Purification and Characterization of a Molecular Weight 100000 Coat Protein from Coated Vesicles Obtained from Bovine Brain†

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ABSTRACT: A protein designated as a 100-kDa protein on the basis of sodium dodecyl sulfate gel electrophoresis was purified from coated vesicles obtained from bovine brain, with uncoated vesicles as starting material. Two gel filtration steps, one involving 0.5 M tris(hydroxymethyl)aminomethane, pH 8.0, buffer, and the other 0.01 M tris(hydroxymethyl)aminomethane, pH 8.0, and 3 M urea buffer, were employed. The purified protein has a native molecular weight of 114000 as determined by sedimentation equilibrium analysis. Circular dichroism data showed that the protein has 28% helical structure, 29% β-structure, and 15% β-turns, and the rest is random coil. Addition of the purified protein to clathrin results in the polymerization of clathrin to homogeneous size baskets of sedimentation velocity 150 S. A scan of the Coomassie Blue stained electrophoresis gels of the polymerized baskets shows that, for every clathrin trimer, there is approximately one 100-kDa protein molecule.

Coated pits and coated vesicles (CVs)[1] are involved in several cellular processes concerned with transport across and between membranes (Brown et al., 1983). The best known process is that of receptor-mediated endocytosis. Other processes that are less well characterized are transcytosis, membrane recycling, and secretion (Herzog, 1983; Pearse & Bretscher, 1981; Rothman & Fine, 1980).

Electrophoretic analysis (in SDS gels) of coat proteins from bovine brain CVs reveals one major protein, i.e., clathrin (180 kDa), and several proteins present in significant amounts: a group of proteins betweens 100 and 110 kDa, a 55-kDa doublet, a 50-kDa protein, and a 33–36-kDa doublet (Pearse, 1975). The latter 33–36-kDa protein is considered to be part of the native structure of clathrin, which has been shown to be a triskelion (640 kDa) composed of three clathrin chains and three light chains of either 33 or 36 kDa (Kirchhausen & Harrison, 1981). The light chains are the binding sites of the uncoating ATPase (Schmid, 1984) and are also necessary for the ATP-dependent phosphorylation of the 50-kDa protein (Pauloin & Jolles, 1984). The 55-kDa doublet is an aggregate of the two tubulin monomers. The 100–110-kDa proteins have been further resolved into two groups, which have been separated on hydroxylapatite columns (Pearse & Robinson, 1984). One group contains the 50-kDa protein as part of a complex whose composition is approximately two 100-kDa proteins and two 50-kDa proteins (Pearse & Robinson, 1984). The unfractionated group of 100–110-kDa proteins has been shown to be necessary for the self-association of clathrin, either in the absence of uncoated vesicles (UVs) when coats (i.e., baskets) are formed (Zaremba & Keen, 1983) or in the presence of UVs to form CVs (Prasad et al., 1985). The complex fraction containing the 50-kDa protein obtained from hydroxylapatite columns gives a uniform distribution of small coats (150 S), whereas the second fraction (lacking 50 kDa) gives mainly larger sized coats (Zaremba & Keen, 1983; Pearse & Robinson, 1984).

We have used the UVs obtained by dissociating clathrin in 10 mM Tris, pH 8.5, as the starting material for purifying one of the 100-kDa proteins. We describe our fractionation procedure and some of the molecular and kinetic properties of a highly purified protein obtained from the 100–110-kDa group, which is necessary for the polymerization of clathrin to a uniform distribution of 150S baskets.

Materials and Methods

Chemicals. Tris-HCl was ultrapure grade from Schwarz/Mann. Sodium dodecyl sulfate (SDS) was a very pure grade from BDH Poole, Ltd. *N*-(1-Anilinonaphthyl)-maleimide (ANM) was obtained from Polysciences. 2-(*N*-Morpholino)ethanesulfonic acid (Mes) was from Sigma. Urea was from Bethesda Research Laboratories. All other reagents were Fisher analytical grade. Glass-distilled water was used throughout.

