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Brain clathrin and clathrin-associated proteins

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The assembly of clathrin into baskets or cages *in vitro* may depend on formation of a complex between clathrin and a polypeptide doublet migrating in the 30000-mol.wt. region. Clathrin with several associated proteins was isolated from coated-vesicle fractions of bovine cerebral cortex. Most associated proteins were separated by Sepharose 4B column chromatography. The eluted clathrin retained only the 30000-mol.wt. doublet and assembled into baskets at pH 6.5. Limited proteolysis of coated vesicles or clathrin assembled as baskets removed these clathrin-associated proteins (CAPs) without detectably altering clathrin. Enzyme-treated clathrin assembled into open-lattice structures but no longer formed baskets *in vitro*. Latex particles with bound enzyme cleaved the CAPs from coated vesicles and clathrin baskets, suggesting that the CAPs protrude from the exterior of the clathrin lattice.

Coated vesicles involved in a variety of endocytotic processes (Friend & Farquhar, 1967; Anderson *et al.*, 1977), most notably the retrieval of membrane from neuromuscular junctions (Heuser, 1978), are formed by the assembly of clathrin molecules subjacent to the membrane (Kadota & Kanaseki, 1969; Heuser, 1980). Clathrin lattices, polymerized as a sheet of hexagons, invaginate appropriate areas of the membrane by transforming certain of their hexagonal lattices into pentagonal ones, until an icosahedral structure is assembled surrounding a resealed vesicle (Heuser, 1980).

Clathrin was partially purified and assembled into lattices and basket structures *in vitro* (Schook *et al.*, 1977, 1978, 1979; Woodward & Roth, 1978; Keen *et al.*, 1979; Puszkin *et al.*, 1979; Schook & Puszkin, 1980; Bloom *et al.*, 1980). In our studies, we found that a polypeptide doublet of approx. 30000 mol.wt. was associated with highly purified clathrin. We postulated that its role may be to regulate clathrin's assembly or closure into baskets (Puszkin *et al.*, 1979; Schook & Puszkin, 1980). Here we report that chymotrypsin, under controlled conditions, removed these CAPs, rendering clathrin unable to assemble into baskets *in vitro*.

Abbreviation used: CAP, clathrin-associated protein.

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Experimental

Buffer solutions

Buffer A consists of 0.1 M-Mes (4-morpholineethanesulphonic acid) (or 0.1 M-cacodylate), pH 6.5, 1 mM-EGTA, 0.5 mM-$MgCl_2$, 7 mM-2-mercaptoethanol and 0.02% NaN_3. Buffer B is the same as buffer A, plus 160 mM-KCl and 5 mM-NaCl. Buffer C consists of 0.1 M-Tris/HCl, pH 7.0, 1 mM-EDTA, 7 mM-2-mercaptoethanol and 0.02% NaN_3. Buffer D consists of 20 mM-Tris/HCl, pH 7.5, 0.5 mM-$MgCl_2$, 7 mM-2-mercaptoethanol and 0.02% NaN_3. Buffer E consists of 0.5 M-Tris, pH 7.0, 1 mM-EDTA, 7 mM-2-mercaptoethanol and 0.02% NaN_3. Buffer F consists of 0.4 M-Mes, pH 6.5, and 0.02% NaN_3. Buffer G consists of 0.1 M-Mes, pH 6.5, 1 mM-EGTA and 0.02% NaN_3.

Reagents

All buffers were from Fisher Co., New York, NY, U.S.A. Pancreatic α-chymotrypsin was from Sigma Chemical Co., St. Louis, MO, U.S.A. Lytron 615 polystyrene particles (approx. 120 mg/ml) were from Monsanto Chemical Co., St. Louis, MO, U.S.A. 4,4'-Dithiobisphenylazide was from Pierce Chemical Co., Rockford, IL, U.S.A.

Tissue preparation

Bovine brains were delivered in ice fresh from a local slaughterhouse. Cerebella and brain stems were

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discarded and the grey matter was separated from the white matter by suction. All steps of the various fractionation procedures were performed at 4°C unless stated otherwise.

