binding near the bulge. Additional counterion release upon intercalation near the bulge might also contribute to the entropy of intercalation.

We can look at the extent of stabilization of the bulged double helix by comparing the products of the equilibrium constant for double-helix formation and the ethidium binding constant for the normal helix with the corresponding equilibrium constant for formation of the bulged double helix and the binding constant to the strong site. For the normal helix at 25 °C, we calculate $K_hK_d = (1.0 \times 10^5)(2.2 \times 10^4) = 2.2 \times 10^9$, whereas for the bulged helix we obtain $(6.2 \times 10^2)(10)(2.2 \times 10^4) = 1.4 \times 10^8$. The difference in free energy between the bulged and normal helices is $-RT \ln [(6.2 \times 10^2)/(1.0 \times 10^5)] = 3.0$ kcal mol^{-1} at 25 °C, whereas for the double helices with one ethidium ion bound the difference is $-RT \ln [(1.4 \times 10^8)/(2.2 \times 10^9)] = 1.6$ kcal mol^{-1}. Thus, the binding of an ethidium ion to one strong site near a bulge cuts the destabilization of the bulge in half. Binding an ethidium to both strong sites makes the difference almost zero. From the thermodynamic results, it is clear that the intercalation of ethidium significantly stabilizes a bulge.

It would be very instructive to study the binding of ethidium into oligonucleotides containing other types of bulged or mismatched bases. For example, in the double helix formed by the self-complementary deoxyoligonucleotide dCGCAGAATTCGCG, the extra adenine base is stacked into the helix (Patel et al., 1982). The extent of ethidium binding to such an extra base could be very different than to the bulged base studied here. Also, in the case of mismatched bases, where there is a base on both strands that presumably forms a nonstandard base pair, it is known that ethidium binding is enhanced (Helfgott & Kallenbach, 1979). One could speculate that the effect should be similar to that observed in the case of the bulged cytosine.

ACKNOWLEDGMENTS

We thank Dr. Neville R. Kallenbach for useful discussions.

Registry No. $dCA_5G + dCT_5G$, 75579-56-5; $dCA_6G + dCT_6G$, 87261-35-6; $dCA_3CA_3G + dCT_6G$, 87261-33-4; ethidium, 3546-21-2.

REFERENCES

Fasman, G. D. (1975) *Biochem., Mol. Biol., 3rd Ed. 1*, 589.
Helfgott, D. C., & Kallenbach, N. R. (1979) *Nucleic Acids Res. 7*, 1011–1017.
Hertzberg, R. P., & Dervan, P. B. (1982) *J. Am. Chem. Soc. 104*, 313–315.
Hertzberg, R. P., & Dervan, P. B. (1984) *Biochemistry 23*, 3934–3945.
Kean, J. M., White, S. A., & Draper, D. E. (1985) *Biochemistry 24*, 5062–5070.
Lee, C.-H., & Tinoco, I., Jr. (1978) *Nature (London) 274*, 609–610.
Morden, K. M., Chu, Y. G., Martin, F. H., & Tinoco, I., Jr. (1983) *Biochemistry 22*, 5557–5563.
Nelson, J. W., & Tinoco, I., Jr. (1984) *Biopolymers 23*, 213–233.
Nelson, J. W., Martin, F. H., & Tinoco, I., Jr. (1981) *Biopolymers 20*, 2509–2531.
Patel, D. J., Kozlowski, S. A., Marky, L. A., Rice, J. A., Broka, C., Itakura, K., & Breslauer, K. J. (1982) *Biochemistry 21*, 445–451.
Streisinger, G., Okada, Y., Emrich, J., Newton, J., Tsugita, A., Terzaghi, E., & Inouye, M. (1966) *Cold Spring Harbor Symp. Quant. Biol. 31*, 77–84.

Coat Formation in Coated Vesicles

Kondury Prasad, Roland E. Lippoldt, and Harold Edelhoch*

Clinical Endocrinology Branch, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

Received February 20, 1985

ABSTRACT: The proteins of M_r 100 000–110 000 present in the protein coat of coated vesicles have been shown to facilitate formation of a homogeneous small-size basket (coat) when added to clathrin [Zaremba, S., & Keen, J. H. (1983) *J. Cell Biol. 97*, 1339]. We have prepared this fraction of coat proteins by two different methods and shown that they are very important for the binding of clathrin to uncoated vesicles to form coated vesicles. By labeling the three components (clathrin, 100 000–110 000 proteins, and uncoated vesicles) with different fluorescent markers and analyzing their distribution on sucrose gradients, we have been able to determine the composition of the products formed. In the presence of the 100 000–110 000 fraction of coat proteins, not only does the size distribution of the clathrin basket become uniform but also the rate of polymerization is strongly increased.

Coated pits and coated vesicles are specific structures in the plasma membrane and cytoplasm, respectively, responsible for numerous transport processes either across or between membranes, i.e., receptor-mediated endocytosis (Brown et al., 1983; Ciechanover et al., 1983; Fine & Ockleford, 1984; Gex-Fabry & DeLisi, 1984), secretion (Rothman & Fine, 1980), membrane retrieval (Heuser & Reese, 1973), and transcytosis (Herzog, 1983). The coat surrounding the pits and vesicles is composed of protein, principally clathrin (Pearse, 1975). Clathrin has the shape of a triskelion and forms the framework of the network of polygons (largely pentagons and hexagons) constituting the coat structure (Ungewickell & Branton, 1981).

We have reported (Nandi et al., 1982b) that the coat proteins, dissociated from CVs[1] at pH 8.5 (0.01 M Tris), can be

reassociated with UVs[1] by reducing the pH to the value where CVs are stable, i.e., 6.5 (0.10 M Mes). The dissociation of CVs is therefore readily reversible under these conditions. However, of the numerous proteins released from CVs at pH 8.5, it is not clear which are needed for reversal, aside from clathrin. SDS gel electrophoretic analysis of the soluble coat proteins shows, in addition to clathrin (three of 180K + three of 33K–36K), two major groups of proteins, 100K and 110K, as well as numerous proteins present in relatively minor amounts (principally 47K and 50K).

