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Stability and Structure of Clathrin†

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ABSTRACT: The effects of urea on the dissociation and structural transitions of clathrin (8 S) have been evaluated by various techniques. The dissociation of the light chains in 3 M urea has been shown by light scattering, ultracentrifugation, and column chromatography. The dissociated components still retain the capacity to form the characteristic polygonal structure of the coat after removal of the urea. At higher concentrations of urea, the secondary and tertiary structures are eliminated, as documented by various spectroscopic techniques, i.e., tryptophan polarization and emission maxima, circular dichroism, and difference spectra. Two distinct transitions are observed by all techniques, one between 3 and 6 M urea and a second one which starts at 7 M but is still incomplete by 9.6 M urea. A concentration-dependent aggregation of clathrin chains occurs in 4 and 5 M urea solutions, as observed by light scattering and sedimentation. The results indicate that there are two large, independent domains in clathrin heavy chains and that each domain may have a single, highly cooperative transition.

Clathrin (8 S, $M_r \simeq 630\,000$) is the principal protein of the coat structure in both coated pits and coated vesicles (Pearse, 1975, 1976; Ungewickell & Branton, 1981; Pretorius et al., 1981). Clathrin dissociates from coated vesicles at pH values above ~7.5 (Pearse, 1978; Schook et al., 1979; Keen et al., 1979; Woodward & Roth, 1978, 1979). It is stable in this form to moderate changes in pH, ionic strength, and temperature (Pretorius et al., 1981). Clathrin can recombine with uncoated vesicles to form coated vesicles (Nandi et al., 1982a) or self-associate to form empty coats when the pH is reduced below ~7.0 (Pearse, 1978; Schook et al., 1979; Keen et al., 1979; Woodward & Roth, 1978, 1979; Nandi et al., 1980; Van Jaarsveld et al., 1981; Crowther & Pearse, 1981). The self-association is inhibited by salt (>0.2 M) whereas the reassociation to coated vesicles is not particularly sensitive to salt concentration (Nandi et al., 1982a).

Various aspects of the structure of native clathrin have been elucidated by electrophoresis in sodium dodecyl sulfate (SDS)[1] gels, electron microscopy, circular dichroism, and hydrodynamic methods. Purified preparations of clathrin contain essentially two distinctive species, i.e., three so-called heavy (M_r ~180 000) and three light (M_r 33 000) chains (Kirchhausen & Harrison, 1981; Ungewickell & Branton, 1981; Kirschhausen et al., 1983; Lisanti et al., 1982). The molecule has a symmetric structure, a triskelion, with three long arms (~450 Å) which have a bend in the middle (160–190 Å from the vertex) and a kink at their ends (Crowther & Pearse, 1981; Kirschhausen et al., 1983). Electron microscopic examination of the binding of antibodies to light chains or of ferritin-conjugated avidin to biotin-labeled light chains suggests that the three light chains are bound to the three heavy chains close to the vertex of the triskelion (Ungewickell et al., 1981; Kirschhausen et al., 1983). The light chains have been isolated by various methods, usually involving denaturation of the heavy chains or of both types of chains (Ungewickell et al., 1982; Lisanti et al., 1982). We do not know what role the light chains serve although clathrin cages can be re-formed without them from clathrin which has been digested with trypsin when in the form of cages (Schmid et al., 1982). Although the composition and structure of native clathrin have received considerable attention, the association of clathrin monomers to form triskelions and its interaction with light chains are awaiting investigation.

We have studied the effects of increasing concentrations of urea on clathrin in order to dissociate the subunits and to evaluate the structural transitions in the heavy chains of clathrin. The latter should afford information on the organization of the polypeptide chains in terms of its domains. Covalently bound substructures which fold independently are referred to as domains.

Materials and Methods

2-(*N*-Morpholino)ethanesulfonic acid (MES) and ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were from Sigma Chemicals. Analytical-grade urea

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[1] Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; Gdn·HCl, guanidine hydrochloride (GdmCl in figures); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

and Tris were obtained from Bethesda Research Laboratory. All other reagents used were Fisher analytical grade. Glass-distilled water was used for all experiments.

Preparation of Clathrin. Coated vesicles were isolated by the procedure of Nandi et al. (1982b) at pH 6.5 containing 0.1 M MES, 1 mM EGTA, 0.5 mM $MgCl_2$, and 3 mM NaN_3. The coated vesicle suspension was dialyzed with three changes of 10 mM Tris–3 mM NaN_3, pH 8, buffer for 12 h in the cold and then centrifuged at 150000*g* for 1 h. The supernatant which contains native clathrin (Irace et al., 1982) was used for further experiments.

