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Configuration of Clathrin Trimers: Evidence from Electron Microscopy

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We used a combination of electron microscopic techniques—negative staining, glycerol spraying with rotary shadowing, and quick-freezing followed by deep-etching—to study the configuration of clathrin trimers. All three approaches provide images indicating that the molecule is nonplanar and rather puckered at its center. Viewed from the convex (cytoplasmic) side, its arms display a consistent *clockwise* slew at their proximal/distal hinge. The most flexible part of the heavy chain may be the links between the distal portion of the leg and the terminal domain.

Clathrin, the protein that forms the outer lattice of coated vesicles, can reassemble rapidly into well-defined and completely closed empty cages (*1*). These cages have a variety of polyhedral structures similar to those found in undisrupted coated vesicles. Rapid and efficient self-assembly is characteristic of viral coat subunits, actin, tubulin, and other cytoskeletal proteins, but in these examples the associating structures are relatively compact and of limited flexibility. Specific protein–protein contacts, of defined directionality, and reliable switching between alternative states of these contacts, determine the accuracy of assembly (*5*). Clathrin is not a compact protein, however (*10, 17*). It is a trimer of three heavy chains and three light chains, and each heavy chain forms an extended leg, 50 nm in length (*10, 17*). The distal end of the heavy chain (180 kDa) folds into a compact terminal domain that comprises about 30% of the total mass (52 kDa) (*11*). This domain can be isolated by limited proteolysis of assembled cages (*11, 14*). The light chains lie along the proximal part of a leg (*12, 16, 18*).

Previous electron microscopic studies of negatively stained samples of clathrin adsorbed to carbon films have suggested that the disposition of its legs displays a constant slew (*1, 10*). In contrast, electron microscopic pictures obtained in several laboratories by the technique of glycerol spray and rotary shadowing showed images of clathrin where the legs had a clockwise and/or counterclockwise slew (*12, 14, 17, 20*). From this, it was inferred that the hinge between the proximal and distal segment of the heavy chain is highly flexible (*14, 20*).

Using the quick freeze–deep etch technique, we have recently reported that clathrin molecules that adsorb to mica chips in solutions containing Tris tend to display their heavy chain legs with a predominantly clockwise orientation; in the absence of Tris, the adsorbed molecules appear less threefold symmetric and display straight or counterclockwise orientation of their legs (8).

Here, we use electron microscopy to study further the configuration of clathrin, in order to determine the constraints on flexibility of its legs. We have used several techniques—negative staining, glycerol spray with rotary shadowing, and quick-freeze–deep-etching—to contrast the molecules, and analysis of the images indicates that

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clathrin trimers are nonplanar and puckered at their center, that the legs have consistent clockwise slew when viewed from their cytoplasmic side, and that the most flexible part of the chain may be the links between distal leg and terminal domain.

MATERIALS AND METHODS

1. Purification of Clathrin

Clathrin was obtained from calf-brain coated vesicles and kept as free trimers (conc. ~ 0.8 mg/ml) in 50 m*M* TEA, pH 7.0, 1 m*M* EDTA, 0.5 m*M* DTT, 0.02% NaN_3, and 0.5 m*M* PMSF (*11*). Clathrin was dissociated and separated into heavy-chain trimers and light chains as described by Winkler and Stanley (*20*), with slight modifications. The dissociation buffer used was 50 m*M* TEA, pH 8.0, 1.4 *M* KSCN, 2 m*M* EDTA, 0.5 m*M* DTT, and 0.02% NaN_3; gel filtration was carried out on a Sephacryl S-300 (Pharmacia) column (0.7 × 60 cm) equilibrated with the same buffer. The extent of dissociation was checked by SDS–PAGE and Coomassie brilliant blue (Sigma) staining. All procedures were carried out at 4°C and samples were used within 1 week of purification.

