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Protein Organization in Clathrin Trimers

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Summary

We have prepared a homogeneous, soluble 8.6S species ("8.6S clathrin") from calf-brain coated vesicles. Crosslinking experiments show that this 8.6S clathrin is composed of three heavy chains (molecular weight 180,000) and three light chains (molecular weights 33,000 and 36,000). Each heavy chain is in close contact with a single light chain, and the light chains appear not to be in contact with each other. Intact 8.6S clathrin can reassemble into cages without participation of additional protein species.

Introduction

Clathrin is the principal protein of coated vesicles (Pearse, 1975; 1976). It forms the cagelike coat, a network of hexagons and pentagons about 140 Å on a side, that surrounds an internal membrane vesicle (Crowther et al., 1976). Several investigators have shown that clathrin, defined as a species of apparent molecular weight 180,000, can be dissociated from the surface of coated vesicles by high concentrations of primary amines (Keen et al., 1979), urea (Blitz et al., 1977) or high pH (Woodward and Roth, 1978). The solubilized protein may be reassembled into cagelike structures with a morphology similar to the exterior of intact coated vesicles (Woodward and Roth, 1978; Keen et al., 1979; Schook et al., 1979). This reassembly constitutes in effect an assay for "native" clathrin. Electron micrographs of the solubilized protein shadowed with platinum and carbon have been obtained by Ungewickell and Branton (1981) showing images of a threefold symmetric structure with a pinwheellike shape. This structure appears to be the assembly unit of the cage.

We show here by crosslinking experiments that this assembly unit is composed of three heavy chains (molecular weight 180,000) and three light chains (of which there are at least two species, with molecular weights 33,000 and 36,000). That is, each heavy chain has one tightly bound light chain, and the complete unit is a trimer of such pairs. Intact trimeric units seem to be required for rapid assembly of cages.

Results

Preparation of Homogeneous, 8.6S Clathrin

Lane 1 of Figure 1 shows the SDS-PAGE pattern of purified clathrin isolated from coated vesicles according to the procedure described in Experimental Procedures. It shows three bands: a heavy chain (MO) with a calculated molecular weight of 180,000, in agreement with previously reported values, and two light chains (LC_a and LC_b) with molecular weights of 36,000 and 33,000. No other species is present in significant amount. The same electrophoretic pattern is observed without β-mercaptoethanol (Figure 1, lane 2), suggesting the absence of interchain disulfide links. The ratio of strength of LC_a to LC_b is approximately 1:2 in all preparations examined.

The sedimentation profile in a 5–20% linear sucrose gradient of an aliquot of the same clathrin sample is shown in Figure 2a. There is a single, symmetrical peak. Its sedimentation coefficient is approximately 8.6S, as calibrated with bromelain-cleaved hemagluttinin and catalase (9.3S and 11.2S respectively, indicated by the arrows in Figure 2a), and the material is denoted "8.6S clathrin" below. This sedimentation profile is characteristic of samples in which all divalent cations have been chelated with either 1 mM EGTA or EDTA, and as a general rule the profile is independent of ionic strength (up to 1 M NaCl), pH (between 6.0 and 8.5) and buffer (Na acetate, Tris, MES, PIPES, imidazole, ammonium carbonate and triethanolamine).

Assembly of Cages from 8.6S Clathrin

At pH 6.2 and at low ionic strength (such as, 20 mM imidazole or MES) addition of 2 mM Ca^{2+} to protein at a concentration of about 0.5 mg/ml transforms the 8.6S species into a rapidly sedimenting complex (Figure 2b). Electron microscopy shows cagelike structures, varying in diameter from 650 to 1250 Å (Figure 3a). A similar association occurs without Ca^{2+} if the protein is concentrated fivefold. These cages are evidently similar to the exterior of coated vesicles. Above about pH 6.7, Ca^{2+} is ineffective in inducing cage formation, and clathrin remains as a 8.6S species. Formation of such cages from solubilized clathrin has been described, but without consideration of the light chains, by Keen and collaborators (1979).