Purified Clathrin. Clathrin was obtained by dissociating the coat proteins from CVs in 10 mM Tris, pH 8.5. This preparation of clathrin contains significant amounts of 100–110-kDa proteins and polymerizes readily to baskets at 0.1 M Mes, pH 6.0–6.5 (Irace et al., 1982). Further purification

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[1] Abbreviations: CVs, coated vesicles; UVs, uncoated vesicles; SDS, sodium dodecyl sulfate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Trp, tryptophan; ANM, *N*-(1-anilinonaphthyl)maleimide; AN, anilinonaphthalene; CD, circular dichroism; RTIC, rotor temperature indicator and control unit; kDa, kilodalton.

of clathrin was achieved by treating the above with 0.5 M Tris, pH 8.0, buffer follwed by gel filtration on a Sephacryl S-300 column (Keen et al., 1979; Pearse & Robinson, 1984; Prasad et al., 1985). The purified clathrin does not polymerize at 0.1 M Mes, pH 6.2, (Prasad et al., 1985).

AN-Labeled 100-kDa Protein. Labeling with ANM has been described elsewhere (Prasad et al., 1985). The 100-kDa protein was labeled to about 1 mol of AN/mol of protein.

Column Chromatography. The gel filtration of proteins was carried out in Sephacryl S-300, 1.5 × 196 cm columns, equilibrated with the appropriate buffers. A flow rate of 10 mL/h was used.

Gel Electrophoresis. Polyacrylamide gradient gels (5–15%) were employed. Electrophoresis (in SDS) was performed according to the procedure of Laemmli (1970). Gels were scanned for Coomassie Brilliant Blue stain at 590 nm with a Beckman DU8 spectrophotometer equipped with a gel scanner.

Fluorescence Measurements. Fluorescence intensities were measured in a Perkin-Elmer MPF-3 spectrofluorometer. Relative protein concentration was obtained by exciting at 290 nm and measuring the emission at 340 nm. AN-labeled 100-kDa protein was determined by exciting at 350 nm and measuring the emission at 426 nm.

Sucrose Gradient Centrifugation. Solutions of polymerized clathrin after overnight incubation were sedimented on a 10–30% linear sucrose gradient in a SW40 rotor at 24000 rpm for 100 min in a Beckman Model L2-65B centrifuge. The fractions from the gradient were collected from the bottom of the tube by using a peristaltic pump. They were assayed by monitoring the fluorescence intensities of the various probes.

Sedimentation Equilibrium. Molecular weights were determined in a Beckman Model E ultracentrifuge equipped with an ultraviolet scanner. Temperature was controlled at 23 °C by a RTIC-type device from Arden Instruments. The sedimentation pattern analyzed here was obtained after 45 h at 13000 rpm. The sample volume was 150 μL. The solution height in the cell was 4.4 mm, and the light path was 1.2 cm. The optical density of the solution was 0.25 at 280 nm.

Circular Dichroism. The circular dichroism spectra were obtained on a Jasco J500C spectropolarimeter equipped with a temperature-controlled cell block set at 25 °C. The light path was 0.20 cm, and the scan was at 1 nm/min with a sensitivity of 1 mdeg/cm. The protein concentration was 0.0912 mg/mL.

Protein Concentration. The concentration of the 100-kDa protein was determined by measuring the ultraviolet absorbance at 215 and 280 nm and using the equation

$$C = (1/11.55)(A_{215} - 3.667A_{280})$$

which is similar to the Scopes equation [Scopes, 1974; see also Edelhoch and Chen (1980)] that was derived by using the ultraviolet absorbance relationship between 205 and 280 nm. The constants in the above equation were evaluated by plotting E_{215} vs. E_{280} for bovine serum albumin, ovalbumin, trypsinogen, α-lactalbumin, α-chymotrypsinogen, and lysozyme. Average values for three to four determinations for each protein were used. With the above method, an extinction coefficient of $E^{1\%}_{280}$ = 7.24 is obtained for the purified 100-kDa protein. For clathrin an extinction coefficient of $E^{1\%}_{280}$ = 10.9 (Nandi et al., 1980) was used. The relative amounts of clathrin reacting with the increasing concentrations of 100-kDa protein were determined by measuring the decrease in the tryptophan fluorescence at fraction 4 in the sucrose gradients (Figure 6) and were verified by SDS gel electrophoresis.

Amino Acid Analysis. The amino acid analysis was determined on a Beckman Model 121 amino acid analyzer with a Model 427 integrator. Tryptophan was calculated from the extinction coefficient after correcting for tyrosine.