Fluorescence. A Perkin-Elmer Model MPF-3 fluorometer with a Hitachi QPD 33 recorder was used. Temperature was controlled at 23 °C with a Lauda K-2/R water bath from Brinkmann Instruments, which was used to circulate water through the fluorometer cell holder.

Polarization. The polarization was measured in an SLM instrument, 4000 Series (SLM Instrument Co., Urbana, IL). This instrument contained two emission grating monochromators at 90° and 270° to the incident beam. The same solutions used for fluorescence measurements were used for polarization experiments. The excitation and emission wavelengths were 290 and 340 nm, respectively. The temperature of the sample was controlled as described above. The value of tryptophan polarization was calculated by using the equation used previously (Gwynne et al., 1975).

Difference Spectra. The difference spectra were measured on a Cary Model 14 spectrophotometer equipped with an expanded slide wire for optical densities less than 0.2. Kinetics of the difference absorption were measured in a Cary 219 spectrophotometer using a 0.02 absorbance unit scale.

Light Scattering. Light scattering was measured in a square cuvette at 90° by using the perkin-Elmer MPF-3 fluorometer.

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

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, $Z(s_w)$, present at time t is given by eq 1 where the $(r/r_0)^2$ factor

$$Z(s_w) = (r/r_0)^2(dc/ds) = (r/r_0)^2\{dc/\{d\{\ln (r/r_0)/[\omega^2(t - t_0)]\}\}\} \quad (1)$$

is simply a correction for radial dilution and t_0 is the effective starting time for sedimentation (Pretorius et al., 1981). The sedimentation data in urea have been corrected to that of water (s_w) by

$$S_w = \frac{s_u\eta_u(1 - \nu\rho_w)}{\eta_w(1 - \nu\rho_u)}$$

where η is the viscosity, ν is the partial specific volume, ρ is the density, u is urea, and w is water.

Protein Concentration. Clathrin concentration was determined by absorption using $E^{1\%,1cm}_{280nm} = 10.9$ (Nandi et al., 1980). Absorbance was measured with either a Cary 14 or a Beckman DU spectrophotometer.

Column Chromatography. The separation of protein components in 3 M urea solutions was carried out in a 1.5 × 196 cm (~350-mL bed volume) Sephacryl S-300 column. A flow rate of 9 mL/h was used.

Circular Dichroism. Dichroic spectra were recorded with a Jasco J 500C spectropolarimeter.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate gel electrophoresis was carried out as reported earlier (Nandi et al., 1980) using 5–15% gradient gels.

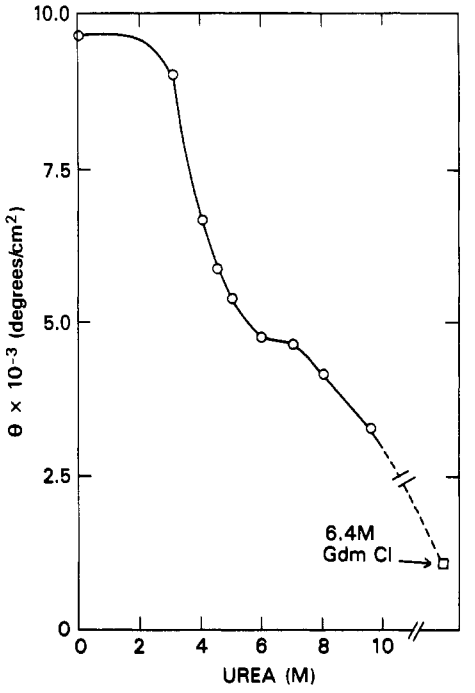

FIGURE 1: Effect of urea concentration on the ellipticity at 220 nm of clathrin. Solutions were measured only after 36 h. One solution was in 6.4 M Gdn·HCl. Solutions were at pH 8.0 in 0.02 M Tris–0.008 M fluoride, T = 23 °C. Protein concentration was 0.10 mg/mL.

Results

Secondary Structure. The far-UV circular dichroic spectrum of native clathrin revealed the double minima at 220 and 208 nm characteristic of the α-helix. Analysis of the spectrum indicated that about 50% of the residues were in α-helical structures, 17% in the β structure, and the remaining residues in an unorganized peptide group (Pretorius et al., 1981). We have used the minimum at 220 nm to follow the loss in secondary structure.

The effect of urea concentration on the ellipticity of clathrin at the first minimum, i.e., 220 nm, is shown in Figure 1. Since significant time effects were observed by other methods at intermediate urea concentrations, ellipticities were measured after 36 h in order to obtain equilibrium values. Only about two-thirds of the ellipticity at 220 nm was eliminated by 9.6 M urea. In 6 M Gdn·HCl, however, the ellipticity was reduced almost to zero (Figure 1). It is known that most proteins lose essentially all of their secondary (and tertiary) structure in 6 M Gdn·HCl (Tanford et al., 1967). In a separate series of experiments, no difference was observed in the CD spectra between water and 2 M urea when measurements were made 2–3 h after urea addition.