2. Electron Microscopy

Samples of clathrin for negative staining (conc. ~10 μg/ml) were adsorbed onto freshly glow-discharged carbon-coated grids, washed with a few drops of 1.5% uranyl acetate, blotted, and air-dried. Samples adsorbed to mica were prepared by either the glycerol spray (*3, 15*) or quick-freeze/freeze-dry (*7*) techniques and visualized by rotary shadowing with platinum. For glycerol spraying, samples were directly diluted 50- to 100-fold (to a conc. of ~10 μg/ml) into a solution of 45% glycerol with the appropriate ionic composition. Alternatively, samples (conc. ~0.8 mg/ml) were first dialyzed against the appropriate buffer and then diluted into the glycerol mixture. At this point, samples were sprayed onto freshly cleaved mica and dried *in vacuo*. With this technique, adsorption at low ionic strength (~1 m*M*) generated clathrin images with a clockwise slew; at higher ionic strength (>25 m*M*), images show a preferential counterclockwise slew. The number of adsorbed molecules did not vary with ionic composition. For freeze-drying, samples were first diluted 50- to 100-fold with the solution of interest and mixed briefly with a slurry of mica chips before quick-freezing. Clockwise images were generated by applying clathrin to mica in low ionic strength buffers (10 m*M* Tris, pH 8), although under these conditions the affinity of the mica was extremely low. Alternatively, clockwise images were generated in 10–50 m*M* Tris, pH 8, 50–100 m*M* NaCl. Counterclockwise images were generated by applying clathrin to mica in 50–100 m*M* TEA, pH 8.0. Grids were examined in a standard transmission electron microscope operating at 80–120 kV. Negatively stained bacteriophage T4 tails were used as magnification standards, taking 4.1 nm as the repeat distance. The micrographs were printed so that the negatively stained or rotary-replicated molecules lie facing the viewer on the "upper" surface of carbon or mica, respectively.

3. Graphic Simulation and Data Analysis

Three-dimensional graphic simulation of negatively stained clathrin trimers was performed with the program GRAMPS (*13*), using an Evans and Sutherland multi-picture system linked to a VAX 11/780. A line representation of a clathrin molecule, with its legs spanning two edges, was placed at each vertex of a simulated cage. The surface lattice of the cage formed the edges of a truncated icosahedron (the "soccer ball" structure), which corresponds to a frequently found design in *in vitro* reassembled cages (*11*), *in situ* visualization of coated vesicles (*6*), and in purified preparations of coated vesicles obtained from calf brain and liver and human placenta (Turkewitz, Kirchhausen and Harrison, unpublished observations). Various parameters that define the shape of the trimer (apical pucker, "bond angle" at the elbow and torsion angle about the proximal leg) and its position and orientation in the lattice were systematically changed until a good visual agreement with electron micrographs of cages was obtained (not shown). The simulated image of the trimer was rigidly rotated to obtain a match with the electron micrographs of negatively stained clathrin trimers.

For analysis of leg lengths and end-to-end distance of terminal domains, images of samples adsorbed to mica were enlarged from negatives and traced with a camera lucida. Contour leg lengths were measured with a digitizing tablet (Evans and Sutherland multi-picture system) and corrected by the average diameter of Pt grains in the background of the same fields (range, 1.7–4 nm).

RESULTS AND DISCUSSION

1. The Nonplanar Shape of a Clathrin Trimer

Clathrin trimers negatively stained on glow-discharged carbon-coated grids present a variety of shapes (Figs. 1–3). We believe that these are best interpreted as flattened projections of triskelions with quite restricted flexibility. Most fields show images of clathrin molecules with a clockwise slew of their distal legs when viewed from the molecule toward the carbon substrate (Fig. 1), as reported in previous negative stain studies (*1, 10*). We cannot find images

that present uniform counterclockwise directionality, but certain fields show primarily trimers with at least one counterclockwise leg (Fig. 2). We have indeed been able to obtain grids from the same clathrin sample (Figs. 1 and 2) showing either largely one kind of field or largely the other, indicating that the two classes of images represent different modes of adsorption to the carbon rather than different states of the trimer. It is difficult to explain those images that do not show uniform slew (e.g., Fig. 2) by assuming a random "flip" of the distal segments. In a complete count of 154 molecules in two different fields, images with two legs apparently counterclockwise and one clockwise are reasonably frequent and are about as numerous as those with one leg counterclockwise and two clockwise (see caption to Figs. 1–3). If these images were due to flexibility of the proximal/distal linkages, fully counterclockwise images should then also occur. Such images were not observed and, in general, are extremely infrequent. All the views can, in fact, readily be interpreted as flattened projections of a three-dimensional object, with uniformly slew legs and significant overall pucker. This is illustrated by the gallery in Fig. 3, which compares trimer micrographs with corresponding computer-graphic simulations of the three-dimensional shape of a clathrin trimer. (A similar comparison between images of clathrin adsorbed to mica and the simulated model was less satisfactory.) The computer-generated views assume that the trimer has a fixed pucker at its apex and fixed angles between proximal and distal segments. The apical pucker and the "bond angle" between proximal and distal segments were chosen to simulate the shape of a triskelion packed into a truncated icosahedral cage (see Materials and Methods). The distal legs hang down from the apex, giving the molecule a strongly curved aspect. The simulation produces an orthogonal projection, which does not adequately model the flattening that occurs when a trimer is dried down in stain. Legs in the images are less foreshortened than in the projected views. Nonetheless, it is clear that a strongly curved trimer with legs of constant slew can produce the entire range of images we have observed. The absence of fully counterclockwise images implies that adsorption to the carbon film is particularly unlikely for certain angles of approach or that clathrin adsorbed from these angles is poorly stained (*1, 10*).

