REFERENCES
Baccarini-Melandri, A., Jones, O. T. G., & Hauska, G. A. (1978) *FEBS Lett. 86*, 151-154.
Bashford, C. L., Prince, R. C., Takamiya, K., & Dutton, P. L. (1979) *Biochim. Biophys. Acta 545*, 223-235.
Bergstrom, J., & Vanngard, T. (1982) *Biochim. Biophys. Acta 682*, 452-456.
Bowyer, J. R., Dutton, P. L., Prince, R. C., & Crofts, A. R. (1980) *Biochim. Biophys. Acta 592*, 445-460.
Cramer, W. A., Widger, W. R., Hermann, R. G., & Trebst, A. (1985) *Trends Biochem. Sci. (Pers. Ed.) 10*, 125-129.
Crofts, A. R. (1985) *Enzymes Biol. Membr. 4*, 347-382.
Crofts, A. R., & Wood, P. M. (1978) *Curr. Top. Bioenerg. 1*, 175-244.
Crofts, A. R., Meinhardt, S. W., Jones, K. R., & Snozzi, M. (1983) *Biochim. Biophys. Acta 723*, 202-218.
Crowder, M. S., Prince, R. C., & Bearden, A. (1982) *FEBS Lett. 144*, 204-208.
Daldal, F., Cheng, S., Applebaum, J., Davidson, E., & Prince, R. C. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2012-2016.
Dutton, P. L. (1978) *Methods Enzymol. 54*, 411-435.
Dutton, P. L., Petty, K. M., Bonner, H. S., & Morse, S. D. (1975) *Biochim. Biophys. Acta 387*, 536-556.
Evans, E. H., & Crofts, A. R. (1974) *Biochim. Biophys. Acta 357*, 89-102.
Glaser, E. G., & Crofts, A. R. (1984) *Biochim. Biophys. Acta 766*, 322-333.
Imhoff, J. F., Truper, H. G., & Pfennig, N. (1984) *Int. J. Syst. Bacteriol. 34*, 340-343.
Jackson, J. B., & Dutton, P. L. (1973) *Biochim. Biophys. Acta 325*, 102-115.
Matsuura, K., Bowyer, J. R., Ohnishi, T., & Dutton, P. L. (1983) *J. Biol. Chem. 258*, 1571-1579.
Meinhardt, S. W., & Crofts, A. R. (1982) *FEBS Lett. 149*, 223-227.
Mitchell, P. (1966) *Chemiosmotic Coupling in Oxidative Phosphorylation*, Glynn Research Ltd., Bodmin, U.K.
Prince, R. C. (1983) *Biochim. Biophys. Acta 723*, 133-138.
Prince, R. C., Baccarini-Melandri, A., Hauska, G. A., Melandri, B. A., & Crofts, A. R. (1975) *Biochim. Biophys. Acta 387*, 212-227.
Prince, R. C., Bashford, C. L., Takamiya, K., van den Berg, W. H., & Dutton, P. L. (1978) *J. Biol. Chem. 253*, 4137-4142.
Prince, R. C., O'Keefe, D. P., & Dutton, P. L. (1982) *Top. Photosynth. 4*, 197-248.
Robertson, D. E., Moser, C. C., Giangiacomo, K. M., deVries, S., & Dutton, P. L. (1985) *Biophys. J. 47*, 240a.
Robertson, D. E., Davidson, E., Prince, R. C., van den Berg, W. H., Marrs, B. L., & Dutton, P. L. (1986) *J. Biol. Chem. 261*, 584-591.
Sandmann, G., Reck, H., Kessler, E., & Boger, P. (1983) *Arch. Microbiol. 134*, 23-27.
Takamiya, K., & Dutton, P. L. (1977) *FEBS Lett. 80*, 279-284.
Takemoto, J., & Bachmann, R. C. (1979) *Arch. Biochem. Biophys. 195*, 526-534.
van den Berg, W. H., Prince, R. C., Bashford, C. L., Takamiya, K., Bonner, W. D., & Dutton, P. L. (1979) *J. Biol. Chem. 254*, 8594-8604.
Wiley, D. L., Auffret, A. D., & Gray, J. C. (1984) *Cell (Cambridge, Mass.) 36*, 555-562.
Wood, P. M. (1980) *Biochem. J. 189*, 385-391.