It is clear from the curve in Figure 1 that there is more than one transition in clathrin. The first transition occurs between ~3 and 6 M urea. A second transition begins at 7 M urea which, however, is not complete at the highest urea concentration used, i.e., 9.6 M. Therefore, the upper limit of the second transition occurs at higher concentations of urea.

Tertiary Structure. We have used the fluorescence properties of the Trp residue and the absorption of the Trp and Tyr residues to follow the loss in tertiary structure of native clathrin. Since the aromatic groups of these two residues are among the least polar side chains, and tend to be located in the interior of the protein, they can serve as an indicator of tertiary interactions.

(*A*) *Fluorescence.* Emission spectra were recorded during the first 2–3 h after solutions were prepared and again after 24 h. We have plotted the variation in emission maxima

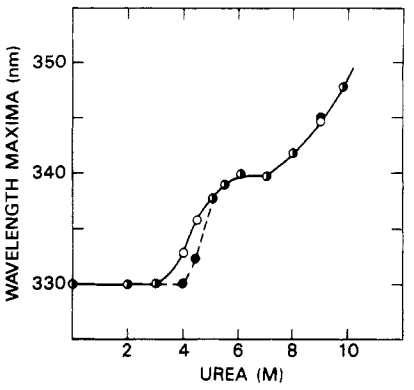

FIGURE 2: Effect of urea concentration on the wavelength of the emission peak of clathrin. The points shown by closed and open circles were measured 2–3 and 24 h, respectively, after addition of urea. Solutions were at pH 8.0 in 0.01 M Tris. Protein concentration was 0.010 mg/mL. T = 23 °C.

against the urea concentration in Figure 2. This parameter should afford a sensitive measure of the extent of structural disorganization since its value depends on the polarity of the environment of the Trp residues.

No change in the wavelength maximum was observed between 0 and 3 M urea. A complete transition was observed between 3 and 6 M urea (after 24 h) in which the peak shifted from 330 to 340 nm. A plateau region in the wavelength maxima curve between 6 and 7 M urea demarcates the first from a second transition. The latter occurs above 7 M urea but is incomplete by 9.6 M urea. No time effects were found in the emission maxima values either between 0 and 3 M urea or between 5 and 9.6 M urea when measurements were made at 2–3 and 24 h, respectively, after urea was added to the aqueous protein solution. Important changes were observed with time at 4 and 4.5 M urea (Figure 2).

The emission intensity values also displayed a biphasic curve above 3 M urea which closely resembled that for the wavelength maxima. Time effects were observed also in 4 and 4.5 M urea solutions. The changes in intensity, however, were relatively small, i.e., about an increase of 20% in each transition.

(*B*) *Polarization.* The polarization of Trp fluorescence depends on the relaxation behavior of clathrin and consequently on properties very different from those responsible for the emission intensities. Measurements were made at 2–3 and 24 h after solutions were prepared. The only significant effect of time was found in the 4 M solution. Two distinct transitions are seen also in the polarization vs. urea concentration curve (Figure 3) although they are not separated by a plateau region as seen in the wavelength maxima curves of Figure 2. Nevertheless, they clearly correspond to the two transitions observed by the fluorescence maxima. In accord with the fluorescence data, the second transition is incomplete by 9.6 M urea.

The magnitude of the decrease in polarization in each transition is clearly too large to represent dissociation of clathrin subunits (or of light chains) without significant unfolding taking place, i.e., an increase in rotational freedom of the polypeptide chains. A monomer of clathrin with the same structure it possesses in the triskelion should have almost the same polarization value since its relaxation time would still be very large compared with the lifetime of the excited state, i.e., 1–4 ns, of Trp residues. Similarly, a decrease in molecular weight from 630000 to 530000 resulting from the loss of light chains should have almost no effect on the polarization unless the light chains strongly modify the folding of clathrin chains