Electron Microscopy of 8.6S Clathrin

Examples of negatively stained images of individual 8.6S clathrin molecules appear in Figure 4. The structure is composed of three arms, bent at their centers in a uniform way and radiating from a single point of contact with an apparent threefold symmetry. The images show a consistent handedness, indicating that these clathrin molecules tend to adsorb to the carbon film on a single side. The inner part of an arm is about 140 Å in length, the outer part about 110 Å, and some views show an additional, short stretch at the apical end (see Figures 4c–4f). The arms appear to be of relatively uniform thickness, between 20 and 40 Å. Each arm is about twice the length of a cage, and the angle at the bend is approximately 120°. Striking images of these structures have been obtained by Ungewickell and Branton (1981), who shadowed molecules deposited from finely divided droplets. They point out that such a pinwheellike shape is called a "triskelion."

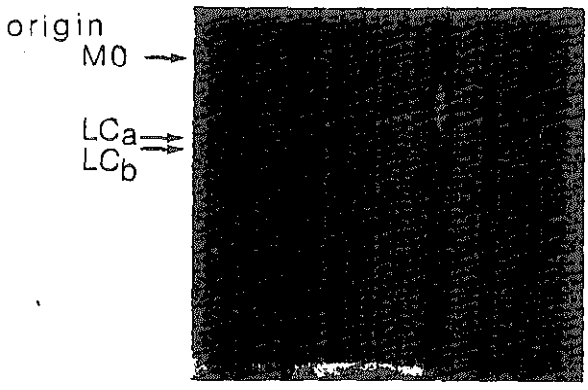

Figure 1. SDS-Polyacrylamide Gel Electrophoresis (15% Slab Minigel) of Calf-Brain Clathrin

(Lane 1) Clathrin, purified as described in Experimental Procedures: observed bands migrate with apparent molecular weights of 180,000 (M0, heavy chain), and 36,000 and 33,000 (LC_a and LC_b, light chains). (Lane 2) Same as lane 1, without β-mercaptoethanol in sample buffer. (Lane 3) Clathrin, digested with elastase: 1 μl elastase (Worthington) 0.03 mg/ml, added to 100 μl clathrin, 0.6 mg/ml, and quenched after 6 hr at room temperature with 1 μl 70 mM phenyl methyl sulfonyl fluoride (Sigma) (diluted with water from a 350 mM solution in ethanol just before adding). (Lane 4) Reassembled clathrin cages (prepared as described in Experimental Procedures) digested with elastase by the same protocol. (Lane 5) Molecular weight standards (phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme; Bio-Rad Laboratories).

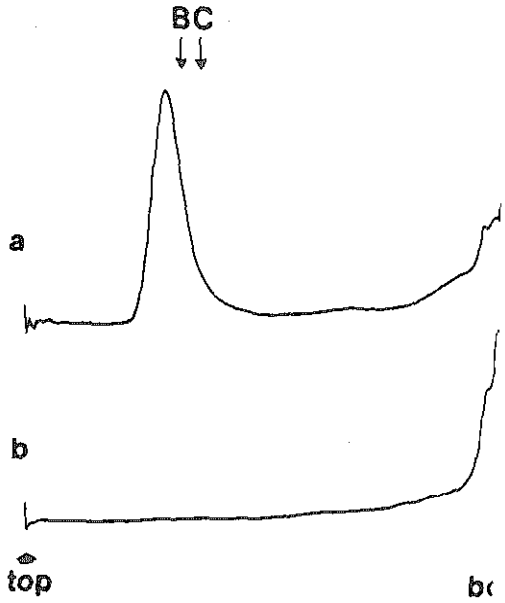

Figure 2. Sedimentation of Clathrin in a 5–20% Linear Sucrose Gradient (see Experimental Procedures)

(a) In 20 mM imidazole (pH 6.2), 1 mM EDTA; (b) in 20 mM (pH 6.2), 2 mM $CaCl_2$. The arrows B and C show positions of bromelain-cleaved haemagglutinin from influenza virus (S) and catalase (11.2S) respectively.

8.6S Clathrin Is a Trimer of Heavy Chains Each Linked to One Light Chain

We have studied the association of heavy and light chains in 8.6S clathrin by crosslinking experiments using DMS, DMTP, or DTSP, with SDS-PAGE analysis of the products. The results are qualitatively the same with any of the three reagents although with DTSP they require a lower ratio of reagent/protein for the same extent of crosslinking. Most of the experiments were carried out at pH 8.0–8.5, and the pattern of crosslinked forms was similar to that observed at pH 6.2 with DTSP.