It is now accepted that native clathrin is a trimer of three 180K chains and three so-called light chains of 33K–36K, located near the vertex of the clathrin triskelion (Kirchhausen & Harrison, 1981; Ungewickell et al., 1982). The second major group of proteins that forms part of the clathrin coat appears in the molecular weight region of 100K–110K in SDS gels (Keen et al., 1979; Pearse, 1982; Irace et al., 1982). One or more of these proteins are involved in controlling the size of the basket that is formed when clathrin is polymerized in the absence of UVs (Irace et al., 1982; Zaremba & Keen, 1983; Pearse & Robinson, 1984).

We have prepared the 100K–110K group of proteins by two different methods and evaluated their effects on clathrin binding to UVs to form the coat structure. This was accomplished by analyzing for both the protein and phospholipid distributions after sedimentation on sucrose gradients (Nandi et al., 1982a). This type of experiment gives information concerning the extent of combination of the coat proteins with the UVs and the size and homogeneity of the products that are formed.

Materials and Methods

Chemicals. Ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and 2-(*N*-morpholino)-ethanesulfonic acid (Mes) were obtained from Sigma. Analytical grade urea, sucrose, and tris(hydroxymethyl)aminomethane were from Bethesda Research Laboratory. Gold label deuterium oxide and 1,6-diphenyl-1,3,5-hexatriene (DPH) were from Aldrich Chemicals. *N*-(1–Anilinonaphthalenyl)-maleimide (ANM) was obtained from Polysciences. Sodium dodecyl sulfate (SDS) was purchased from BDH Chemicals, Ltd.

Preparative Procedures. CVs were prepared from bovine brains as previously described (Nandi et al., 1982a). They were stored in 0.10 M Mes, pH 6.5, the buffer used for their preparation and referred to as standard buffer.

We have prepared coat proteins by three methods. (1) The first was to dissociate CVs by raising the pH from 6.5 (standard buffer used for their preparation) to 8.5 (in 0.01 M Tris) and to separate the soluble coat proteins from the UVs by centrifugation (Nandi & Edelhoch, 1984). The supernatant contains most of the clathrin and the light chains and about half of the 100K–110K proteins. The UVs contain the 55K doublet (tubulin) and the remaining half of the 100K–110K proteins. (2) The second procedure corresponds to that used by Zaremba & Keen (1983), i.e., 0.50 M Tris, pH 8.0, which dissociates most of the 100K–110K proteins from clathrin and UVs. (3) The third method follows that of Edelhoch et al. (1984) in dissociating most or all of the coat proteins from each other in 2.8 M urea, pH 8.0. The various proteins are then separated by gel filtration on Sephacryl S-300 columns containing 2.8 M urea. The protein composition of the SDS gel eluates has been published elsewhere (Edelhoch et al., 1984).

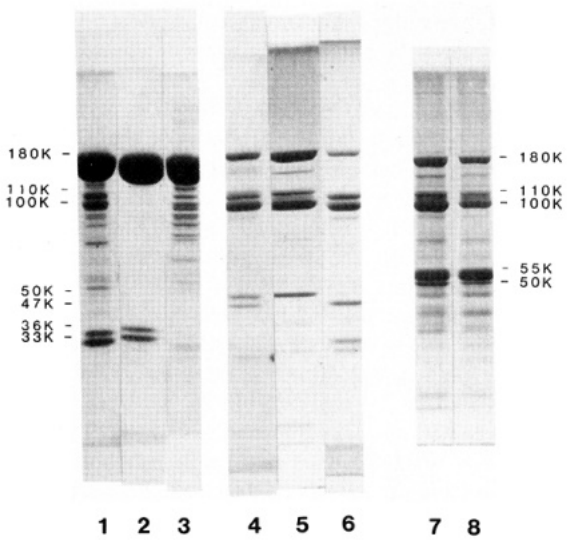

FIGURE 1: SDS gel electrophoretic analysis: (1) Clathrin, from 0.01 M Tris, pH 8.5; (2) clathrin, from 0.50 M Tris, pH 8.0; (3) clathrin, from 2.8 M urea, pH 8.0; (4) 100K–110K proteins, from 0.50 M Tris, pH 8.0; (5) 100K–110K proteins, from UVs extracted with 0.50 M Tris, pH 8.0; (6) 100K–110K proteins, from 2.8 M urea, pH 8.0; (7) UVs, from 0.01 M Tris, pH 8.5; (8) UVs, pellet from CVs extracted first with 0.01 M Tris, pH 8.5, and then with 0.50 M Tris, pH 8.0.

Description of Preparations. SDS gel electrophoretic analyses of various preparations used in reassociation experiments are shown in Figure 1. The lane numbers correspond to the following preparations: (1) clathrin prepared by dissociating CVs in 0.01 M Tris, pH 8.5 (supernatant after centrifugation); (2) clathrin prepared by adjusting (1) to 0.50 M Tris, pH 8.0, and by gel filtration on Sephacryl S-300 in the same solvent; (3) clathrin prepared by adjusting (1) to 2.8 M urea, pH 8.0, 2 mM DTT, and 0.01 M Tris and then by gel filtration on Sephacryl S-300 in the same solvent; (4) 100K–110K proteins prepared simultaneously with clathrin as described in (2); (5) 100K–110K proteins prepared simultaneously with UVs as described in (8) (supernatant after centrifugation); (6) 100K–110K proteins prepared simultaneously with clathrin as described in (3); (7) UVs prepared simultaneously with clathrin as described in (1) (pellet after centrifugation); (8) UVs prepared from UVs in (7) by bringing to 0.50 M Tris, pH 8.0, and sedimenting out of solution (pellet after centrifugation).