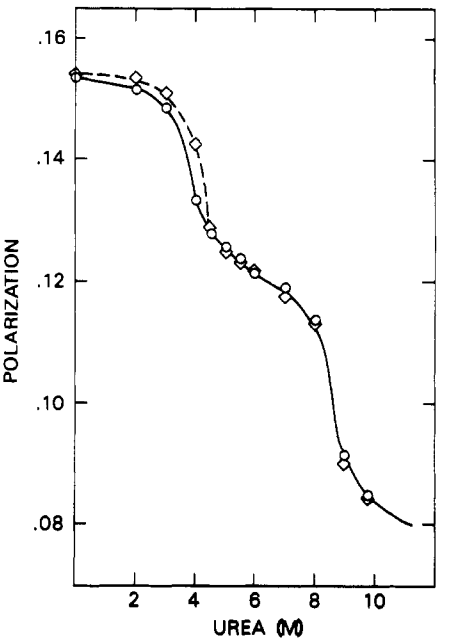

FIGURE 3: Effects of urea concentration on the polarization of clathrin tryptophan fluorescence. The points shown by (◊) and (O) were measured 2–3 and 24 h, respectively, after addition of urea. Solutions were at pH 8.0 in 0.01 M Tris. Protein concentration was 0.010 mg/mL. T = 23 °C.

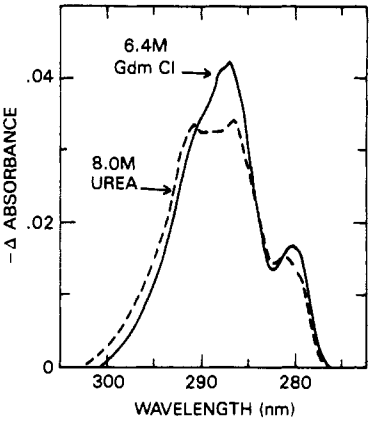

FIGURE 4: Effect of 8.0 M urea and 6.4 M Gdn·HCl on the difference absorbance spectra of clathrin at pH 8.0 in 0.01 M Tris. Protein concentration was 0.46 mg/mL. T = 23 °C.

or represent a much higher percentage of the total emission intensity than is represented by its relative mass.

(*C*) *Difference Absorption.* We have also evaluated the absorption properties of clathrin since this parameter reflects the behavior of the ground state rather than that of the excited state of the Trp (or of Tyr) residues. Moreover, all the Trp residues have similar extinction coefficients whereas their quantum yields can vary almost from 0 to 1.

The difference spectra of clathrin in 8 M urea and in 6.4 M Gdn·HCl, when measured against aqueous solutions, are shown in Figure 4. The negative difference peak at 291 nm in 8.0 M urea is characteristic of the indole chromophore while those at 280 and 287 nm, seen in both denaturants, are representative of the phenolic group (Donovan, 1969). The negative difference spectra in 8 M urea and 6.4 M Gdn·HCl closely resemble each other except for a small additional absorption in the phenol region in 6.4 M Gdn·HCl. Evidently there is a region or regions of clathrin which contain Tyr but not Trp residues which are refractory to denaturation by 8 M urea but not by 6.4 M Gdn·HCl.

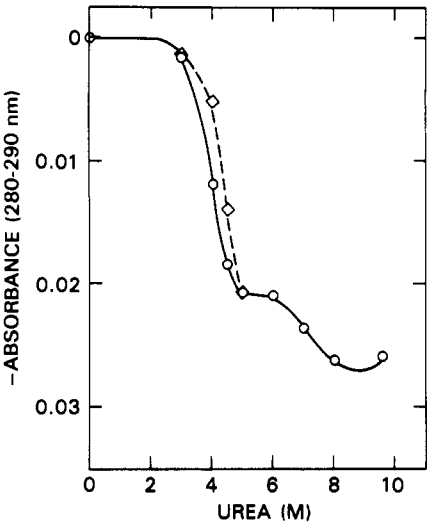

FIGURE 5: Effect of urea concentration on the difference absorbance (280–290 nm) of clathrin. The points shown by (◊) and (O) were measured 2–3 and 24 h, respectively, after addition of urea. Solutions were at pH 8.0 in 0.01 M Tris. Protein concentration was 0.50 mg/mL. T = 23 °C.

The dependence of the difference absorption between the 280- and 290-nm peaks on urea concentration is shown in Figure 5. Two distinct transitions occur between ~3 and 5 M urea and between 6 and 9 M urea. Measurements at early (2–3 h) and late (24 h) times were made for solutions between 3 and 5 M urea. Significant time-dependent changes were observed only at 4 and 4.5 M urea.

Quaternary Structure. (A) Light Scattering. None of the above methods depends on mass and therefore does not distinguish between subunit dissociation and polypeptide unfolding. We have obtained, therefore, light scattering and velocity ultracentrifugation data in order to provide some informaton on the degree of clathrin dissociation. The light-scattering intensity depends on the weight-average molecular weight and therefore can be used to follow reactions involving a change in mass. It has distinct disadvantages in mixed solvent systems, however, in that the function on which the molecular weight depends, dn/dc, is not necessarily the value one reads in a refractometer since this assumes that there is no preferential interaction between urea and water for the solute (Cassasa & Eisenberg, 1964). We have circumvented this effect by making many measurements at early times and extrapolating to zero time. Since solvent interactions with the solute which modify dn/dc are instantaneous, the extrapolating values represent the light scatter in the mixed solvent before dissociation. Fortunately, the time effects were sufficiently slow at 4 M urea and below to permit precise extrapolation. A linear negative dependence of the scatter (Ir^2/I_0) on urea concentration was observed between 0 and 4 M urea concentration at time zero (Figure 6).