An Intermediate Polymer in the Assembly of Clathrin Baskets

Kondury Prasad,* Roland E. Lippoldt, and Harold Edelhoch‡
Clinical Endocrinology Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892
Marc S. Lewis
Biomedical Engineering and Instrumentation Branch, Division of Research Services, National Institutes of Health, Bethesda, Maryland 20892

Received January 6, 1986; Revised Manuscript Received April 2, 1986

ABSTRACT: Clathrin (8 S) is known to polymerize into two varieties of basket structures (150 S or 300 S) under the normal buffer conditions [100 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes), pH 5.9–6.7] used for the isolation of coated vesicles. However, it is now observed that under very low salt conditions (2 mM Mes, pH 5.9), it forms a homogeneous species with a sedimentation coefficient of 27 S. Increasing the salt concentration to 50 mM Mes completely converts all the 27S species into 150S baskets. Sedimentation equilibrium data show that this 27S species has a molecular weight that is 6 times that of the clathrin protomer and is the result of highly cooperative reversible self-association of the 8S protomer. Light-scattering studies show that the stabilities of 27S species and baskets (150 S or 300 S) are comparable. Fluorescent labeling of sulfhydryl groups with *N*-(1-anilinonaphthalenyl)maleimide indicates that the conformation of clathrin in 27S species and baskets (150 S or 300 S) is similar. Trypsin digestion reveals that in the 27S species clathrin has a conformation differing from that in both the 8S species and baskets.

Clathrin is the structural protein responsible for the polygonal organization of the coat in coated pits and coated vesicles (CVs)[1] (Pearse, 1976). The clathrin protomer (M_r ≈630 000), as isolated from CVs, contains three identical chains of 180 kDa and three light chains of two sizes, i.e., 33 or 36 kDa (Kirchhausen & Harrison, 1981), and has a sed-

* Correspondence should be addressed to this author.
‡ Deceased January 15, 1986.

[1] Abbreviations: AN, anilinonaphthalene; ANM, *N*-(1-anilinonaphthalenyl)maleimide; CV(s), coated vesicle(s); kDa, kilodalton; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Trp, tryptophan; SDS, sodium dodecyl sulfate.

imentation coefficient of 8.2 S (Nandi et al., 1980). Hydrodynamic methods indicated that the 8S molecule was very asymmetric (f/f_0 = 3) (Pretorius et al., 1981). Electron micrographs revealed a pinwheel arrangement with three arms; this form of clathrin was referred to as a triskelion (Ungewickell & Branton, 1981).

When clathrin polymerizes to form a basket (also referred to as a cage or coat), the size of this structure depends on whether a second protein with a molecular weight of 100K–110K is present or absent (Irace et al., 1982; Zaremba & Keen, 1983; Pearse & Robinson, 1984). In its presence, clathrin forms baskets which are usually quite homogeneous and have a sedimentation coefficient of 150 S. In the absence of the 100–110-kDa protein, the baskets are much larger (≥300 S) and less homogeneous. In our earlier studies on the self-association of 8S clathrin to form baskets, no intermediate-size polymers were observed either by velocity centrifugation or by sedimentation on sucrose gradients (Irace et al., 1982; van Jaarsveld et al., 1981). However, under the special conditions of very low Mes buffer concentrations, we have observed the formation of a stable intermediate with a sedimentation coefficient of 27 S. We now report on the properties of this intermediate species of clathrin polymer.

Materials and Methods

Chemicals. Tris(hydroxylmethyl)aminomethane (Tris) was from Bethesda Research Laboratory. 2-(*N*-Morpholino)-ethanesulfonic acid (Mes) was from Sigma. *N*-(1-Anilinonaphthalenyl)maleimide (ANM) was obtained from Polysciences.

Preparation. (*A*) *8S Clathrin.* Clathrin was prepared as previously described and stored in 0.01 M Tris, pH 8.5 (Irace et al., 1982). This preparation contained significant amounts of the 100–110-kDa proteins as seen in SDS gel electrophoretic analysis (Figure 5, lanes 1 and 5). When analyzed in the ultracentrifuge, about 85–90% of the protein sedimented as an 8S species with a symmetrical boundary; the rest of the protein trailed behind the 8S component. These proteins may be undergoing a slow dissociation from the clathrin during centrifugation.

(*B*) *27S Species.* The clathrin used for preparing the 27S species was obtained by purifying 8S clathrin further by gel filtration in 0.5 M Tris as previously described by Keen et al. (1979) and by Prasad et al. (1985). This buffer dissociates the 100-kDa proteins from clathrin. The 27S species was prepared from this purified clathrin by reducing the Tris concentration from 0.5 M, first by extensive dialysis against 0.01 M Tris, pH 8.5, and then against 2 mM Mes, pH 5.9, with repeated changes of the dialysate. For sedimentation equilibrium studies, the solution was sedimented at 30000 rpm for 30 min in a Ti 70.1 rotor to remove a small percentage (5–10%) of aggregates that are frequently seen after dialysis.

(*C*) *Baskets.* Baskets were prepared by dialyzing 8S clathrin against 0.10 M Mes, pH 5.9. The baskets were dialyzed against 2 mM Mes, pH 5.9, for several hours, with repeated changes of buffer so that they would be present in the same solvent conditions as the 27S species. The baskets were found to remain intact, since no dissociation was observed during sedimentation in the analytical ultracentrifuge.

