Clathrin Cubes: An Extreme Variant of the Normal Cage

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Abstract. Clathrin triskelions form polyhedral cages with hexagonal and pentagonal faces when dialyzed against suitable assembly buffers. However, when the buffer is made 12% saturated in ammonium sulfate and the dialysis is performed at 4°C, clathrin polymerizes into cubes. The cube is constructed from eight triskelions with one at each corner. The edge length of the cube is ~45 nm, equivalent to the length of the leg of a triskelion. Thus, each edge of the cube is composed of two antiparallel legs overlapping over their whole length. The interactions between the legs in the cube are a subset of those postulated to occur in cages. Indeed, the cube can be derived from a pentagonal dodecahedron by removing 12 of the 20 triskelions with only slight adjustment of the legs of the remaining triskelions. The cube forms regular arrays and appears to be a favorable species for crystallization of clathrin.

Clathrin forms the characteristic polyhedral lattice of hexagons and pentagons on the cytoplasmic surface of coated regions of cell membrane (Kanaseki and Kadota, 1969; Pearse, 1975; Crowther et al., 1976; Heuser, 1980). Soluble clathrin exists as a trimer of three identical 180-kD heavy chains, to each of which is bound one of two types of 30-40-kD light chains (Pearse, 1978; Ungewickell and Branton, 1981; Kirchhausen and Harrison, 1981). When visualized in the electron microscope after negative staining or rotary shadowing, the trimer is seen to be a triskelion consisting of three thin jointed legs radiating from a mass at the apex (Ungewickell and Branton, 1981; Kirchhausen and Harrison, 1981). Each 44-nm long leg can be divided into at least three domains based on shape and proteolytic sensitivity: a slender proximal segment that binds light chains, an equally slender distal segment, and a globular terminal domain (Schmid et al., 1982; Ungewickell et al., 1982; Kirchhausen and Harrison, 1984). Under suitable conditions triskelions assemble in vitro into cages of heterogeneous size with pentagonal and hexagonal faces like the surface lattices seen in vivo on coated vesicles (Ungewickell and Branton, 1981; Crowther and Pearse, 1981). Triskelions are centered at vertices in the cage lattice and each leg spans two edges (Crowther and Pearse, 1981). Thus, each edge in a cage is composed of two antiparallel proximal leg regions and two antiparallel distal leg regions, the precise arrangement of which is unknown. The globular terminal domains lie in groups of three beneath each vertex (Vigers et al., 1986) and, in conjunction with other 100 and 50-kD coat proteins, are believed to mediate interactions with certain membrane receptors (Pearse and Bretschger, 1981; Pearse, 1985).

We describe here the isolation in high yield of a previously unreported form of clathrin assembly that is constructed from eight triskelions in a cubic framework. For the sake of brevity, we will refer to this structure as a cube, reserving the name cage for assemblies with pentagonal and hexagonal faces. Cubes are produced by dialyzing extracted triskelions against buffers containing ammonium sulfate at pH 6.0. Cubes have approximately twice the edge length of cages and the cube edge is therefore probably composed of two antiparallel clathrin legs. This suggests that a subset of the allowable leg-leg interactions in cages occurs in the cube and that these are sufficient to hold a structure together.

At higher ammonium sulfate concentration cubes aggregate to form two- and three-dimensional ordered arrays consisting of up to several thousand unit cells. These results indicate that clathrin packed in a cubic framework may form crystals suitable for structure determination.

