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Molecular Characterization of Human Clathrin†

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ABSTRACT: Clathrin extracted from coated vesicles at pH 8.0 sediments as a single boundary with 8.1S sedimentation constant ($s^0_{20,w}$) of 8.1 ± 0.1 S. Sedimentation equilibrium gave a molecular weight (M_r) of 610 000 ± 30 000. The clathrin frictional ratio (pH 7.5) computed from $s^0_{20,w}$ and M_r is very large, i.e., 3.06 ± 0.18. Analysis of the circular dichroic spectrum in the far-ultraviolet showed that about half of the peptide residues are in a α-helical conformation. The molecular weight of a preparation of clathrin purified to homogeneity on a Sepharose CL-4B column in 6 M guanidine hydrochloride was 170 000 ± 26 000 by sedimentation equilibrium, which is in agreement with the values we and others obtained by sodium dodecyl sulfate gel electrophoresis. The 8.1S clathrin species may be regarded as the "native" promoter since (1) it is extracted from coated vesicles by an extremely mild procedure, (2) it is stable over considerable ranges of pH, temperature, and ionic strength, and (3) it readily polymerizes into characteristic closed lattice structures resembling those observed in coated vesicles in the electron microscope. The 8.1S clathrin molecule self-associates at pH 6.3 to form two very high molecular weight species with average sedimentation coefficients of 150 and 300 S. The sedimenting boundaries of both of these species have been analyzed to reveal their molecular heterogeneity. The two species observed by sedimentation velocity may correspond to the two sizes of coated vesicles previously reported to be present in some cells when observed by electron microscopy. Analysis of the sedimentation pattern in the ultracentrifuge also gives the amount of unreacted 8.1S clathrin from which the yield of polymerizable clathrin is obtainable. This methodology can therefore be employed to estimate the quality of the 8.1S preparation of clathrin and thereby affords an assay of its activity.

Coated pits are specialized regions of the plasma membrane that are characterized by an invagination of the cytoplasmic surface. Such regions have been recognized in many eukaryotic cells (Roth & Porter, 1964; Fawcett, 1965; Friend & Farquhar, 1967; Palade & Burns, 1968; Heuser & Reese, 1973; Pearse, 1975, 1976; Ockleford & Whyte, 1977; Goldstein et al., 1979) and have been referred to also as bristle coat areas. The bristle coat is believed to be the same coat observed in intracellular coated vesicles (Franke et al., 1976) which are thought to form by endocytosis of coated pits. Current evidence suggests numerous functions for coated pits and coated vesicles, including receptor-mediated endocytosis (Pearse, 1976; Ockleford & Whyte, 1977; Goldstein et al., 1979; Anderson et al., 1977; Gorden et al., 1978), specific exocytosis of newly synthesized protein (Ericsson, 1965; Dumont, 1969; Franke et al., 1976), and transfer of proteins (Maxfield et al., 1978; Roth et al., 1976) and hormones (Rodewald, 1973; Bradshaw, 1978; Hemmaplardh & Morgan, 1976; Anderson et al., 1978) among certain cellular organelles.

Evidence of the dynamic cellular physiology occurring at the coated pit and/or bristle coat areas has understandably led to much interest in these regions of membranes. Immunochemical and electron microscopic methods suggest the presence of clathrin in the coat of coated pits (Goldstein et al., 1979; Hemmaplardh & Morgan, 1976). Coated vesicles purified from tissue homogenates by differential centrifugation and sedimentation in sucrose gradients contain one major protein which is clathrin (Pearse, 1976) and several minor protein components (Goldstein et al., 1979; Blitz et al., 1977; Woods et al., 1978; Woodward & Roth, 1978; Keen et al., 1979).

The major protein present in the coated vesicle, and the one responsible for its coat structure, has been identified as clathrin by Pearse (1975, 1976) and verified by other investigators (Ockleford & Whyte, 1977; Blitz et al., 1977; Woods et al., 1978). Although the preparations of clathrin have been analyzed extensively by sodium dodecyl sulfate ($NaDodSO_4$)[1] gel electrophoresis, almost no description is available con-

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[1] Abbreviations used: Gdn·HCl, guanidine hydrochloride; $NaDodSO_4$, sodium dodecyl sulfate; NaMES, sodium 2-(*N*-morpholino)ethanesulfonic acid; EDTA, disodium ethylenediaminetetraacetate; Ag–Ab, antigen–antibody.

cerning the molecular, structural, or stability properties of the clathrin molecule isolated from coated vesicles. The purpose of this report is to provide this type of information so that one can proceed to understand how the properties of the clathrin protomer molecule enable it to form the coat structure of coated vesicles.