Methods. (*A*) *Fluorescence Measurements.* Fluorescence spectra were obtained by using a Perkin-Elmer MPF-3 spectrofluorometer. Fluorescent labeling of the clathrin with *N*-(1-anilinonaphthalenyl)maleimide was performed as previously described (Prasad et al., 1984). The labeling was 1–2 mol/mol of clathrin (M_r 630000). Excitation of this fluorochrome was at 350 nm, and emission was at 426 nm. Tryptophan fluroescence was measured by excitation at 290 nm and reading the emission at 340 nm.

(*B*) *Gel Electrophoresis.* Polyacrylamide gels with a 5–15% gel gradient were used. The SDS gel electrophoresis was performed according to Laemmli (1970). The gels were fixed and stained with Coomassie Brilliant Blue R250 in 50% trichloroacetic acid.

(*C*) *Light Scattering.* Light scattering was measured at a wavelength of 340 nm, a temperature of 22 °C, and a scattering angle of 90° by using a square cuvette in the Perkin-Elmer MPF-3 fluorometer.

(*D*) *Analytical Ultracentrifugation.* A Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanner was used for both velocity and equilibrium studies. Double-sector cells, with a 12-mm optical path length, were used for all experiments. Scans were taken at a wavelength of 280 nm.

Velocity experiments were carried out in an AN-F-Ti rotor at a speed of 20000 rpm and a temperature of 23 °C. The radial position of the 50% concentration point of the boundary as a function of time was used to compute the sedimentation coefficient.

Equilibrium experiments were carried out at 5 °C using an AN-D rotor at a speed of 3000 rpm. Ultracentrifugal equilibrium was considered to have been attained when successive scans became invariant with time. Data were obtained by manually digitizing the scans and converting the x–y coordinates to concentration in the form of optical density at 280 nm as a function of radial position in the cell. These were analyzed by mathematical modeling by nonlinear least-squares curve fitting using MLAB, an interactive system operating on a DEC-10 computer (Knott, 1979). Since the development of appropriate mathematical models is intimately related to the results obtained, this will be described in detail under Results.

(*E*) *Sucrose Density Gradient Centrifugation.* Solutions of the 27S species were sedimented on a 5–20% linear sucose density gradient in an SW 40 rotor at 30000 rpm for 6.5 h in a Beckman Model L2-65B centrifuge at 23 °C. The fractions from the gradient were collected from the bottom of the tube by using a peristaltic pump and were analyzed for clathrin concentration by the fluorescence measurements described previously.

Results

Formation of the 27S Species from 8S Clathrin. In studies on the self-association of clathrin to baskets, we have not observed the formation of intermediate-size polymers during analysis by velocity ultracentrifugation either in aqueous solvents (van Jaarsveld et al., 1981) or on sucrose gradients (Irace et al., 1982; van Jaarsveld et al., 1981). This type of result is characteristic of a number of self-associating systems involving a slow nucleation and then a rapid growth step, usually to a cylindrically shaped particle (Wegner & Engel, 1975; Frieden & Goddette, 1983; Ferrone et al., 1980; Carlier & Pantaloni, 1978). In the case of clathrin, a polyhedral shell is formed consisting of open pentagons and hexagons. As with other systems (Borisy & Olmsted, 1972; Durham et al., 1971) showing this type of nucleation mechanism, we have also been able to isolate an intermediate species in high yield when we have markedly reduced the concentration of the buffer. When 8S clathrin at pH 8.5, 0.01 M Tris was dialyzed against a 2 mM Mes buffer at pH 5.9 for 15 h, a single symmetrical sedimenting boundary with a sedimentation coefficient of 27 S was observed in velocity experiments in the ultracentrifuge. The homogeneity of the 27S species formed in 2 mM Mes buffer was examined by sucrose density gradient sedimentation