Materials and Methods

Assembly and Purification of Clathrin Cubes

Clathrin was purified from bullock brains as described previously (Pearse and Robinson, 1984), except that low and high speed pellets normally discarded were re-extracted once with the appropriate buffer to increase the final yield from twelve brains to 100-120 mg clathrin. Clathrin was stored as assembled cages in 0.1 M 2-(N-morpholino)ethane sulfonic acid (MES)-NaOH, pH 6.5, 0.2 mM EDTA, 0.5 mM MgCl2, 0.02% NaN3, 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Cages were disassembled by addition of an equal volume of 2 M Tris-HCl, pH 7.0, 2 mM EDTA, 0.1% 2-mercaptoethanol, 0.2 mM PMSF, dialyzed overnight against 40 mM Tris-HCl, pH 7.0, 1 mM EDTA, 0.01% 2-mercaptoethanol, 0.2 mM PMSF (low Tris buffer), and clarified by centrifugation at 200,000 g for 45 min at 5°C. Trimers were then concentrated to a protein concentration of 8-10 mg/ml by externally coating a dialysis bag containing the dilute solution of trimers with dehydrated carboxy-methyl cellulose (Aquacide II, Calbiochem-Behring Corp., La Jolla, CA) for several hours, and then re-dialyzing against low Tris buffer.

Abbreviations used in this paper: MES, 2-(N-morpholino)ethane sulfonic acid; PMSF, phenylmethylsulfonyl fluoride.
Cubes were assembled by dialyzing concentrated trimers in low Tris buffer, pH 7.5, against cube assembly buffer: 0.1 M MES-NaOH, pH 6.0, 1 mM EDTA, 0.2 mM PMSF, 12% saturated ammonium sulfate. They were purified by layering up to 3 ml of sample onto 12.5 ml 5-30% (wt/vol) linear sucrose gradients, made up in 0.1 M MES-NaOH, pH 5.5, 1 mM EDTA, 12% saturated ammonium sulfate, and centrifuged at 200,000 g for 3 h at 5°C. Fractions containing cubes were identified by electron microscopy, pooled, and dialyzed against cube storage buffer: 0.1 M MES-NaOH, pH 6.0, 1 mM EDTA, 20% saturated ammonium sulfate. The resulting dialysate was centrifuged for 15 min in an Eppendorf centrifuge to remove any precipitate. Cubes were concentrated 1.5-fold in dialysis bags coated with Aquacide II as described above and then dialyzed against cube storage buffer. Several cycles of concentration and dialysis, each 2-3 h in duration, were required to achieve a final concentration of 5-8 mg/ml protein. Attempts to concentrate cubes by vacuum dialysis or by pelleting in an ultracentrifuge resulted in the formation of intractable precipitates.

Protein concentration was routinely determined using a Bradford assay (Bio-Rad Laboratories, Richmond, CA) with clathrin trimers as a standard assuming E ̇ₐ₅₉₅ (280 nm) = 11.0 (Winkler and Stanley, 1983). Clathrin samples were analyzed by electrophoresis on 10% SDS polyacrylamide gels (Laemmli, 1970).

**Molecular Weight Determination of Cubes**

Samples of cubes (purified by sucrose density gradient centrifugation) in 100 mM MES-NaOH, pH 6.0, 1 mM EDTA, and 12% saturated ammonium sulfate at a concentration of 0.09-0.50 mg/ml were sedimented at a speed of 25,000 rpm at 20°C in a Beckman L8 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA) with a Prep UV Scanner interfaced to an Apple III microcomputer (Apple Computer Inc., Cupertino, CA) for data collection and analysis. Sedimentation coefficients at various concentrations were calculated from linear regression of ln(r) against t², where r is the weight average position of the boundary. Similar samples of cubes at a concentration of 0.07 mg/ml in the same buffer were filtered through 0.22-μm millipore filters and used for the determination of their diffusion coefficient in a Malvern Instruments laser light-scattering system (Malvern, Worcestershire, England).