Figure 5 displays an example of the crosslinked bands determined by reacting 8.6S clathrin with increasing amounts of DMS. We refer to these bands as M1, D0, D1, D2 and T. M1 is readily observed at low concentrations of crosslinker; its mobility indicates a molecular weight of 215,000. The following observations show that this band corresponds to an association of one heavy chain (M0) and one light chain (LC_a or LC_b). First, there are no bands between the 36,000 and 180,000 regions of the gel, suggesting the absence of dimers or trimers of the light chains. It is therefore unlikely that M1 is an aggregate composed solely of light chain (for example, pentamer or hexamer). Second, the appearance of M1 is simultaneous with the disappearance of the light and heavy chains (see, for example, Figure 5a line 2), strongly suggesting a crosslinking event between them. Finally, M1 can be regenerated into light and heavy chains in a ratio similar to that found in noncrosslinked 8.6S clathrin (gel not shown). This experiment was done by crosslinking with DTSP; after separation by SDS-PAGE under nonreducing conditions, bands were eluted for 24 hr in sample buffer in the presence of 6 M urea, 3% β-mercaptoethanol and 10 mM dithreitol and again submitted to electrophoresis, this time in reducing conditions.

The molecular weights of bands with lower mobilities were estimated by extrapolation of the mobilities of "known" markers: M0, M1 and crosslinked trimers and tetramers of catalase (189,000 and 252,000 respectively, see Figure 5b). D0, D1 and D2 be species of molecular weight 360,000, 395,(430,000, suggesting that these forms corres the three possible dimer species generated and M1 (two heavy chains with 0, 1 and 2 ligh respectively). By measuring the relative am D0, D1 and D2, for any given degree of cro of heavy chains to light chains, we can deter total number of light chains in an 8.6S unit scribed in the caption to Table 1, the ratio o the dimer bands can be used, together with of stain in the monomer bands, to estimate th of heavy chains that have a light chain bou method is in principle equivalent to measurin ratios of heavy to light chain in a second dim electrophoresis after reversal of crosslinkin however, the decisive advantage of using comparable strength and of similar polypep position (principally heavy chain, either as or dimer) rather than bands of very unequa and of different composition (heavy to ligh

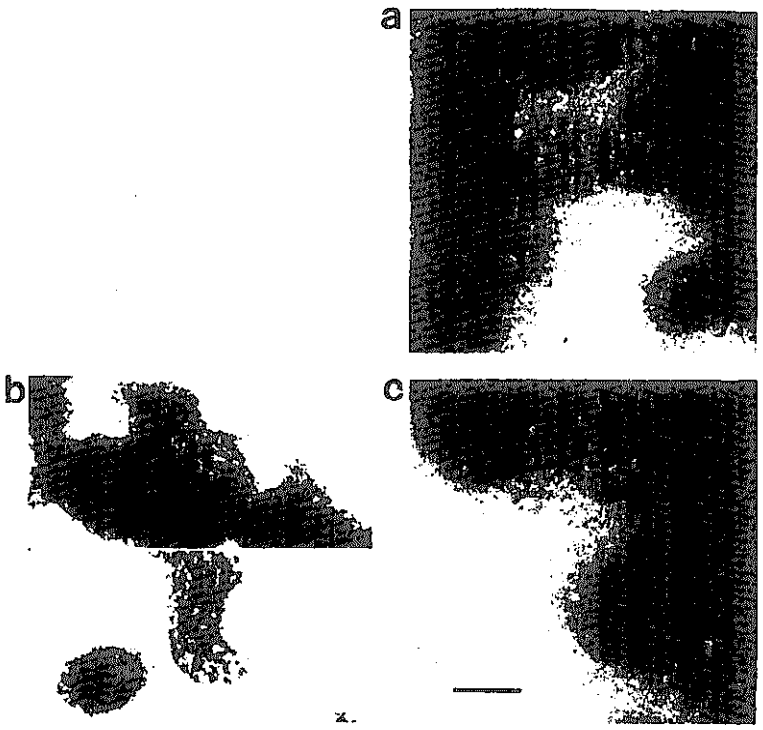

Figure 3. Electron Microscopy of Reassembled Clathrin Cages, Negatively Stained with 2% Uranyl Acetate