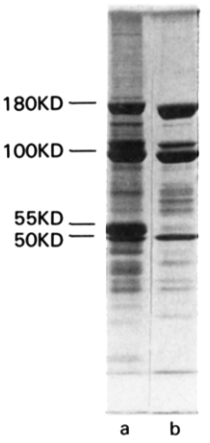

FIGURE 1: SDS gel electrophoretic pattern of (a) uncoated vesicles (UVs) after dissociating coated vesicles (CVs) in 0.01 M Tris, pH 8.5, and (b) supernatant after treating UVs (a) with 0.5 M Tris, pH 8.0, and centrifuging for 1 h in a Ti70.1 rotor at 23 °C and at 40000 rpm.

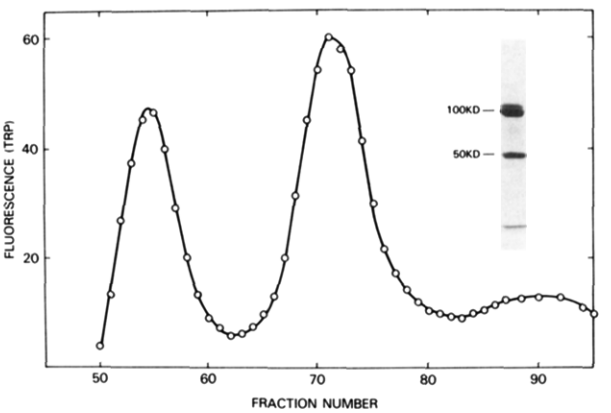

FIGURE 2: Sephacryl S-300 chromatography elution profile of supernatant of UVs after treating with 0.5 M Tris, pH 8.0. Flow rate is 10 mL/h. Fractions, $2^1/_2$ mL, were collected. (Inset) SDS gel electrophoretic pattern of the second peak (fractions 68–75).

RESULTS

Purification of the 100-kDa Protein. CVs were isolated from bovine brains by the procedure described by Nandi et al. (1982), which was a modification of the one published by Pearse (1975). They were prepared and stored at pH 6.5 in 0.10 M Mes buffer. UVs were dissociated from CVs by dialyzing against a large volume of 10 mM Tris, pH 8.5, buffer for 15 h at 4 °C. The solution was then centrifuged at 50000 rpm for 1 h (in a Ti70.1 rotor), yielding a pellet containing the UVs. The supernatant contained most of the clathrin and about 40% of the 100–110- and 50-kDa proteins present in CVs [see Figure 1, lane 1 in Prasad et al. (1985)]. The pelleted UVs containing the remainder of the clathrin and the 100–110- and the 50-kDa proteins (Figure 1a) were dispersed in 0.5 M Tris, pH 8.0, for 18 h and centrifuged at 40000 rpm for 70 min (in a Ti70.1 rotor). The supernatant was enriched in the faster migrating 100-kDa proteins and 50-kDa protein as well as the remaining clathrin (Figure 1b). It was then filtered on a Sephacryl S-300 column equilibrated with 0.5 M Tris, pH 8.0 (Figure 2), which removed most of the clathrin (fractions 50–62) and other minor components (fractions 80–95) (Figure 2). It should be noted from the gel electrophoretic pattern illustrated in Figure 2 that, of the two bands representing the group of 100–110-kDa proteins, the protein