The density of the buffer in which the measurements of the sedimentation and diffusion coefficients of the cubes were made and that of a concentrated sample of clathrin cages, exhaustively dialyzed against this buffer, was determined on a Parr density meter DMA02D (Anton Parr, Graz, Austria). The concentration of cages used for the density measurements was estimated by amino acid analysis of samples of known weight and volume. From the density measurements and the estimated protein concentration, the density increment of the clathrin in the cube buffer was calculated to be 0.119 mg/ml, corresponding to an apparent partial specific volume of 0.7 ml/g with a measured solvent density of 1.0417 g/ml. The molecular weight of the cubes was calculated from the sedimentation and diffusion coefficients using the standard equation (Svedberg and Pedersen, 1940)

\[ M = \frac{RTS}{D(k^2 - 1)} \]

**Characterization of Cube Stability and Solubility**

To characterize conditions in which cubes disassemble, 10-μl samples of cubes at 3-5 mg/ml were diluted into 50 μl of buffers containing 3-12% saturated ammonium sulfate, 1 mM MgCl₂, and either 0.1 M MES in the pH range 6.5-5.5 or 0.1 M sodium acetate in the pH range 5.5-5.0. Samples were withdrawn for electron microscopy after 1 and 48 h, and randomly selected fields were examined for the presence of cubes. The precipitation point of cubes was determined in the pH range 5.5-6.5 by adjusting the ammonium sulfate concentration to 18-30% saturation in 50 μl samples of cubes at 3.5-5 mg/ml in small glass vials containing 0.1 M MES and either 1 mM MgCl₂ or 1 mM EDTA. The vials were stored at 4°C and examined periodically over a 6 h period. Below pH 5.5, 20-μl samples were dialyzed against 0.1 M sodium acetate, 1 mM MgCl₂, 5-20% saturated ammonium sulfate in small plastic buttons. Samples for crystallization, 5-6 mg/ml in protein, were prepared in the same way. They were examined in a dissecting microscope fitted with polarizing filters, and samples were periodically withdrawn for electron microscopy.

**Electron Microscopy**

For shadowing, the crystal sample was diluted by 1/30 in the appropriate ammonium sulfate solution and a drop applied to freshly cleaved mica. After a few seconds the mica surface was washed with a 0.1% solution of uranyl acetate in 12% ammonium sulfate, thoroughly washed with water, air dried, and shadowed with platinum at an angle of ~25°. Carbon was evaporated and the replica floated from the mica onto water, picked up on 400-mesh copper grids. No regular images were found in the absence of the uranyl acetate step, which thus seemed to act as a fixing procedure. For negative staining, samples were applied to carbon-coated grids, washed with water, and stained with a few drops of 1% uranyl acetate. Specimens were examined in a Philips EM300 or EM301.

**Results**

**Formation of Cubes**

When clathrin trimers are dialyzed against assembly buffer, normal cages consisting of hexagons and pentagons are formed (Ungewickell and Branton, 1981; Crowther and Pearse, 1981). However, when the buffer is made 12% saturated in ammonium sulfate and the dialysis performed at 4°C, a substantial proportion of the clathrin assembles into a quite distinct structure. Specimens photographed in the electron microscope (Fig. 1) show a population of particles, many of which display features characteristic of an open cube. The clearest class of image shows a square profile with dots at the corners, corresponding to the view of a cube along a fourfold axis of symmetry. Other common images resemble a buckle or a circle with a dot in the middle and correspond to views of a cube along a two- or threefold axis, respectively. The edge length of the cube is ~45 nm, which is approximately equal to the length of leg of a triskelion. A natural way to construct the cube would thus be to have a triskelion at each of the eight vertices. Each leg of a triskelion would then run from one vertex to another and interact along its length with an oppositely directed leg from a neighboring vertex. This packing is to be contrasted with that in normal cages, in which the edge length is only 18.5 nm and in which each leg of a triskelion runs along two neighboring polygonal edges.

**Isolation and Characterization of Cubes**

Optimum conditions for forming cubes were found to be dialysis overnight of clathrin trimers against 0.1 M MES-NaOH, pH 6.0, 1 mM EDTA, 0.2 mM PMSF, made 12% saturated in ammonium sulfate (cube assembly buffer) at 4°C. Lower concentrations of ammonium sulfate in the assembly buffer raised the proportion of cages, and higher concentrations increased nonproductive precipitation at the expense of assembly. Assembly is most efficient at 4°C, roughly half as efficient at 15°C, and does not occur at room temperature. The proportion of trimers that forms cubes increases with protein concentration. In optimal conditions of buffer and temperature, cubes are not formed at protein concentrations <0.2 mg/ml but above this concentration the yield of cubes increases steadily to a maximum of 50% at 1.0 mg/ml and above. The replacement of EDTA by either 1 mM MgCl₂ or CaCl₂ had no effect on cube formation but increased the formation of small aggregates of cubes.

