

# Structural Domains of Clathrin Heavy Chains

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**ABSTRACT** We used a combination of electron microscopy and proteolytic dissection to study the substructure of the clathrin trimer. The fragments of a heavy chain generated by limited proteolysis of cages were examined by rotary shadowing after disassembly. Correlation of lengths and molecular weights allowed us to map certain cleavage points along an arm and to assign them to positions in a model for a cage. We found that a particularly stable fragment of 52,000–59,000  $M_r$  (depending on the enzyme) corresponded to the knob-like terminal domain at the tip of each arm.

Clathrin trimers are the assembly units of a polyhedral framework that is believed to coat and engulf defined regions of membrane, together with proteins, receptors, and their ligands (1–5). Coated pits represent the first stage of clathrin assembly that can be visualized by electron microscopy, and coated vesicles are the “final product”—that is, complete clathrin-enclosed vesicles (6–8).

Clathrin trimers are composed of three identical heavy chains, each with an attached light chain (9). They are pinwheel-like structures with three extended arms (9, 10), suitable for efficient assembly into a coat with hollow pentagonal and hexagonal facets (9, 11, 12). Electron microscopy has shown that the arms are ~45–50 nm in length and that there is a bend at ~16–18 nm from the apex, separating the proximal and distal segments (10, 12). The most distal part of the arm sometimes appears in images of shadowed or negatively stained molecules as a distinct structure (9, 10). We shall refer to it here as the terminal domain. The dimensions and designs of cages—the empty, coat-like structures into which pure clathrin reassembles *in vitro*—suggest that a clathrin arm spans at least two of the 19 nm long edges (9, 12).

Limited tryptic digestion of cages reassembled *in vitro* from purified calf brain clathrin has been shown to cleave heavy chains into two major families of peptides. The larger peptides, of  $M_r$  110,000–125,000, remain associated as cages; the smaller ones ( $M_r$  53,000 and 41,000) separate from the cages as soluble proteins (13). By studying rotary shadowing of trimers from trypsin-treated cages, Schmid et al. (13) concluded that the cleavage point between these peptides was at the elbow between the proximal and distal arms. Similar proteolytic dissection experiments led Ungewickell et al. (14) to suggest that a single arm might span only one edge, and that its distal portion, corresponding to the released fragment, might not be involved in assembly. However, this picture is at variance with packing models proposed by Crowther and

Pearse (12), on the basis of electron microscopy of partially assembled cages.

To establish a more precise correspondence between chemical and morphological fragments, we have used various proteases to generate subfragments of clathrin arms. Here we show that the released fragments include the terminal domain, which can be cleaved from trimers regardless of the assembly state. When clathrin participates in the lattice of a cage, the distal arm can be further degraded, but the proximal arm remains protected. In contrast, arms of isolated clathrin trimers are cleaved at various points throughout their length. Correlation of lengths and molecular weights allows us to map certain cleavage points along the arms of a trimer and to assign them to positions in a model for a cage.

## MATERIALS AND METHODS

**Purification of Clathrin:** Clathrin was obtained from calf-brain coated vesicles using a modification of protocols previously described (9, 15). A crude homogenate of five or six brains in 800 ml 0.1 M Na-2-(*N*-morpholino)ethane sulfonic acid, pH 6.5, 1 mM EGTA, 0.5 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol (DTT),<sup>1</sup> 0.02% NaN<sub>3</sub>, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) (solution A) was first centrifuged in a JA-10 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 10,000 rpm (30 min). The pellet (rehomogenized with 200 ml of solution A) and the supernatants were further clarified in a JA-14 rotor (Beckman Instruments) at 14,000 rpm (30 min). The supernatant was then centrifuged at 33,000 rpm for 70 min in a 35 rotor (Beckman Instruments) to yield a pellet of crude coated vesicles. These were resuspended in 60 ml of solution A, applied to the top of six sucrose step gradients (12 and 9 ml of 10% and 30% sucrose in solution A) and centrifuged in a SW 27 rotor (Beckman Instruments) at 25,000 rpm for 45 min. The 10% fraction was pooled, diluted with 2 vol of solution A and concentrated by centrifugation in a 35 rotor at 33,000 rpm for 70 min. Pellets were resuspended with 20 ml solution A and applied to the top of a second sucrose step gradient (12, 15, and 3 ml of 5%, 30%, and 60% sucrose in solution A, respectively) and centrifuged in a SW 27 rotor (Beckman Instruments) at 25,000 rpm for 25 min. The 5% fractions were

<sup>1</sup> Abbreviations used in this paper: DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride.

pooled, diluted twofold with solution A, and concentrated as before. To solubilize clathrin, we resuspended the coated vesicles in 2 M urea, 0.02 M Tris-HCl, pH 7.4 (measured at room temperature), 0.1 M NaCl, 1 mM EDTA, 0.5 mM DTT, 0.02% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, 0.5 mM PMSF (solution B), and incubated overnight. After onefold dilution with water, vesicles were separated from solubilized proteins by two sequential centrifugations on a 42 rotor (Beckman Instruments) at 40,000 rpm for 60 min. The supernatant was concentrated by rapid addition of one volume of 100% saturated ammonium sulfate, pH 7.0. The pellet obtained after 10 min of centrifugation at 10,000 rpm in a 35 rotor was rapidly resuspended with 4–5 ml solution B and applied to a Sepharose CL4B column (2 × 80 cm) equilibrated with solution B. The included clathrin was concentrated by ammonium sulfate precipitation as before, and dialyzed against 0.05 M triethanolamine, pH 7.0, 1 mM EGTA, 0.5 mM DTT, 0.02% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, 0.5 mM PMSF. The yield was 20–25 mg of purified clathrin. Unless otherwise stated, all procedures were carried out at 4°C.

**Assembly of Cages:** Clathrin cages were assembled by overnight dialysis of 0.8 mg/ml clathrin against 0.025 M Na-2-(*N*-morpholino)ethane sulfonic acid, pH 6.5, 0.5 mM DTT, 0.02% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, 0.5 mM PMSF (solution C). The pH of the dialysate was then lowered to 6.3, and dialysis was continued for a few hours. Self-assembly of the lattice was initiated by addition of 2 mM CaCl<sub>2</sub> to the dialysate (solution D), and dialysis was continued for at least 24 h. All dialysis steps were carried out at 4°C.