In order to determine the quality of the clathrin preparation, we have also evaluated the properties of the products formed by its polymerization, usually referred to as baskets or cages. We have shown the formation of two distinct sizes of baskets, each with its own size distribution. Moreover, the yield of baskets is readily measured. Almost nothing is now known of the yield, i.e., activity, of clathrin preparations since analysis has heretofore been performed exclusively by electron microscopy (Woodward & Roth, 1978; Keen et al., 1979; Schook et al., 1979). Elsewhere, we are describing in much greater detail information on the molecular weight and size of the two species of baskets (Nandi et al., 1980) and on the effects of pH and other variables on the rate and yield of basket formation (Van Jaarsveld et al., 1981).

Materials and Methods

Chemicals. Tris-HCl, ammonium sulfate, Gdn·HCl were ultrapure grade from Schwarz/Mann. Phenylmethanesulfonyl fluoride (PMSF) was the Boehringer Mannheim crystallized product. Merck, Sharp & Dohme was the source of D_2O of 99.7% minimum isotopic purity. Coomassie brilliant blue R-250 was electrophoresis purity grade from Bio-Rad. Na-DodSO_4 was a specially pure grade from BDH Chemicals Ltd. Dithiothreitol and dithioerythritol were from Sigma. All other chemicals were reagent grade. Distilled water which had been exposed to metal pipes was redistilled in glass. All pH measurements were made with a Radiometer Model 26 pH meter.

Dialysis Tubing. Union Carbide dialysis tubing of molecular weight cutoff about 20000 was washed and boiled several times in 1% $KHCO_3$ and stored at 4 °C in 50% ethanol. Collodion membranes (Schleicher & Schuell) of molecular weight cutoff about 25000 and about 75000 were rinsed with distilled water and with buffer prior to use.

Na-DodSO_4 Gel Electrophoresis. Polyacrylamide slab gels were run by techniques similar to those of Weber et al. (1972) or Laemmli (1970) in 5–30% preformed gradient gels or in 7.5% gels with 5% stacking gels. Cross-linked hemocyanins (*Limulus polyphemus*, from Sigma) with monomer M_r = 70000 were used as molecular weight markers.

Fluorescence and Absorbance. A Perkin Elmer Model MPF-3 fluorometer with a Hitachi QPD 33 recorder was used. Temperature was controlled at 25 ± 0.1 °C with a Lauda K-2/R water bath from Brinkmann Instruments, which was used to circulate water through the fluorometer cell holder. Fluorescence emission maxima obtained with this instrument agreed within 2–3 mm with those found with a corrected instrument, i.e. Turner 210.

Absorbance was measured on a Cary 14 spectrophotometer equipped with an expanded slide wire for optical density differences less than 0.2. According to standard neutral density filters (Gilford), the optical density readings were accurate to within 0.003. When the turbidity of samples prevented direct measurement of absorbance, the samples were clarified by dilution into concentrated (about 6 M) Gdn·HCl.

Protein Concentration. A series of protein absorbance spectra recorded in this laboratory (Edelhoch & Chen, 1980) was used to determine the average peptide absorbance for wavelengths less than 220 nm. The absorbance spectrum of 8.1S clathrin in 0.1 M NaF/10 mM sodium borate, pH 8.0, was compared with the average peptide absorbance at 215, 210, and 205 nm. The best estimate of protein concentration was taken to be the average of values from each wavelength. Measurement of the absorption at 280 and 205–215 nm gave an extinction coefficient of 10.9 for a 1% solution at 280 nm.

Computer Analysis. Sedimentation equilibrium measurements were analyzed by a nonlinear least-squares computer program, NONLIN, described elsewhere (Johnson & Yphantis, 1978). The remaining least-squares fits were accomplished with an on-line modeling program, MLAB, developed at the National Institutes of Health (Knott & Reese, 1972).

Numerical differentiation was done by dividing sets of at least 150 data points into approximately 100 overlapping intervals, each containing 5 data points. Each of these intervals was then fit (least-squares sense) to a quadratic polynomial, which was analytically differentiated.

Sedimentation. A Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanner was used with either 12- or 30-mm optical path length double-sector cells.

