIGURE 10: Types of basket structures formed under varying conditions as analyzed by velocity centrifugation. Concentration ratios of APs to 8S in (a), (b), (c), (d), and (e) are 0, 0.16, 0.33, 0.5, and 1.0, respectively. The 220S baskets in (b) and (c) are dissociable to 150S on raising the pH from 6.0 to 6.5 whereas those in (e) are not dissociable.

were observed, whereas in the reacted mixture there were two peaks at fractions 16 and 19 with almost equal tryptophan fluorescence. Electron microscopy of the baskets is shown as insets in Figure 9. Inset A corresponds to the control 150S baskets, and inset B corresponds to fraction 19 of the reassociated products. All baskets were of the 150S size. However, the baskets corresponding to the second peak (fraction 19) in the reacted products (Figure 9, squares) were associated with a large amount of stain-excluding material and resemble coated vesicles isolated from bovine brain. They have a sedimentation velocity coefficient of 220 S, corresponding to intermediate-size baskets, but the mechanism of their formation and their appearance in the electron microscope are different. Similar results were obtained when clathrin and an excess of APs were mixed in 0.01 M Tris, pH 8.0, and then dialyzed against 0.1 M Mes, pH 6.0.

The types of baskets that are formed under various conditions are summarized in Figure 10.

Interaction of APs with Coated Vesicles. To examine whether the APs react only with baskets or also with coated vesicles, AN-labeled APs (0.15 mg/mL) were incubated with coated vesicles (0.6 mg/mL) at 0.1 M Mes, pH 6.5. The sucrose gradient pattern showed that the label was superimposable on the tryptophan peak of the coated vesicles (data not shown). The pattern was similar to that observed with baskets (see Figure 8).

Aggregation of APs under Clathrin Polymerization Conditions. We reported earlier (Prasad et al., 1986) that the purified 100-kDa AP aggregates in the absence of clathrin under clathrin polymerization conditions. Pearse et al. (1982) reported the same phenomenon with the total APs, but the results were not conclusive since a significant amount of clathrin was present in their preparation. Robinson and Pearse (1984), however, did not observe aggregation with their purified preparation of the 100–50-kDa complex when the protein concentration was less than 0.1 mg/mL. Ahle and Ungewickell (1986) and Manfredi and Bazari (1987), on the other hand, observed aggregation of APs under clathrin polymerization conditions. We investigated this question using either total APs (Figure 1, lane 1) or a pure preparation of 100–50 kDa (Figure 1, lane 2) [prepared according to Prasad et al. (1986)]. AN-labeled protein solutions ranging from 0.01 to 0.10 mg/mL in 0.01 M Tris, pH 8.5, were dialyzed into 0.1 M Mes, pH 6.5 or 6.0, for 15 h and were analyzed by sucrose gradient ultracentrifugation. In 0.01 M Tris, pH 8.5, all the protein remained at the top of the gradient whereas in 0.1 M Mes, pH 6.5, about 80–90% of the sample aggregated and sedimented to the bottom of the tube (data not shown). Separate aliquots dialyzed from 0.01 M Tris, pH 8.5, to 0.1 M Mes, pH 6.5, were tested for their ability to polymerize pure clathrin. Sucrose gradient ultracentrifugation showed that the aggregated material did not react with clathrin to form baskets but sedimented to the bottom of the gradient. On the other hand, the aggregated material was dissociable in 0.01 M Tris, pH 8.5, and recovered the ability to bind to baskets and to polymerize clathrin.

When a small quantity of a concentrated solution of APs was added to baskets at a final concentration of 0.05 mg/mL APs and 0.2 mg/mL clathrin baskets at pH 6.5, the APs did not aggregate but remained soluble and bound to the baskets (data not shown). Similar results were obtained with coated vesicles. When the ratio was increased to 0.2 mg/mL APs to 0.15 mg/mL baskets, the baskets no longer show a sharp distribution on sucrose gradients, implying heterogeneity in the masses of the resulting baskets or aggregation of the baskets themselves (data not shown).