Cages were obtained by incubation for 24 hr in 20 mM imidazole (pH 6.2), 2 mM $CaCl_2$. Scale = 1000 Å. (a) Cages reassembled from 8.6S clathrin trimers. (b) Incorrectly assembled cages from clathrin trimers previously treated with elastase: complete digestion of light chains had occurred in this sample (see Figure 1, lane 3). (c) Cages, formed from intact trimers and treated with elastase subsequent to assembly (see Figure 1, lane 4).

mass ratios of 5 or 10 to 1, for D2 and D1 respectively). Optical density traces of four stained SDS-PAGE gels from independent crosslinking experiments with increasing amounts of DMS are given in Figure 5c, and the corrected areas of peaks in Table 1. Relative amounts of the different dimers agree well with a model in which each heavy chain is in contact with one light chain.

T behaves as a species of molecular weight ≅40,000, that is, a trimer of M1. No additional bands of lower mobility can be detected with higher amounts of DMS (see, for example, Figure 5a lane 5), even when most of the 8.6S clathrin has been crosslinked to T (in this case we have used DTSP for the extensive crosslinking; gel not shown).

Selective Proteolysis of the Light Chains

It is possible to digest the light chains completely into peptides smaller than 10,000 with elastase in low molar ratio, as given in Figure 1 lane 3. Under the conditions outlined in the figure caption, the heavy chain remains undigested. Crosslinking of elastase-treated 8.6S clathrin (Figure 5a lane 6) shows that heavy-chain contacts in the trimer are still maintained. Band M1 (one heavy chain linked to one light chain) has disappeared, and D1 and D2 are absent; only D0 (dimer of heavy chains) and T (trimer) are clearly seen. Elastase-treated clathrin aggregates in the presence of Ca^{2+}, but it appears to have lost the ability to form regular cages (electron micrograph, Figure 3b). Elastase treatment of intact cages has no apparent effect on their morphology (Figure 3c), although the light chains have been completely digested (Figure 1 lane 4).

Discussion

Purification and Composition of 8.6S Clathrin

Previous reports of clathrin purification identify a series of bands on gel electrophoresis, many of which can be separated from solubilized clathrin by gel filtration or by centrifugation (Woodward and Roth, 1978; Keen et al., 1979). The experiments described here show clearly that under all nondenaturing conditions examined, only two classes of components are tightly associated: the 180,000 molecular weight heavy chain originally designated as clathrin by Pearse (1975) and the 33,000 and 36,000 light chains, also observed by Pearse (1978) in cholate-extracted vesicles.

These two components appear to be sufficient for reformation of stable, coated vesiclelike cages in the low ionic strength (ca 20 mM), Ca^{2+}-containing buffers we employ. No other protein species appear, even in overloaded SDS gels. Moreover, our results suggest that light chains may be required for correct reassembly, since elastase treatment of solubilized clathrin prevents formation of regular cages. We cannot, however, rule out that cleavage of a significant small peptide from one end of the heavy chain, rather than loss of light chains, is responsible for the effect of elastase on reassembly. Our reassembly buffers are essentially the same as those used by Keen et al. (1979), but these authors do not address the question

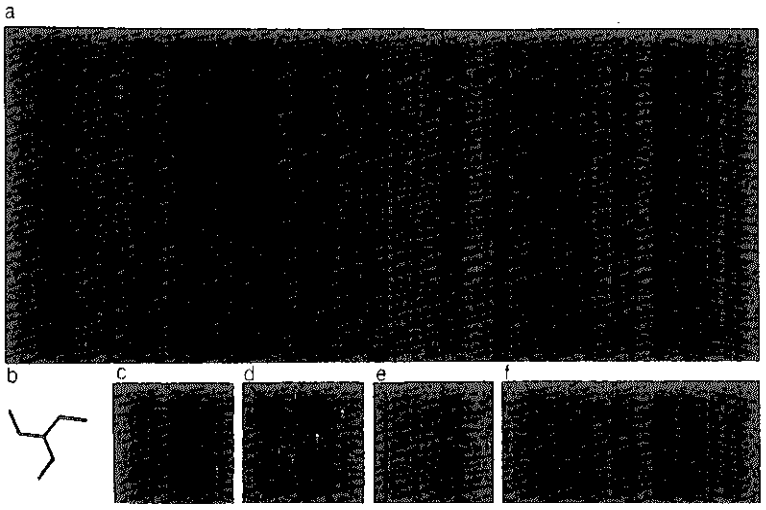

Figure 4. Electron Microscopy of 8.6S Clathrin

(a) Field of 8.6S clathrin negatively stained with 2% uranyl acetate. Trimers are contrasted most clearly in relatively deep stain, ensured by addition of tomato bushy stunt virus to the clathrin solution before adsorption to the carbon film. Clathrin concentration, 5 μg/ml; scale = 1000 Å. (b) Idealized triskelion. (c)–(f) Selected views of 8.6S trimers: scale = 500 Å. In (f) two trimers appear to be partially embedded in stain that trails off in a gradient from the neighboring virus particle.