The agreement of simulated views with the shape of negatively stained trimers (Fig. 3) shows that clathrin legs have a uniform slew and suggests that the molecule may be relatively stiff, with a few joints of restricted angular freedom. The intrinsic curvature of such a structure implies that only small changes in the three-dimensional organization of its legs are needed in order to accommodate to different packing environments in the assembled lattice of the coat (*1, 10*). It is also consistent with the rapid and efficient reassembly of coats that is observed *in vitro* (*1*) and that is believed to occur *in vivo* during formation of coated vesicles and of their intermediates, coated pits (for reviews, see *2, 4, 6, 9*).

2. Clathrin Can Absorb to Mica with Two Different Orientations

Figures 4 and 5 show fields of clathrin that have been adsorbed to mica and visualized by the glycerol spray–rotary shadowing technique. Figure 4 illustrates that when the experiment is carried out at a very low ionic strength (<1 m*M*) most of the trimers display a uniform clockwise slew, with a relatively sharp bend at points halfway along their legs. By contrast, Fig. 5 shows that a similar adsorption at a higher ionic strength (25 m*M* or more) generates images of clathrin trimers with extended legs that are frequently bent in a counterclockwise orientation. A consistent correlation between the apparent handedness of the image and the ionic strength (same number of adsorbed molecules) can be obtained with various buffers: TRIS–HCl (pH 7.1), am-

monium acetate (pH 6.6–7.5), and triethanolamine (pH 7.0–8.0).