**Proteolytic Cleavage of Clathrin Trimers and Clathrin Cages:** Clathrin trimers and clathrin cages were digested at room temperature with chymotrypsin, elastase, subtilisin BPN, thermolysin, trypsin (all from Sigma Chemical Co., St. Louis, MO) and papain (Worthington Biochemicals, Freehold, NJ) at various clathrin:protease ratios. One batch of subtilisin gave reproducible cleavage patterns and was kept frozen in solution D. With other batches (including one from Boehringer Mannheim Biochemicals, Indianapolis, IN), the cleavage patterns were variable. All other enzyme solutions were freshly prepared. Before digestion with subtilisin and thermolysin, free clathrin trimers (in solution C) were made 2 mM CaCl<sub>2</sub> and 0.2 M NaCl (to inhibit self-assembly) and aggregates were removed by centrifugation in the airfuge (Beckman Instruments) at 100,000 rpm for 5 min (room temperature). For digestion with elastase, free trimers were kept in solution C. Cages were pelleted by a brief airfuge centrifugation and resuspended in solution D for incubation with protease. The proteolytic digestion was stopped by adding PMSF (50 mM in isopropanol) to a final concentration of 1 mM, or (for thermolysin) by adding phenanthroline to 0.1 mM. The extent of the reaction was followed by SDS PAGE (16) or by electron microscopy (see below).

**Purification of Terminal Domain:** Clathrin cages (10–20 mg) were digested with protease (according to the conditions outlined in the caption of Fig. 2). The released fragments were separated by high speed centrifugation (usually for 1 h at 60,000 rpm in a 70.1 rotor) and concentrated with 50% ammonium sulfate. The pellet was resuspended with 0.1 ml 50 mM Na-2-(*N*-morpholino)ethane sulfonic acid, pH 6.5, 1 mM EGTA, 0.5 mM DTT, 0.02% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, and 0.5 mM PMSF, and applied to a Sephadex G-100 column (0.5 × 40 cm) equilibrated with the same solution.

**Electron Microscopy:** For rotary shadowing and visualization with platinum, samples were suspended either in 1 vol of 1 M Tris, pH 10.0, or in 1 vol of solution B, diluted to ~10 µg/ml in 45% glycerol—50 mM ammonium acetate, pH 7.5, sprayed onto freshly cleaved mica, and dried *in vacuo* (17). Negatively stained samples were prepared by adsorption onto freshly glow-discharged carbon-film coated grids. Uranyl acetate (1.5%) was used for negative staining, and grids were examined at 80 kV with a nominal magnification of 36,000 in a JEOL 100CXII electron microscope. Tropomyosin tactoids and T4 bacteriophage tails were used as magnification standards, taking 39.5 and 4.1 nm as the repeat distance, respectively.

## RESULTS

### Electron Microscopy of Clathrin

Images of clathrin, rotary shadowed with platinum after spraying onto mica, show a planar structure with radiating arms, flattened onto the mica substrate (Fig. 1*a*). The arms appear curled, and the direction of bend at the elbow can vary substantially both within a field and within a trimer (see, for example, Fig. 1*a* and Fig. 5*a*). From two independent experiments, the arm lengths not corrected for Pt decoration of the tip were  $53.9 \pm 0.6$  nm ( $n = 117$ ) and  $52.0 \pm 0.9$  nm ( $n = 24$ ). Since the average diameters of Pt grains in the backgrounds of the same fields were 2.7 and 3.8 nm, an estimate for the corrected length is 50 nm. A prominent feature of the

arm is a terminal domain that appears to be somewhat thicker than the rest (Fig. 1, *a* and *b*, arrows).

With heavy unidirectional platinum coating, clathrin arms cast shadows that are relatively uniform along their length (Fig. 1*c*). The thickness of an arm estimated from the shadow length is 2.7–3.3 nm. With light shadowing the Pt accumulation is uneven along the arm, and sometimes a longer shadow appears at the terminal domain (Fig. 1*b*).

### Proteolytic Dissection of Clathrin in Cages

Clathrin cages are susceptible to proteolytic degradation, which generates definite fragments of the arms (13, 14). We studied digestion by a series of proteases in order to map points of accessibility and to correlate length and molecular weight. Fig. 2*a* shows SDS PAGE analysis of cleavage patterns obtained by treating clathrin cages with a series of proteases (see also Fig. 4*b*, which includes data with chymotrypsin). As previously shown (9), the light chains are readily accessible and extremely susceptible to proteolytic degradation. In contrast, the heavy chains are more resistant to proteolytic attack, and their cleavage produces a distinct pattern. After brief proteolysis, two families of peptides appear. The first one contains high molecular weight fragments of 130,000, 116,000, 105,000, and 90,000, and their relative proportions vary with the protease used. The simplest explanation of these data is that all proteases except papain attack preferentially the same subset of four cleavage regions. The second group contains peptides of smaller size, ~59,000, 52,000, and 41,000 *M<sub>r</sub>*. Subtilisin (and also chymotrypsin, data not shown) produces primarily a 52,000-*M<sub>r</sub>* fragment that is relatively resistant to further degradation. Thermolysin yields first a 59,000-*M<sub>r</sub>* fragment and subsequently a 52,000-*M<sub>r</sub>* fragment. Elastase produces first a fragment of 52,000 followed on further digestion by a fragment of 40,000 *M<sub>r</sub>*. A similar time course has been reported with trypsin (13).

The time course of thermolysin proteolysis is shown in Fig. 2*b*. The heavy chain is cleaved preferentially to the 105,000-*M<sub>r</sub>* species (Fig. 2*b*, lanes 8–11), which upon further digestion is cleaved in turn to the 90,000-*M<sub>r</sub>* fragment (Fig. 2*b*, lanes 10–12). The 59,000-*M<sub>r</sub>* fragment appears simultaneously with initial cleavage of the 180,000-*M<sub>r</sub>* heavy chain. A 52,000-*M<sub>r</sub>* fragment is also produced, which becomes more pronounced at later times in digestion. Partial proteolysis shows that the 59,000- and 52,000-*M<sub>r</sub>* fragments contain largely the same peptides (data not shown), and it is reasonable to suppose that one derives from the other.

The time course of subtilisin proteolysis is shown in Fig. 2*d*. After short intervals, only the heaviest fragments are produced (Fig. 2*d*, lane 8), while after longer proteolysis, these heavy fragments are chased into the 90,000-*M<sub>r</sub>* fragment (Fig. 2*d*, lane 9). Close inspection of the cleavage pattern shows that some of the 105,000- and 90,000-*M<sub>r</sub>* fragments are further cleaved into species of ~103,000 and 88,000 *M<sub>r</sub>*. The 52,000-*M<sub>r</sub>* fragment appears simultaneously with cleavage of the 180,000-*M<sub>r</sub>* heavy chain, and, in contrast to the other fragments, it is extremely resistant to further degradation. From this time course, we conclude that initial cleavage of the 180,000-*M<sub>r</sub>* heavy chain usually generates the 52,000-*M<sub>r</sub>* fragment and its complement and that the latter is extremely susceptible to further proteolysis, generating the series of fragments that vary in molecular weight down to 90,000 *M<sub>r</sub>*.