Sedimentation equilibrium data analysis was done by a nonlinear least-squares method (see Computer Analysis) by using the exponential form of the concentration distribution:

$$C = \sum_i C_{i0} \exp[\sigma_i(r_i^2/2 - r_0^2/2)] \quad (1)$$

where

$$\sigma_i = \frac{M_i(1 - \bar{v}\rho)\omega^2}{RT} \quad (2)$$

C is the total concentration of solutes at radius r, C_{i0} is the concentration of the ith solute at a reference radius, r_0, ω is the angular velocity of the rotor, ρ is the density of the solution, R is the ideal gas constant, T is the absolute temperature, M_i is the molecular weight of the ith solute, and $\bar{v}$ is the apparent partial speific volume.

Solvent densities were measured with an Anton-Parr densitometer, which had an instrument constant reproducible to within 1 part in 500000 with temperature controlled to within 0.05 °C. Solvent viscosities were measured with a 4.0-mL viscometer in a 30-L water bath whose temperature was maintained constant to within 0.01 °C. The usual method of correcting sedimentation coefficients to standard conditions has been used.

Sedimentation velocity experiments were analyzed according to eq 3, where r_i and s_i are the radical position and sedimentation constant, respectively, of the ith boundary at time t. The

$$r_i = r_0 \exp[s_i\omega^2(t - t_0)] \quad (3)$$

radial position used is the half-height of the sedimenting boundary, where boundary height (optical density across boundary) has been assumed proportional to protein concentration.

The distribution of sedimentation constants in sedimentation velocity experiments was calculated from the dependence of concentration upon radial position. With the assumption that diffusion is negligible, the distribution of sedimenting particles, Z_s, present at time t is given by eq 4 where the $(r/r_0)^2$ factor

$$Z_s = (r/r_0)^2(dc/ds) = (r/r_0)^2\{dc/d[\ln (r/r_0)/\omega^2(t - t_0)]\} \quad (4)$$

is simply a correction for radial dilution and t_0 is the effective starting time for sedimentation. The Z_s distributions found from several successive scanner traces (Δt = 8 min) superimposed when plotted as optical density vs. sedimentation coefficient, i.e., $\ln (r/r_0)/\omega^2(t - t_0)$. This procedure shows that the boundary spreading is due to the sedimentation of a heterogeneous sample rather than a consequence of the dif-

fusion of a homogeneous sample. In the latter case, the Z_s patterns would not superimpose, since boundary spreading due to diffusion is proportional to the square root of the time, rather than the first power of the time (Van Holde & Wolfgang, 1978).

Reduction and Alkylation. Protein was chemically reduced in 2 mM dithioerythritol solutions at room temperature, pH 8.0, in 6.0 M Gdn·HCl. After 1 h, the reduced groups and unreacted dithioerythritol were alkylated with iodoacetic acid (2.2 mM) while maintaining the pH of 8.0 (Haeberli et al., 1975). Solutions were protected from exposure to light until excess iodoacetic acid was removed by negative pressure dialysis, about an additional 2 h.

Circular Dichroism. Ultraviolet circular dichroic spectra were recorded with a Cary Model 60 spectropolarimeter equipped with a Pockel cell. The mean residue ellipticity, $[\theta]$, of the peptide groups was calculated by eq 5 where 113 is the

$$[\theta] = 113(\theta_{obsd})/10Cl \quad (5)$$

mean residue molecular weight of clathrin calculated from the amino acid analysis data (Pearse, 1976), θ_{obsd} is the observed ellipticity, C is the concentration in grams per milliliter, and l is the path length in centimeters. The CD data between 200 and 240 nm were analyzed by the equations of Chen et al. (1974) for the distribution of α-helical structure, β structure, and unordered peptide groups. The program was constrained so that the sum of these three forms equalled 100%. An additional parameter which is derived from this analysis is the mean length of the α-helical segments.

Electron Microscopy. Negative staining was done with 1% uranyl acetate. Samples used for the preparation of electron micrographs contained principally volatile or dilute (<0.1 M) salts at pH 6.3. The microscope used was a Phillips 300.

Preparation of Clathrin. Human clathrin was extracted from brain tissue obtained from two autopsies performed at the National Institutes of Health. Pathology was not described for the tissues used in this study except for slight edema. One of the patients succumbed to pancreatic adenocarcinoma and the other to complications of hypertrophic cardiomyopathy. The second patient had elevated serum triglycerides, but both patients had normal serum cholesterol. The first and second patients' ages were 67 and 49 years, respectively.