Crude coated vesicles

The grey matter of three brains was homogenized with 500ml of buffer A in a Waring blender in three 10-s bursts and centrifuged at 30000*g* for 15min to remove large debris. The supernatant was centrifuged again at 80000*g* for 60min to obtain a crude coated-vesicle pellet and used to prepare pure coated vesicles. Buffer C was substituted during the initial homogenization when clathrin was purified by the method of Schook *et al.* (1979).

Purified coated vesicles

A variation of the procedure of Keen *et al.* (1979) was used to purify coated vesicles. The crude coated vesicles were resuspended in 35ml of buffer B and loaded on six discontinuous-sucrose-gradient tubes prepared with buffer B (w/v) as follows: 4.5ml of 5%; 9.0ml of 10%; 9.0ml of 40%; 4.5ml of 50%; 4.5ml of 60% sucrose. After centrifugation at 100000*g* for 60min using an SW-27 rotor, the upper pink-red layer and lower pad were discarded; the middle layers of gradients were pooled, diluted three times with buffer B and sedimented at 80000*g* for 60min. The pellets were resuspended in 20ml of buffer B and loaded on three discontinuous sucrose gradients prepared with buffer B (w/v). These gradients consisted of 10ml of 5%, 15ml of 30% and 5ml of 60% sucrose. After centrifugation at 50000*g* for 45min, the 5% layer was collected, diluted twice with buffer B and centrifuged at 80000*g* for 60min. The pearly-white pellets were resuspended in buffer G, stored at 4°C and used within 7 days.

Clathrin extraction

For preparation of clathrin, the crude coated-vesicle pellets were used. Vesicles were extracted by stirring for 16h in 150ml of buffer D containing 1mM-EDTA in place of $MgCl_2$ and centrifuged at 80000*g* for 60min. Solubilized clathrin was gel-filtered by column chromatography on Sepharose 4B (Schook *et al.*, 1979; Bloom *et al.*, 1980). Also, clathrin was extracted with buffer E and purified further by the method of Keen *et al.* (1979).

The biological activity of clathrin preparations was determined by their ability to form baskets at pH6.5, as viewed using negative staining in the electron microscope. Proteins were stored frozen in buffer D containing 10% sucrose. Clathrin was purified further by the method of Bloom *et al.* (1980) to remove several co-purifying proteins. This was accomplished by re-chromatography on Sepharose 4B columns (2.5cm × 80cm) equilibrated with buffer D.

Preparation of plain synaptic vesicles

Plain synaptic vesicles were prepared from bovine brain by the method of DeLorenzo & Freedman (1978). Each preparation was screened by sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis. Vesicles were stored suspended in buffer G at 4°C and used within 3 days.

Limited proteolysis of vesicles and cleavage of CAPs

Chymotrypsin (2mg) was dissolved in 300μl of buffer D. A portion (20μl) was removed from this stock, diluted to 1ml with buffer A and utilized for enzymic digestion; 5μl portions were used per 400μg of protein (1.65μg of proteinase/mg of protein).

Immobilized chymotrypsin was prepared as follows. Chymotrypsin (1mg) was dissolved in 500μl of buffer D and added to 30μl of a Lytron particle suspension (120mg/ml). After the solutions were mixed, Lytron particles were sedimented at 30000*g* for 15min and washed twice with buffer A. The resulting Lytron pellet was resuspended in 100μl of buffer A and 40μl was diluted to 1ml with buffer A. Approx. 5μl of this diluted solution was used per 400μg of protein. The amount of chymotrypsin adsorbed on the Lytron particle surface was calculated from protein remaining in the supernatant (Puszkin *et al.*, 1975). This Lytron particle suspension, termed 'immobilized chymotrypsin', contained three times the enzyme activity of the free chymotrypsin solution.

To obtain limited proteolysis the various preparations were incubated at pH6.5 with periodic mixing for 1h at room temperature. Before incubation, the concentration of depolymerized clathrin was adjusted with buffer F to promote formation of baskets. Enzymic digestion was halted by addition of 10% sodium dodecyl sulphate to yield a final concentration of 3.2%. When the biological activity of enzyme-treated clathrin was assayed, reactions were halted with 1mM-phenylmethanesulphonyl fluoride. Clathrin was recovered by 50% $(NH_4)_2SO_4$ saturation and dialysis against 20vol. of buffer D containing 2M-urea instead of Mg^{2+}. Further dialysis was carried out in buffer D. In some instances, the $(NH_4)_2SO_4$-precipitation step was omitted.