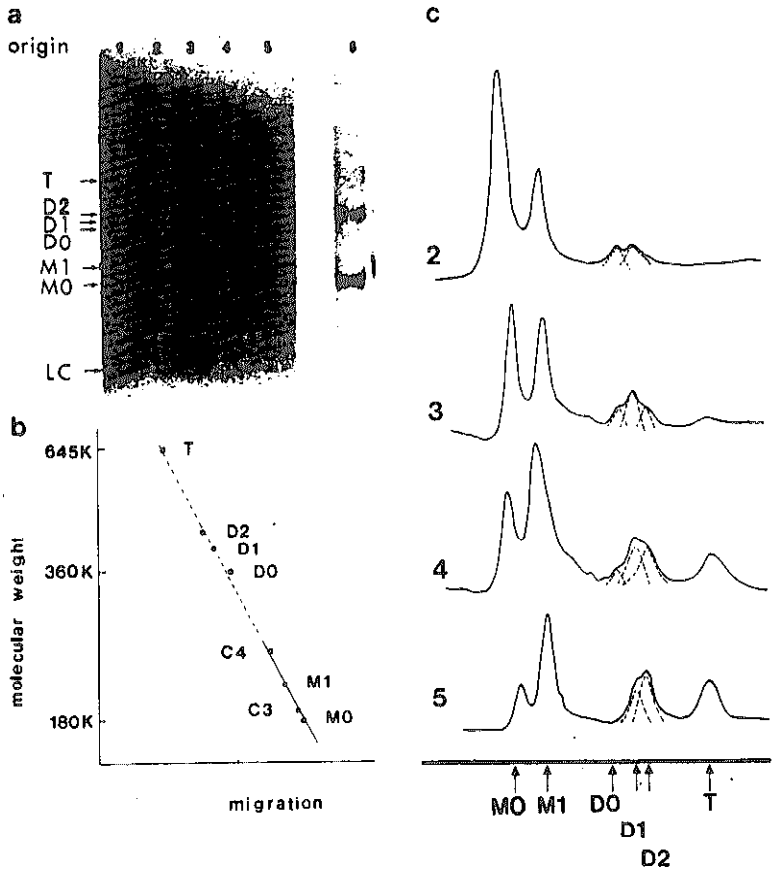

Figure 5. DMS Crosslinking of 8.6S Clathrin

(a) Electrophoresis of crosslinked species on a composite polyacrylamide (3%)-agarose (1%) slab gel in the presence of SDS. Lane 1: purified clathrin, no DMS. Lanes 2–5: results of an experiment with 0.1, 0.2, 0.4 and 0.8 mg/ml DMS and 0.5 mg/ml clathrin (for details, see Experimental Procedures); about 10 μg protein were electrophoresed in each lane. Lane 6: an experiment with 0.8 mg/ml DMS and 0.5 mg/ml elastase-treated clathrin.

(b) Logarithm of apparent molecular weight versus mobility for bands labeled in (a). C3 and C4 are DMS-crosslinked trimer (189,000) and tetramer (252,000) of catalase, used for calibration. The line was drawn through these points and through M0 and M1, assuming molecular weights of 180,000 and 215,000 respectively. Mobilities of bands D0, D1, D2 and T then place them on the extrapolated line at positions corresponding to 360,000, 395,000, 430,000, and 640,000 respectively.

(c) Optical density scans of lanes 2–5 of gel in (a). Decomposition of the composite peak into D0, D1 and D2 is shown by dashed lines. Areas under the various peaks are given in Table 1.

of a requirement for the 33,000 and 36,000 species. They report that assembly is inhibited in higher ionic strength buffers and that the inhibition can be overcome by addition of a further protein fraction. This fraction is probably distinct from the light chains described here, since the latter appear to be very tightly bound to heavy chains.