Autopsies were performed within 18 and 8 h, respectively, of the death of the first and second patients. The cerebral cortex was cut in 1-cm-thick sections in the frontal plane and taken from the autopsy room in an ice-chilled beaker. Subsequent steps were principally carried out in a cold room at 4 °C, or on ice, and consisted of a modification of the procedure of Schook et al. (1979). Clathrin was extracted from a crude coated vesicle pellet with 50 mM Tris-HCl (pH 8.0) and then isolated on a Sepharose CL-4B column as reported in detail elsewhere (Nandi et al., 1980). All the data reported refer to human clathrin except where otherwise stated. A few experiments on the pH and temperature dependence of sedimentation were performed with similarly prepared bovine clathrin since much larger amounts of this material were available.

Results

Native Clathrin. The preparation of human clathrin obtained by column chromatography with Sepharose CL-4B sediments as a single boundary with a sedimentation constant in 10 mM Tris-HCl, 0.25 M NaCl, and 3 mM NaN_3, pH 7.5, at 25 °C of 9.10 ± 0.06 S. Extrapolation to zero concentration was accomplished by least-squares fit of data obtained with three concentrations of clathrin, all of which were less than 0.1%. At these low concentrations, the measured sedimentation constants were all within 1% of the extrapolated value. When correction is made to water at 20 °C, the sedimentation constant of clathrin ($s^0_{20,w}$) becomes 8.1 ± 0.1 S. Clathrin purified from bovine brain by the same procedure gave a sedimentation pattern and rate indistinguishable from that of human clathrin. Schook et al. have reported $s^0_{20,w} = 8.76$ for a major sedimenting peak of bovine clathrin (Schook et al., 1979) which had been exposed to 2 M urea during its preparation. No further centrifugal characterization of their preparation was given.

A value of the apparent partial specific volume of human 8.1S clathrin was found by velocity sedimentation experiments as a function of solvent density in 10 mM Tris-HCl, 0.20 M KCl, 0.05 M NaCl, and 3 mM NaN_3, pH 7.5. A linear dependence of sedimentation velocity on solvent density was found for solvent compositions of 0, 50, 75, and 99% D_2O. Viscosities and densities of these solvents were measured at the temperature (25 °C) of the velocity sedimentation runs. Extrapolation of the weighted (by the uncertainty in each sedimentation rate) least-squares line to the density corresponding to zero sedimentation rate yields the reciprocal of the apparent partial specific volume, since the buoyancy term, $1 - \bar{v}\rho$, must be zero at zero sedimentation rate. The apparent partial specific volume determined in this way was 0.744 ± 0.006 mL/g.

The sedimentation equilibrium pattern of clathrin in 10 mM Tris-HCl, 1.5 mM dithioerythritol, 0.25 M NaCl, and 3 mM NaN_3, pH 7.5, at 20 °C was measured at 7200 rpm in a column height of 3.80 mm after 80 h of sedimentation. The initial clathrin concentration ($t = 0$) was 0.16 mg/mL. The data were analyzed between 0 and 0.53 mg/mL which occurred at distances of 0.13 and 3.70 mm from the meniscus in the cell.[2] The sedimentation pattern was consistent with the presence of a single molecular species with a molecular weight of 610000 ± 30000. By use of the above value of the apparent partial specific volume, i.e., 0.744 ± 0.006 mL/g, the frictional ratio of 8.1S clathrin was calculated to be 3.06 ± 0.18.

The CD spectrum of 8.1S human clathrin at pH 7.5 was measured since nothing is known of the conformation of the polypeptide backbone. The double minima characteristic of an α-helical structure are observed. Computer analysis gave the distribution of peptide residues as 49% α-helical structure, 17% β structure, and 34% random structure (Figure 1), and an average length of the α-helical segments of 12 residues. Thus, two-thirds of the peptide groups appear to be in α-helical or β conformations.

The CD spectrum of bovine clathrin in the near-ultraviolet shows two major peaks at 292 and 285 nm with molecular ellipticity values of 2.0×10^5 and 1.8×10^5 deg·cm^2/dmol and, in addition, several minor peaks in the 270–280-nm region. The two major peaks belong to tryptophanyl and tyrosyl residues, respectively, while the ones at lower wavelengths are presumably due to phenylalanine (Edelhoch & Chen, 1980).