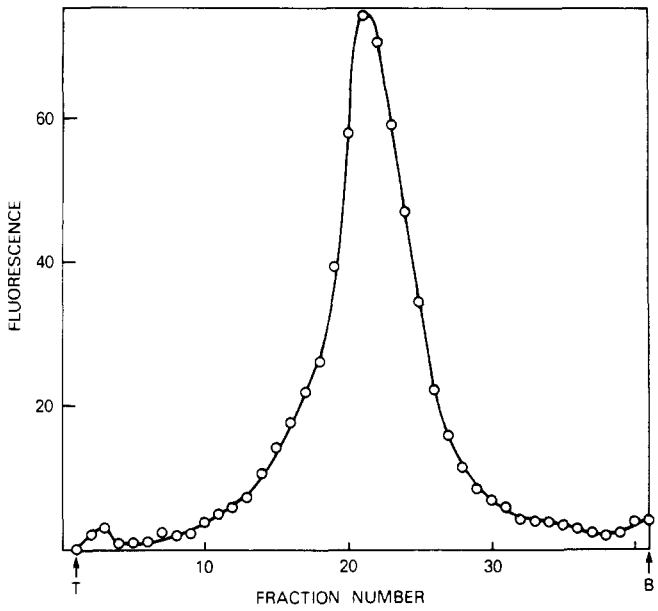

FIGURE 1: Sucrose density gradient centrifugation of 27S clathrin in 2 mM Mes, pH 5.9. The gradient was between 5% and 20% sucrose. Sedimentation was at 30000 rpm for 6.5 h at 23 °C. Tryptophan fluorescence was measured at 340 nm, and excitation was at 290 nm.

analysis. Figure 1 shows the sedimentation profile of the 27S species on a 5–20% gradient. No significant amounts of species with sedimentation coefficients different from 27 S appear to be formed. When the same clathrin solution was dialyzed against 10 mM Mes, pH 5.9, about one-third of the material sedimented at 150 or 300 S, characteristic for baskets, while the remainder sedimented at 27 S. Little or no clathrin was observed to sediment at velocities between 27 and 150–300 S. In 20 mM Mes, pH 5.9, somewhat more than half of the clathrin sedimented as baskets and the rest as 27S species. At 50 mM Mes (pH 5.9), all the clathrin polymerized as baskets, with both 150S and 300S species present in a ratio of 2 to 1, respectively.

In order to ascertain whether the 27S species was an intermediate in the formation of clathrin baskets, the Mes concentration of a solution containing the 27S species, formed in 2 mM Mes, pH 5.9, was increased to 50 mM, while maintaining the pH constant at 5.9. The 27S species was found to form homogeneous 150S baskets. When the 8S clathrin was polymerized directly in 50 mM Mes, pH 5.9, both 150S and 300S baskets were observed. It appears that the pathway of polymerization is very important, since the preformed 27S intermediate gives only 150S baskets whereas if the 8S clathrin used to prepare the 27S species is directly polymerized with 50 mM Mes (pH 5.9), it gives both 150S and 300S baskets.

There is an additional point to be noted at this stage. While the 27S species is capable of forming from 8S clathrin devoid of 100–110-kDa proteins, the baskets obtained from such a preparation are not homogeneous in size as are the baskets that are obtained in the presence of 100–110-kDa proteins. In the experiment described above, we have used 8S clathrin that contains the 100–110-kDa proteins. When a similar experiment was done using 8S clathrin that was devoid of these proteins, the result was a heterogeneous distribution of faster sedimenting baskets as described previously (Irace et al., 1982; Zaremba & Keen, 1983; Pearse & Robinson, 1984).

Equilibrium between 8S and 27S Species. As the first step in the mathematical modeling of an association reaction, it is necessary to know what molecular species are present. Thus, for a self-association reaction, we may expect to observe the presence of monomer and varying amounts of reversibly formed polymers depending on the total concentration. The concentration of any molecular species as a function of radial position at sedimentation equilibrium is given by

$$c_r = c_b \exp[AM(r^2 - r_b^2)] \quad (1)$$

and the total concentration at any radial position is given by

$$c_r = \sum_{i=1}^{n} c_{b,i} \exp[AiM_1(r^2 - r_b^2)] + \epsilon \quad (2)$$

Equation 2 contains a term for each molecular species which might be present: M_1 = the molecular weight of the monomer, $A = (1 - \bar{v}\rho)\omega^2/2RT$, $\bar{v}$ = the partial specific volume of the protein, ρ = the solution density, ω = the rotor angular velocity, R = the gas constant, T = the absolute temperature, and ϵ = a small error term to adjust for base-line error (Johnson et al., 1981). Implicit in this equation is the assumption that the partial specific volume is equal for all molecular species present. We have no basis for predicting whether or not a volume change occurs with self-association and what the possible magnitude of such a change might be. This point is moot since we are here assuming a partial specific volume of 0.73 cm^3/g in the absence of data that would enable us to determine a better value.

Equation 2 can be used as a mathematical model for fitting the data in order to determine what species are present by using M_1, ϵ, and $c_{b,i}$ for values of i from 1 through 8 as fitting parameters, subject to the constraint that all values of $c_{b,i}$ must be equal to or greater than zero. When this was done, it was found that the only species detectable were monomer and hexamer. The sensitivity of this method is such that very small quantities of dimer or trimer (>2% of the total concentration) would have been detectable; larger quantities of pentamer or heptamer would have had to be present to be detectable in the presence of a relatively large quantity of hexamer. This enables us to write a mathematical model for reversible self-association of a monomer–hexamer system without the need for concern for intermediate species.