A more detailed description of the above preparations follows: (1) Preparation of clathrin. Clathrin was obtained by dissociating CVs in 0.01 M Tris, pH 8.5. The soluble coat proteins were separated from the particulate UVs by centrifugation. The supernatants were used to further purify clathrin. The pellet contained the UVs. (2 and 3) Clathrin prepared in (1) was treated further with either 0.50 M Tris, pH 8.0, and 3 mM NaN_3 [for (2)] or 2.8 M urea, 0.01 M Tris, pH 8.0, 2 mM DDT, and 3 mM NaN_3 [for (3)] by dialyzing for 18 h. Solutions were then placed on a 2-m column of Sephacryl S-300 gel equilibrated with the respective solvents. The column eluates, corresponding to clathrin, were dialyzed for several hours against 0.01 M Tris, pH 8.0, before being used for reassociation studies. The column profile for 2.8 M urea has been published elsewhere (Edelhoch et al., 1984). The eluates of the 0.50 M Tris sample were similar to those reported by Zaremba & Keen (1983). (4 and 6) The 100K–110K group of proteins was also obtained from the Sephacryl

[1] Abbreviations: CV(s), coated vesicle(s); UV(s), uncoated vesicle(s); DPH, 1,6-diphenyl-1,3,5-hexatriene; AN, 1-anilinonaphthalene; ANM, AN-maleimide, *N*-(1-anilinonaphthalenyl)maleimide; Tris, tris(hydroxymethyl)aminomethane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; SDS, sodium dodecyl sulfate; DTT, dithiothreitol.

S-300 column, as described in (2) and (3), respectively. (5) A third preparation of 100K–110K proteins was obtained from the UVs prepared in (1). The latter was extracted with 0.50 M Tris, pH 8.0, and the supernatant contained the 100K–110K proteins. (7) UVs were prepared as the pellet in (1). (8) UVs were prepared as the pellet in (5).

Comparison of Major Components in UVs. The UVs prepared (7 and 8) were scanned for Coomassie blue staining intensity. Assuming that the tubulin bands (55K) are not extracted by 0.5 M Tris (Pfeffer et al., 1983), we can calculate the relative amounts of 100K–110K and 50K proteins extracted by 0.50 M Tris from the 0.01 M Tris preparation of UVs. In two preparations of 0.5 M Tris UVs, the reduction in 100K–110K and in 50K proteins was approximately 50%.

Fluorescence Labels. In studies on the polymerization of clathrin in the absence of UVs, the 100K–110K proteins were labeled with small amounts (1–2 mol/mol) of AN[1] by reacting with AN-maleimide as described elsewhere (Prasad et al., 1984). Thus the relative amounts of clathrin and 100K–110K proteins were resolvable on sucrose gradient fractions from their different fluorescent properties. We have used the intrinsic fluorescence of clathrin to evaluate it in solution. The phospholipid was identified by its interaction with DPH, a fluorescent marker for phospholipid bilayers (Nandi et al., 1982a).

Methods. The CVs, UVs, and clathrin baskets could be differentiated by their sedimentation rates. The homogeneity of these organelles could be assessed by the distribution of their sedimentation constants, i.e., width and symmetry of the sedimenting band on sucrose gradients. The sucrose gradient analyses [described elsewhere (Nandi et al., 1982a)] therefore served as an analytical tool that permitted us to determine the relative amounts of reactants and products.

(*A*) *Sucrose Gradient Centrifugation.* Solutions of UVs or CVs were sedimented on a 10–30% linear sucrose gradient in a SW40 rotor at 27000 rpm for 110 min in a Beckman Model L2-65B centrifuge. Solutions of clathrin or baskets were sedimented on a 10–30% linear sucrose gradient in a SW27 rotor at 24000 rpm for 110 min. The fractions from the gradient were collected from the bottom of the tube by using a peristaltic pump. The elution was monitored by the fluorescence intensities of the various probes.

(*B*) *Fluorescence Measurements.* Fluorescence intensities were measured in a Perkin-Elmer MPF3 spectrofluorometer. Relative protein concentrations were obtained by exciting at 290 nm and measuring the emission at 340 nm. Relative phospholipid concentrations were determined from the fluorescent intensity of membrane-bound diphenylhexatriene (DPH)[1] by exciting at 360 nm and measuring the emission at 430 nm. The AN-labeled proteins were excited at 350 nm, and their emission was measured at 425 nm.

(*C*) *Gel Electrophoresis.* Gradient gels (5–15%) were employed everywhere. The SDS gel electrophoresis was done according to Laemmli (1970).

(*D*) *Light Scattering.* Light scattering was measured at 90° to the incident beam in a polished cylindrical cell obtained from Precision Cell, Inc., in a Brice-Phoenix universal light scattering photometer. A blue filter was used on the exciting beam. Other details are described elsewhere (Van Jaarsveld et al., 1981).

(*E*) *Protein Concentrations.* Clathrin concentration was measured by its absorption at 280 nm; $E^{1\%}_{280nm} = 10.9$ was used. The concentration of protein in UVs was measured by Lowry analysis. The concentration of the proteins in the 100K–110K group of proteins was estimated by measuring the optical