Extraction of clathrin from chymotrypsin-treated crude vesicles

Crude coated vesicles from one bovine brain were incubated in a total volume of 20ml of buffer A with 1.65μg of chymotrypsin per mg of protein. Digestion was carried out at room temperature for 1h with stirring. Phenylmethanesulphonyl fluoride (1mM)

was added to the suspension, which was centrifuged at 80000*g* for 60min. Pellets were resuspended in 50ml of buffer E containing 0.5mM-phenylmethanesulphonyl fluoride and left to stir overnight. This suspension was centrifuged at 80000*g* for 1h. Proteins from the clear supernatant were fractionated by addition of solid $(NH_4)_2SO_4$ to 50% saturation. The precipitate was collected by centrifugation at 20000*g* for 10min, resuspended in 4ml of buffer E containing 0.5mM-phenylmethanesulphonyl fluoride and dialysed against two changes of 20vol. of buffer E with no phenylmethanesulphonyl fluoride. The dialysate was loaded on a Sepharose 4B (2.5cm × 80cm) column previously equilibrated with buffer E and eluted at a rate of 6–7ml/h. Column fractions were monitored by their absorbance at 280nm. Those comprising a broad plateau were pooled, adjusted to 70% saturation in $(NH_4)_2SO_4$, and left at 4°C for 30min. The precipitate obtained after centrifugation at 20000*g* for 10min was resuspended in 2ml of buffer E, dialysed against 20vol. of buffer E followed by two changes of 20vol. of buffer D. Protein obtained by this method was stored frozen in 10% sucrose. Samples were tested on sodium dodecyl sulphate/polyacrylamide slab gels and biological activity (basket formation) was determined by electron microscopy.

Photochemical cross-linking

A solution of 0.01M-4,4′-dithiobisphenylazide in ethanol was prepared with the aid of a red safety lamp. Photolysis was performed essentially as described previously for erythrocyte and ciliary membranes (Mikkelsen & Wallach, 1976; Dentler *et al.*, 1980). Coated vesicles suspended in buffer G (700μg in 200μl) and dispersed into 1-ml microcuvettes were irradiated for 1h with an unfiltered UVL-56 lamp (Ultraviolet Products, San Gabriel, CA, U.S.A.) at a distance of approx. 3cm, after addition of 10μl of reagent (5% ethanol/0.5mM reagent). Vesicles were allowed to equilibrate for 30min before photolysis. Aluminium-foil reflectors were placed approx. 3cm behind each sample. All processes were performed in the dark or under a red safety light. The reaction was terminated by the addition of 10% sodium dodecyl sulphate to a final concentration of 3.2%.

Protein determinations

Protein concentrations were determined by using the Folin phenol reagent (Lowry *et al.*, 1951). A standard curve was constructed with bovine serum albumin. Sodium dodecyl sulphate was used to solubilize organelle preparations before protein determination by using the phenol reagent method.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Electrophoresis runs were performed using 1.5-mm-thick slab gels containing a 5–15% (w/v) linear gradient of polyacrylamide, a 4% polyacrylamide stacking gel and a discontinuous buffer system (Laemmli, 1970). Samples were made 3.2% in sodium dodecyl sulphate and reduced by addition of an equal volume of dye mixture containing 0.8% 2-mercaptoethanol, Phenol Red and glycerol. Current was applied at 7.5mA for a period of 14–16h. Gels were fixed [methanol/water/acetic acid (9:9:2, by vol.)], stained with Coomassie Brilliant Blue (R-250) and destained in fixer. Samples from cross-linking experiments were not reduced with 2-mercaptoethanol.

Electron microscopy

Appropriate solutions of clathrin (0.1–0.5mg/ml) were placed on Formvar carbon-coated grids, stained with aq. 1% (w/v) uranyl acetate and air dried. Grids were examined in a JEOL JEM 100-B electron microscope at 80kV. Transmission electron microscopy also was performed on certain samples (Schook *et al.*, 1979).