The rate of decreases in light scatter between 0 and 4 M urea is shown for the first 10 min and after 24 h in Figure 7. In Figure 6A, the scattering intensity values observed after 10 min and 24 h are illustrated as well as the values obtained by extrapolating to zero time. The percent decrease in scatter after 10 min and 24 h is also plotted in Figure 6B. The extent of dissociation is similar at 10 min in 3 and 4 M urea but is much greater in 4 M urea after 24 h.

When clathrin was sedimented in 4 and 5 M urea solutions, the sedimenting boundary became broader than that observed in 3 M urea, and some species sedimented at rates faster than that of native clathrin, i.e., 8 S (see the next section). Since unfolded (or dissociated) protein molecules normally sediment

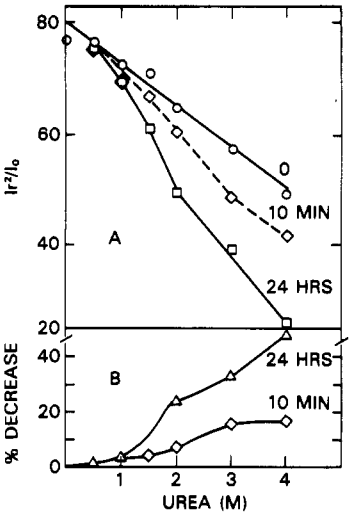

FIGURE 6: (A) Effect of urea concentration on the decrease in light scatter after 0 min, 10 min, and 24 h. Original data are in Figure 7. (B) Percentage decrease in light scatter of data in Figure 6A after 10 min and 24 h.

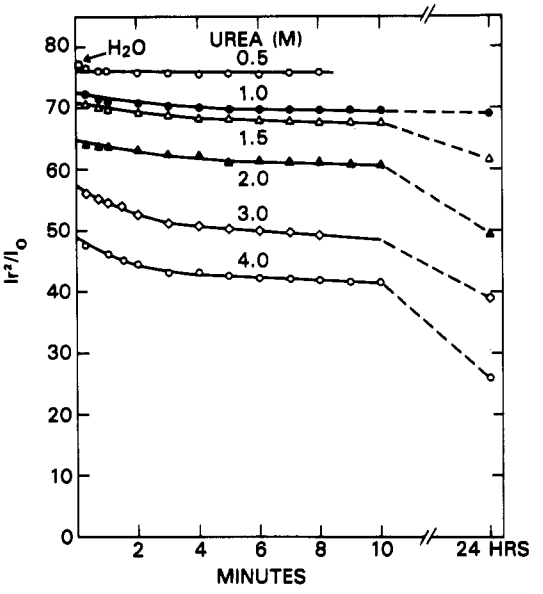

FIGURE 7: Kinetics of the decrease in light scatter of clathrin solutions as a function of urea concentation between 0 and 4.0 M urea. Scatter was measured continuously for about 10 min after addition of protein and then after 24 h. Solutions were at pH 8.0 in 0.01 M Tris. Protein concentration was 0.140 mg/mL. T = 23 °C.

slower than their native forms, we suspected that the unfolded molecules were self-associating to form faster sedimenting species. We therefore attempted to show the self-association by light scattering by following the reaction closely for 3 h and then after 18 and 21 h. We compared 3 M with 4 and 5 M urea solutions, since 3 M solutions did not show faster sedimenting species. The light scatter in 3 M urea, at about the same clathrin concentration as in the velocity ultracentrifugation experiments, continued to decrease for the entire time observed, i.e., 21 h, although almost all of the decrease occurred by 3 h (Figure 8). The light scattering from 4 M urea solutions decreased for about 2 h and then increased slowly for 18 h, whereas that from 5 M urea solutions began to increase 2–3 min after an initial fall. To document further that the reaction was due to self-association of unfolded species, the light scatter was observed from a 0.2 mg/mL instead of a 0.4 mg/mL solution of clathrin in 4 M urea. At the lower concentration, the increase in light scatter observed after 2 h at the higher concentration was not observed. The scatter