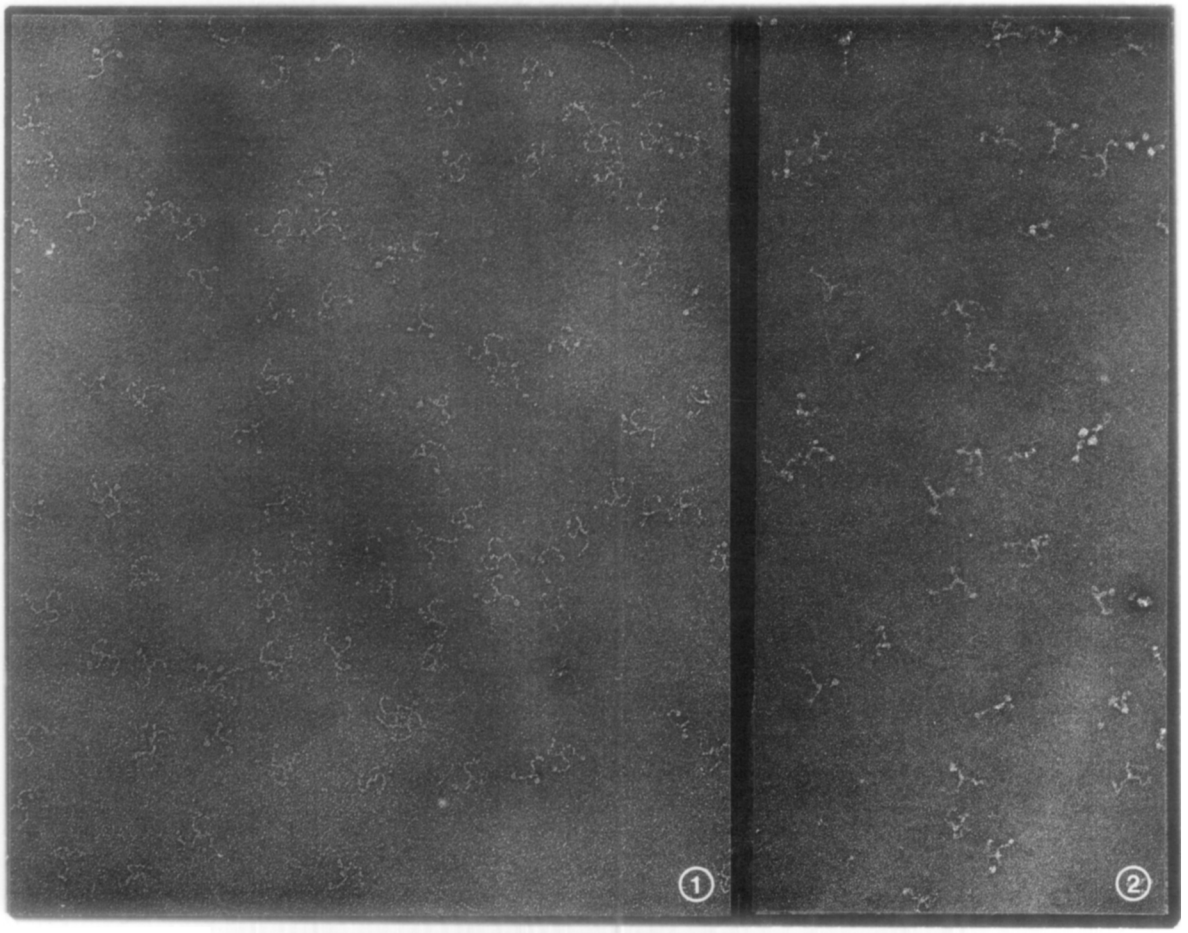
The simplest explanation for these two classes of images is that ionic conditions determine which side of the trimer attaches to the mica. To test this interpretation, we have prepared specimens by quick-freezing combined with freeze-etching (Figs. 6–8). Advantages of this method include (1) the absence of glycerol, (2) a reduction of the distorting effects upon drying, and (3) a better preservation of structural details in the molecules. This approach yields the same two classes of images that we see with the glycerol-spray technique. These are (1) images with legs slewed in a uniformly clockwise direction and (2) images with legs that appear straight or bent in a counterclockwise orientation. Unlike the observation made with the glycerol-spray technique, in this case, we do not observe a simple correlation between ionic strength and apparent handedness of the images, but rather a dependence in the presence of Tris-containing solutions for the generation of clockwise slewed images of clathrin. This effect appears to be independent of ionic strength. In addition, we note that there are some differences between images in each slew class, as seen by the two methods. With the glycerol-spray technique, the apparent contour length of a leg is slightly larger and the tendency of molecules to spread out (as measured by the distance between terminal domains within a trimer) appears to be greater. The qualitative characteristics of the two classes of images are the same with both methods, however, and we believe that the interpretation outlined below applies to both.

Legs in the first (clockwise) group are generally sharply bent. Their contour length, measured from the center of the trimer to the most distal part of the terminal domain, is about 47 nm in the quick-freeze images and about 50 nm in glycerol spray (Table

FIGS. 1–3. Electron micrograph of clathrin trimers (in 1 m*M* Tris–HCl, pH 7.2), adsorbed to glow-discharged carbon-coated grid and negatively stained with 1.5% uranyl acetate (× 105 000). Most of the images show uniform clockwise slew of the legs. (Fig. 1) Similar micrograph of a field (same clathrin sample) in which most images show at least one leg with counterclockwise slew (Fig. 2). We have analyzed the slew of distal legs on all the trimers in several fields, showing both kinds of images. Molecules with 3, 2, 1, and 0 clockwise directed legs account for 53, 14, 14, and 0% of 154 trimers; 19% have one or more legs with indiscernible slew. Figure 3 shows a gallery of views of simulated trimers compared with images of negatively stained clathrin. For the purpose of the comparison, a simulated clathrin trimer with no terminal domain and with fixed pucker, torsion, and bond angles was rigidly rotated in three dimensions and graphically displayed. The views show the best agreement between the model and various negatively stained clathrin trimers. The parameters used to simulate the trimer correspond to those that best fit a cage with "soccer ball" design (truncated icosahedron with 12 pentagonal hollow facets, each surrounded by 5 hexagonal ones, giving a total of 32 facets). In some other cases (not shown), the agreement between the simulated trimer and the negatively stained molecule could be improved by an increase of up to 55° in the torsion angle of the distal leg about the proximal leg.