The 8.1S human clathrin appears to be stable in dilute alkali since there is little pH dependence of the far-ultraviolet CD spectrum, which shows no change between pH 7.5 and 9.0 and

[2] A sedimentation equilibrium experiment of bovine clathrin (0.10 mg/mL) in a solution of much higher ionic strength (0.25 M phosphate, Na, 3 mM NaN_3, and 0.25 M Tris-HCl, pH 6.24) sedimented at 4400 rpm for 160 h gave a molecular weight of 610000 ± 6000. Increasing the rpm to 7200 for 80 h gave a molecular weight of 590000 ± 12000. No polymerization of clathrin occurred at pH 6.24 because of the high ionic strength. The column height was the same as that for the human clathrin experiment.

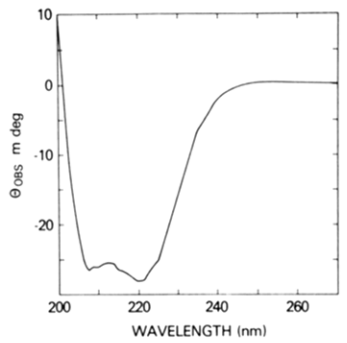

FIGURE 1: Circular dichroism spectrum of clathrin. A 1-mm cell was used. Protein concentration = 0.21 mg/mL. Computer regression analysis predicted 49 ± 1% α helix, 17 ± 2% β structure, and 33 ± 2% random structure. The ordinate gives the observed ellipticity. The minimum value of the mean residue ellipticity, $[\theta]$, is at 220 nm and equal to 15 500 units. The solution contained 0.1 M sodium fluoride and 0.01 M sodium borate, pH 7.5.

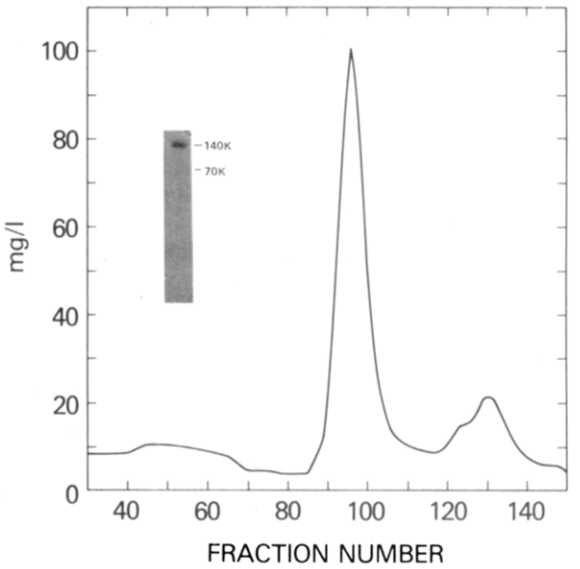

FIGURE 2: Clathrin isolation on Sepharose CL-4B in 6 M Gdn·HCl. Column buffer was 6 M Gdn·HCl and 0.05 M Tris-HCl, pH 8.0. The sample consisted of clathrin isolated from a Sepharose CL-4B column which was concentrated by precipitation with 50% saturated ammonium sulfate, reduced by dithioerythritol, and alkylated by iodoacetic acid. Fractions were 3.5 mL each, and the flow rate was 20 mL/h. The ordinate gives the clathrin concentration in mg/L which would produce the fluorescence signal observed at 360 nm, excited at 295 nm. The main peak is clathrin by $NaDodSO_4$ gel electrophoresis. Inset: $NaDodSO_4$ gel electrophoresis of isolated clathrin. The peak fraction from the major peak was dialyzed against urea to remove Gdn·HCl and then run on a 5–30% gradient slab polyacrylamide gel in the Weber and Osborne buffer system (Nandi et al., 1980). A single band was observed which comigrated with clathrin. The positions of hemocyanin monomer (70 000) and dimer (140 000) are indicated.

only about a 10% decrement in ellipticity at 220 nm between pH 9.0 and 10.0. The sedimentation coefficient ($s_{20,w}$) of bovine clathrin was independent of pH between pHs 6.0 and 9.0 in 0.25 or 0.50 M NaCl and Tris–glycine buffers and of temperature between 5 and 35 °C at pH 7.5 in 10 mM Tris-HCl, 0.25 M NaCl, and 3 mM NaN_3.

The fluorescence emission spectrum of 8.1S human clathrin has a peak at 335 nm in water at neutral pH and at 350 nm in 6 M Gdn·HCl. The blue shift in water, relative to the spectrum in 6 M Gdn·HCl, suggests that there is considerable shielding of tryptophanyl residues from the solvent due to tertiary structure. In 6 M Gdn·HCl, a distinctive peak is observed at 305 nm in the spectrum which represents emission from tyrosyl groups. In water, tyrosyl emission is obscured by the stronger emission of the tryptophanyl residues. In accord with these results, the CD spectrum of human clathrin in 6 M Gdn·HCl does not show the double minima characteristic of α-helical structure and is similar to that of other proteins unfolded in this strongly denaturing solvent.