Organization of Chains in the 8.6S Clathrin Trimer

We conclude from our crosslinking experiments that 8.6S clathrin is a trimer of heavy chains, each in close contact with a light chain. The light chains do not appear to be in contact with each other. The triskelion structure revealed by electron microscopy (Ungewickell and Branton, 1981) therefore contains three heavy-

Table 1. Ratios of Corrected Integrated Stain Densities of Crosslinked Species

	M1′/M0′ + M1′	D0′	D1′	D2′
Observed lane 2	0.30	0.45	0.55	~0
Calculated model 1		0.49	0.42	0.09
Calculated model 2		0.63	0.34	0.03
Observed lane 3	0.42	0.27	0.53	0.20
Calculated model 1		0.33	0.49	0.18
Calculated model 2		0.50	0.44	0.06
Observed lane 4	0.61	0.13	0.54	0.33
Calculated model 1		0.15	0.48	0.37
Calculated model 2		0.31	0.57	0.12
Observed lane 5	0.68	~0	0.44	0.56
Calculated model 1		0.10	0.44	0.46
Calculated model 2		0.25	0.60	0.15

As outlined in the text, we interpret M1 as a crosslinked complex of one heavy chain and one light chain, and we ascribe the presence of three dimer species, D0, D1, and D2, to crosslinking of 2 M0, 2 M1 and M0 + M1 respectively. Let M0′, D0′, etc. indicate the total stain in the corresponding band, corrected to represent *molar* ratios of the various species. In model 1, we assume that in a 8.6S clathrin trimer, each heavy chain has one closely associated light chain. If we further assume no correlation between heavy:light crosslinks, we calculate for a ratio $\alpha = M1'/(M0' + M1')$ that $D0':D1':D2' = (1-\alpha)^2:\alpha(1-\alpha):\alpha^2$. The predictions for observed values of α are labeled "model 1." As an example of an alternative, we assume that in each trimer, one heavy chain systematically lacks a light chain. Calculated ratios for D0′:D1′:D2′ are $(3 - 4\alpha + \alpha^2)/3:(4\alpha - 2\alpha^2)/3:\alpha^2/3$, and predictions for observed α are shown as "model 2." The ratio of integrated peak densities, corrected by 0.83 for M1 and D1 and by 0.71 for D2, are from Figure 3c and correspond to lanes 2–5 of Figure 3a.

chain arms in limited association at the center, with a light chain positioned somewhere along each arm. The light chains are not required to maintain the threefold contacts, suggesting that they may be at some distance from the center. A light chain and heavy chain may have extensive interaction, since the efficiency of their crosslinking is greater than that between two arms. The molecular weight of solubilized clathrin determined by sedimentation equilibrium and by quantitative electron microscopy (Ungewickell and Branton, 1981) agrees with the composition required by this model.

Two classes of light chains have been shown to be present in a variety of different species and tissues (Pearse, 1978), although the precise sizes seem to vary. The general presence of such chains suggests a significant but presently unknown function, and the meaning of two classes is likewise obscure. The susceptibility of light chains to proteolysis both in trimers and in cages suggests a readily accessible position. The crosslinking of elastase-treated trimers shows that proteolytic degradation leads to dissociation of light-chain fragments from the heavy chains. The elastase-treated trimers appear unable to reassemble correctly into cages; this observation may point to some function of light chains in assembly, although their degradation in situ on the cages does not lead to disassembly.

If we assume the heavy-chain mass to be distributed uniformly along its length, we calculate approximately six residues per angstrom. Since a single α-helix contains 0.67 residues per angstrom, the clathrin arms are unlikely to be simple fibrous structures such as α-helical coiled coils. We note the case of the T4 phage tail fiber, studied by Earnshaw et al. (1979), which appears to be a rigid concatenation of tightly folded domains.