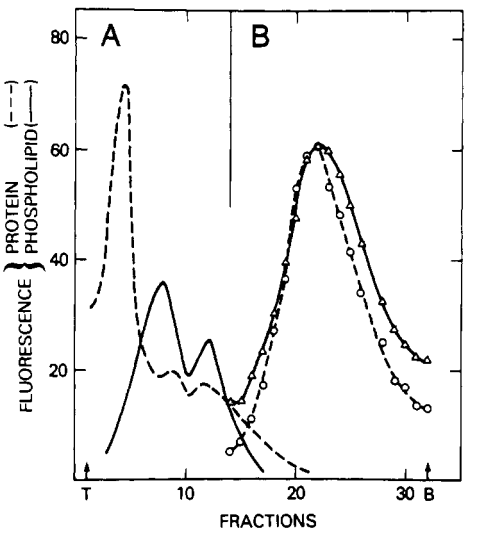

FIGURE 2: (A) Lines without points: Sucrose gradient of CVs (1 mg/mL) in 0.50 M Tris, pH 8.0. (B) Lines with points: Sucrose gradient of reassociated CVs in standard buffer (0.10 M Mes, pH 6.5). The dissociated CVs in 0.50 M Tris, pH 8.0, were dialyzed for 15 h at 4 °C against standard buffer before putting on sucrose gradients. Less than 10% of the protein or phospholipid remained dissociated at pH 6.5 (not shown). Dashed lines show protein fluorescence; full lines show phospholipid probe fluorescence. Centrifugation was done in 10–30% sucrose gradient (B = bottom, T = top) for 110 min at 40000 rpm at 23 °C. All gradients in this figure and Figures 3–5 were performed under the same conditions.

density at 280 nm and at 215 nm according to the method of Stokes (1974).

(*F*) *Column Chromatography.* The separation of protein components was carried out in Sephacryl S-300 on a 1.5 × 196 cm column. A flow rate of 10 mL/h was used in each case.

(*G*) *Densitometer Scanning of Gels.* UVs were scanned for Coomassie blue staining of the gels at 590 nm on a Beckman DU8 spectrophotometer with a gel scanning apparatus.

(*H*) *Electron Microscope.* Electron microscopic analysis was by negative staining with 1% uranyl acetate and was performed by Dr. B. Kramarsky (Electro Nucleonics, Silver Spring, MD).

RESULTS

We have studied the dissociation of CVs and the reassociation reaction by two types of experiments. In the first we have dissociated CVs and reassociated the components without separating the solubilized coat proteins from the particulate UVs. In this way we could ascertain if the conditions used to dissociate the various coat components affected their ability to recombine since all components of the CVs were still present. The same experiment was then performed with the several purified components in order to determine which components were needed for full reassociation of clathrin with UVs.

Dissociation of CVs in 0.50 M Tris, pH 8.0, and Reassociation. The protein and phospholipid profiles of CVs dissociated in 0.50 M Tris, pH 8.0, on sucrose gradients containing 0.50 M Tris, pH 8.0, are shown in Figure 2A. The major protein peak at fraction 4 is similar to that found by dissociation of CVs at low Tris (0.01 M) at pH 8.5 (Nandi et al., 1982b). The positions of the two smaller protein peaks agree with those of the two phospholipid peaks. The phospholipid probe pattern, which defines the UVs, consists of two overlapping bands with peaks at fractions 8 and 12. This result is somewhat different from that observed in low Tris (0.01 M) where only a single band is found centered near fraction 10 (Nandi et al., 1982b). The peaks for UVs in 0.50 M Tris

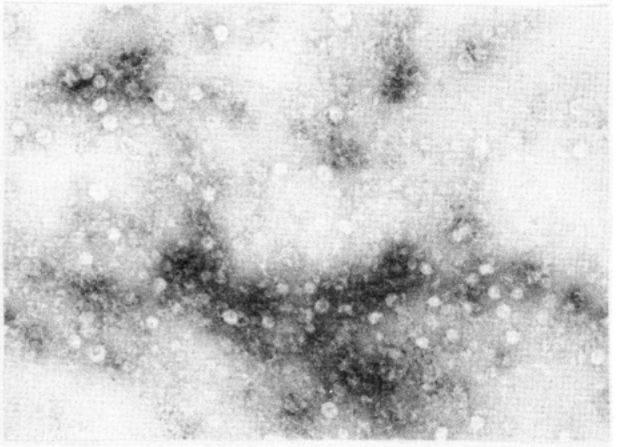

FIGURE 3: Electron micrograph of UVs obtained from fraction 12 in Figure 2A. Magnification is 47250×.

contain a larger percentage of the protein fluorescence than those for UVs prepared in low Tris (0.01 M). The larger protein to phospholipid ratio in the UV boundaries appears to result from the binding of clathrin to UVs in the high Tris concentration (0.50 M); however, it is not present as polygonal coat structure when observed in electron micrographs (Figure 3).

To determine the reversibility of the dissociation, the CV solution in 0.50 M Tris (pH 8.0) was dialyzed against 0.10 M Mes, pH 6.5 (for 15 h at 4 °C), to allow the coat proteins to reassociate with the UVs. Fluorescence analysis of sucrose gradient fractions revealed protein and phospholipid profiles similar to those of native CVs with the same sedimentation rates (Figure 2B).

Influence of 100K–110K Proteins on Reassociation. In order to evaluate the effect of the 100K–110K proteins on the binding of clathrin to UVs, we first isolated clathrin and UVs by raising the pH of a CV solution from 6.5 to 8.5 (0.01 M Tris) by dialysis for 15 h at 4 °C. After separating the clathrin and UVs by centrifugation, each was separately treated with 0.50 M Tris to dissociate the bound 100K–110K proteins. The clathrin was separated from the 100K–110K proteins by gel filtration on a Sephacryl S-300 column in 0.5 M Tris and then dialyzed against 0.01 M Tris, pH 8.0. The UVs were separated from the 100K–110K proteins by centrifugation and then resuspended in 0.01 M Tris. The supernatant containing the 100K–110K proteins was dialyzed against 0.01 M Tris, pH 8.0. When these preparations of clathrin and UVs (in 0.01 M Tris, pH 8.0) were mixed and dialyzed against standard buffer (0.10 M Mes, pH 6.5), no CVs were formed as shown either by sucrose gradient analysis (Figure 4A) or by electron micrography (not shown). When the reaction mixture included the 100K–110K proteins (isolated from the UVs), clathrin reassociated with UVs to form CVs since most of the protein and phospholipid now sedimented together with a rate only slightly less than that of native CVs (Figure 4B). The small displacement of the phospholipid relative to the protein peak may be due to some residual phospholipid that did not recombine with clathrin and the 100K–110K proteins. Similar results were obtained when the 100K–110K proteins were isolated from clathrin.