We used electron microscopy to map the principal cleavage sites to positions on the clathrin arm. With all enzymes, the

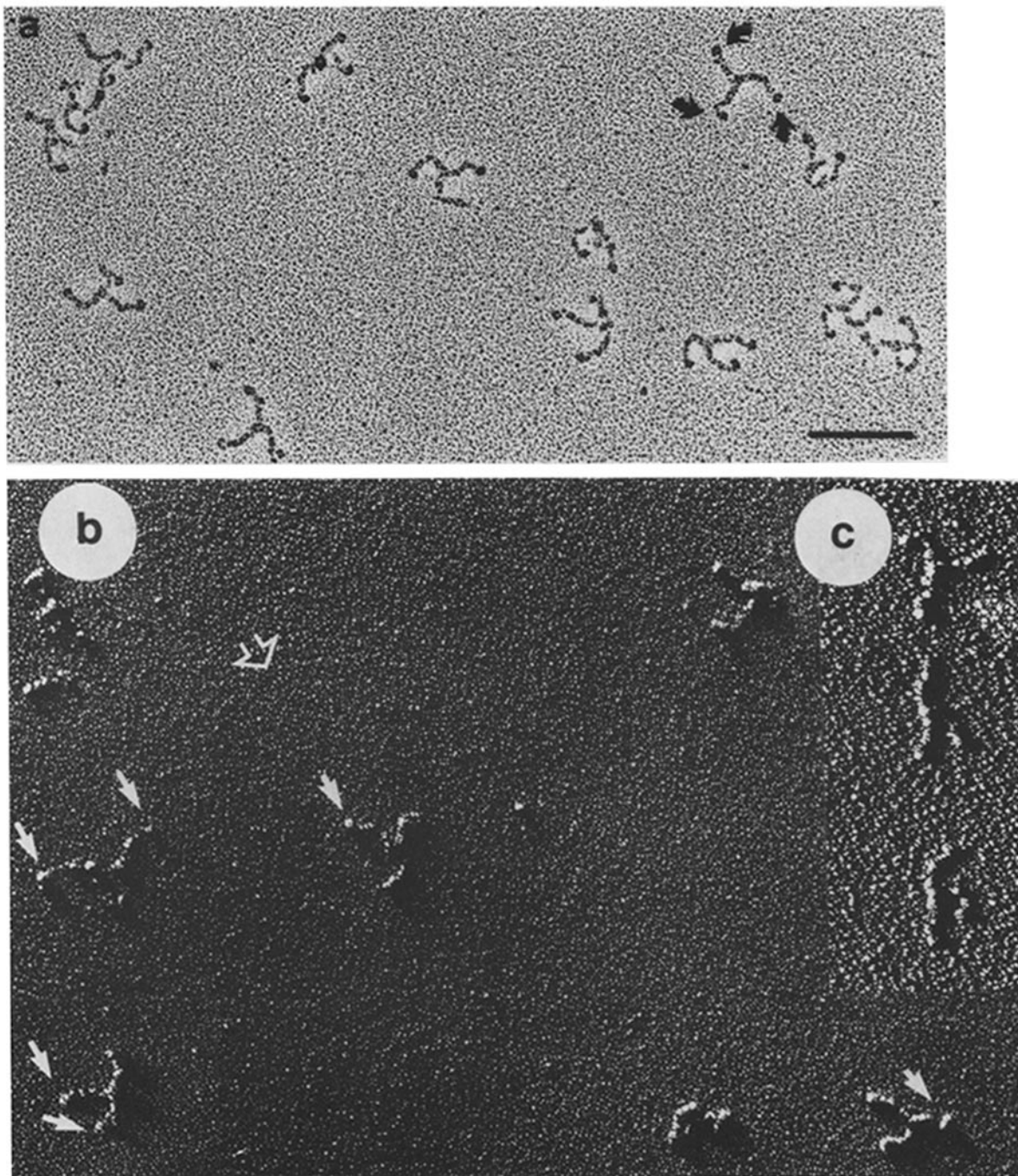
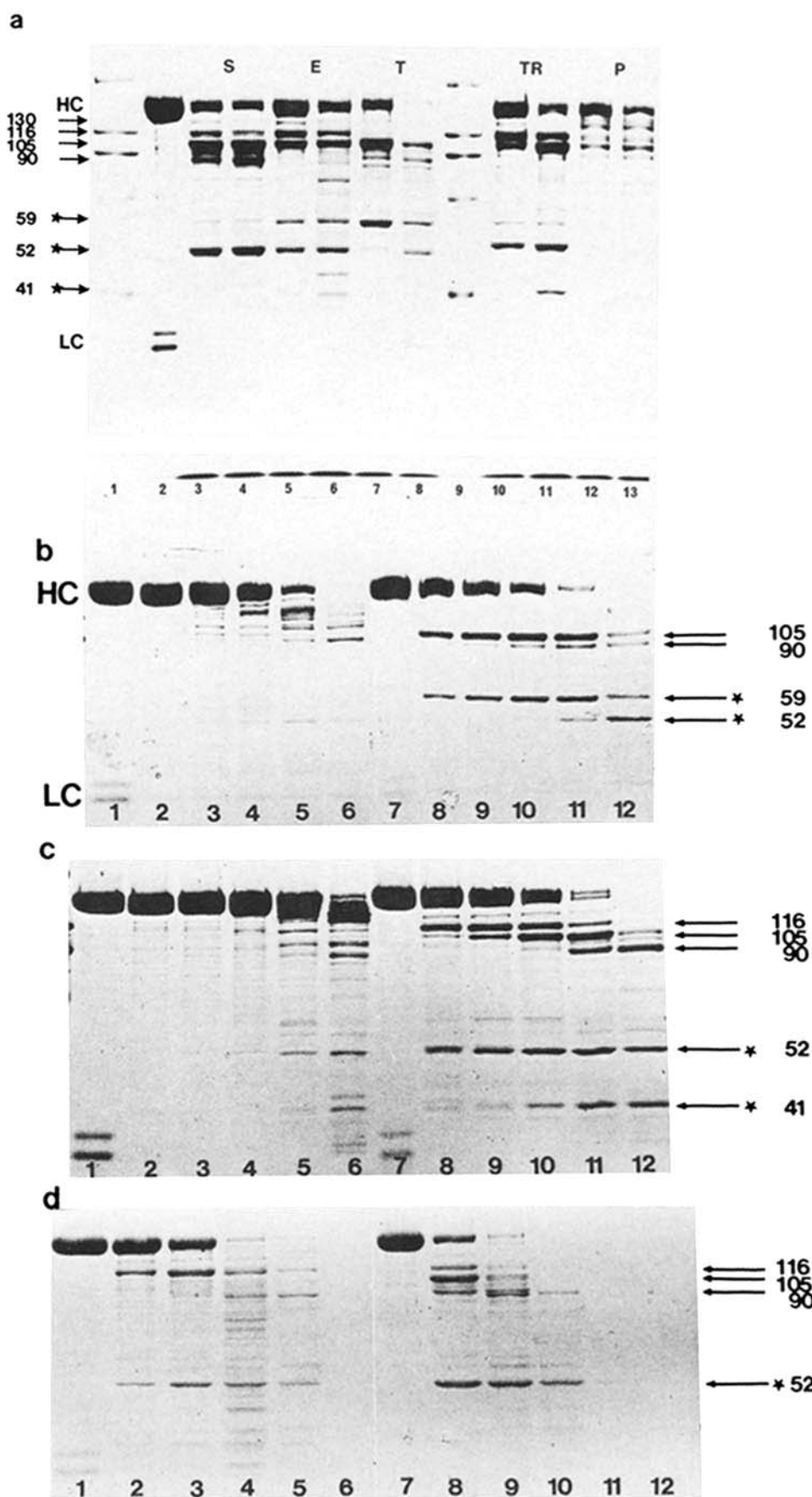