Relation of Trimers to Cages

Association of 8.6S clathrin under our conditions appears always to lead to complete cages having a small variety of regular designs, based on polyhedra with hexagonal and pentagonal faces. This self-assembly implies similar contacts between the structure units. In all cages we have seen, three edges meet at a vertex. It is plausible to locate the center of a clathrin triskelion at each of these local threefold positions, with every arm spanning two edges. An important property of this distribution is that it allows conservation of contacts between clathrin molecules in pentagonal as well as in hexagonal arrangement, with only small conformational changes to satisfy both cases (Caspar and Klug, 1962). Other modes of placing clathrin in a cage (such as, with triskelion centers at every *second* local threefold position) require special reorganization to obtain pentagonal faces. We note that Crowther et al. (1976) estimated the molecular weight of a cage with 54 edges to be 22×10^6. This corresponds to a lower limit of 400,000 daltons per edge or four half-arms per edge, consistent with the arrangement just described. Indeed, the edges of cages appear substantially thicker in negative stain than the arms of isolated molecules.

The vertices of a cage are positions of curvature. We indeed have some evidence that isolated clathrin triskelions are not planar structures. Most micrographs show trimers with incomplete arms: a plausible interpretation is that the trimer arms are not coplanar and that the missing portion is protruding from the stain. A few favorable images of molecules embedded in clear stain gradients are consistent with this view (Figure 4f).

Experimental Procedures

Preparation of Clathrin

Clathrin was purified from gray matter of calf brains according to the procedure of Keen et al. (1976) with the following modifications. For each preparation, ten brains were taken within 1 hr of slaughter. During this period, the brains were either chilled or kept at 37°C, as suggested by Pearse (1980). Unless otherwise stated, the rest of the purification was done at 4°C. The tissue was homogenized in a blender for 1 min at high speed with 2 vol of solution A [0.1 M NaMES (pH 6.2), 10^{-3} M EGTA, 5×10^{-4} M $MgCl_2$, 0.02% NaN_3]. Cell debris was separated by centrifugation in a JA-14 rotor (Beckman) for 30 min at 14,000 rpm, and a crude vesicle fraction was obtained from the supernatant by centrifugation in a type 35 rotor (Beckman) at 35,000 rpm for 60 min. The vesicle pellets were resuspended in solution A with the aid of a Dounce homogenizer, and 16 ml samples were applied to the tops of six discontinuous sucrose gradients (4,

13 and 4 ml of solution A containing 5, 10 and 40% sucrose, respectively). These gradients were centrifuged in a SW 27 rotor (Beckman) at 20,000 rpm for 2 hr, and the 10% sucrose fractions were pooled, diluted with 3 vol of solution A, and centrifuged in a type 35 rotor at 35,000 rpm for 60 min. The pellets were resuspended in buffer A with the Dounce homogenizer, and 6.5 ml were applied to the tops of six discontinuous sucrose gradients (19.5, 10 and 3 ml of solution A with 5, 30 and 60% sucrose, respectively) and centrifuged in an SW 27 rotor at 20,000 rpm for 45 min. The 5% fractions were pooled, diluted with 1 vol of buffer A, and centrifuged in a type 35 rotor at 35,000 rpm for 60 min. The pellets of purified coated vesicles were resuspended with a homogenizer in a final volume of 25 ml solution B (0.75 M Tris, 0.025 M NaMES (pH 6.2), 2.5×10^{-4} M EGTA, 1.2×10^{-4} M $MgCl_2$, 0.02% NaN_3), and clathrin and other proteins were allowed to solubilize for 30 min at room temperature. Membrane vesicles were then removed by centrifugation in a type 42 rotor (Beckman) at 42,000 rpm for 60 min; the supernatant was brought up to a concentration of 30% in $(NH_4)_2SO_4$, and after 30 min, a pellet was obtained by centrifugation in an SW 27 rotor for 10 min at 5000 rpm. This pellet was resuspended in solution B, the suspension clarified by centrifugation at 42,000 rpm, and soluble proteins were concentrated with $(NH_4)_2SO_4$ as before. The pellet was resuspended in about 5 ml of solution C (0.5 M Tris, 0.05 M NaMES (pH 6.2), 5×10^{-4} M EGTA, 2.5×10^{-4} M $MgCl_2$, 0.02% NaN_3). Clathrin was separated from other, smaller proteins by gel filtration on Biogel A-5M (Bio-Rad) or Ultrogel AcA 22 (LKB) columns (2.5 × 75 cm or 2.5 × 86 cm respectively, equilibrated with solution C). A single peak emerged near the void volume; pooled fractions corresponding to this peak yielded approximately 30 mg of protein (Lowry, 1951) and constituted the purified clathrin sample. This solution had an OD_{280}/OD_{260} ratio of 1.7, $E^{1\%}_{cm}$ (280) = 10.0, and electron microscopy showed it to be free of membranous vesicles.