FIGS. 4 AND 5. Effect of ionic composition on the appearance of glycerol spray-Pt rotary-shadowed clathrin trimers. Two microliters of stock solution of clathrin (0.8 mg/ml, in 10 m*M* ammonium acetate, pH 6.7) was mixed with 0.1 ml of 45% (v/v) glycerol, sprayed onto mica, and rotary-shadowed with platinum. In this low ionic strength condition 75% of trimers display a uniform clockwise slew of their legs. (Fig. 4) Two microliters of same stock clathrin solution was mixed with 0.1 ml of buffer containing 45% (v/v) glycerol and 50 m*M* ammonium acetate, pH 6.7. Less than 1% of trimers display a uniform clockwise bent of their legs (Fig. 5). × 62 500.

FIGS. 6–8. Appearance of clathrin trimers adsorbed to mica and visualized after quick-freezing, freeze-drying, and rotary-replication. Clathrin (conc. 10 μg/ml) was adsorbed to mica flakes in the presence of 10 m*M* Tris–HCl, pH 8.0, 100 m*M* NaCl (Fig. 6), or 100 m*M* TEA, pH 8.0 (Figs. 7 and 8). Note that in Fig. 7, one molecule (arrow) has attached in the clockwise configuration that predominated in Tris-containing solutions (e.g., Fig. 6). These stereo figures can be viewed with a pocket stereo viewer. × 261 500.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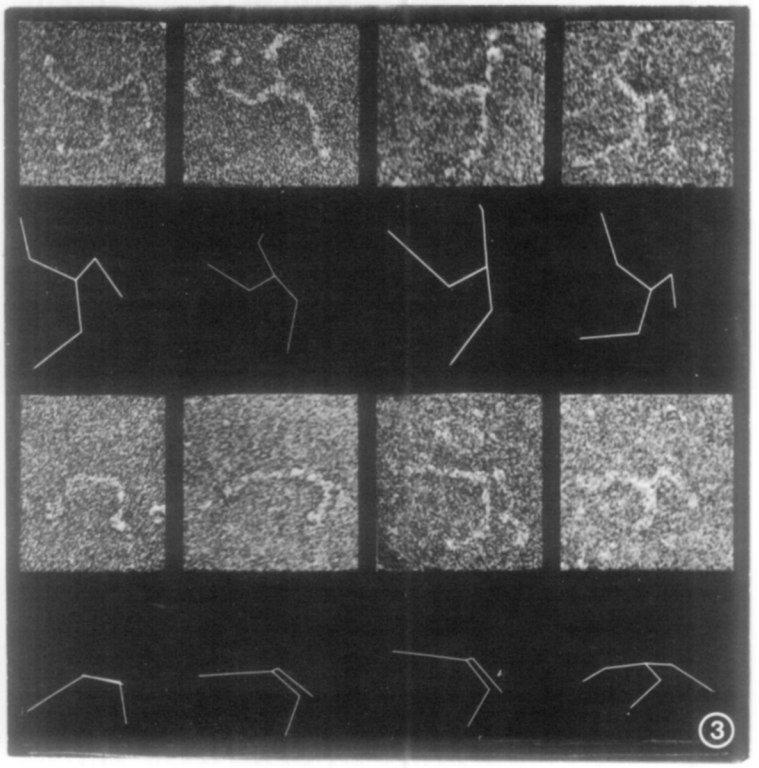
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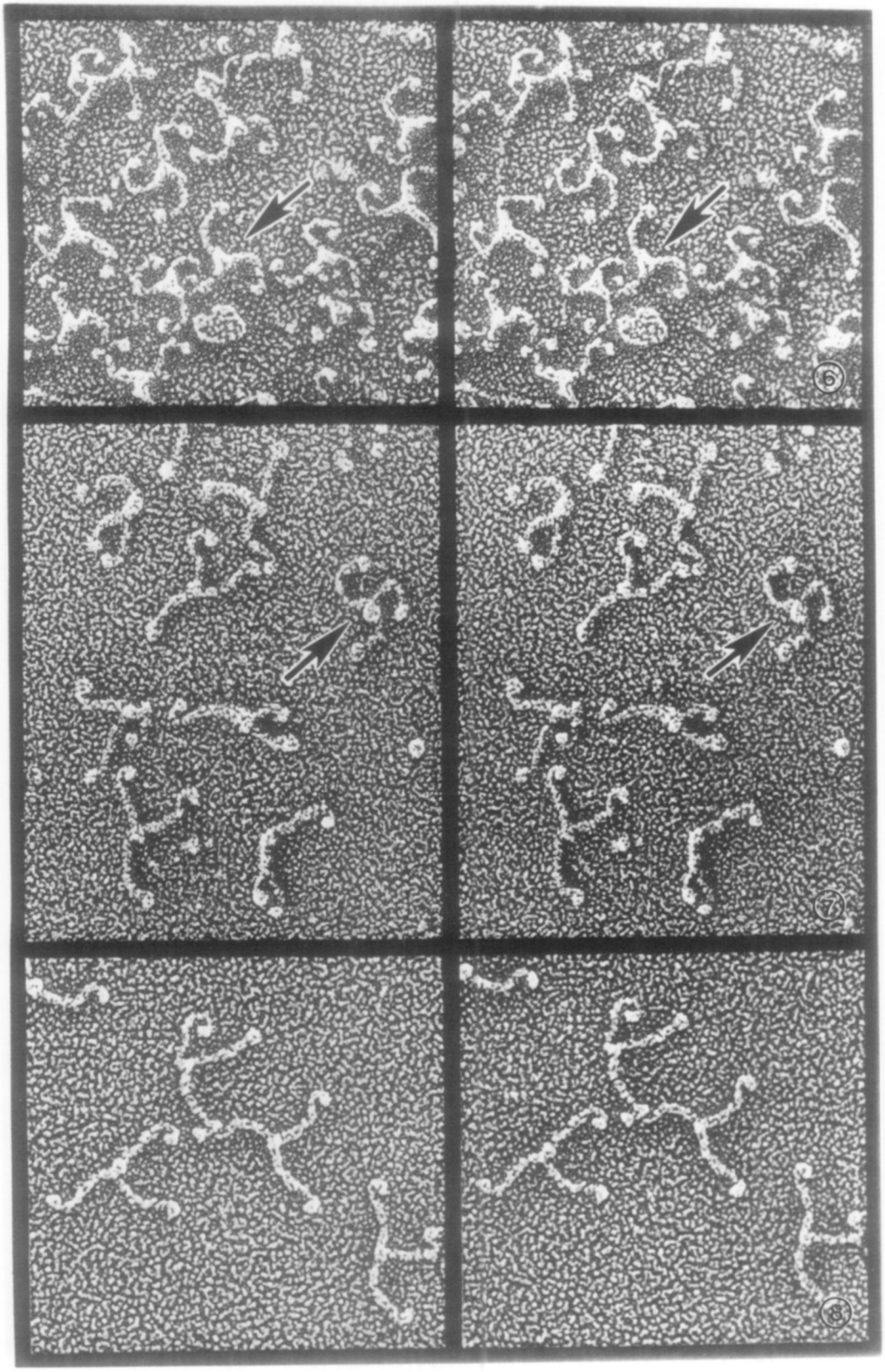
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TABLE I