FIGURE 1 Electron micrographs of clathrin trimers, shadowed with Pt. (a) Rotary-shadowing with the field viewed from the mica towards the molecule; (b) light unidirectional shadowing; (c) heavy unidirectional shadowing. Note that in *b*, the Pt accumulation is uneven along the arm, with a longer shadow appearing at the terminal domain. Heavier shadowing (*c*) obscures these variations. Arrows point to terminal domains. The angle and orientation of unidirectional shadow (indicated by empty arrow in *b*) were calibrated with latex beads (85-nm diameter, E. Fullam) sprayed together with the clathrin. Bar, 100 nm.  $\times 160,000$ .

high molecular weight species are retained in the lattice of the cage, whereas the smaller molecular weight species are released into solution, as found previously for trypsin cleavage by Schmid et al. (13) and by Ungewickell et al. (14). The retention of the large fragment and the release of the 52,000  $M_r$  species generated by subtilisin are documented in Fig. 3. Therefore, we have prepared proteolyzed cages free of smaller species by centrifugation, dissociated the cages, and prepared the cleaved trimers for electron microscopy by rotary shadowing. The histograms in Fig. 4 summarize measured length

distributions, and some of the micrographs are shown in Fig. 5. Before cleavage, the average length of a clathrin arm is 48–50 nm. Since under appropriate conditions, chymotrypsin and thermolysin produce primarily the 105,000- $M_r$  heavy chain fragments, digestion with these enzymes was used to estimate its length. The cleaved arms remain associated as trimers, and their length distribution is centered at about 31.5 nm (see the histograms in Fig. 4, *b* and *c*, and the corresponding SDS PAGE). Trimers from subtilisin-cleaved cages show images whose appearance (Fig. 5) and arm-length distribution



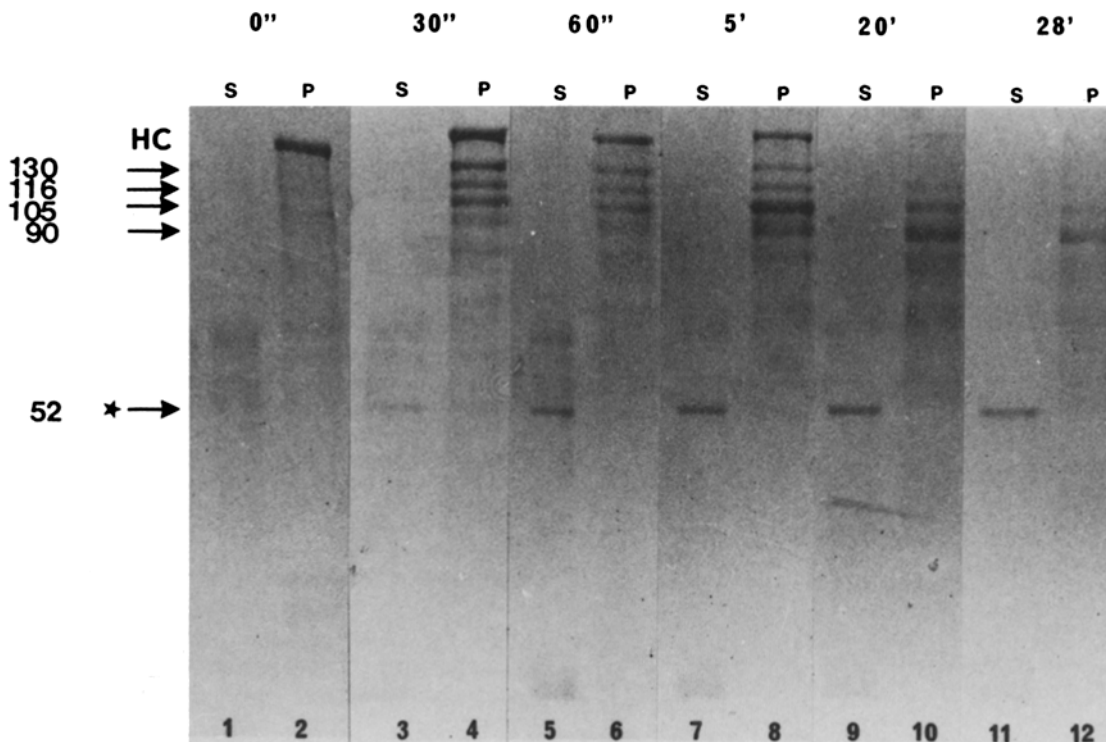


FIGURE 3 Proteolytic release of terminal domain. Time course of the release of terminal domain obtained from subtilisin-treated clathrin cages. Clathrin cages (0.8 mg/ml) were incubated with subtilisin at a ratio of 30:1 (wt/wt). Aliquots were taken at various times, and the reaction was stopped with PMSF. The samples were centrifuged for 10 min at 100,000 rpm in the airfuge and the supernatants (odd numbered lanes) and pellets (even numbered lanes) were analyzed by 12% SDS PAGE. Proteolysis times shown in the figures are before addition of subtilisin (lanes 1 and 2), 30 s (lanes 3 and 4), 1 min (lanes 5 and 6), 5 min (lanes 7 and 8), 20 min (lanes 9 and 10), and 28 min (lanes 11 and 12). Symbols as in Fig. 2.

(Fig. 4) varies with the extent of cleavage. After 5 min, the significant cleavage products are 130,000, 116,000, and 105,000  $M_r$ , and there is a broad distribution of trimer arm lengths (Fig. 4, *d-h*). After extensive cleavage, the arms of trimers show further shortening to a final length of about 21.5 nm (Figs. 4*g* and 5*e*). At this point dimeric structures and rod-like fragments also appear. The length distribution of the rods is indistinguishable from that of extensively cleaved trimer arms (Fig. 4, *g* and *h*). It is likely, therefore, that the rods and the two-armed structures are the result of an additional cleavage very near the center of the trimer. Indeed, the 90,000- and 105,000- $M_r$  bands appear as doublets on SDS PAGE (see, for example, Fig. 2*d*, lane 9, and Fig. 5, *d* and *e*). In the time series shown in Fig. 4, SDS PAGE shows mostly the lower member of the doublet near 90,000  $M_r$  after cleavage for 120 min, while the micrographs show mostly rod-like fragments with an average length of 21.5 nm.

The micrographs in Fig. 5 also show a correlation between the extent of digestion and the absence of the knob-like domain at the distal end of the arm (thin arrows in Fig. 5*b*). For example, after 5 min of digestion, ~60% of the arms have a distal knob (field sampled in Fig. 5*b*), and indeed about half the heavy chains are still intact. We show in the next section that the knob corresponds to the released 52,000- $M_r$  fragment.