We can define an equilibrium constant for this reversible reaction on the concentration scale of optical density at 280 nm by the equation

$$k = c_6/c_1^6 \quad (3)$$

and if we substitute the relationship $c_6 = kc_1^6$ in eq 2 we obtain

$$c_r = c_{b,1} \exp[AM_1(r^2 - r_b^2)] + kc_{b,1}^6 \exp[6AM_1(r^2 - r_b^2)] + \epsilon \quad (4)$$

Equation 4 can be used as a mathematical model for fitting the data with k, M_1, $c_{b,1}$, and ϵ as fitting parameters. Unless k and $c_{b,1}$ are constrained to nonnegative values, it is possible to obtain convergence in a false local minimum in parameter space. To avoid the need for such constraints, one may fit for the natural logarithms of the equilibrium constant and the concentration of monomer at the cell bottom (Johnson et al., 1981; Lewis et al., 1984) by using the equation:

$$c_r = \exp[\ln c_{b,1} + AM_1(r^2 - r_b^2)] + \exp[\ln k + 6 \ln c_{b,1} + 6AM_1(r^2 - r_b^2)] + \epsilon \quad (5)$$

where $\ln k$, $\ln c_{b,1}$, M_1, and ϵ are now the fitting parameters. It is particularly appropriate to use $\ln k$ as a fitting parameter, as the value of the change of standard free energy of the reaction is given by

$$\Delta G^\circ = -RT \ln K \quad (6)$$

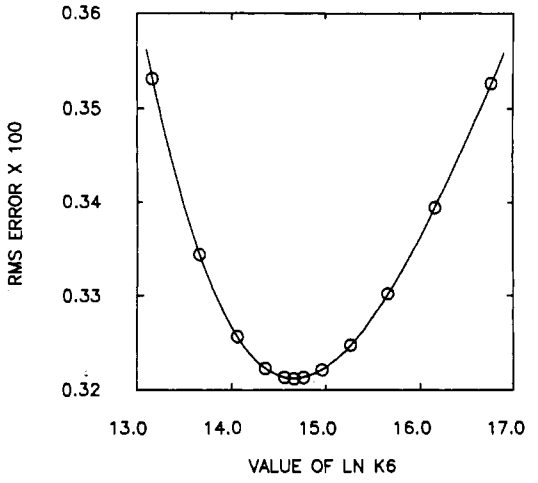

FIGURE 2: Root mean square (RMS) error as a function of the value of ln k when the other fitting parameters are allowed to assume optimum values of ln k (denoted LN K6 in the figure). The smooth curve fitting the points is a polynomial with different coefficients on each side of the minimum; it is for illustrative purposes and has no other significance.

Since the molar equilibrium constant K is directly related to the optical density equilibrium constant k as shown below, we are, in effect, using the change of standard free energy of the reaction as a fitting parameter.

When this is done, we obtain the values $M_1 = 730\,000 \pm 13\,200$ and ln $k = 14.66 \pm 0.62$. The dependence of the value of the root mean square error upon the value of ln k in the nonlinear least-squares fitting procedure is shown in Figure 2. This was obtained by determining the values for the root mean square error obtained with various fixed values of ln k when the other parameter values were allowed to attain optimum values for fitting the data with that value of ln k. It can be seen that the root mean square error is close to being symmetrical on either side of the optimum value of ln k and that the minimum value is quite well-defined. Figure 3A,B shows the quality of the fit to the data with Figure 3A showing a direct fit and Figure 3B showing the deviation of the data points from the fitting line. The root mean square error of this fit is 0.0032 optical density unit at 280 nm, indicative of a very good fit.

The values of the natural logarithm of the molar equilibrium constant and the change in standard free energy for the association can now be readily calculated. With an extinction coefficient for a concentration of 1 g/L, $E_1 = 1.09$ at 280 nm, the molar extinction coefficient for monomer $E_M = E_1M_1$, the molar concentration of monomer $C_1 = c_1/E_M$, and the molar concentration of hexamer $C_6 = c_6/6E_M$. Using these, we obtain

$$K = C_6/C_1^6 = (c_6/6E_M)/(c_1/E_M)^6 = kE_M^5/6 = k(E_1M_1)^5/6 \quad (7)$$

Transforming this equation to a logarithmic form then gives

$$\ln K = \ln k + 5 \ln (E_1M_1) - \ln 6 \quad (8)$$

We then obtain a value of ln $K = 80.81 \pm 0.62$ which gives a value for the molar equilibrium constant of $K = 1.242 \times 10^{35}$ for the monomer–hexamer association and a change of standard free energy of $\Delta G° = -44.66 \pm 0.34$ kcal/mol of hexamer formed.