Discussion

We have studied the interaction of purified clathrin with the 180-, 110-, 100-, 50-, and 47-kDa associated proteins (APs) of coated vesicles under conditions in which pure clathrin does not or does polymerize by itself. It was reported earlier from this laboratory that clathrin polymerizes into predominantly two size classes with sedimentation coefficients of 150 and 300 S. In the present investigation there appear to be three size classes of 150, 220, and 300 S. The concentration of APs present in a particular clathrin preparation determines the size distribution of the basket structures. Low ratios of clathrin to APs give rise to smaller sizes, whereas higher ratios give rise to predominantly the larger sizes. This observation is somewhat in variance with the existing information that APs induce polymerization of clathrin into only smaller size baskets (Zaremba & Keen, 1983; Pearse & Robinson, 1984; Ahle & Ungewickell, 1986; Prasad et al., 1985). The difference is presumably due to the reason that in the present report the effect of APs on the nature of clathrin polymerization is being investigated whereas in most of the earlier reports the ability of APs to induce polymerization of clathrin was studied. However, at pH values of 6.5 or above, only small-size baskets (150S) were formed, and clathrin associated stoichiometrically with the APs (Figure 3). This result is in general agreement with the limited amount of data reported in the literature.

To understand further the nature of the polymerization reaction, we investigated the stability of the various-size baskets. Both the 300S and 220S baskets dissociate readily when the pH is increased from 6.0 to 6.5. When these structures were examined by using AN-labeled APs, it was found that both structures dissociate into 150S baskets and free clathrin, with all the AN label present with the 150S baskets. This observation lends support to the idea that the 150S basket is probably an intermediate in the formation of the larger size baskets. Further support to the idea comes from the observation that pure clathrin reacts with the 150S baskets and forms larger structures when the pH is reduced from pH 6.5 to 6.0 (Figures 6 and 7). Under the latter conditions, 150S baskets alone did not form 300S baskets or the aggregates. Finally, electron microscopic examination revealed that the products of the above reaction are larger structures, confirming

the view that an additional coat is probably formed on the 150S baskets to give rise to larger structures (inset, Figure 7). We speculate that pure clathrin reacts with the APs present in the smaller baskets (150S) and polymerizes on their surface. However, at this point it is not clear whether the large-size baskets obtained directly from the polymerization of pure clathrin or clathrin with limited amounts of APs are any different from those obtained through the 150S intermediate. A more detailed examination of these structures by methods such as rotary shadowing (Heuser & Kirchhausen, 1985) is required to answer this question. The growth of the basket structure may be analogous to microtubule formation, where it was shown that microtubule subunits can attach to microtubule fragments when they are predecorated with polycations such as DEAE, dextran, etc. (Olmsted et al., 1974).

We also investigated the nature of the interaction between the associated proteins and preformed baskets. Baskets of all sizes can react with the associated proteins to form heavier species, the sedimentation coefficient depending on the concentration ratio of basket to the associated proteins. Similar observations were made with isolated coated vesicles. on the other hand, the APs do not react in such a way as to alter the size distribution of the preformed larger baskets when added to them (Figure 8). The above data showing that the small baskets have binding sites for both clathrin and APs seem to be in disagreement with the observation of Vigers et al. (1986) that the APs form an inner layer of the clathrin coat in the reassembled baskets or CVs. Until more work is done on the electron microscopic analysis of the present data, we offer no explanation for this discrepancy.

Finally, the observation that more APs can bind to the 150S baskets and shift their sedimentation coefficient value, inconsistent with their size alone (Figure 10), has an interesting consequence. First, they look like isolated coated vesicles with large amounts of stain excluding material present on them. Second, if, as currently believed, coated vesicles that are isolated from bovine brain belong to the smaller size range and have large contamination of baskets, and these baskets have substantial amounts of APs associated with them, then the sedimentational analysis on sucrose gradients with superimposable protein and phospholipid peaks (Nandi et al. 1982b) is highly unlikely to differentiate the two types of particles.

Acknowledgments

We thank Dr. Jan Wolff for guidance of this work and Dr. Jacob Robbins for constant encouragement throughout this work. Efficient typing of the manuscript by Valerie deGrange is deeply appreciated.

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