### Terminal Domain

The time course of release of the 52,000- $M_r$  fragment from association with cages appears in Fig. 3, which shows SDS PAGE analysis of pellets and corresponding supernatants obtained by centrifugation of cages treated for various times with subtilisin. All the high molecular weight fragments sediment. The 52,000- $M_r$  species appears only in the supernatant, showing that it is released as soon as generated. Similar results

FIGURE 2 Electrophoretic analysis of proteolytic digestion of clathrin. (a) Comparison of cleavage pattern of clathrin cages with different proteolytic enzymes. Clathrin cages (lane 2) were treated with subtilisin (lanes 3 and 4), elastase (lanes 5 and 6), thermolysin (lanes 7 and 8), trypsin (lanes 10 and 11), and papain (lanes 12 and 13), for 30 min at room temperature at enzyme/clathrin ratios of 1:45, 1:30; 1:100, 1:50; 1:250, 1:100; 1:100, 1:50; 1:500, and 1:250, respectively. The reactions were stopped with PMSF or with phenanthroline, and products were analyzed by 12% SDS PAGE. Molecular weight markers (lanes 1 and 9) were myosin,  $\beta$ -galactosidase, phosphorylase B, bovine serum albumin, and ovalbumin (Sigma Chemical Co.). (b-d) Time course of proteolysis of free clathrin and of clathrin cages. Samples of clathrin trimers (lanes 1-6) and clathrin cages (lanes 7-12) at 0.7 mg/ml were treated at room temperature with thermolysin (b), elastase (c), and subtilisin (d) at enzyme/clathrin ratios of 1:40 (or 1:200 for cages), 1:50, and 1:35, respectively, for various times and the products analyzed by 12% SDS PAGE. Cleavage was stopped with phenanthroline or PMSF at the following times: before addition of protease (lanes 1 and 7); 5 min (lanes 2 and 8); 25 min (lanes 3 and 9); 1 h (lanes 4 and 10); 3 h (lanes 5 and 11); 8 h (lanes 6 and 12). HC and LC indicate clathrin heavy and light chains. High molecular weight fragments (arrows) are 130,000, 116,000, 105,000 and 90,000. Lower molecular weight fragments (arrows with star) are 59,000, 52,000-53,000, and 41,000.

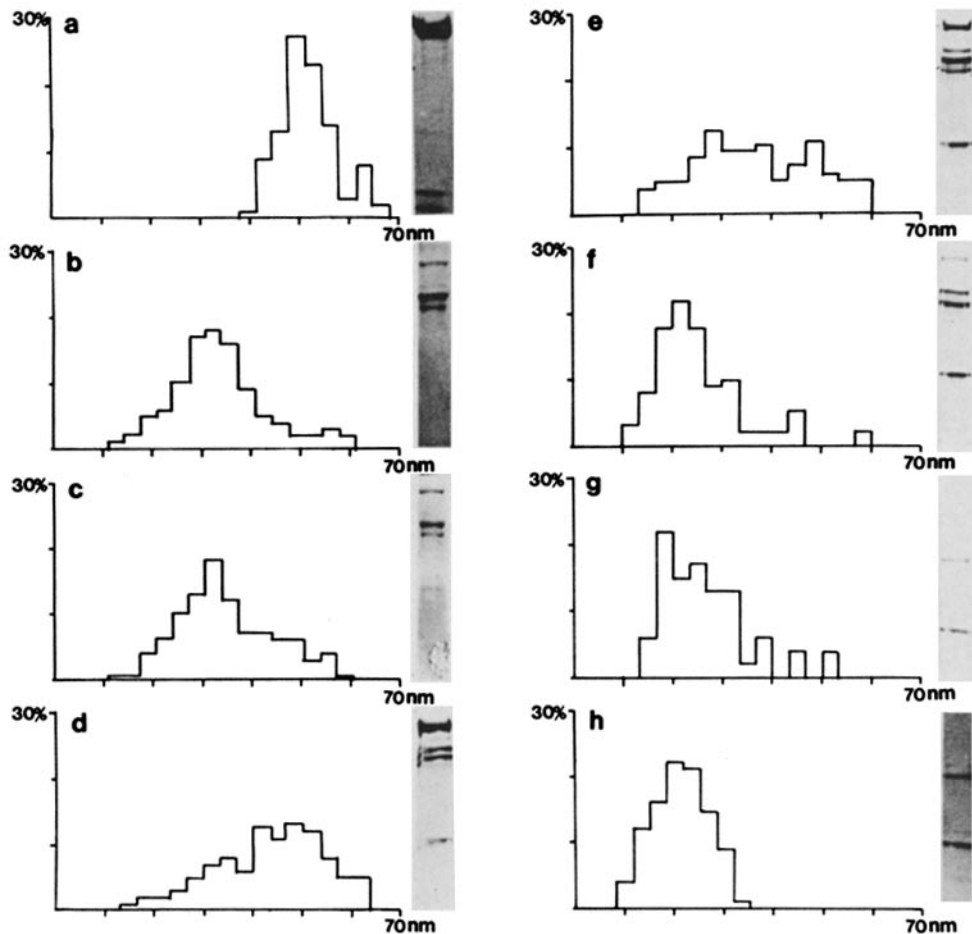


FIGURE 4 Histograms showing distribution of arm lengths on clathrin trimers obtained from cages digested with chymotrypsin, thermolysin, and subtilisin. The pellets of proteolyzed clathrin cages were resuspended in assembly buffer and then dissociated and prepared by rotary-shadowing for electron microscopy. Arm lengths of trimeric molecules were measured with a digitizer (Numonics Corp. or Evans and Sutherland Multi-Picture System [Salt Lake City, UT]) on projections of negatives of nominal magnification 36,000. Length counts were accumulated in 3.3-nm increments. Digestion with chymotrypsin or with thermolysin (*b* and *c*) produces one principal fragment of  $M_r$  105,000, corresponding to cleavage site 4. The histograms (*d*–*h*) correspond to length measurements from the subtilisin experiment shown in Fig. 5 (*b*–*e*). The arm length distribution of extensively cleaved trimeric structures (*g*) and the one-arm rods produced by extensive subtilisin digestion (*h*) define the centroid for cleavage site 5. The number of arms measured were, respectively, 120, 300, 97, 339, 105, 144, 54, and 99. The lengths are corrected for grain size; for the one-arm rods of *h*, the diameter of two grains has been subtracted to correct for the presence of a second free end. Insets show lanes from 12% SDS PAGE of samples before (*d*–*h*) or after (*a*–*c*) centrifugation.

were obtained with the soluble fragments generated by treatment of cages with thermolysin or with chymotrypsin (data not shown). These fragments were also released from coated vesicles (data not shown). Examination of rotary-shadowed fields of the released 52,000- $M_r$  species shows globular structures, and control experiments (not shown) indicate that the fragment does not correspond to the protease nor is it released from intact nondigested cages.