It is very important to note that the absence of any experimentally detectable concentration of any species other than monomer and hexamer implies that the association is very highly cooperative. Ring closure (i.e., hexagon formation) without the presence of other effects would not be sufficient

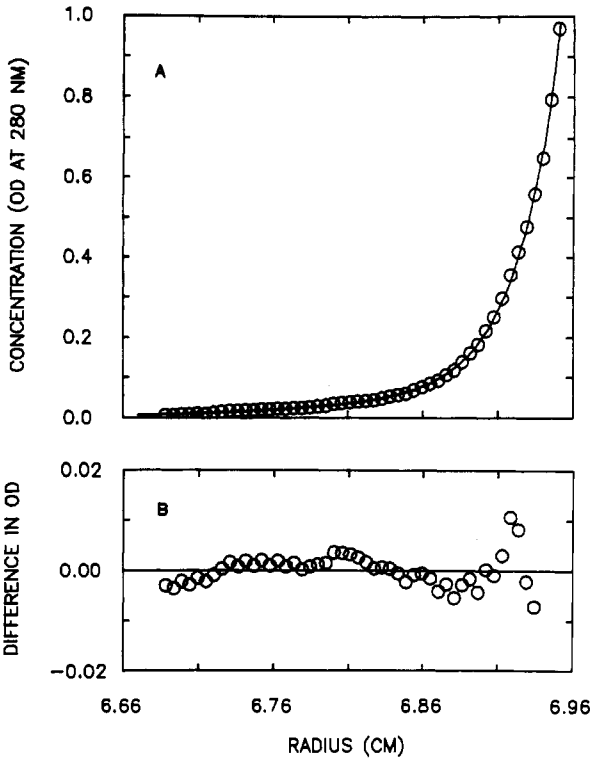

FIGURE 3: (A) Fit of eq 5 to the experimental data. The fitting parameters are the following: $M_1 = 730\,000 \pm 13\,200$; ln $k = 14.66 \pm 0.60$; ln $c_{b,1} = -2.467 \pm 0.101$; $\epsilon = -0.0100 \pm 0.0025$. (B) Deviation of the data points shown in Figure 3A from the fitting line. The root mean square error = 0.0032 optical density unit at 280 nm.

to explain the high degree of cooperativity that we have observed. Thus, the total free energy change which was measured must include a free energy change attributable to these other effects as well as a free energy change due to bond formation, and it is not possible in an experiment of this type to determine these separately.

Stability of the 27S Species to Alkaline Dissociation. We have compared the pH dependence of dissociation of the 27S intermediate with that of the 150S baskets by light scattering. The 150S baskets were formed in 0.10 M Mes, pH 5.9, and were then dialyzed against 2 mM Mes, pH 5.9, so that the solvent was the same in both cases. It can be seen in Figure 4 that the final scattering intensities at high pH were the same, indicating that the products were similar, presumably 8S clathrin. The large decrease in scattering for both species occurs in approximately the same pH range (~6.2–7.0), showing that the stabilities are comparable. The changes in scattering intensities between the initial structures (i.e., 27 S and 150 S) and the final protomer (i.e., 8 S) are not proportional to the changes in their molecular weights. This is to be expected with large polymers which do not scatter symmetrically with respect to the angle of observation. Our measurements were performed at 90° to the incident beam, and clearly, there is a significant loss of scattering with angle since the changes in intensities are much smaller than the changes in molecular size.

Tryptic Digestion. We have compared the extent of tryptic digestion of the 27S species with that of 8S clathrin as well as that of baskets in order to evaluate the conformational state of the 8S triskelion in its protomeric and two polymeric forms, i.e., the 27S species and baskets. It has been reported by Schmid et al. (1982) and Ungewickell et al. (1982) that trypsin readily digested the clathrin monomer (180 kDa) to an ~100-kDa species when it was in the form of baskets. In contrast, very little digestion occurred when clathrin was present