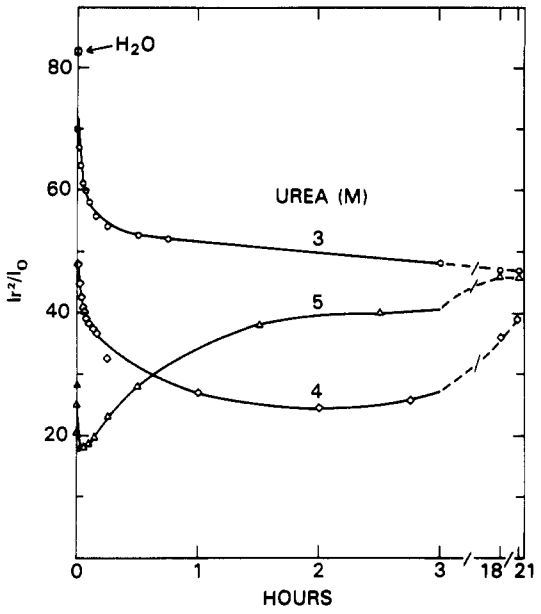

FIGURE 8: Kinetics of the change in light scatter of clathrin solutions in 3, 4, and 5 urea solutions. Scatter was measured continuously for about 30 min, then intermittently for about 3 h, and then after 18 and 21 h. Solutions were at pH 8.0 in 0.01 M Tris. Protein concentration was 0.36 mg/mL. T = 23 °C.

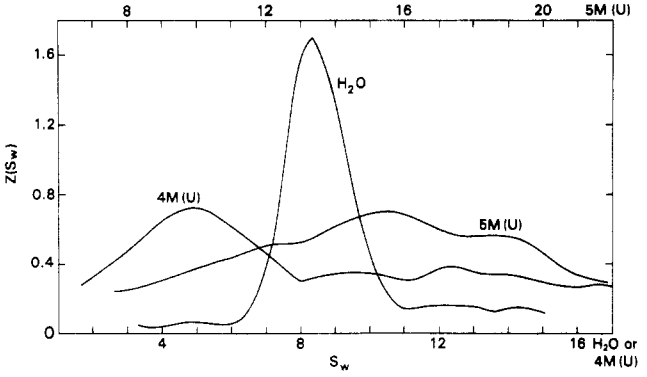

FIGURE 9: Distribution of sedimentation constants, $Z(s_w)$, as a function of the sedimentation constant of clathrin in H_2O, 4 M urea, and 5 M urea. All solutions were at pH 8.0 in 0.01 M Tris. T = 23 °C. The values of $Z(s_w)$ were obtained by applying eq 1 to the data obtained by the scanner. Protein concentration was 0.40 mg/mL.

remained constant between 2 and 18 h.

(*B*) *Ultracentrifugation.* In order to observe the change in molecular weight in urea solutions, we have made velocity ultracentrifuge and gel chromatography experiments. The sedimentation patterns of clathrin in 3 M urea after 40 min were almost superimposable on those of clathrin in the absence of urea after 24 min except for a slower sedimenting boundary representing about 25% of the total asorption. The faster sedimenting boundary had the same sedimentation coefficient ($s_{20,w}$) as that of native clathrin, i.e., 8.3 S (Figure 9).

Sedimentation of clathrin in 4 and 5 M urea solutions gave more complex patterns. We have analyzed these patterns with eq 1 in order to obtain a better description of the number and/or size distribution of the sedimenting species since the molecular unfolding occurring in 4 and 5 M urea solutions leads to association. In 4 M urea, about half the protein sedimented with a rate near 5 S whereas the remaining protein showed faster sedimentating boundaries with rates greater than 8 S (see Figure 9). The slower sedimenting component all but disappeared in 5 M urea, with only faster moving boundaries accounting for most of the protein. The maximum value of the sedimentation constant (s_w) was near 15 (see Figure 9).

(*C*) *Column Chromatography.* The sedimentation and

Table I: Percent Distribution of Protein Bands from Sephacryl S-300

		100–110K[a]					
fraction	clathrin	S	I	F	LC_S	LC_F	15K
53	83	2	64	2	4	0	0
61	60	4	8	2	11	6	0
64	20	9	20	3	30	17	0
66	7	6	22	3	32	25	0
68	5	2	17	1	35	34	0
70	3	0	6.2	0	40	48	3
73	3	0	1	0	17	63	4
75	3	0	1	0	3	58	10
control	80	2	6	1	3	6	0

[a] S, I, and F denote slow, intermediate, and fast components, respectively, in the same group of bands.