We purified the released fragment by sizing chromatography. Its behavior is consistent with a relatively compact structure. As shown in Fig. 6, gel filtration on Sephadex G-100 of the supernatant from subtilisin digested cages gives for the 52,000- $M_r$  fragment a symmetrical peak at a position corresponding to an apparent Stokes radius of 3.4 nm. This hydrodynamic behavior is similar to that of the hemoglobin tetramer, a globular protein of 65,000  $M_r$ . Electron micrographs of the purified terminal fragment (Fig. 6, inset) confirm that it is a globular structure ~5–8 nm in diameter, corresponding to the terminal domain at the end of intact clathrin

arms. We obtained similar results with 52,000  $M_r$  terminal domain generated with chymotrypsin (data not shown).

#### *Proteolytic Dissection of Free Clathrin*

The thermolysin, elastase, and subtilisin cleavage patterns of isolated clathrin trimers (Fig. 2, *b*–*d*) show a much broader spectrum of bands than those found when proteolysis is performed with clathrin molecules assembled into cages. None of the fragments accumulates to an appreciable extent. The only clearly identifiable bands are the ones with molecular weight corresponding to the terminal domain. Many of the larger fragments are probably products of cleavage in the unprotected proximal arm. Support for this interpretation is found in electron micrographs of shadowed trimers after 5 min of cleavage. There are reasonable numbers of two-armed structures as well as almost complete single arms (data not shown). In contrast, electron micrographs of trimers dissociated from proteolyzed cages show such structures only after

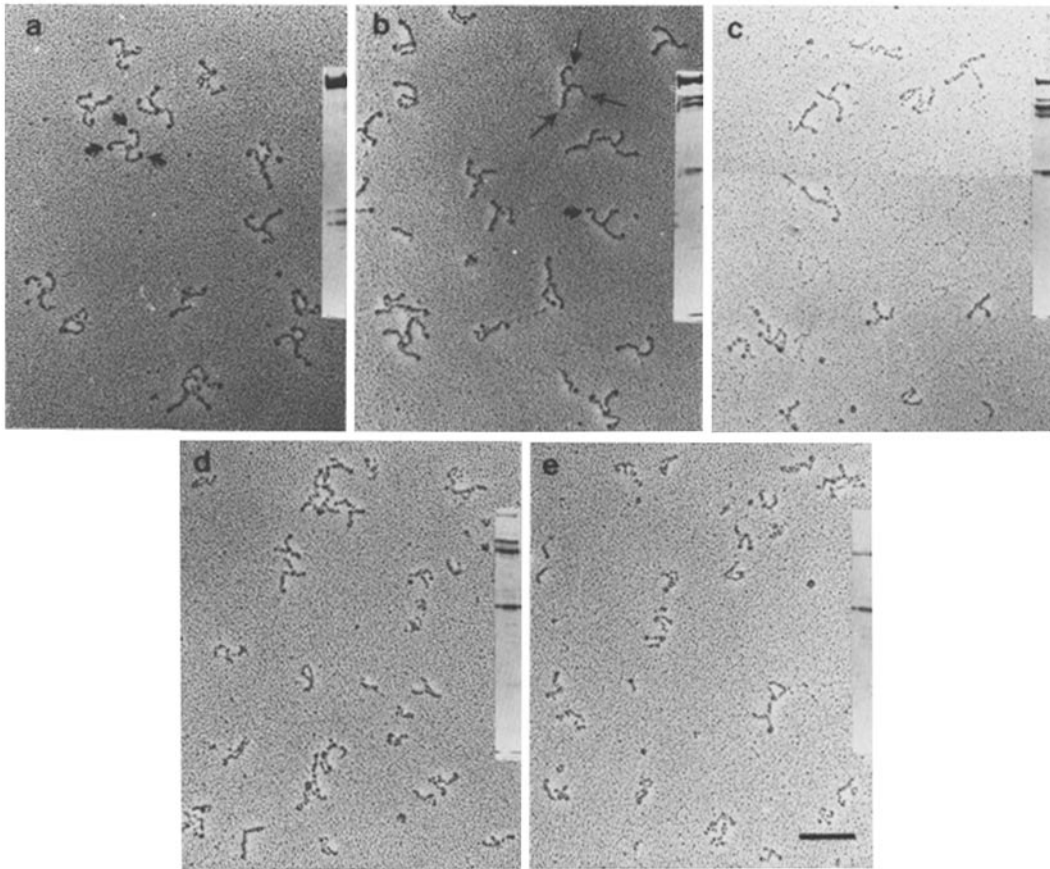


FIGURE 5 Clathrin trimers dissociated from subtilisin cleaved cages and analyzed by electron microscopy. Digestion was stopped with PMSF after the indicated time; cages were separated from soluble fragments by centrifugation in the airfuge; the cleaved cages were resuspended in solution D and dissociated by addition of one volume of 1 M Tris, pH 10.0. (a) Before addition of subtilisin; (b) 5 min; (c) 20 min; (d) 1 h; and (e) 2 h. Terminal domain is shown by full arrows. Its absence on an arm is indicated by the thin arrow in *b*. Insets show lanes from 12% SDS PAGE of sample before airfuge centrifugation. Bar, 100 nm.  $\times 73,000$ .

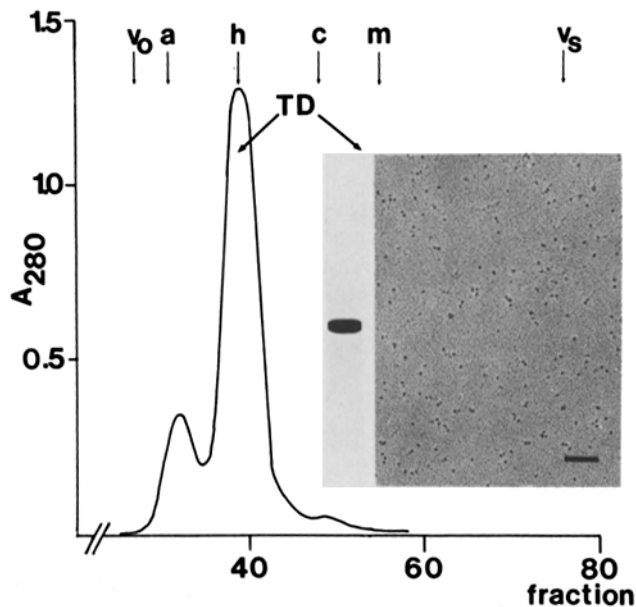


FIGURE 6 Purification of terminal domain by sizing chromatography on Sepharose G-100. Clathrin cages were digested with subtilisin and the released terminal domain applied to the column. The main component of the peak fraction (>95%) is the  $M_r$  52,000 fragment. The column was calibrated with aldolase (*a*), hemoglobin (*h*), carbonic anhydrase (*c*), and myoglobin (*m*);  $V_0$  and  $V_s$  correspond to void and separation volume. Inset shows 12% SDS PAGE and electron microscopy performed on fraction 36. Bar, 100 nm.  $\times 44,000$ .

extensive proteolysis (see, for example, Fig. 5*e*).