DETERMINATION OF LEG CONTOUR LENGTHS AND END-TO-END DISTANCE BETWEEN TERMINAL DOMAINS OF CLATHRIN TRIMERS

	Appearance of clathrin legs			
	Clockwise		Counterclockwise	
Type of sample	Leg length (nm)	End-to-end distance (nm)	Leg length (nm)	End-to-end distance (nm)
Clathrin[a] (quick-freeze)	47.2 ± 0.7 (75)	45.5 ± 1.0 (75)	50.0 ± 0.5 (90)	60.7 ± 1.7 (90)
Clathrin[b] (glycerol spray)	50.7 ± 1.1 (72)	57.2 ± 1.2 (72)	52.2 ± 0.7 (60)	61.4 ± 2.2 (60)
Clathrin heavy chain[c] (glycerol spray)	47.9 ± 0.6 (72)	56.6 ± 1.1 (72)	52.1 ± 0.7 (60)	66.6 ± 2.1 (60)
	50.8 ± 0.7 (84)	55.8 ± 1.3 (84)	51.7 ± 0.7 (84)	64.9 ± 1.7 (84)

Note. Unambiguous images were scored as indicated. Clockwise refers to images of clathrin where the three legs display a clockwise slew. The counterclockwise category includes all other molecules with one or more legs which either display counterclockwise slew or had rather extended disposition. Leg lengths were measured from tracings along the leg from center of trimer to distal end of its terminal domain and were corrected for average platinum grain diameter in the background of same field. End-to-end distance between terminal domains is the linear distance between the center of terminal domains in a single trimer. Measurements are expressed as average ± SE.