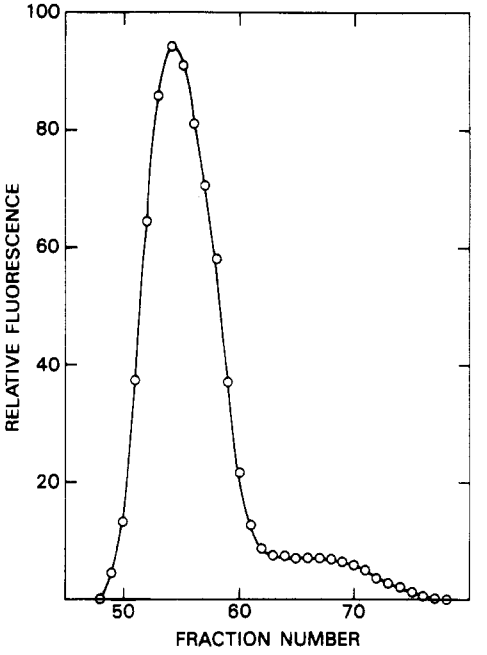

FIGURE 10: Elution pattern of clathrin on Sephacryl S-300 in 3 M urea, pH 8.0, and 0.01 M Tris.

light-scatter data in 3 M urea clearly indicate that some components with smaller sedimentation rates than native clathrin are dissociating without significantly changing the sedimentation behavior of the major component. In order to identify the dissociating species, we have chromatographed solutions of clathrin in 3 M urea on a sizing gel, i.e., Sephacryl 300 (Table I). Protein analysis by fluorescence of the various fractions showed a major peak followed by a minor shoulder (Figure 10). SDS gel electrophoresis revealed that clathrin was contained almost entirely in the major peak whereas the light chains were concentrated almost completely in the shoulder fractions (Figure 11).

Densitometric tracing of the gel patterns of various fractions gave the distribution of proteins shown. It is clear that the light chains are almost completely separated from the heavy chains but are somewhat contaminated with the M_r 110 000 proteins (Figure 11).

Discussion

We have used several parameters to detect the molecular transitions in clathrin. Since the light and heavy chains are noncovalently bound to each other, we have tried to see whether their dissociation preceded or occurred concomitantly with unfolding of the heavy chains. Since only 16% of the mass of clathrin is due to the light chains, we cannot evaluate their structural transitions in the presence of the heavy chains. They

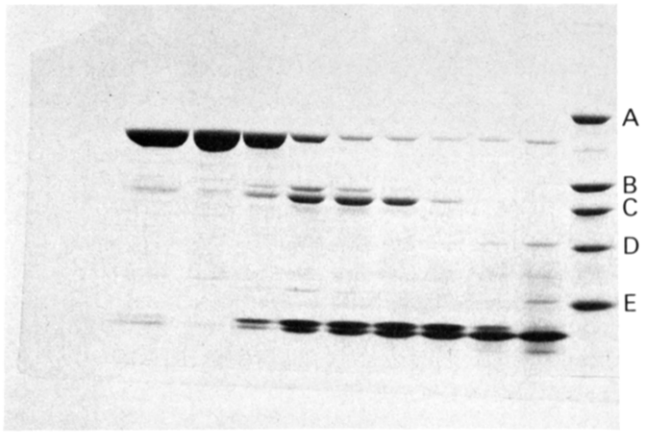

FIGURE 11: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the different fractions shown in Figure 10. From left to right, the lanes are clathrin before being loaded on the column, fractions 53, 61, 64, 66, 68, 70, 73, and 75, and the following molecular weight standards: (A) 200K; (B) 116K; (C) 92.5K; (D) 66.2K; (E) 45K.

need to be isolated in their native state before they can be studied by the same procedures.

It is convenient to consider the data obtained in urea solutions which are below 3 M as one group and those which are above as a secondary group, since the dissociation of light chains occurs in the former without much change in the structure of the heavy chains. This is evident from the spectroscopic techniques, which are sensitive to conformation but show very little, if any, change below 3 M urea. In contrast, the techniques which depend on mass, i.e. light scatter and centrifugation, reveal significant changes in properties. Above 3 M urea, all the spectroscopic procedures show important losses of secondary and tertiary structure. Some proteins dissociate before they unfold. It has been shown that phosphofructokinase dissociates first into subunits at Gdn·HCl concentrations below 0.8 M and then unfolds at higher Gdn·HCl concentrations (Parr & Hammes 1975).

In 3 M urea, clathrin sediments with essentially the same sedimentation coefficient that it possesses in water. Components representing about 25% of the absorption sediment more slowly than 8 S. As observed by gel permeation chromatography, this slower moving boundary consists largely, but not exclusively, of the light chains of clathrin. The decrease in light scatter after 24 h, however, is almost twice as large as can be accounted for by the mass of the light chain, if the extinction per unit weight at 280 nm is the same for the light and heavy chains. Much of the difference in scatter can be accounted for by the dissociation of the M_r 100 000–110 000 proteins which are separated with the light chains as is evident by column chromatography in 3 M urea. It is unlikely that the heavy chains are dissociating from the triskelion structure of clathrin since the small amount of clathrin chains found with the light chains appears to result from incomplete resolution of the heavy and light chains on the column.