#### Electron Microscopy of Cleaved Cages

After treatment with trypsin or with subtilisin sufficient to reduce most of the heavy chains to the 116,000- and 105,000- or to the 105,000- and 90,000- $M_r$  fragments, clathrin cages have a characteristically collapsed appearance when negatively stained with uranyl acetate (Fig. 7, *b* and *c*). The cage indicated by an arrow in Fig. 7*b* is particularly striking, showing at its periphery a folded-over hexagonal facet. Notice that both sides of the flattened cages are seen in the stain. Many well-contrasted edges of such flattened cages appear as distinct double lines,  $\sim 3.5$  nm apart. Images of uncleaved cages prepared by identical negative staining procedures at exactly the same time have edges that appear much more "solid," although particular views show some with a double- or triple-line character (Fig. 7*a*). We interpret these results as indicating that the distal arm, part or all of which is separated from the proximal arm by cleavage, normally contributes to an edge (see Fig. 8), reinforcing the apparent stain exclusion. The double-line appearance of edges in cleaved cages confirms the anti-parallel relationship of proximal arms.

## DISCUSSION

### Divisions of the Clathrin Arm

The results of proteolytic cleavage experiments and correlated length measurements can be used to obtain a picture of

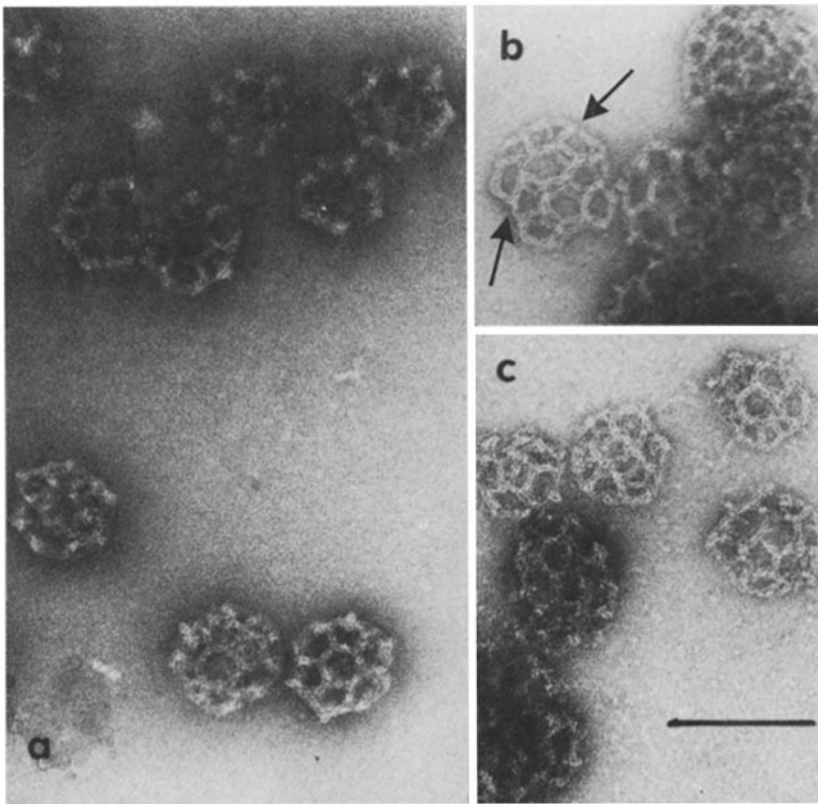


FIGURE 7 Effect of limited proteolysis on the appearance of negatively stained cages. (a) Untreated cages. (b) Trypsin-cleaved cages. (c) Subtilisin-cleaved cages. Cleaved cages were separated from terminal domain and other small fragments by centrifugation. The extent of digestion was determined by SDS PAGE; it was similar to that shown in Fig. 2a, lane 11, and Fig. 2d, lane 9, respectively. Before digestion, the edges of negatively stained cages do not show easily recognizable substructure; after digestion, the cages seem to collapse onto the carbon and their edges often appear as two fine, parallel lines (arrow). The absence of knobs at the periphery of intact cages suggests that terminal domains do not project outwards. Bar, 100 nm.  $\times 195,000$ .

the domain organization of the heavy chain and how it corresponds to the structure of a clathrin arm. The enzymatic digestions define a restricted number of strong cleavage points. Since these points are approximately protease independent, their lability must be due to the folding of the chain or to its packing in the lattice. Three points are defined by the central fragments retained in proteolyzed cages (116,000, 105,000, and 90,000  $M_r$ ), and at least two more by the released distal peptides (52,000 and 59,000  $M_r$ ). The sharpness of the 52,000- and 59,000- $M_r$  bands and the general absence of other strong bands in this region suggest that the released fragments have one common terminus. Moreover, the globular structure of the terminal domain is consistent with the assumption that "nibbling" is not likely to occur at the distal end. The simplest view is therefore that the released fragments and the intact heavy chains have essentially identical distal terminus. This picture is shown diagrammatically in Fig. 8a. We emphasize that we cannot yet completely rule out more complicated patterns—e.g., that the 52,000- $M_r$  peptide is not co-terminous at either end with the 59,000- $M_r$  peptide. We also point out that points 2 and 3 in Fig. 8a are only 4,000  $M_r$  apart (assuming exactly 180,000 for the intact chain) and that they may not, therefore, be structurally distinct.

These cleavages do not all occur at boundaries of the three obvious morphological divisions of a clathrin arm—the proximal segment, the distal segment, and the terminal knob. Nonetheless, the relationship between the lengths of arms cleaved at positions 4 and 5 and the molecular weights of the corresponding fragments shows that the polypeptide chain does not double back over great distances within the distal segment. Our measurements of negatively stained trimers show that the proximal arm extends 16.6 nm outward from the apex of the trimer (see also reference 12). The nearest significant subtilisin cleavage, yielding a 90,000- $M_r$  fragment,

is at position 5, 21.5 nm from the center. This cleavage must therefore occur  $\sim 5$  nm beyond the elbow. Assuming a relatively uniform distribution, the mass per unit length of the proximal segment is  $\sim 4.2$  kDa/nm. This corresponds to a diameter of  $\sim 2.5$  nm for a protein of density  $1.4$  g/cm<sup>3</sup>. In fact, we might expect some extra concentration of mass at the proximal end, if a specialized domain is needed to stabilize interactions between the three heavy chains. On intact clathrin, light chains are also bound to the proximal segment (18), further increasing its mass. The distal segment spans  $\sim 26$  nm between the elbow and the terminal domain. We can estimate its mass per unit length as follows. Cleavage point 5 is  $\sim 5$  nm beyond the elbow. Cleavage point 1 can lie no farther from the tip of the arm than the diameter of the released terminal domain ( $\sim 7$  nm). A minimum estimate for the distance between these points is, therefore, 21.5 nm, giving an upper limit to the mean diameter of the distal segment of only 1.6 nm.