Results

Polypeptides associated with vesicles and purified clathrin

A typical coated-vesicle preparation (Plate 1*a*) displayed numerous coated vesicles, empty shells, some large vesicles and membrane fragments. Approx. 10mg of total coated-vesicle protein was obtained from one beef brain. Sodium dodecyl sulphate/polyacrylamide-gel analysis of these coated vesicles revealed especially prominent clathrin and protein bands co-migrating with purified tubulin and actin (Fig. 1). There were several minor bands in the 100000-mol.wt. region and a doublet in the 30000-mol.wt. region (Fig. 1, lane 3). The predominent protein bands in plain vesicles co-migrated with purified tubulin and actin (Fig. 1, lane 2). Clathrin was present in small amounts, an indication that coated vesicles had separated from plain vesicles. A typical plain synaptic-vesicle preparation is illustrated in Plate 1(*b*). A yield of 2mg of total protein in plain vesicles from 20g of bovine brain grey matter was obtained.

All preparations obtained after the various protocols described yielded clathrin as the major constituent. Small amounts of other proteins co-migrating with tubulin and actin plus polypeptides in the 100000- and 30000-mol.wt. regions (Fig. 2, lane 4) co-purified with clathrin. Most other polypeptides were separated from clathrin by re-chromatography on Sepharose 4B. Two discrete peaks were eluted;

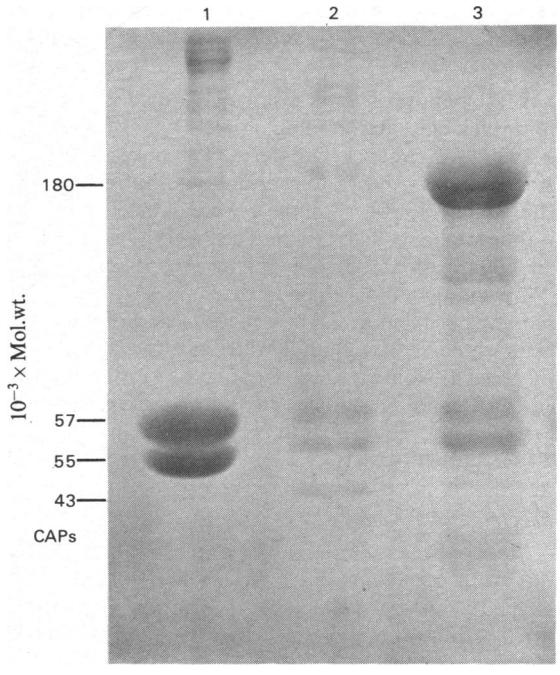


Fig. 1. ***Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis revealing proteins of plain synaptic vesicles and coated vesicles***

Lane 1, 100μg of purified bovine brain tubulin (M_r = 55000–57000). Lane 2, 30μg of total proteins of a purified plain synaptic vesicle preparation. Only traces of clathrin are seen. Lane 3, 100μg of total proteins of a purified coated-vesicle fraction. Note the high content of clathrin present.

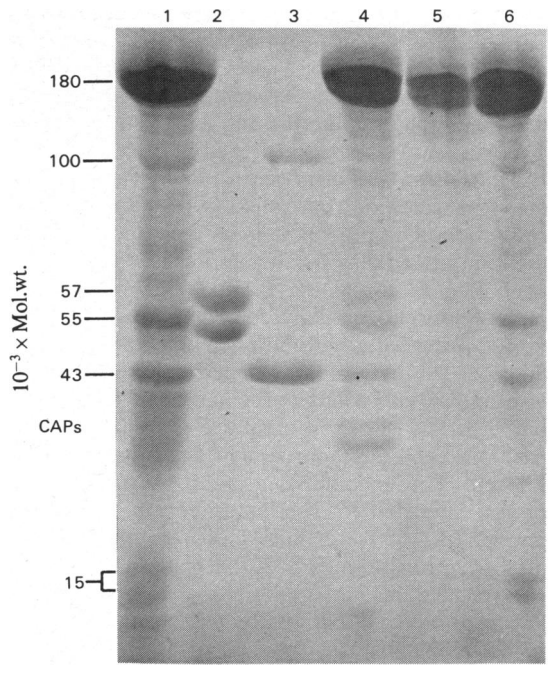


Fig. 2. ***Sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis of various clathrin preparations***