Measurement of Sedimentation Velocity

Sedimentation velocity was estimated by rate zonal centrifugation in 5–20% linear sucrose gradients according to the procedure of Martin and Ames (1961). Samples (50–100 μl) were loaded on top of preformed gradients and centrifuged in an SW 50.1 rotor (Beckman) at 48,000 rpm for 3 1/2 hr at 18–20°C. The concentration profile was determined with a flow cell, monitoring OD_{280} through the gradient by upward displacement with Fluorinert (DuPont). Bovine liver catalase (Sigma) and bromelain-cleaved hemagglutinin from influenza virus (gift from J. Skehel and D. C. Wiley) were used as standards.

Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed in 15% polyacrylamide slab minigels (1:37.5 (w/w) bisacrylamide:acrylamide) containing 0.1 M Tris, 0.1 M Bicine (pH 8.3) and 0.1% SDS (Earnshaw et al., 1979). Samples were mixed with 1 vol of sample buffer (0.01 M Tris, 0.01 M Bicine (pH 8.3), 30% glycerol, 1% SDS, 3% β-mercaptoethanol), boiled for 1 min, run at 100 V for 15 min and stained with Coomassie Blue. Crosslinked products were analyzed on composite 1% agarose-3.5% polyacrylamide slab gels (1:12 (w/w) bisacrylamide:acrylamide; gel 100 mm × 100 mm × 1 mm) with Tris–Bicine buffer as above. When required, gels were scanned for optical density at 525 nm with a Helena Lab gel scanner.

Crosslinking

Crosslinking experiments were performed at room temperature with the bifunctional reagents dimethyl suberimidate (DMS) (Davies and Stark, 1970), dimethyl 3–3′-dithio-bis-propionimidate (DMTP), and dithio-bis-succinimidyl-propionate (DTSP) (Lomant and Fairbanks, 1976) purchased from Pierce Chemical Co. The first two are rapidly hydrolysable imidoesters that crosslink primary amines with an optimum reactivity around pH 8–9. The third acylates primary and secondary amines, hydrolyses at a much slower rate and is reactive at pH 6.2. Crosslinking experiments were performed in the following way: to provide adequate buffering capacity, 1 vol of 1 M triethanolamine (pH 8.5) was added to 4 vol protein at 0.6 mg/ml in 0.02 M imidazole or Na acetate (pH 6.2), 0.001 M EDTA (or EGTA), 0.02% NaN_3. The imidoesters were dissolved in 0.5 M triethanolamine (pH 8.5) at 8 mg/ml, and an appropriate volume was added within 1 min to the protein sample. An equal amount of freshly prepared reagent was added after 30 min. The final concentration of the imidoesters was between 0.1 and 3.2 mg/ml. Dithio-bis-succinimidyl-propionate was first dissolved in acetone (28 mg/ml), diluted ten times with either 0.1 M triethanolamine (pH 8.5) or 0.02 M Na acetate (pH 6.2) and the turbidity was eliminated by heating at 37°C for 30 sec. The reagent was added to the protein sample to a final concentration between 0.025 and 0.8 mg/ml, and the crosslinking reaction was carried out for at least 30 min. The samples were then mixed with one volume of sample buffer without β-mercaptoethanol, incubated at 37°C for 30–45 min and subjected to electrophoresis. When dimethyl suberimidate was used as the crosslinking reagent, the sample buffer contained β-mercaptoethanol, and the sample was boiled for 1 min prior to electrophoresis. Gels were run at 100 V for 2 1/2 hrs.

Electron Microscopy

Clathrin was negatively stained using the flotation technique of Wrigley et al. (1977). A carbon film, previously deposited onto freshly cleaved mica, was floated onto the surface of a solution of the sample to be examined. After 10–30 sec, the carbon film was withdrawn onto the same mica and floated onto a 2% unbuffered uranyl acetate solution. After a few seconds, the carbon film was picked up from above with a 400-mesh copper grid and dried by blotting with filter paper.

Clathrin cages were used at a concentration of 0.6–1.3 mg/ml and solubilized trimers at 5 μg/ml, sometimes with added tomato bushy stunt virus at the same concentration. Samples were examined at 80 KV with a magnification of 45,000 in a Phillips EM-301 microscope.

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