[a] Clockwise images, obtained by incubation of clathrin with mica in 50 m*M* Tris, pH 8.3, and 50 m*M* NaCl. Counterclockwise images obtained in 50 m*M* TEA, pH 8.3.

[b] Data from two independent experiments: Clathrin stock solution (~0.8 mg/ml) in either 10 m*M* Tris-HCl, pH 7.1, 0.1 m*M* EDTA, or 10 m*M* ammonium acetate, pH 6.6; 0.1 m*M* EDTA was diluted 50 fold into 45% glycerol (60 and 75% of images with clockwise slew, respectively), or 45% glycerol, 50 m*M* Tris, pH 7.1, or 50 m*M* ammonium acetate, pH 6.6, respectively (98% counterclockwise images in both cases).

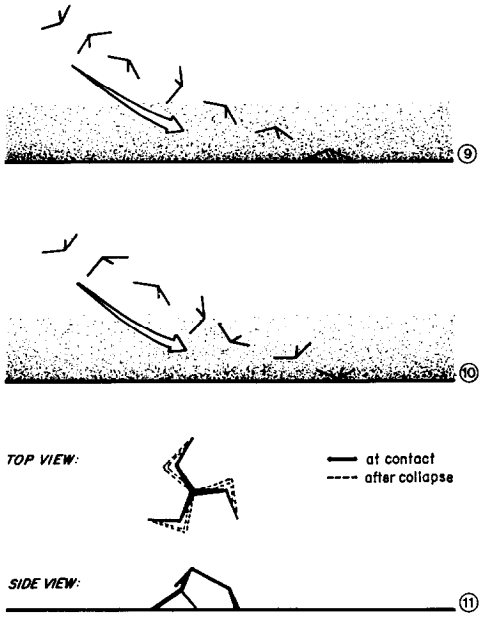
[c] At least 95% of light chains were removed as determined by SDS–PAGE. Heavy chains (conc. 0.5 mg/ml in 25 m*M* Tris–HCl, pH 7.2) were diluted into 45% glycerol (27% of images show clockwise slew) or 45% glycerol, 50 m*M* ammonium acetate, pH 6.6 (95% of images with counterclockwise slew).

I). (Variability in apparent contour length can arise from differences in the configuration of the link between distal leg and terminal domain, as discussed in more detail below.) The separation between terminal domains within a given trimer is a measure of how extended are its legs at the"elbow." This separation is about 45.5 nm in clockwise freeze-etched images and about 57 nm in clockwise glycerol sprayed images. Comparison of Figs. 4 and 6 illustrates this difference directly; legs of freeze-etched molecules do indeed appear more "curled." Furthermore, freeze-etched trimers in the clockwise configuration show an additional deposition of platinum at the center or vertex of each molecule (arrow, Fig. 6, see also 7). This decoration does not seem to reflect the presence of light chains at the vertex, because we find that clathrin depleted of light chains by the KSCN procedure of Winkler and Stanley (*20*) still displays this feature when adsorbed in the clockwise orientation (picture not shown).

These observations on clockwise images are consistent with the notion that a puckered and relatively stiff clathrin trimer has struck the mica like a spider (terminal domains first) when approaching from its concave side (Figs. 4, 6, and 9). It would then collapse internally, perhaps with a screwing motion that would retain the threefold symmetry, until its vertex also contacted the mica (see schematic drawing, Fig. 11). Thus, the ~45-nm separation of terminal domains in the quick-freeze images would represent a three-point landing or "footprint" and should reflect how extended the molecule was in solution. We note that this value is very close to the distance between any two of the three noncontiguous vertices occupied by the distal ends of a single trimer in a clathrin lattice. (For this calculation, we constructed a plane that intercepted all three

of these vertices, and then measured their linear separation. In the case of the soccer-ball design—truncated icosahedron—a 45-nm spread was obtained.) This observation is thus consistent with the notion that terminal domains would tend to "hang down" under the vertices of an assembled cage. This in fact appears to be the case as nicely shown in the recent work of Vigers *et al.* (*19*), who used a three-dimensional reconstruction made from electron micrographs of frozen hydrated samples to study the structure of clathrin cages. Under each vertex of the coat they find finger-like projections that are assigned to the terminal domains.