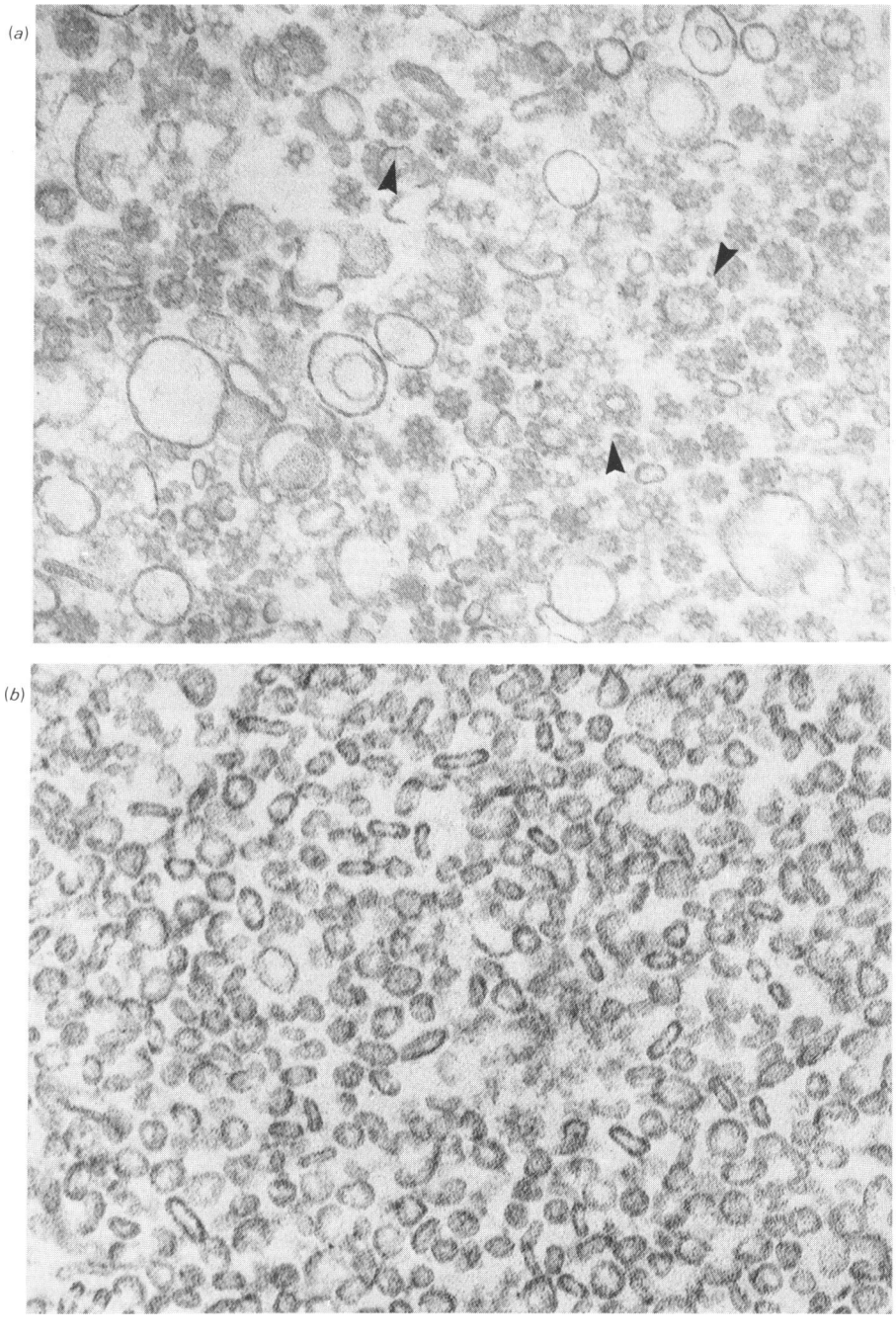
Lane 1, 110μg of proteins of partially purified clathrin from crude coated vesicles pretreated with chymotrypsin. Lane 2, 25μg of purified bovine brain tubulin used as a molecular-weight marker (M_r = 57000 and 55000). Lane 3, 3μg of dog muscle α-actinin (M_t = 100000) and 10μg of rabbit muscle actin (M_r = 43000) as markers. Lane, 4, 110μg of clathrin purified from untreated coated vesicles and after gel filtration on a Sepharose 4B column. This preparation assembled into baskets at pH 6.5. Lane 5, 100μg of clathrin as shown in lane 4, but after chymotrypsin treatment at pH 6.5. This preparation could not form baskets. Lane 6, 110μg of a clathrin preparation as shown in lane 1, but after column chromatography on Sepharose 4B. Note the absence of the polypeptide doublet of mol.wt. 30000. A smaller polypeptide doublet (M_r = 15000) was present. This clathrin preparation assembled into lattices of various sizes but not into baskets.