In Figure 5 can be seen the electron micrograph obtained on the UVs after the addition of clathrin and 100K–110K proteins. The formation of normal coats on all or most particles is clearly visible.

Dissociation of CVs in 2.8 M Urea, pH 8.0, and Reassociation. CVs were dialyzed against 2.8 M urea and 0.01 M Tris, pH 8.0, for 15 h at 4 °C and then sedimented on sucrose

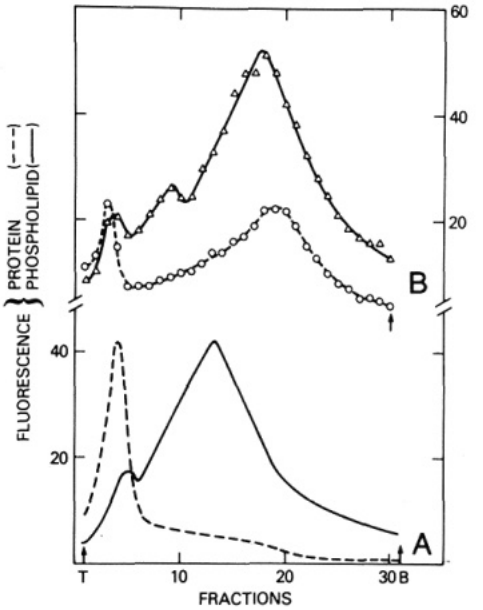

FIGURE 4: (A) Lines without points: Sucrose gradient of a mixture of purified clathrin (0.78 mg/mL) and UVs (0.12 mg/mL) (extracted with 0.5 M Tris) sedimented in standard buffer (0.10 M Mes, pH 6.5). (B) Lines with points: Same as (A) (clathrin, 0.43 mg/mL; UVs, 0.12 mg/mL) with 100K–110K proteins (0.23 mg/mL) (extracted from UVs with 0.50 M Tris). Solutions were dialyzed against 0.01 M Tris, pH 8.0, before mixing. After mixing, solutions were dialyzed for 15 h at 4 °C against standard buffer as in Figure 2. Dashed lines show protein fluorescence; full lines show phospholipid fluorescence.

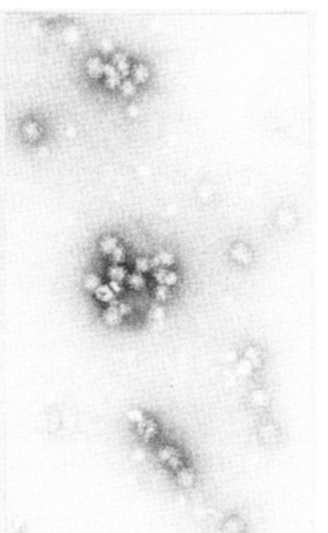

FIGURE 5: Electron micrograph by CVs re-formed from UVs, clathrin, and 100K–110K proteins. Magnification is 35000×.

gradients formed in the same solvent. The protein fluorescent curve (Figure 6A) is similar to that observed when CVs are dissociated in 0.01 M Tris, pH 8.5. The phospholipid band, however, is displaced to much lower fractions, being centered at fraction 5 instead of 10. The slower sedimentation rate of UVs probably results from a lower proetin to phospholipid ratio although the rate may also be reduced by the greater viscosity and density of the sucrose gradient in 2.8 M urea. The size of the UVs probably remains the same as in aqueous solutions since, upon removal of urea by dialysis against 0.10 M Mes, pH 6.5, the fluorescence profiles of the protein and phospholipid correspond rather closely to those of the original, native CVs (Figure 6B). If the UVs had suffered some change in size, it seems unlikely that they would have recovered their

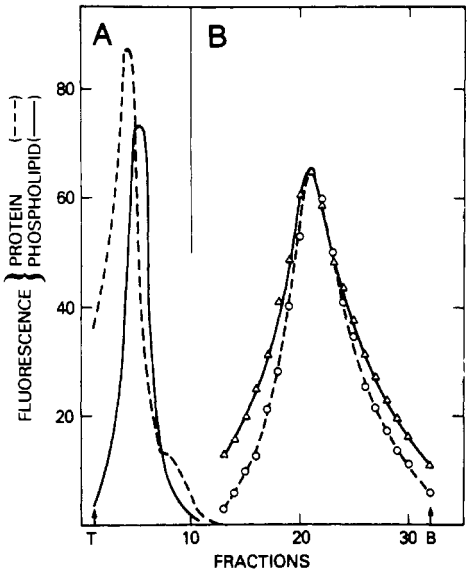

FIGURE 6: (A) Lines without points: Sucrose gradient of CVs (1 mg/mL) dissociated and sedimented in 2.8 M urea and 0.01 M Tris, pH 8.0. (B) Lines with points: Sucrose gradient of reassociated CVs in standard buffer (0.10 M Mes, pH 6.5). Dashed lines show protein fluorescence; full lines show phospholipid probe fluorescence.

original size distribution after reassociation with the dissociated coat proteins. It was also evident that the coat proteins did not undergo any irreversible damage during exposure to 2.8 M urea where they readily dissociate from each other.