As judged by circular dichroism and fluorescence, little or no secondary or tertiary structure survives in 6 M Gdn·HCl. We can assume, therefore, that only the structureless monomeric chains of clathrin survive in this denaturing solvent. Consequently clathrin was purified on a Sepharose CL-4B column containing 6 M Gdn·HCl (Figure 2). Clathrin was first reduced and alkylated in this solvent to be sure that no cross-links survived to connect smaller chains to each other (see Materials and Methods). Protein from the leading peak of the column (fraction 95, Figure 2) was electrophoresed on $NaDodSO_4$ gels and gave a single band which comigrated with clathrin. This material was dialyzed against 6.5 M Gdn·HCl/10 mM Tris-HCl, pH 8.0, which was prepared both in water and in D_2O. Dialysis was continued for 1 week at room temperature in solutions protected from light. Both protein solutions were then simultaneously sedimented to equilibrium at 25 °C and 18 000 rpm. From the sedimentation equilibrium patterns in H_2O and in D_2O and the measured solvent densities, the apparent partial specific volume, 0.722 ± 0.016 mL/g, and the molecular weight, 170 000 ± 26 000, of the clathrin monomer were calculated. The molecular weight obtained by this thermodynamic method is in agreement with values reported by other investigators obtained by $NaDodSO_4$ electrophoresis (Pearse, 1975; Ockleford & Whyte, 1977; Woods et al., 1978). The partial specific volume is somewhat less than that found in water solutions, but it is known that the apparent partial specific volumes of most proteins decrease slightly in 6 M Gdn·HCl (Lee & Timasheff, 1974).

Formation and Heterogeneity of the 150S and 300S Polymers of Clathrin. The 8.1S preparation of clathrin was readily polymerized to the typical structure that these molecules possess when they form the coat of coated vesicles (Woodward & Roth, 1978; Keen et al., 1979; Schook et al., 1979). Polymers of clathrin were found in sedimentation velocity experiments after dialysis of 8.1S clathrin particles from pH 7.5–8.0 to pH 6.3–6.8 into either 0.10 M NaMES/8 mM $MgCl_2$/3.0 mM NaN_3 or 0.20 M ammonium acetate/3.0 mM NaN_3 containing several millimolar concentrations of calcium or magnesium chloride.

Two principal polymeric species, representing about two-thirds of the protein (Figure 3A), were found with average sedimentation rates near 150 and 300 S. These two sedimenting boundaries were analyzed for the heterogeneity of the sedimentation constant (s) from the radial dependence of concentration on s, i.e., Z_s (see Materials and Methods). This analysis assumes that there is no diffusion of the various components during sedimentation. The curve of protein concentration (i.e., OD) vs. s is shown in Figure 3A, and the distribution of molecular sizes derived from it appears in Figure 3B.

If the polymerized clathrin were not heterogeneous, then differentiation of the original curve of OD vs. radius (r) would provide a direct measure of the diffusion constant of the protein in each sedimenting boundary. The differentiated curve with respect to r is very similar in appearance to that with respect to s and is not shown. Apparent diffusion constants of 4.7×10^{-7} cm^2/s (for the 150S boundary) and 11×10^{-7} cm^2/s (for the 300S boundary) were calculated. When the distances between the half-heights of the differentiated curve (with respect to r) of 8.1S clathrin were analyzed in the same

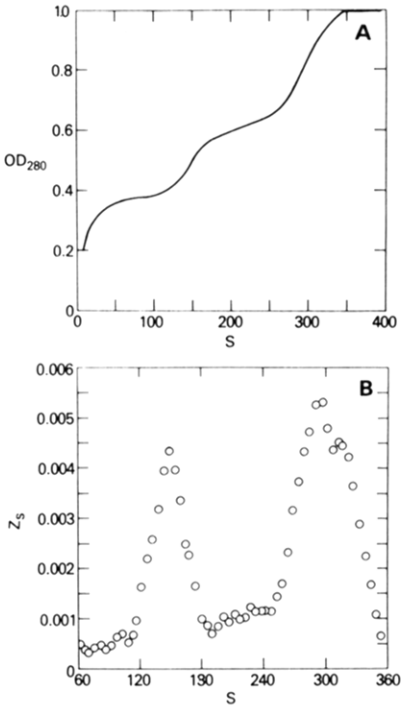

FIGURE 3: Molecular heterogeneity of clathrin latticelike coat. (A) Plot of the observed optical density against the sedimentation rate(s) of clathrin calculated from eq 3. Solution contained 0.1 M MES, 0.01 M $MgCl_2$, and 3 mM azide, pH 6.32. (B) Distribution of sedimentation rates calculated according to eq 4.