the first contained small amounts of clathrin and most accompanying proteins (Fig. 3, lane 1). The second peak was highly enriched in clathrin and contained the 30000-mol.wt. doublet, the CAPs (Fig. 3, lane 2). Clathrin preparations assembled into basket structures at pH 6.5 (Plate 2*a*). Baskets formed in the absence of chelating agents without addition of other polypeptides.

Cofactor requirement

Limited proteolysis of clathrin baskets at pH 6.5 resulted in cleavage of CAPs (Fig. 2, lane 5; Fig. 3, lanes 3–6; Fig. 4, lane 4). After chymotrypsin treatment the clathrin polypeptide remained intact. Decreasing concentrations of enzyme-treated clathrin loaded on sodium dodecyl sulphate/polyacrylamide slab gels revealed the clathrin 180000-mol.wt. polypeptide migrating at the same molecular weight, devoid of smaller clathrin fragments (Fig. 3, lanes 6–3). Clathrin baskets remained assembled after the enzyme treatment, but failed to reassemble after their depolymerization.

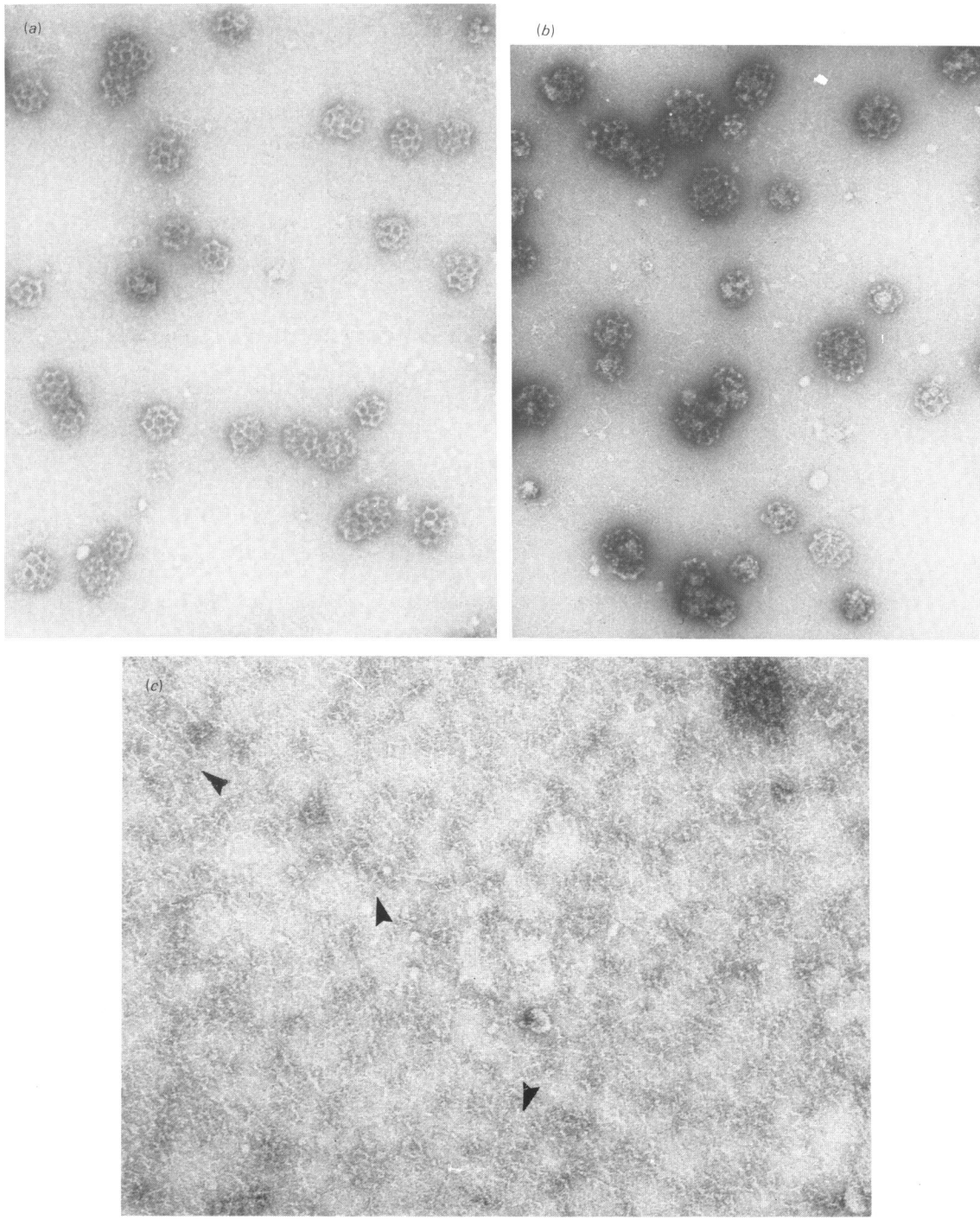
Because of chymotrypsin's ability to cleave clathrin's associated proteins a similar digestion was carried out on crude coated vesicles in suspension. Under these conditions, purified clathrin showed small content of actin- and tubulin-like polypeptide bands (Fig. 2, lane 6). The CAPs were absent, coinciding with the appearance of a doublet of mol.wt. 15000. This preparation did not assemble into baskets. Instead, partially assembled lattices of clathrin were seen. Also clathrin appeared polymerized in smaller units of pentagons and hexagons



EXPLANATION OF PLATE 1