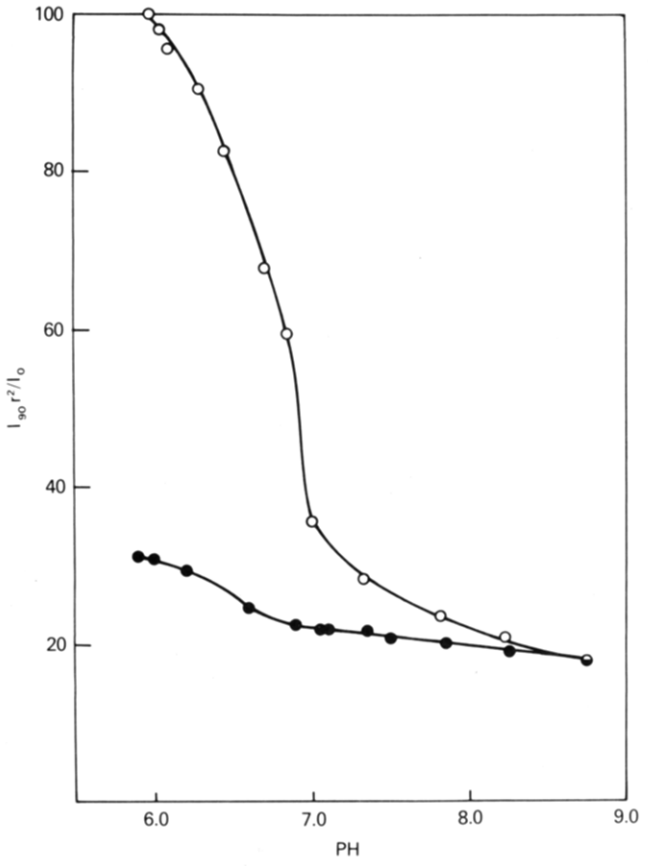

FIGURE 4: Light scattering of the 27S species and of baskets as a function of pH. The pH titration was performed by adding very small volumes of a concentrated solution of NaOH to the light-scattering cuvette with continuous stirring.

as the 8S protomer. Figure 5 compares the results of the digestion of the 27S species with those of 8S clathrin and 150S baskets in either 1% or 2% ratios (by weight) of trypsin to clathrin. In accordance with the above references, it can be seen that almost all of the 180-kDa clathrin monomer in 150S baskets is digested in 2% trypsin and the major product is the 100-kDa fragment. Under the same conditions of digestion, the 180-kDa monomer in 8S clathrin was hardly affected. The mobility profile of the 27S species is clearly different from that of either 150S baskets or 8S clathrin, since an intermediate amount of 180-kDa monomer is digested and the principal product is smaller than the 100-kDa fragments observed in the case of 150S baskets. Other significant changes in the distribution of the remaining proteins and fragments can also be readily distinguished between the three different types of polymer species.

Fluorescent Labeling with ANM. The 27S species was labeled with ANM in order to compare the environment of the AN label in the intermediate polymer with those of protomer and baskets. We have previously reported (Prasad et al., 1984) that the emission peak of the AN label in baskets (or coated vesicles) was shifted toward shorter wavelengths by 3 nm when compared with the same label in native 8S clathrin. A similar shift was observed when the 27S species was labeled with ANM. An experiment with 150S baskets gave results identical with those obtained with the 27S species [Figure 1; see Prasad et al. (1984)].

Discussion

We have observed an intermediate molecular species composed of six protomers and having a sedimentation coefficient of 27 S when clathrin is polymerized at very low concentrations

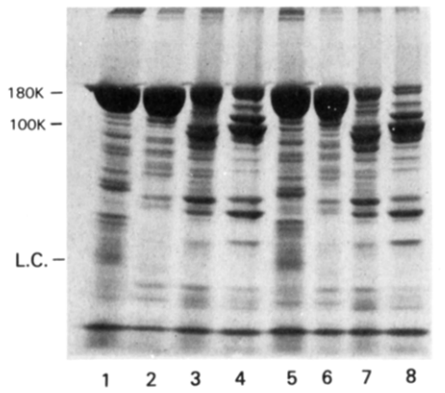

FIGURE 5: SDS gel electrophoretic pattern of 8S clathrin, 27S intermediate, and 150S baskets after trypsin digestion for 2 h at 23 °C. The trypsin digestion is with either 1% or 2% by weight ratio of trypsin to clathrin. The digestion was stopped by addition of soybean trypsin inhibitor (in the weight ratio of 2:1 of trypsin). Lane 1, 8S clathrin without trypsin (control). Lane 2, 8S clathrin with 1% trypsin. Lane 3, 27S species with 1% trypsin. Lane 4, baskets (150 S or 300 S) with 1% tyrpsin. Lane 5, 8S clathrin without trypsin (control). Lane 6, 8S clathrin with 2% trypsin. Lane 7, 27S intermediate with 2% trypsin. Lane 8, baskets with 2% trypsin.

of Mes, i.e., 2 mM at pH 5.9. Analysis of the sedimentation equilibrium data attributed a specific size to the 27S species, since smaller polymer species could be excluded as significant components. This result suggests that the self-association reaction is highly cooperative and very little of the smaller polymers is formed. When the hexamer size is reached, there is a very large decrease in free energy. It appears likely that the hexamer is in a hexagonal configuration and that the change in conformation indicated by the tryptic digestion experiments occurs with formation of this stable hexameric ring. This conclusion is in accord with the AN labeling results which show an environment resembling that of the basket structure rather than that of the triskelion protomer structure. The basket structure, of course, contains closed rings of clathrin either in a pentameric or in a hexameric form. The similarity in pH stability of the 27S species and that of the 150S baskets (see Figure 4) also favors a closed-ring structure rather than a random polymer of six protomers.