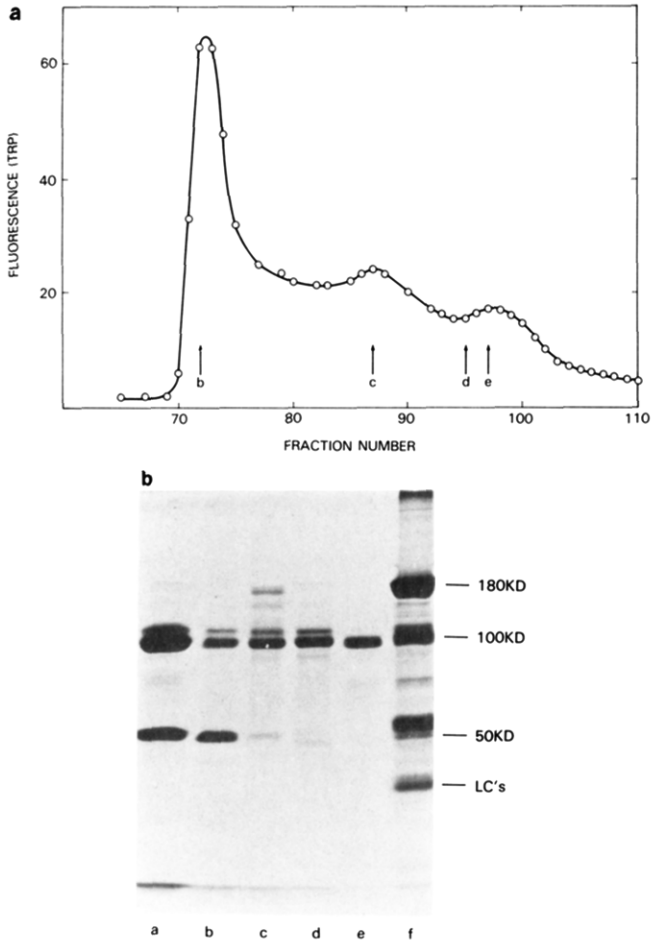

FIGURE 3: (a) Sephacryl S-300 chromatography elution profile of fractions 68–75 in Figure 2 in 3 M urea and 0.01 M Tris, pH 8.0. Fractions, 2 mL, were collected. (b) SDS gel electrophoretic pattern of various fractions of the above column: lane a, total material put on the column; lane b, fraction 72; lane c, fraction 87; lane d, fraction 95; lane e, fraction 97; lane f, purified coated vesicles.

designated as 100-kDa is now the predominant one and is in the fractions 68–75.

Fractions 68–75 were pooled and concentrated by vacuum dialysis. After removing a small amount of aggregated protein by centrifugation, the solution was dialyzed against 3 M urea, pH 8.0, and 10 mM Tris for 15 h. It was then filtered on a Sephacryl S-300 column equilibrated with 3 M urea, pH 8.0, and 10 mM Tris. The elution profile and the SDS electrophoretic patterns of the peak fractions are shown in Figure 3. The third peak (fraction 97) contains primarily the protein designated as the 100-kDa protein and is the material we have characterized (fractions 97–102). All three peaks contain the 100-kDa protein(s). These probably represent different 100-kDa species, which migrate too close to each other for resolution (Robinson & Pearse, 1986). The first and major peak (fraction 73) contains most of the 50-kDa protein. The second peak (fraction 87) contains several minor bands migrating in the vicinity of clathrin.

Characterization of the Protein Designated as 100 kDa. Sedimentation Analysis. The purified 100-kDa protein (fractions 97–102) sediments with a symmetrical boundary with a coefficient $s_{20,w}$ of 5.5 ± 0.5 S in solutions of concentrations ranging from 0.14 to 0.70 mg/mL, in 0.1 M Tris, pH 8.0.

Sedimentation Equilibrium. In Figure 4 is shown the sedimentation equilibrium pattern of the purified 100-kDa protein in 0.01 M Tris, pH 8.0 (circles). The line is a best

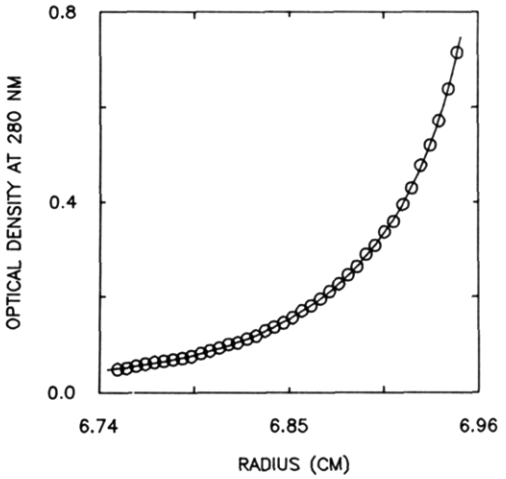

FIGURE 4: Equilibrium centrifugation pattern of the purified 100-kDa protein. The circles represent the measured optical density. The line represents the fit of the data for a two-component, noninteracting system analyzed according to the method of Gladner et al. (1981).