The stability of cubes is strongly dependent on ammonium sulfate concentration and pH. Cubes appear to be most stable in 0.1 M MES-NaOH, pH 5.5, 1 mM EDTA, 20% saturated in ammonium sulfate (storage buffer) and under these conditions have a tendency to form small multimeric aggregates. The rationale for our purification of cubes is to isolate them.
rapidly as a monomer species on a 5–30% linear sucrose gradient and then to shift them to stabilizing conditions for concentration and subsequent analysis. Concentration of purified cubes proved difficult because of their tendency to form intractable precipitates. Effective concentration was achieved by covering dialysis bags containing dilute cube solutions with dehydrated carboxymethyl-cellulose for several hours.

A typical sucrose gradient separation of a crude preparation is shown in Fig. 2 a. The major peak corresponds to monomer cubes, as judged by electron microscopy of the corresponding fraction, and the main contaminant is free triskelions, which remain at the top of the gradient. Sucrose gradients can also be used for monitoring the stability of the isolated cubes (Fig. 2, b and c). Samples of cubes stored in 12% saturated ammonium sulfate decay over the course of several days into an increasingly heterogeneous mixture of small cages and cage fragments. However this rearrangement does not occur at 20% saturated ammonium sulfate and above. Cubes are stable at these higher ammonium sulfate concentrations for many months, though in equilibrium with triskelions.

The integrity of the clathrin polypeptides in gradient-purified cubes was analyzed by electrophoresis on SDS polyacrylamide gels. Cubes consist of intact heavy chains associated with a normal complement of light chains (Fig. 2 d). Thus formation of cubes is not due to proteolysis or the loss of light chains.

As discussed above, the cubic aggregate would be expected to contain eight triskelions. The molecular mass of a triskelion is 635,000 D (Ungewickell and Branton, 1981), yielding a predicted molecular mass for the cube of ~5 × 10^6 D. This is too large for conventional equilibrium sedimentation on our Beckman L8 centrifuge, and such analysis would be complicated by the presence of free triskelions. We therefore estimated the molecular weight from measured diffusion and sedimentation coefficients. Laser light scattering gave a $D_{20w}$ of 7.5 × 10^{-8} cm^2 s^{-1}. Velocity sedimentation gave an $S_{20w}$ of 42S. Combined with a measured partial specific volume of 0.7 ml gm^{-1}, the calculated molecular mass of the cube is 5.0 × 10^6 D. This is consistent with the idea that the cube is the major species present and is constructed from eight triskelions. Assuming a protein hydration of 0.3 g/g, the frictional ratio $f/f_o$ is calculated to be 2.3. This frictional ratio is incompatible with a compact structure even of extreme ellipticity (Oncley, 1941). It implies that the cube in solution is an open free draining object, as indeed it appears to be in the electron micrographs of negatively stained samples (Fig. 1 a).

**Formation of Aggregates of Cubes**

The cube represents an attractive species for producing crystals of clathrin, which are needed if the detailed structure and function of the molecule is to be understood. Indeed when working with various cube preparations, small regular aggregates were sometimes observed (see Figs. 4 and 6). To understand the factors governing the stability of cubes and their aggregation, the effect of varying ammonium sulfate concentration and pH were investigated. The results are summarized in the phase diagram in Fig. 3.