Further evidence for the concept of ring closure as the terminal event in this self-association is the fact that the value of the frictional coefficient (f/f_0) for the 27S species is the same as that for the 8S clathrin. The evidence for this is that the ratio of the sedimentation coefficients of the two species is virtually identical with the two-thirds power of the ratio of their molecular weights ($27/8.2 = 3.29$; $6^{2/3} = 3.30$). This identity can occur only if the value of the frictional coefficient is the same for both species. Lateral aggregation without ring closure would be expected to give a greater frictional coefficient, while aggregation involving stacking would be expected to decrease the frictional coefficient.

The similarity in stability suggests that the clathrin hexamer, probably in hexagonal configuration, may be the unit which polymerizes to baskets at higher Mes concentrations. The formation of baskets from 8S clathrin probably follows the same type of nucleation and growth kinetics reported for many self-associating proteins (Wegner & Engel, 1975; Frieden & Goddette, 1983; Ferrone et al., 1980; Carlier & Pontaloni, 1978). In this case, the hexameric species we have isolated under conditions where it is stable is probably the nucleus which polymerizes very rapidly in the growth phase at higher Mes concentrations. In the nucleation theory of self-associating reactions, the series of bimolecular associations leading to the nucleus species are too unfavorable to yield significant

amounts of species smaller than the nucleus. In accord with this mechanism, we have not observed any species between the 8S protomer and 27S hexamer.

It has been shown in several studies that the 100–110-kDa proteins are necessary to form the smallest size baskets, i.e., 150 S (Irace et al., 1982; Zaremba & Keen, 1983; Pearse & Robinson, 1984). In the absence of 100–110-kDa proteins, larger size baskets, which are frequently heterogeneous in size, are the dominant species formed. When 8S clathrin was polymerized at pH 5.9, we obtained a mixture of 150S and 300S baskets. However, when the same 8S clathrin was first polymerized to the 27S intermediate and then polymerized to baskets by increasing the Mes concentration, only 150S baskets were formed. It would appear that, by fixing the structure of the nuclei at the 27S intermediate, the growth phase is directed so that only a single product is formed. When the nucleus is not formed as a stable intermediate at very low Mes concentrations, the reaction mechanism is more ambiguous, since the baskets formed under these conditions are more heterogeneous in distribution of size.

We have also shown that the 27S species is formed by 8S clathrin itself and that the associated 100–110-kDa proteins of coated vesicles are not required. However, for the formation of baskets of homogeneous size, the presence of these 100–110-kDa proteins is required. This appears to imply that a hexameric structure, or any other discrete structure that is formed as the initial product from 8S clathrin, is essentially incapable of orienting its structure for the uniform formation of basket structures. The key to such uniform structures lies only in the associated proteins. Although a variety of basket structures can form from clathrin, it is probable that only one variety of species is the building block. The mechanism by which the 100–110-kDa proteins accomplish the proper orientation of these species to form homogeneous baskets has yet to be determined. A detailed study of the interactions of these proteins with clathrin appears essential for an understanding of this mechanism.

Added in Proof

Since the manuscript was accepted, we have been in communication with Dr. John Heuser (Washington University School of Medicine, St. Louis, MO) with regard to his subsequent electron microscopic studies on the 27S species of clathrin. In his observations, the 27S species appears to be a tetramer rather than the hexamer we have observed. We have not been able to achieve an acceptable fit for a monomer–tetramer model to our data, and this discrepancy remains unresolved to date.

References

Borisy, G. G., & Olmsted, J. B. (1972) *Science (Washington, D.C.) 177*, 1196.

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

Durham, A. C. H., Finch, J. T., & Klug, A. (1971) *Nature (London), New Biol. 229*, 37.

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

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

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

Johnson, M. L., Correia, J. J., Yphantis, D. A., & Halvorson, H. (1981) *Biophys. J. 36*, 575.

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.

Knott, G. D. (1979) *Comp. Prog. Biomed. 10*, 271.

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

Lewis, M. S., Carmassi, F., & Chung, S. I. (1984) *Biochemistry 23*, 3874.

Nandi, P. K., Pretorius, H. T., Lippoldt, R. E., Johnson, M. L., & Edelhoch, H. (1980) *Biochemistry 19*, 5917.

Pearse, B. M. F. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1255.

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

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

Prasad, K., Lippoldt, R. E., & Edelhoch, H. (1985) *Biochemistry 24*, 6421.

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

Schmid, S. L., Matsumoto, A. K., & Rothman, J. E. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 91.

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.

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