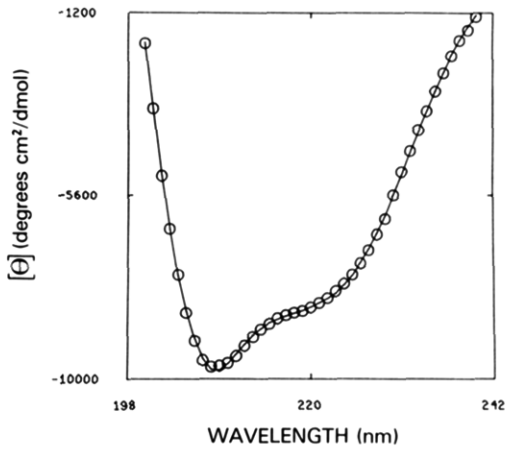

FIGURE 5: Circular dichroism spectrum of the purified 100-kDa protein. The ordinate gives the peptide molar ellipticity. The buffer was 0.001 M Tris and 0.01 M sodium fluoride, pH 8.0. The circles represent the measured ellipticity. The line represents the analysis according to the modeling program of Provencher (1982a,b).

fit curve for a two-component noninteracting system (Gladner et al., 1981), with the major component, representing 85% of the protein, having a molecular weight of 114000 ± 2700. A partial specific volume $\bar{v}$ of 0.742 was approximated by calculation from the amino acid composition that we determined. (The amino acid composition in number of moles per 114000 g is as follows: Trp, 9; Asp + Asn; 117; Thr, 50; Ser, 63; Glu + Gln, 115; Pro, 62; Gly, 59; Ala, 79; Cys, 7; Val, 81; Met, 20; Ile, 56; Leu, 123; Tyr, 26; Phe, 36; Lys, 64; His, 19; and Arg, 40.) This $\bar{v}$ was used in the calculation of the molecular weights. The molecular weight of the second component, 496000 ± 55000, presumably represents an average of several proteins, probably aggregates of the lower molecular weight species faintly visible on SDS gels, each of which is present in a minor amount. The root mean square error for the fit was 0.0019. When the molecular weight of 114000 was combined with a sedimentation coefficient $s_{20,w}$ of 5.5 S, a frictional ratio of 1.5 was calculated.

Circular Dichroism. The molecular ellipticity of the purified 100-kDa protein in the far-ultraviolet wavelength is shown in Figure 5. Analysis of the curve by the modeling program of Provencher (1982a,b) gave the following composition of the polypeptide backbone: helix, 28%; β-structure, 29%; β-turns, 15%; and random peptides, 28%. The average deviation of

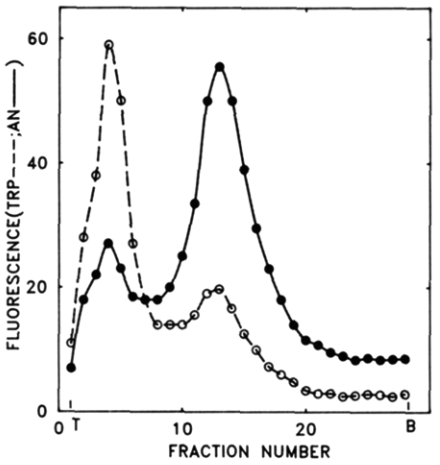

FIGURE 6: Sucrose gradient centrifugation of baskets obtained with the use of purified AN-labeled 100-kDa protein. Clathrin, 0.4 mg/mL, and AN-labeled 100-kDa protein, 0.15 mg/mL, in 0.1 M Mes, pH 6.2, were centrifuged for 100 min at 23 °C in a Beckman SW40 rotor at 24000 rpm. Open circles are tryptophan fluorescence, with excitation, 290 nm, and emission, 340 nm. Closed circles are AN-fluorescence, with excitation, 350 nm, and emission, 426 nm. T and B represent the top and bottom of the gradient, respectively.

the data points frm the fitted curve was ±0.33% of the value of the full curve height.

Polymerization of Clathrin. In the absence of the purified 100-kDa protein or the 100–50-kDa complex, clathrin at a concentration of 0.4 mg/mL did not polymerize at 0.10 M Mes, pH 6.2. Addition of the purified 100-kDa protein to a final concentration of 0.15 mg/mL resulted in the polymerization of clathrin to baskets in the same buffer. Velocity centrifugation in 0.1 M Mes, pH 6.2, and at 23 °C showed that the baskets formed sediment as a single symmetrical boundary with a sedimentation coefficient of 150 S. The product of polymerization was also analyzed by sucrose gradients. The purified 100-kDa protein was AN-labeled by reaction with *N*-(1-anilinonaphthyl)maleimide to distinguish it from clathrin. Most of the AN label was present at fractions 12–14, the position of the 150S baskets (Figure 6). However, a small amount of labeled 100-kDa protein as well as the excess of clathrin was sedimented as unpolymerized species.