Influence of 100K–110K Proteins on Reassociation. Since the sedimentation rate of the UVs was very similar to that of the coat proteins in 2.8 M urea, they cannot be separated readily from each other by centrifugation (see Figure 6A). Consequently, we first dissociated clathrin from the UVs by dialyzing CVs against 0.01 M Tris, pH 8.5, for 15 h at 4 °C and then separated the UVs by centrifugation. The supernatant, which contained the coat proteins, was then dialyzed against 2.8 M urea, pH 8.0, for 15 h at 4 °C. Clathrin and the 110K–100K proteins were then separated by gel filtration on a Sephacryl S-300 column equilibrated with 2.8 M urea and 0.01 M Tris, pH 8.0. The fractions containing clathrin and 100K–110K proteins were separately dialyzed against 0.01 M Tris, pH 8.0 buffer. The UV pellet was dissolved in the same solvent, i.e., 0.01 M Tris, pH 8.0.

A mixture of clathrin and UVs was then dialyzed for 15 h at 4 °C against standard buffer. Analysis of the protein and phospholipid probe fluorescence on the sucrose gradient fractions showed that most of the clathrin had not reacted (Figure 7A). A small amount of protein (10–20%) was present at the fraction corresponding to CVs along with phospholipid. Most of the phospholipid had sedimented to the bottom of the gradient as seen by the rising phospholipid fluorescent curve and a large loss of phospholipid probe fluorescence intensity. In a control experiment at 0.10 M Mes, pH 6.5, with only the UVs, nearly all of the phospholipid sedimented to the bottom of the tube with only a small amount left in the last 10 fractions. When the pH of the UVs was kept at 8.5 (0.01 M Tris), the phospholipid probe fluorescence band was centered at fraction 10.

When the same experiment was performed with the 100K–110K proteins obtained from the 2.8 M urea column, a much larger percentage of the protein, as well as the phospholipid probe, fluorescence was present in the CV region. In Figure 7 we have superimposed the two protein patterns and the two phospholipid patterns in order to reveal the increase in the CV composition (fraction ~20) of these two components when

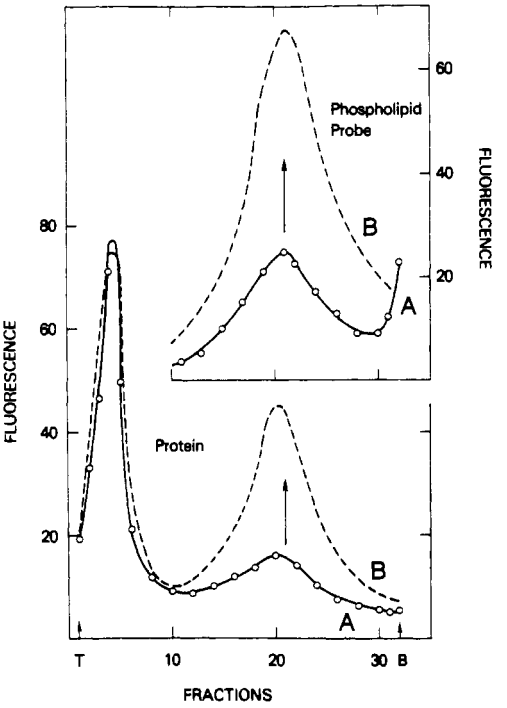

FIGURE 7: (A) Full lines with points: Sucrose gradient of purified clathrin (0.68 mg/mL) (dissociated in 2.8 M urea) and UVs (0.21 mg/mL) (obtained from CV solutions in 0.01 M Tris, pH 8.5) in 0.10 M Mes, pH 6.5. (B) Dashed lines without points: Same as (A) with 100K–110K proteins (0.15 mg/mL) (obtained from clathrin separated in 0.01 M Tris, pH 8.5, which was then put on a 2.8 M urea column). Solutions were dialyzed against 0.01 M Tris, pH 8.0, mixed, and then dialyzed for 15 h at 4 °C against standard buffer (0.10 M Mes, pH 6.5). Bottom left scale shows protein fluorescence; top right scale shows phospholipid probe fluorescence.

the 100K–110K proteins are added to UVs and clathrin. In this UV preparation (0.01 M Tris, pH 8.5), significant amounts of 100K–110K proteins are left in the UVs, which accounts for the small CV boundary observed in the absence of added 100K–110K proteins. When purified 100K–110K proteins were added, more of the protein and most of the phospholipid sediment in the position of the original CVs. These data demonstrate the importance of the 100K–110K proteins for clathrin coat formation on UVs. Using binding measurements, Unanue et al. (1981) have measured the binding of clathrin (prepared in 0.5 M Tris) to UVs (prepared in 0.01 M Tris, pH 8.5) and found that only about 20% of clathrin is bound in comparison to the amount of clathrin present in native CVs. These results are in accord with our experiments performed in the absence of the 100K–110K proteins with similar preparations of UVs and clathrin.

Influence of 100K–110K Proteins on the Polymerization of Clathrin in the Absence of UVs. (A) 100K–110K Proteins Prepared in 0.50 M Tris. Zaremba & Keen (1983) observed the formation of large baskets in the absence of 100K–110K proteins at pH values as high as 6.7. We did not observe any polymerization of clathrin to form baskets in standard buffer or at pH values as low as 6.20 (0.10 M Mes) in the absence of the 100K–110K proteins. At pH 6.00, however, we observed clathrin baskets sedimenting on sucrose gradients that were similar in size to those observed by Zaremba & Keen (1983) at pH 6.2. This difference in behavior could result from the two-step procedure we used to isolate clathrin and the 100K–110K proteins. We first dissociated the CVs in 0.01 M Tris, pH 8.5. After separation of the clathrin from the UV pellet by centrifugation, the supernatant containing the coat proteins was brought to 0.50 M Tris (pH 8.0). Following the procedure