manner, an apparent diffusion constant of 1.9×10^{-7} cm^2/s was calculated. This value is only about 50% larger than the value of the diffusion constant calculated for 8.1S clathrin from the sedimentation equilibrium molecular weight. The values of the apparent diffusion constants obtained for 150 and 300S clathrin are thus greater than that for 8.1S clathrin, and comparable to the diffusion constants found for small protein molecules, such as ceruloplasmin ($s_{20,w} = 7.5$) and lysozyme ($s_{20,w} = 1.9$), respectively. Since the method gives a reasonable value for 8.1S clathrin and very unreasonable values for the 150S and 300S species, we conclude that the large apparent diffusion constant values found for these two species indicate that the observed boundary spreading is almost completely due to molecular heterogeneity. The plot of Z_s (Figure 3B), therefore, should be approximately representative of the heterogeneity of the molecular species present in the 150S and 300S boundaries.

Electron Microscopy. A minimal concentration of about 0.2 mg/mL clathrin was required for the formation of cagelike structures similar to those found with intact coated vesicles (Figure 4). These structures were observed only in samples whose concentrations were sufficient to allow identification of the 150S and 300S boundaries. The structures appeared to be porous with pentagonal and hexagonal surfaces similar to those observed with coated vesicles. The particle size range was large and similar to that found by others (Friend & Farquhar, 1967; Pearse, 1975, 1976; Woods et al., 1978; Schook et al., 1979; Crowther et al., 1976). Some aggregates of individual particles were also observed.

Discussion

We have prepared clathrin from coated vesicles and possibly from bristle coat areas of membrane fragments by extracting in slightly alkaline buffers (pH 8.0) of low (less than 50 $\Gamma/2$) ionic strength. Other workers have used cholate (Pearse, 1976) or 0.5 M Tris-HCl, pH 7 (Keen et al., 1979), to extract

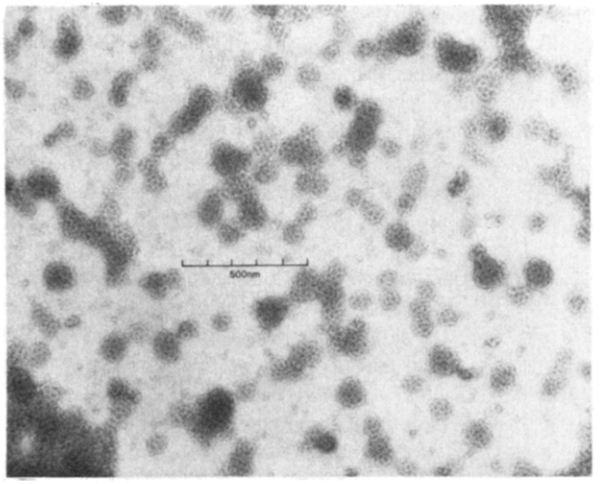

FIGURE 4: Electron micrograph of clathrin latticelike coat. Clathrin was polymerized at pH 6.8 by dialysis into 0.20 M ammonium acetate, 3 mM NaN_3, and 0.5 mM $CaCl_2$. The particles formed are similar to coated vesicles but lack the lipid bilayer. Particle diameters are in the range of 30–150 nm. The total length of the bar shown in the figure is 500 nm.

clathrin from coated vesicles. The ease of extraction with pH and the inability to extract clathrin with the nonionic detergent Tween (Woodward & Roth, 1978; Keen et al., 1979) suggest that clathrin is not imbedded in the membrane and may be held in place primarily by electrostatic interactions.

Almost no molecular characterization is available of the clathrin promoter. The 8.1S molecule can be considered as the primary protomer since (1) it is extracted from clathrin vesicles by extremely mild procedures, (2) it is conformationally stable to variations in pH, ionic strength, and temperature near physiological values, and (3) it readily polymerizes to baskets near physiological pH values.