The reaction between clathrin and the 100-kDa protein was quantitated by using a fixed concentration of clathrin at 0.1 mg/mL and varying the concentration of 100-kDa protein from 0.02 to 0.08 mg/mL. As the concentration of the 100-kDa protein increased, the amount of polymerized clathrin increased as evidence by an increase in light scattering and as seen in sucrose gradient centrifugation (data not shown). The relative amount of clathrin reacting with the 100-kDa protein was determined by measuring the decrease in tryptophan fluorescence at fraction 4 in the sucrose gradients. Since a part of the tryptophan fluorescence may be due to unreacted 100-kDa protein remaining at the top of the gradient, the values obtained should be considered as approximate; however, it was observed in a number of experiments that the unreacted 100-kDa protein aggregates and sediments to the bottom of the gradient tube, with only a small percentage remaining at the top. This was validated by gel electrophoresis showing that the supernatant of the reaction mixture contained little if any 100-kDa protein. The composition of the baskets isolated from the sucrose gradients was analzyed by SDS gel electrophoresis (Figure 7). The molar stoichiometry of clathrin heavy chains (180 kDa) to 100-kDa protein and to light chains (33–36 kDa) is 3:0.91 (±0.16):1.7 (±0.15) (mean ± SD of four determinations).

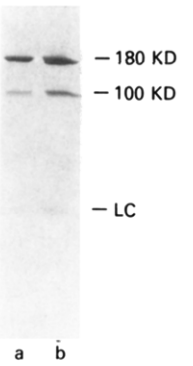

FIGURE 7: SDS gel electrophoretic pattern of baskets polymerized at 0.1 M Mes, pH 6.2: lane a, clathrin, 0.1 mg/mL, plus 100 kDa, 0.058 mg/mL; lane b, clathrin, 0.1 mg/mL, plus 100 kDa, 0.104 mg/mL. Baskets were isolated from sucrose gradients (see Figure 6), pelleted by centrifuging at 40000 rpm for 45 min in a Ti70.1 rotor, and washed twice with 0.1 M Mes, pH 6.2.

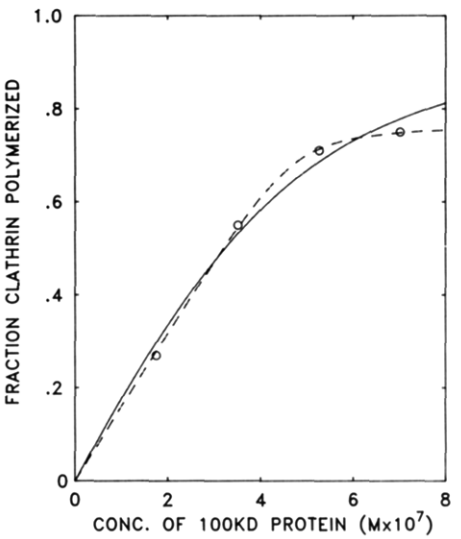

FIGURE 8: Fraction of clathrin polymerized as a function of the concentration of 100-kDa protein. Clathrin, 0.1 mg/mL, and AN-labeled 100-kDa protein at various concentrations were mixed in 0.01 M Tris, pH 8.5, and titrated to 0.1 M Mes, pH 6.2; (with 1 M Mes, pH 6.2). The sample was then analyzed by sucrose gradient centrifugation as in Figure 6. The ordinate was calculated from arbitrarily assigned fluorescence units for the clathrin peak at fraction 4, Figure 6. The solid curve was calculated for one site with $K = 10^{7.4}$ and the dashed curve for 0.77 site with $K = 10^{8.6}$ (see text).

The data of Figure 8 may be evaluated to provide an apparent association constant for the binding of the 100-kDa protein to each triskelion in the formation of baskets with the following assumptions: (1) The concentrations of unpolymerized complex of the triskelion and 100-kDa protein is small compared to that of baskets. (2) About one 100-kDa protein binds to one triskelion in the formation of baskets (see above).