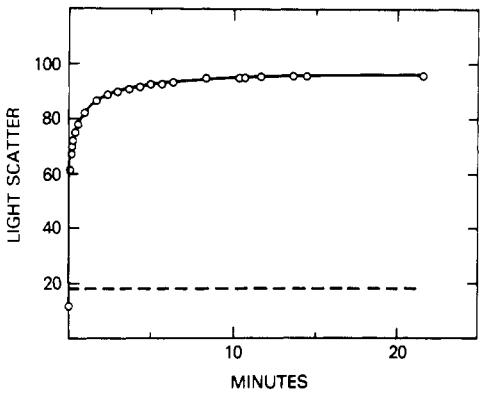

FIGURE 8: Rates of increase in light scatter at pH 6.25 in 0.10 M Mes. Dashed line (without points): purified clathrin (0.50 mg/mL) at pH 6.25 in 0.10 M Mes. Full line (with points): purified clathrin and 100K–110K (0.20 mg/mL) proteins at pH 6.25 in 0.10 M Mes.

of Zaremba & Keen (1983), clathrin was then purified on a Sephacryl S-300 column in 0.50 M Tris. We would therefore surmise that our two-step preparation gave a product that was somewhat purer than that of Zaremba and Keen since it had a smaller tendency, i.e., required a much lower pH, to form the larger sized, heterogeneous baskets observed by them in the absence of 100K–110K proteins.

In the presence of sufficient 100K–110K proteins, only a single, sharp, symmetric sedimenting band of baskets was formed at pH 6.2. The distribution of the 100K–110K proteins, as revealed by AN fluorescence, agreed very well with that of clathrin, as shown by Trp fluorescence. In the velocity ultracentrifuge, in the absence of sucrose, it had the sedimentation rate of the 150S baskets we have described earlier (Pretorius et al., 1981). In the presence of smaller amounts of 100K–110K proteins, a slightly faster sedimenting species was also present, usually as a shoulder to the 150S species. These results resemble those of Irace et al. (1982), where a purified 110K protein inhibited the formation of the faster sedimenting baskets and all of the clathrin was polymerized to the 150S species.

We have also measured the rate of clathrin polymerization by light scattering. In the absence of the 100K–110K proteins, no change in scattering was observed with clathrin at pH values as low as 6.20, in accord with the sucrose gradient analysis. In the presence of 100K–110K proteins at pH 6.20, the light scatter increased very rapidly, reaching 60% of its final value in 6 s and then its final value in about 10 min (Figure 8). A similarly rapid reaction was also observed at pH 6.78 with somewhat larger concentrations of 100K–110K proteins. It is thus clear that the 100K–110K proteins not only change the size distribution of baskets but also have a very large effect on the rate of polymerization.

(B) 100K–110K Proteins Prepared in 2.8 M Urea. We have also used clathrin and the 100K–110K proteins prepared in 2.8 M urea to evaluate the effect of the 100K–110K proteins on the formation of baskets. In contrast to the clathrin prepared from 0.50 M Tris, the light chains are dissociated from clathrin in 2.8 M urea and are eluted last in the Sephacryl S-300 column (in 2.8 M urea) (Edelhoch et al., 1984).

In agreement with the results obtained with clathrin dissociated in 0.50 M Tris, which contained the light chains, no polymerization occurred after clathrin was titrated from pH 8.0 to pH 6.20. When the light chains, isolated from the 2.8 M urea column, were included in the above reaction, no effect was observed. When the 100K–110K proteins were added to the clathrin–light chain mixture and the pH was adjusted in the same way to 6.20, the 150S baskets were found by sucrose gradient analysis. A similar result was obtained when the light chains were omitted. It is clear, therefore, that the light chains are not necessary for the polymerization of clathrin and the dissociating conditions, i.e., 2.8 M urea, pH 8.0, do not produce any irreversible effects on the structure of either clathrin or the 100K–110K proteins.

Discussion

We have examined the interactions in the three-component system of UVs, clathrin, and 100K–110K proteins to form CVs by fluorescence analysis for protein and phospholipid. The various species were resolved by sucrose gradient sedimentation and the components identified by their distinctive fluorescence behavior. We could thereby obtain an approximate measure of the extent of combination and of the composition of the products formed by the interactions. We have also evaluated the two-component system, clathrin and 100K–110K proteins, by the same methods in order to compare the influence of the 100K–110K proteins on clathrin polymerization to form baskets. We have used electron microscopic characterization of organelles to supplement our gradient analysis rather than as a primary analytical tool in describing the nature of the products formed in the reaction of clathrin with 100K–110K proteins.

Two very different methods have been used to prepare both clathrin and the 100K–110K proteins. In the method using 2.8 M urea, clathrin is prepared without light chains. When 0.50 M Tris is used, the light chains remain bound to clathrin. The two preparations of clathrin behaved similarly, however, indicating that the light chains do not play a significant role in clathrin self-association. Winkler & Stanley (1983) have obtained similar results; they removed the light chains from clathrin by a different method and could polymerize clathrin to baskets. In contrast to the light chains, the 100K–110K proteins have an important function in facilitating clathrin binding to UVs to form CVs.

In the absence of 100K–110K proteins, clathrin does not bind to UVs in standard buffer, i.e., the conditions used to prepare clathrin from brain tissue. In fact, in the absence of clathrin, UVs have a strong tendency to aggregate under these conditions. It is quite possible that one of the functions of clathrin is to maintain CVs in a dispersed form shortly after their formation from coated pits since they coalesce with each other (and probably other smooth vesicles) soon after they lose their coats to form endosomes (Brown et al., 1983; Gex-Fabry & DeLisi, 1984).

The importance of the 100K–110K proteins in polymerizing clathrin at neutral pH values has been pointed out by Zaremba & Keen (1983) and further discussed by Pearse & Robinson (1984). It seems very likely, although it is not yet adequately documented, that clathrin polymerization occurs by a nucleation process, as is found with many self-associating proteins that form supramolecular structures, usually cylindrical fibriles, i.e., actin (Wegner & Engle, 1975; Frieden & Goddette, 1983), sickle cell hemoglobin (Ferrone et al., 1980), tubulin (Carlier & Pantaloni, 1978), and other proteins. One function of the 100K–110K proteins would then be to increase the rate of coat formation by increasing the equilibrium constant for either nucleation or elongation or possibly for both steps. A second function could be to regulate the size of the coat by modulating the angles between the arms of the clathrin triskelion. Crowther et al. (1976) and Crowther & Pearse (1981) have described three different types of coat structures by electron microscopic examination of CVs and the arrangement of clathrin triskelions in the coat structure. This

property of the 100K–110K proteins could also control the size of the coated pits and CVs in cells since these are known to vary in different types of cellular membranes (Friend & Farquhar, 1967; Geisow & Burgoyne, 1984).