Morphology of brain vesicle fractions

(*a*) Coated-vesicle-enriched fractions were prepared by differential centrifugation and a discontinuous sucrose gradient. The fraction separated from the gradients was diluted and pelleted. Coated vesicles and clathrin shells were the most numerous organelles. Resealed vesicles and membrane fragments are present in lesser numbers. Magnification 88000×. (*b*) The plain synaptic-vesicle preparation from bovine brain cortex was prepared by homogenization with 0.32M-sucrose followed by osmotic shock and differential centrifugation under conditions of iso-osmolarity (DeLorenzo & Freedman, 1978). After high-speed centrifugation the pellet was processed for transmission electron microscopy. It was free of coated vesicles and large membrane fragments. Magnification 80000×.



EXPLANATION OF PLATE 2

Ultrastructure of clathrin assemblies

(*a*) Negatively contrasted brain clathrin baskets after enzyme treatment. The protein preparation used is that shown in Fig. 3, lane 2 (before hydrolysis) and lanes 3–6 (after hydrolysis). Basket structures remained structurally and morphologically intact. Magnification 80000×. (*b*) Clathrin baskets before enzyme treatment. Magnification 80000×. (*c*) Negatively contrasted lattices of clathrin molecules after enzyme treatment. Clathrin assembled baskets were enzyme-treated and depolymerized in buffer D. The protein molecules were tested then for reassembly at pH 6.5. Note the polydispersed distribution of partial molecular assemblies; the lattices seem partially attached in trimers, pentagons and hexagons. Magnification 120000×.