Since the formation of baskets is a polymerization process with a degree of polymerization of about 36 (Crowther et al., 1976), the apparent association constant will be a function of both the concentrations of the triskelion and the 100-kDa protein. A perturbation factor, α, for formulating an apparent simple association, T + D = TD (where T and D are the triskelion and the 100-kDa protein, respectively), could have a form such as

$$\alpha = 1 + rk_r k_D{}^{r-1} c_D{}^{r-1} c_T{}^{r-1}$$

where r is the degree of polymerization, k_D is the intrinsic association constant for the binding of one 100-kDa protein

to a triskelion, k_r is a polymerization constant, c_D is the concentration of free (uncomplexed) 100-kDa protein, and c_T is the concentration of free triskelion. In applying this perturbation factor, the concentrations c_D and c_T (only for the factor, α) are assumed to have some constant average value for the conditions of the experiment. With these limiting assumptions, the following two simultaneous equations may be employed:

$$p = Kc_D/(1 + Kc_D) \quad (1)$$

$$c_2 = c_1[Kc_D/(1 + Kc_D)] + c_D \quad (2)$$

where p is the fraction polymerized, $\alpha k_D = K$, the apparent association constant, c_1 is the total concentration of the clathrin in units of the triskelion, and c_2 is the total concentration of 100-kDa protein.

In fitting these two equations to the data, an additional factor, f, must be employed because the total amount of 100-kDa protein is not equilibrated with the total amount of clathrin in the sucrose gradient centrifugation, thus changing eq 2 to

$$c_2 f = c_1[Kc_D/(1 + Kc_D)] + c_D \quad (3)$$

The two fitting parameters K and f were evaluated by the method of least squares to give $K = 10^{7.4}$ and $f = 0.35$. Given that eq 1 and 3 represent a model consistent with the data, the introduction of a third parameter for the number of sites n on the triskelion available for the binding of the 100-kDa protein should improve the fit. The dashed line of Figure 8 represents the best fit for a value of $n = 0.77$, $K = 10^{8.6}$, and $f = 0.33$. Thus an apparent association constant of $K = 10^{7.4}$–$10^{8.6}$ with $n = 0.77$–1.0 represents reasonable values for the binding of the 100-kDa protein to the triskelion of clathrin under the present conditions of basket formation.

Discussion

We have extensively purified one of the active 100–110-kDa proteins of CVs using two gel filtration steps: the first column contained 0.50 M Tris, pH 8.0, the buffer used by Keen et al. (1979) to dissociate coat proteins from CVs and to resolve the 100–50-kDa complex of coat proteins; the second column contained 3 M urea and 0.01 M Tris, pH 8.0, which dissociates the 100–50-kDa complex into its constituent proteins. We have isolated the 100-kDa protein from UVs rather than from CVs since the former contain predominantly the 100–50-kDa complex and very little of the 110-kDa proteins (or clathrin).

The 100-kDa protein(s) is (are) found in all three eluting peaks from the 3 M urea column: the first peak (fraction 73, Figure 3a) contains most of the 50-kDa protein; the second peak (fraction 87, Figure 3a) contains several slowly migrating proteins (165–190 kDa); the third peak (fraction 97, Figure 3a) contains the purified 100-kDa protein and traces of both slower and faster migrating proteins. The latter 100-kDa protein has a large effect on the rate of polymerization of clathrin at pH 6.2 and is roughly twice as active as the 100–50 kDa complex isolated from the 0.50 M Tris column. Moreover, the purified protein from the third peak produces only the small size baskets (150 S) when reacting to polymerize clathrin. It seems likely, but has not been proven, that the 100-kDa proteins present in the various peaks have slightly different properties, although they may be related.

The purified protein has a molecular weight of 114 000 in its native state as analyzed by the thermodynamic method of sedimentation equilibrium. It has a sedimentation coefficient $s_{20,w}$ of 5.5 S and has about 28% helical structure. It reacts in approximately stoichiometric amounts with clathrin triskelions in 0.1 M Mes, pH 6.2, to form homogeneous size baskets with a sedimentation coefficient of 150 S. The composition of the reconstituted baskets shows that for every triskelion of clathrin, one molecule of 100 kDa is required for polymerization (Figure 8). Interestingly, this stoichiometric ratio is consistent with the composition of the baskets polymerized in the presence of mannose 6-phosphate receptor, where it was shown that the receptor, clathrin triskelion, and 100-kDa proteins exist in a ratio of 1:1:1 (Pearse, 1985).