The 100K–110K protein fraction isolated in 0.50 M Tris contains at least six proteins in this size range as well as a 50K protein. Cross-linking experiments with this fraction indicate that these proteins are associated in complexes of 300K–350K that appear to contain two 100K and two 50K proteins (Pauloin & Jolles, 1984; Pearse & Robinson, 1984). The 50K protein has been shown to be phosphorylated when this fraction is incubated with ATP. The light chains stimulate the kinase present in this complex (Pauloin & Jolles, 1984).

The 100K–110K group of proteins has been fractionated on hydroxyapatite columns into two fractions, one containing three of the proteins of this size and the other containing the remaining three and also a 50K protein (Pearse & Robinson, 1984). The fraction containing the 50K protein was shown to produce the small, homogeneous preparation of baskets from clathrin when polymerized at pH 7.0 whereas the other fraction yielded the larger, heterogeneous baskets. The chemical relationships of the proteins in the range of 100K–110K to one another, to clathrin self-association, and to binding to UVs still remain to be elucidated. Work is continuing along these lines.

References

Brown, M. S., Anderson, R. G. W., & Goldstein, J. L. (1983) *Cell (Cambridge, Mass.) 32*, 663.

Carlier, M. F., & Pantaloni, D. (1978) *Biochemistry 17*, 1908.

Ciechanover, A., Schwartz, A. L., Dautry-Varsat, A., & Lodish, H. F. (1983) *J. Biol. Chem. 258*, 9681.

Crowther, R. A., & Pearse, B. M. F. (1981) *J. Cell Biol. 91*, 790.

Crowther, R. A., Finch, J. T., & Pearse, B. M. F. (1976) *J. Mol. Biol. 103*, 785.

Edelhoch, H., Prasad, K., Lippoldt, R. E., & Nandi, P. K. (1984) *Biochemistry 23*, 2314.

Ferrone, F. A., Hofrichter, J., Sunshine, H. R., & Eaton, W. A. (1980) *Biophys. J. 32*, 361.

Fine, R. E., & Ockleford, C. D. (1984) *Int. Rev. Cytol. 91*, 1.

Frieden, C., & Goddette, D. W. (1983) *Biochemistry 22*, 5836.

Friend, D. S., & Farquhar, M. G. (1967) *J. Cell Biol. 35*, 357.

Geisow, M. J., & Burgoyne, R. D. (1984) *FEBS Lett. 169*, 127.

Gex-Fabry, M., & DeLisi, C. (1984) *Math. Biosci. 72*, 245.

Herzog, V. (1983) *J. Cell Biol. 97*, 607.

Heuser, J. E., & Reese, T. S. (1973) *J. Cell Biol. 57*, 315.

Irace, G., Lippoldt, R. E., Edelhoch, H., & Nandi, P. K. (1982) *Biochemistry 21*, 5764.

Keen, J. H., Willingham, M. C., & Pastan, I. H. (1979) *Cell (Cambridge, Mass.) 16*, 303.

Kirchhausen, T., & Harrison, S. C. (1981) *Cell (Cambridge, Mass.) 23*, 755.

Laemmli, U. K. (1970) *Nature (London) 227*, 680.

Nandi, P. K., & Edelhoch, H. (1984) *J. Biol. Chem. 259*, 11290.

Nandi, P. K., Irace, G., Van Jaarsveld, P. P., Lippoldt, R. E., & Edelhoch, H. (1982a) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5881.

Nandi, P. K., Prasad, K., Lippoldt, R. E., Alfsen, A., & Edelhoch, H. (1982b) *Biochemistry 21*, 6434.

Pauloin, A., & Jolles, P. (1984) *Nature (London) 311*, 265.

Pearse, B. M. F. (1975) *J. Mol. Biol. 97*, 93.

Pearse, B. M. F. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 451.

Pearse, B. M. F., & Robinson, M. S. (1984) *EMBO J. 3*, 1951.

Pfeffer, S. R., Drubin, D. G., & Kelly, R. B. (1983) *J. Cell Biol. 97*, 40.

Prasad, K., Alfsen, A., Lippoldt, R. E., Nandi, P. K., & Edelhoch, H. (1984) *Arch. Biochem. Biophys. 235*, 403.

Pretorius, H. T., Nandi, P. K., Lippoldt, R. E., Johnson, M. L., Keen, J. H., Pastan, I., & Edelhoch, H. (1981) *Biochemistry 20*, 2777.

Rothman, J. E., & Fine, R. E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 780.

Stokes, R. K. (1974) *Anal. Biochem. 59*, 277.

Unanue, E. R., Ungewickell, E., & Branton, D. (1981) *Cell (Cambridge, Mass.) 26*, 439.

Ungewickell, E., & Branton, D. (1981) *Nature (London) 289*, 420.

Ungewickell, E., Unanue, E. R., & Branton, D. (1982) *Cold Spring Harbor Symp. Quant. Biol. 46*, 723.

Van Jaarsveld, P. P., Nandi, P. K., Lippoldt, R. E., Saroff, H., & Edelhoch, H. (1981) *Biochemistry 20*, 4129.

Wegner, A., & Engel, J. (1975) *Biophys. Chem. 3*, 215.

Winkler, F. K., & Stanley, K. K. (1983) *EMBO J. 2*, 1393.

Zaremba, S., & Keen, J. H. (1983) *J. Cell Biol. 97*, 1339.