There have been only a limited number of studies on the reassembly of clathrin into structures resembling the coat of coated vesicles (24–26) (Woodward & Roth, 1978; Keen et al., 1979; Schook et al., 1979). These studies used the latticelike appearance of coated vesicles in the electron microscope to document the formation of the coat or basket structures. No attempt was made to characterize further the properties of these latticelike structures which lack the interior membrane of coated vesicles.

We have shown that these reassembled molecules sediment as two distinct boundaries with rates near 150 and 300 S. Each boundary has been shown to consist of a distribution of sedimenting species. We report elsewhere that the conversion of clathrin to baskets is pH dependent between 6.0 and 6.7 and exceeds 90% at pH 6.10 (Van Jaarsveld et al., 1981). In this paper, we found that the yield of baskets is near two-thirds at pH 6.3 (Figure 3A). Thus, ultracentrifugal characterization of clathrin polymerization affords a means for the first time of determining the quality of a clathrin preparation in terms of its ability to polymerize to specific, defined structures. Thus, it would not be necessary, except as a control, to run routine electron micrographs of reassembled baskets. This type of analysis constitutes an evaluation of one of its functional properties and may be a better indicator of activity than analytical $NaDodSO_4$ gel electrophoresis since the latter only documents the relative amounts of clathrin subunits ($M_r \simeq$ 175 000) present.

There is some uncertainty in the molecular composition of the 8.1S particle. Its molecular weight by sedimentation equilibrium is 610 000 ± 30 000. This could imply that the extracted form of clathrin is either a trimer or a tetramer of

the clathrin monomer, for which we found M_r = 170000 ± 26000. Another possibility that could explain the nonintegral value of the number of monomers in the 8.1S species is that minor amounts of proteins other than clathrin may be present. From the appearance of coated vesicles in electron micrographs, there is reason to suspect a trimer since the vertex of each polygonal face of the latticelike surface is an intersection of three dense-staining (positive strains) structures. Alternatively, a fourth monomer may reach up or down from the trigonal vertex on the surface. Cross sections of coated vesicles appear to have a star-shaped protein coat, which would be in harmony with the latter interpretation.

Clathrin isolated from coated vesicles is a molecular with a M_r of 610000. The very high frictional ratio, i.e., 3.06, found for clathrin indicates that the molecular form of this molecule deviates strongly from that of a compact sphere. A random-coil polypeptide chain could have a very high frictional ratio, but this type of structure is excluded since about two-thirds of the peptide groups are in rigid, organized structures, i.e., α helical and β. Moreover, considerable tertiary structure must also exist in clathrin since many of the aromatic groups are not exposed to the solvent. The types of organized structures which have very high frictional ratios are either very rigid, elongated molecules, such as myosin or tropomyosin (Rainford & Rice, 1970), or molecules with rigid domains connected by flexible segments of polypeptide chains, such as fibrinogen (Johnson & Mihalyi, 1965) and immunoglobulins (Green, 1969; Yguerabide et al., 1970; Metzger, 1970). The latter type of structure seems to be a more likely possibility since the elongated rigid molecules are usually highly helical although myosin has only about two-thirds of its residues in α-helical structures. Other types of structures which are combinations of the above two can also be envisioned as fitting the above constraints. However, considering the basketlike structures that are formed by the self-association of clathrin, it seems more likely that clathrin consists of several globular (perhaps α-helical) domains attached by flexible polypeptide sequences to a common locus. The flexibility could explain the two sizes of the polymeric species seen by electron microscopy (Crowther et al., 1976) that are formed from clathrin by self-association. The flexibility of the immunoglobulins allows for different size ring structures in the Ag–Ab complexes (Metzger, 1970).

The size of empty (membrane-free) coats present in preparations of coated vesicles, as well as that of clathrin polymers reconstituted from "native" clathrin (8.1 S), is very similar to that of intact coated vesicles (Schook et al., 1979; Woodward & Roth, 1978). The structural similarity of the clathrin polymers and the isolated coated vesicles suggests that clathrin, the major component of the protein coat, plays an important role in determining the structure of the coated vesicles. Our data do not permit unequivocal correlation of the sedimenting boundaries with the structures seen in electron micrographs, although there is considerable evidence of two discrete size ranges of coated vesicles (Friend & Farquhar, 1967; Crowther et al., 1979). The reported sedimentation constant of 220 S for coated vesicles (Pearse, 1975) is consistent with the 300S value for the polymerized clathrin since the density of the lipid parts of the membrane is much less than that of the protein coat. This difference in density would reduce the sedimentation rate.

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