The functions and properties of 100-kDa proteins both in vivo and in vitro are yet to be elucidated. Robinson and Pearse (1986), using antibodies to the family of 100-kDa proteins and immunofluorescence analysis, concluded that coated vesicles containing different members of the 100-kDa family are embarked on different pathways of membrane traffic. Clearly, in vitro, one function of one of the 100-kDa proteins is to polymerize clathrin under conditions where it otherwise does not polymerize (Zaremba & Keen, 1983; Pearse & Robinson, 1984). We had shown earlier (Prasad et al., 1985) that the complexes of 100- and 50-kDa proteins are involved in the reassociation of clathrin to the membrane under the conditions of coated vesicle isolation. Unanue et al. (1981) and recently Manfredi et al. (1985) and Mosley and Branton (1985) have reached similar conclusions using a variety of experimental methods and employing antibodies to these proteins. Since the present pure protein shares the same characteristics as its parent 100- and 50-kDa complex in its ability to polymerize clathrin to a homogeneous size baskets, it probably is also involved in reassociating clathrin with the stripped vesicles, though such a conclusion is yet to be proved. The nature of the molecular interactions between clathrin and the 100-kDa protein as well as among the three components, clathrin, 100-kDa protein, and the stripped vesicle, awaits future research.

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Preferred Sites on Cytochrome *c* for Electron Transfer with Two Positively Charged Blue Copper Proteins, *Anabaena variabilis* Plastocyanin and Stellacyanin†

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ABSTRACT: Rate constants for the reactions of horse cytochrome *c* (E'_0 of +260 mV) with the copper proteins *Anabaena variabilis* plastocyanin (E'_0 of +360 mV) used as oxidant and stellacyanin (E'_0 of +187 mV) used as reductant have been determined at 25 °C, pH 7.5 and 7.0, respectively, and an ionic strength of 0.10 M (NaCl). These rate constants were also measured with eight different singly substituted 4-carboxy-2,6-dinitrophenyl (CDNP) horse cytochrome *c* derivatives, modified at lysine-7, -13, -25, -27, -60, -72, -86, or -87 and with the trinitrophenyl (TNP) derivative modified at lysine-13. The influence of the modifications on the bimolecular rate constants for these reactions defines the region on the protein that is involved in the electron-exchange reactions and demonstrates that the preferred site is at or near the solvent-accessible edge of the heme prosthetic group on the "front" surface of the molecule. Both reactions are strongly influenced by the lysine-72 modification to the left of the exposed heme edge and, to this extent, behave similar to the earlier studied reaction with azurin. These effects span only an order of magnitude in rate constants and are thus many times smaller than those for the physiological protein redox partners of cytochrome *c*. While the preferred sites of reaction on the surface of cytochrome *c* for small inorganic complexes appear to be dependent only on the net charge of the reactants, with the copper proteins additional factors intervene. These influences are discussed in terms of hydrophobic patches and the distribution of charges on the surface of the four copper proteins so far examined.

A variety of singly modified lysine derivatives of horse cytochrome *c* have provided a valuable means of mapping out regions on the protein that react with different redox partners (Staudenmayer et al., 1976, 1977; Smith, et al., 1977; Ng et al., 1977; Ferguson-Miller et al., 1978; Kang et al., 1978; Ahmed et al., 1978; Speck et al., 1979, 1981; Stonehuerner et al., 1979; Webb et al., 1980; Osheroff et al., 1980; Smith, et al., 1980; Konig, et al., 1980; Butler et al., 1981, 1982, 1983; Ahmed & Millett, 1981; Koppenol & Margoliash, 1982; Augustin et al., 1983). In addition to studies with natural mitochondrial electron-exchange partners, other reactions examined (Butler et al., 1983) include those with nonphysiological protein partners, such as parsley plastocyanin, estimated to have a charge of −7 for the Cu(II) state at pH 7, and *Pseudomonas aeruginosa* azurin, with a charge of −1 for the Cu(II) state and a p*I* of 5.4, assuming only one of the two free histidines carries a +1 charge. In the case of plastocyanin, it was observed that replacement of the +1 lysine charge at neutral pH, by the −1 charge of the 4-carboxy-2,6-dinitrophenyl-(CDNP-) substituted derivatives resulted in a decrease in rate constant, as expected on the basis of simple electrostatic considerations. Remarkably, with azurin, the rate constants of the modified cytochromes *c* increased, suggesting that azurin behaves as a positively charged oxidant and/or that factors besides charge are contributing. Therefore, it was decided to investigate further these phenomena by employing two other blue (type 1) single copper proteins (Lappin, 1981), namely plastocyanin from the blue-green algae *Anabaena variabilis*,

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