#### *Relationship of Heavy-Chain Domains to the Packing of Clathrin in Cages*

The diagram in Fig. 8 shows the principal proteolytic cleavage points mapped onto a simulation of part of a cage. The packing shown in the drawing is based on the analysis of partial assemblies by Crowther and Pearse (12), on the micrographs of cleaved cages shown in Fig. 7, and on the dimensions from Fig. 4. The lattice of the simulated cage is a truncated icosahedron—a structure found among the larger cages reassembled from calf-brain clathrin (T. Kirchhausen and S. C. Harrison, unpublished results). All trimers are identical in such a structure although there are small local packing differences for each of the three arms. The distal arm segments are shown lying just beneath proximal segments of another tri-



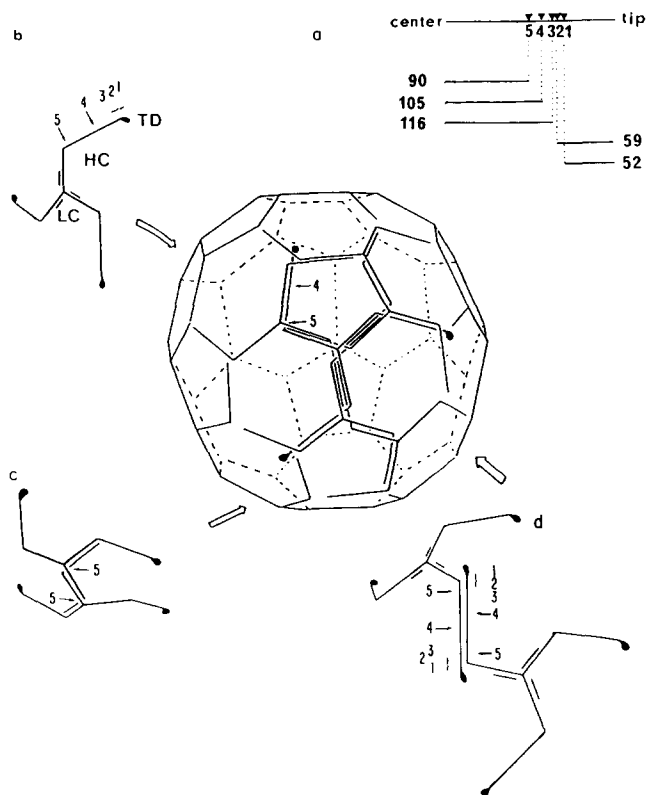


FIGURE 8 (a) The simplest arrangement of cleavage points along the polypeptide chain that is consistent with observed digestion patterns. The positioning of points 1 and 2 depends on the assumption that the  $M_r$  59,000 and 52,000 peptides both terminate at the distal end. See text for a discussion of this assumption. Note that the chemical polarity (amino- to carboxy- terminus) of the heavy chain has not yet been determined. (b) Position of these sites along an arm. (c and d) Relationship of these sites to other trimers in an assembled cage. We do not intend to imply in the central drawing that distal arm segments are closer together than proximal segments, but merely that they run beneath them. See the second section of the Discussion. The clockwise slew of arms when viewed from outside a cage has been chosen arbitrarily, but examination of images of shadowed trimers absorbed to mica under various conditions suggests that this may indeed be the actual hand of the molecule (T. Kirchhausen and J. Heuser, unpublished observations).

mer, and terminal domains are shown pointing inwards. Our evidence for this representation is the appearance of edges of negatively stained cleaved cages (see description of Fig. 7). We have also observed that edges forming the periphery of negatively stained *coated vesicles* have a markedly doubled character (data not shown). Since these structures are much less collapsed than empty cages, peripheral edges are seen side-on, and a doubled edge therefore indicates an inner layer. We believe that this inner layer corresponds to interacting distal arms.

Arrows defining the cleavage positions in Fig. 8 are of course subject to some experimental uncertainty. The location of cleavage site 5 is relatively well determined, since it depends on measurements of a unique population (the subtilisin-produced single arms, Fig. 4, *g-h*). The location of cleavage site 4 is also relatively well measured, since it corresponds to the principal chymotrypsin and thermolysin endpoints (Fig. 4, *b* and *c*). The location of site 3 is not accurately known, however, since no unique population of the 116,000- $M_r$  fragment can be obtained for microscopy. Likewise, the location

of cleavage sites 1 and 2 can only be estimated from the difference in length between the intact arm and the diameter of the terminal domain. Some suggestions can be made about the functional significance of the divisions shown in Fig. 8, based on the positions of cleavage sites with respect to packing interactions. Proximal contacts are clearly sufficient to maintain integrity of a cage, but the collapsed appearance of negatively stained cages after proteolysis (Fig. 7) suggests that distal contacts may be needed for rigidity. The drawing shows that cleavage at position 4 leaves little overlap of remaining distal arms and that cleavage at position 5 completely eliminates distal interactions. These are the major sites of endpoint proteolysis with trypsin and subtilisin, respectively. In both cases, the collapsed appearance of cleaved cages is similar. Reassembly of cages from trypsin-cleaved trimers has been reported (13, 14), but the extent of cleavage in those trimers competent to reassemble has not yet been determined.

### Terminal Domain

Previous studies of clathrin have clearly shown the division of an arm into proximal and distal segments (9, 10), but there has been uncertainty about a distinct structure at the distal end. Favorable images of negatively stained clathrin showed an extra segment at the tip (9), and platinum rotary-shadowing appeared to reveal a "hairpin loop" structure at the distal end (10). Subsequent studies of negatively stained trimers gave no evidence for such a domain, however, and it was suggested that the images obtained by shadowing had artifacts due to Pt decoration effects (12). We can now assert with confidence on the basis of electron microscopic and proteolytic cleavage experiments that there is indeed a distinct terminal domain. By using volatile buffers and a lighter deposition of Pt, we find a clear and apparently globular feature at the distal end of each arm (Figs. 1 and 5*a*). Moreover, we have shown that trimers derived from lightly proteolysed cages have lost these structures. Finally, we have taken advantage of its release from cages to purify the fragment that corresponds to the terminal domain and have shown that it behaves as a globular structure.

The relative ease with which the terminal domain can be cleaved away from the rest of the arm may indicate a somewhat flexible connection. Although small in linear extent, the terminal domain in fact accounts for ~30% of the heavy chain mass. If it indeed points inwards as we suggest in the model in Fig. 8, then it might interact with some component of a coated vesicle membrane. Since the corresponding 52,000 or 59,000  $M_r$  fragments dissociate from coated vesicles as well as from cages, uniform tight interactions with the membrane are not likely (unless proteolysis of the presumed binding site accounts for the release). But it is possible that a few specific, strong contacts with the terminal domain do occur, for example at the sites that nucleate coated vesicle formation, or that there is weaker binding, direct or indirect, of the terminal domain to cytoplasmic portions of integral membrane proteins.

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