Broadly speaking, cubes dissociate at ammonium sulfate concentrations below ~12% saturation, stabilize at moderate concentrations, and precipitate at higher concentrations. Higher concentrations of ammonium sulfate appear to promote the formation of small multimers of cubes and to precipitate out any free triskelions. Cube solubility decreases gradually near the precipitation boundary; typically there is a difference of 4–6% in ammonium sulfate saturation between conditions producing light and heavy precipitates (dark stippling in Fig. 3). The solubility of cubes is strongly dependent on pH and is decreased in the presence of divalent cations. For example, at pH 6.0 in the presence of 1 mM EDTA the precipitation occurs at 34–40% saturation in am-
Sucrose gradient profiles of cube preparations. Cubes at a concentration of 2.5 mg/ml were adjusted to either 12 or 20% saturated ammonium sulfate in 0.1 M MES, 1 mM EDTA, pH 6.0 and stored at 4°C. All samples were readjusted to 12% saturated ammonium sulfate immediately before analysis on 5–30% linear sucrose gradients. The vertical scale represents the OD_{595} as determined on gradient fractions using a Bradford protein microassay (Bio-Rad Laboratories). Roughly 70% of the material applied to each gradient was recovered in the fractions; the remainder is presumed to have sedimented to the bottom of the tube. (a) Freshly prepared cubes. Peak 1 contains cubes (75% of recovered protein) and peak 2, triskelions (25% of recovered protein). The heavy line shows the continuous trace of OD_{280} (peak value OD_{cmm} [280 nm] = 0.6) and demonstrates the symmetry of peak 1, supporting the conclusion that cubes are a single species. (b) Cubes after 7 d storage in 20% saturated ammonium sulfate. The main peak, contains 80% of recovered protein. (c) Cubes after 7 d storage in 12% ammonium sulfate. The main peak indicated by a dashed line contains 45% of recovered protein. (d) 10% SDS gel of 5 μg of purified cubes (lane 1) and the original clathrin preparation (lane 2).

Figure 3. A structural phase diagram showing the relationship between trimers and trimer precipitates, monomeric cubes, and various cube aggregates. Buffers contained 1 mM MgCl₂, 5–30% saturated ammonium sulfate, and either 0.1 M MES in the pH range 6.5–5.5 or 0.1 M sodium acetate in the pH range 5.5–5.0. Vertical lines indicate conditions explored in crystallization trials. The best ordered arrays were seen in the range denoted by hatching, and consisted of up to 1,000 or more cubes. In the lightly stippled region, the tendency of cubes to aggregate into small multimers increased with rising ammonium sulfate concentration.

Figure 2. Sucrose gradient profiles of cube preparations. Cubes at a concentration of 2.5 mg/ml were adjusted to either 12 or 20% saturated ammonium sulfate in 0.1 M MES, 1 mM EDTA, pH 6.0 and stored at 4°C. All samples were readjusted to 12% saturated ammonium sulfate immediately before analysis on 5–30% linear sucrose gradients. The vertical scale represents the OD_{595} as determined on gradient fractions using a Bradford protein microassay (Bio-Rad Laboratories). Roughly 70% of the material applied to each gradient was recovered in the fractions; the remainder is presumed to have sedimented to the bottom of the tube. (a) Freshly prepared cubes. Peak 1 contains cubes (75% of recovered protein) and peak 2, triskelions (25% of recovered protein). The heavy line shows the continuous trace of OD_{280} (peak value OD_{cmm} [280 nm] = 0.6) and demonstrates the symmetry of peak 1, supporting the conclusion that cubes are a single species. (b) Cubes after 7 d storage in 20% saturated ammonium sulfate. The main peak, contains 80% of recovered protein. (c) Cubes after 7 d storage in 12% ammonium sulfate. The main peak indicated by a dashed line contains 45% of recovered protein. (d) 10% SDS gel of 5 μg of purified cubes (lane 1) and the original clathrin preparation (lane 2).