Legs in the second (counterclockwise) class of images have a comparatively larger spread (Figs. 5, 7, and 8, Table I). The average distance between terminal domains in a trimer is about 61 nm (quick-freeze) or 62 nm (glycerol spray), both values significantly larger than the corresponding values for clockwise images. The average apparent contour length of an leg is about 50 nm (quick-freeze) or 52 nm (glycerol spray). The interpretation we propose for this class of images is that they have been generated by trimers that approach and strike the mica from their convex side (vertex first, see Fig. 10). The legs are not constrained during collapse and thus undergo more distortion, limited only by their intrinsic stiffness. Indeed, these images show relatively greater loss of threefold symmetry at the vertex and loss of the sharp bend at the proximal/distal joint. The overall spread between adjacent terminal domains is thus larger and more variable than in images of the clockwise and more symmetrical class. Molecules oriented in this way do not display any additional platinum decoration at their vertices (whether or not light chains are present), and thus they appear smooth (see Figs. 7 and 8). This striking difference in the vertex appearance between the two classes of images further substantiates the notion that they correspond to views from opposite sides of the clathrin trimer.



FIGS. 9–11. Schematic representation of the adsorption of clathrin to mica. Puckered clathrin trimers are shown approaching a mica surface from their concave (Fig. 9) or convex sides (Fig. 10). (For simplicity, the drawings show only the proximal legs. The stippling is intended to suggest that some sort of "charge cloud" may extend from the surface of the mica and affect the orientation of the molecules just before they strike.) The diagram in Fig. 11 provides a top and side view of a trimer showing the consequences of adsorption to mica. The dotted line illustrates the outcome of the internal collapse that would result from a three-point landing (e.g., approach from the concave side, as in Fig. 8). This collapse would probably include a "screwing" motion that would increase the observed bends of the proximal/distal hinges.

These experiments constitute the first direct evidence for the absolute hand of the clathrin triskelion. Our analysis indicates that clathrin legs display a clockwise slew when viewed from what would be the cytoplasmic (convex) side of the intact coat. This interpretation is in agreement with a previously postulated orientation of the legs, based on the inhibition of assembly of clathrin lattices while adsorbing to an electron microscope grid (*1*).

We wish to emphasize the significance of ionic conditions during adsorption to mica for the orientation of adsorbed molecules and for the consequent appearance of their

legs. Apparent random direction of slew and loss of threefold symmetry should not be taken as evidence for extensive flexibility at the center or at the elbow, since those distortions correlate with adsorption from the convex face. We also observe that the light chains do not appear to change the apparent stiffness of the leg as judged by the spread of distal ends (Table I).

3. A Flexible Hinge between the Distal Leg and the Terminal Domain

The total apparent length of a leg, in images from any of the mica adsorption techniques we have used, varies between 47 and 52 nm. We believe that this variation is due to flexible disposition of the terminal domain. We find that the best images to illustrate this point are obtained with the quick-freeze, deep-etch samples (*8*). Improved preservation obtained with this technique confirms our previous assertion that the terminal domain is a distinct, globular structure (*11*). In trimers that attach to mica from their concave side (clockwise slew), legs are on the average ~47 nm long and usually end with no clear demarcation between the terminal domain and the rest of the leg (Fig. 6). In trimers that attach from their convex side (counterclockwise slew), legs are ~50 nm long and end with a short and narrow segment to which the globular terminal domain is attached (Figs. 7 and 8). We presume that in concave-side adsorption, the terminal domain tends to fold back against the leg, making the apparent contour length shorter; in convex-side adsorption, the terminal domain lies beside the leg, the connection with the leg can be traced unambiguously, and the apparent total leg length is greater. Analysis of a number of fields shows no strong correlation of terminal-domain orientation and slew of the leg. The various orientations of the terminal domain suggest a flexible joint. The same suggestion has previously been made because of the relative ease with which the terminal domain can be cleaved from the rest of the leg (*11*).

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