The organized structure of clathrin appears to be largely or completely unaltered by 3 M urea, pH 8.0. The changes in all the structural parameters (excluding mass) are very small and cannot represent a significant modification in the folding of the polypeptide chains of clathrin. They could reflect structural effects in the light chains since they may become unfolded when they are released from the triskelion. We have checked the stability of clathrin by evaluating its ability to form baskets after exposure to 3.0 M urea, pH 8.0, and 0.01 M Tris for various periods of time between 15 min and 24 h. Clathrin solutions in 3 M urea were first diluted to 1 M urea to stop the reaction and then dialyzed to pH 6.0 in 0.10 M MES for 18 h. All solutions gave normal basket preparations, as analyzed by velocity ultracentrifugation, and clathrin was completely polymerized.

The spectroscopic data appear to be self-consistent in that they all show time dependence in the region of the first transition, i.e., between 3 and 6 M urea, and do not show any time effects in the second transition, i.e., >7 M urea. The two transitions are also clearly separated in most of the methods used. Only the polarization data did not reveal a distinct plateau region between the two transitions. This behavior can be understood if the polarization also senses incipient unfolding or loosening of the surface polypeptide chains prior to the penetration of solvent into the interior of the molecule.

We have used several methods to follow the transitions of clathrin which depend on changes either in the conformation (CD and polarization) or in the environment of two types of chromophoric residues (fluorescence and absorption). Since all four methods give essentially the same transition curves, they must all be responding to the same unfolding processes. The use of multiple parameters has revealed multiphasic (overlapping) transitions in the denaturation of pepsinogen and several other small globular proteins by Gdn·HCl (Wada et al., 1983). In the case of H5 histone protein, three different (overlapping) transitions were found by three spectroscopic techniques (Crane-Robinson & Privalov, 1983).

The sedimentation data in 4 M urea reveal a major component with a s_w of ~5. This value is clearly too large to represent the sedimentation of the light chains since even a globular protein of M_r 33 000–36 000 would not have a coefficient of this magnitude even if it were spherical and unhydrated. The 5S component probably therefore represents the sedimentation of monomeric clathrin (i.e., M_r 180 000) if it were still as asymmetric as in the triskelion. Unfortunately, the presence of several aggregated species makes it difficult to analyze further the properties of the 5S species. The large decrease in light scatter observed between 3 and 4 M urea after 24 h (Figure 6) is in harmony with the interpretation of the ultracentrifugation data.

The important dependence of aggregation on protein concentration seen by centrifugation was confirmed by our light-scattering data. The extent of aggregation considerably increases in 5 M urea since the various components have much higher sedimentation values and very little, if any, of the 5 S remains. One sees evidence in Figure 9 of three broad boundaries with S_w values of ~12, 15.5, and 19 S. It should be noted that the fluorescence and CD data were obtained at much lower protein concentration than the sedimentation data and therefore should be free of aggregation effects.

The unfolding that occurs in the first transition, i.e., 3–6 M urea, results in partially unfolded structures which have a limited solubility in 4 and 5 M urea solutions. The species that are formed may be successive polymers of clathrin or higher aggregates. Since we have no further description of these associated species, it is not possible to describe them further at this time.

Protein domains may be resolved by following the molecular transitions resulted from changes in temperature, pH, or solvent composition. It may be necessary to evaluate several structural parameters, however, since a protein may have several transitions but each one may appear as a smooth curve without inflections. In the case of the H5 histone protein, three different transition temperatures (and van't Hoff enthalpies) were found by ellipiticity measurements at 222 and 288 nm and by the chemical shifts of the C-3,5 protons of the tyrosyl

residues. Interestingly, scanning calorimetric measurements clearly indicated a one-step process which agreed with the transition at 222 nm. Since the other measurements respond to the behavior of only the Tyr residues, these groups reveal motions of the side chain prior to the unfolding of the backbone. A multiphasic change was observed in the acid transition of *Staphylococcus* nuclease by comparing Tyr and Trp emissions and His chemical shifts (Anfinsen et al., 1972).

The data on clathrin indicate that there are two distinct transitions, each representing about half of the structured elements of the heavy chains. The time dependence of the first transition and the lack of time dependence of the second transition, observed in all four spectroscopic parameters, indicate that there are two large, independent domains in the heavy chains and that each domain may have only a single, highly cooperative transition.

Registry No. Urea, 57-13-6.

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