The most striking type of aggregate found (Figs. 4 and 5) consists of an open square packing of cubes, producing a pattern reminiscent of the foundations of an ancient building. The structure, which is visualized most clearly by shadowing (Figs. 4 a and 5 a), is seen very rarely in negatively stained preparations and is then poorly preserved (Fig. 4 b). The
Figure 4. Open square packing of cubes, (a) unidirectionally shadowed, (b) negatively stained. In each row, cubes appear to be successively rotated by 30° about an axis parallel to that row, so that every third cube, positioned at the intersection of two rows, is viewed down a fourfold axis of symmetry. In (a) the direction of unidirectional shadowing (arrow) is approximately along the diagonal of each square, giving good contrast on both sets of parallel rows of cubes. Bar, 0.2 μm.

packing appears to arise from having a fourfold view of the cube at the intersections of rows, with two intervening cubes rotated relative to this about an axis parallel to the row. This is seen most clearly in the filtered image (Fig. 5 c), which shows a dimpled fourfold view at intersections and a pair of views resembling the "buckle" view in between. This very unusual arrangement is consistent with the cubic symmetry of the particle, as it is hard to imagine any other class of symmetry giving rise to such a packing. The buckle views must be close to twofold views of the cube and so one might expect them to be rotated respectively by 30° and 60° relative to the fourfold view, to preserve equivalent packing along a row. A further 30° rotation would then produce the fourfold view at the next intersection of rows. Such a packing, incorporating successive 30° rotations about an axis parallel to the row, is shown diagrammatically in Fig. 5 d. However, although the two different buckle views within the pair do appear to be slightly staggered, it is not clear that they are sufficiently different to represent the 30° and 60° views. The orientations of the intervening pair of cubes may therefore be somewhat displaced from the perfectly equivalent packing shown in Fig. 5 d, possibly by drying or shadowing artifacts.

Further stacking of such layers of cubes may be responsible for the thicker aggregates of the type shown in Fig. 6, a and b. The stripes and checkerboard patterns seen in such aggregates are on a much coarser scale than the size of an individual cube and represent complicated superposition patterns arising from many layers of cubes.

Figure 5. Open square packing of cubes. (a) An example in which the direction of unidirectional shadowing (arrow) is roughly parallel to one set of rows of cubes and perpendicular to the other set. Bar, 0.2 μm. (b) Computed diffraction pattern of the array shown in a. The fourfold symmetry is broken because the shadowing does not contrast ridges on the specimen parallel to the shadowing direction. The resulting weak line in the transform (Smith and Kistler, 1977), perpendicular to the direction of shadowing, is indicated by the arrow. (c) Filtered image of a, which shows clearly the pattern of the dimpled fourfold view at the intersection of the rows and the rotated "buckle" type views presented by the intervening cubes. (d) Schematic diagram of packing of cubes along a row, showing successive 30° rotations about an axis parallel to the row, so that each cube makes equivalent interactions with its neighbors. However each of the buckle views in the filtered image (c) appears to be closer to the 45° view, so the packing may not be strictly equivalent.
Figure 6. Ordered aggregates of clathrin. (a) Unidirectionally shadowed aggregate, showing a coarse spacing of ~120 nm. Bar, 0.5 μm. (b) Negatively stained aggregate, showing an intermediate spacing of ~63 nm. Bar, 0.2 μm. (c) Negatively stained aggregate, showing fine spacings of ~20 nm. Bar, 0.2 μm.

A third type of aggregate is shown in Fig. 6 c. Here the scale of detail is smaller than an individual cube and the packing appears much more compact than those seen in Figs. 5 a and 6, a or b. We do not know whether this pattern arises from staggered layers of close-packed cubes or from some other arrangement of triskelions with a similar appearance. This type of array would be more favorable for x-ray diffraction, assuming large crystals could be grown, since the unit cell is likely to be much smaller than in the more open packings.

Discussion

In this paper we report the isolation of a previously un-described type of clathrin assembly, the cube. Cubes form when triskelions are dialyzed at 4°C against buffers of pH 5.5–6.2 made 12% saturated in ammonium sulfate. They sediment as a homogeneous species on sucrose velocity gradients (Fig. 2), allowing their separation from free triskelions and larger aggregates. Electron micrographs of fields of particles show views characteristic of a cube (Fig. 1). The edge length of the cube is 45 nm, roughly the length of a clathrin leg. The triskelions in cubes contain a normal complement of intact light and heavy chains (Fig. 2 d). The molecular weight determination is consistent with a structure containing eight triskelions and on the basis of the symmetry of the particle there would be a triskelion vertex at each corner of the cube (Fig. 7, b and c). The symmetry of the open square packing (Figs. 4 and 5) reinforces this interpretation. The cube thus is a very open structure containing only ~5% protein by volume and this is certainly the impression given by some negatively stained preparations (Fig. 4 b).

The formation of cubes is dependent upon protein concentration. Below 0.2 mg/ml protein, no cubes are formed, suggesting that this represents the critical concentration for formation of a nucleating structure. Assembly of discrete cubes approaches a maximum of 50% efficiency at 1 mg/ml, the rest of the material remaining as triskelions or forming larger aggregates. It appears that addition of further triskelions to the putative nucleating structure is not highly cooperative and that cubes exist in equilibrium with unassembled trimers. The sensitivity of cube formation to temperature suggests that the change in entropy during assembly is negative and has a relatively high absolute value.

Above 12% saturation in ammonium sulfate, free triskelions precipitate and cubes aggregate to form small multimers (Fig. 3), making discrete cubes relatively difficult to concentrate. However, multimeric cubes are more stable than individual cubes (Fig. 2). Thus, the contamination of cube preparations by triskelions and their tendency to become an increasingly heterogeneous mixture of small cages are minimized at ammonium sulfate concentrations high enough to promote aggregation.

Triskelions must have a variable conical angle at the vertex to enable them to form cages of different size and a flexible kink in the leg to form either hexagons or pentagons (Crowther and Pearse, 1981). We were at first surprised that clathrin could form small cubic cages. Micrographs indicate that isolated triskelions possess a characteristic skew and may be nonplanar. Thus the legs of a triskelion in a cube are unlikely to be mutually perpendicular or to run straight along the edges of an idealized cube. Instead, they probably bulge outwards and retain a bend between proximal and distal segments, as suggested by the rounded appearance of cubes in shallow stain. The relationship between cubes and cages constructed from hexagonal and pentagonal faces is revealed by a simple exercise. A cage with pentagonal dodecahedral symmetry (Fig. 7 a) can be transformed into a cubic assembly of eight trimers by the removal of 12 triskelions, followed by rotation of the legs at either the apical–proximal or proximal–distal junctions, so that pairs of legs come into contact and run symmetrically between the cube vertices, removing the skew of the triskelions.

This hypothetical transformation reveals four features of cubes: (a) Triskelion cubes could have roughly the same degree of curvature and conical angle as in small cages. (b) For the eight triskelions to form a cube they cannot possess skew.
In a cubic 432 arrangement of eight skew triskelions, the two legs along an edge would splay apart from the vertices to the edge center: in the cubes it is evidently energetically more favorable for the legs to make continuous side by side contact than to maintain skewness (Fig. 7b). (c) The interactions between triskelions in a cube are a subset of those present in cages with pentagonal and hexagonal faces. Cubes can be considered as "sparsely populated" cages; a pentagonal dodecahedron is built from a cube by adding a triskelion vertex at the proximal/distal junctions present in each cube edge. This geometric similarity suggests that the intramolecular contacts stabilizing cubes and cages may be very similar. (d) Each edge of a cube (Fig. 7c) consists of two overlapping legs rather than the four overlapping half-legs that occur in the edges of cages.

In conclusion, we have demonstrated the isolation of homogeneous preparations of a cubic cage of clathrin. These cubes are constructed from eight intact triskelions and are stable in conditions favoring multimeric association. Aggregates of cubes containing microcrystalline arrays can be prepared by precipitation with ammonium sulfate in the presence of magnesium. The strong tendency of cubes to form ordered arrays suggests that they are promising candidates for the formation of crystals suitable for x-ray diffraction.

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