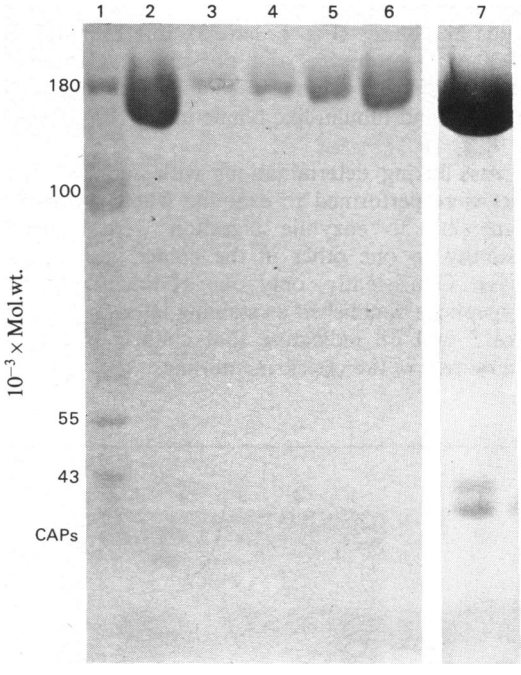


Fig. 3. *Further purification of clathrin utilizing a combination of rechromatography and chymotrypsin treatment*

Lane 1, 60 μg of the first peak eluting during rechromatography on Sepharose 4B columns. Various proteins co-migrating with actin and tubulin, as well as polypeptides migrating in the 100000-mol.wt. region, are seen. There are small amounts of clathrin. The 30000-mol.wt. doublet is absent. Lane 2, 90 μg of the second peak eluted from the same column. It consists almost exclusively of clathrin. The doublet of mol.wt. 30000 remained bound to and emerged with clathrin. This preparation displayed basket formation. Lanes 3–6, chymotrypsin-treated clathrin loaded with 10 μg (lane 3), 20 μg (lane 4), 40 μg (lane 5) and 80 μg (lane 6) of total protein. Clathrin molecules do not show cleavage. Lane 7 shows the clathrin preparation in lane 2 run at higher protein concentration (110 μg) on another gel.

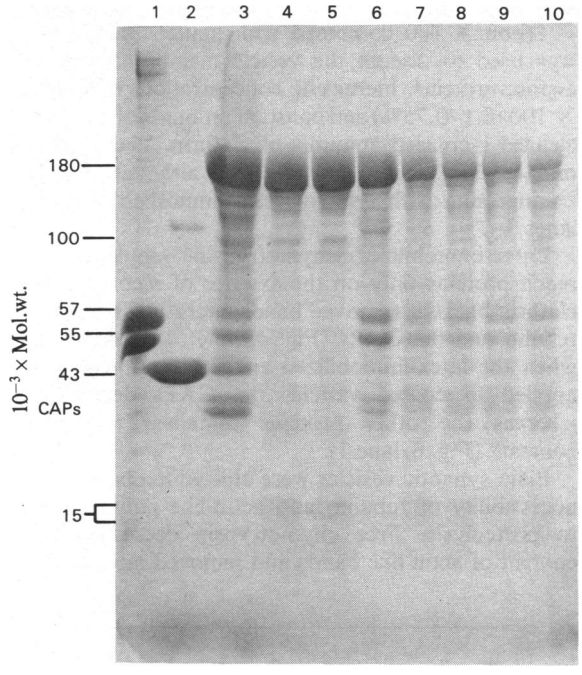


Fig. 4. *Polypeptide composition of coated vesicles and clathrin after enzymic treatment with chymotrypsin free and bound on the surfaces of Lytron polystyrene latex particles*

Lane 1, 50 μg of purified bovine brain tubulin marker. Lane 2, 5 μg of dog muscle α-actinin and 25 μg of rabbit muscle actin markers. Lane 3, polypeptide profile of 125 μg of the clathrin extracted from crude coated vesicles and purified by passage through a Sepharose 4B column. Lane 4, same clathrin shown in lane 3 after treatment with free chymotrypsin at pH 6.5. Lane 5, clathrin prepared as in lane 3, but treated with latex-immobilized chymotrypsin at three times the ratio of enzyme/protein used in lane 4. Note the appearance of a polypeptide doublet around the 15000-mol.wt. region. Lane 6, polypeptide profile of 90 μg of proteins of a purified coated-vesicle fraction. Lanes 7–10, protein profile of coated vesicles shown in lane 6 after treatment with increasing amounts of free chymotrypsin (once, twice, three times and four times the usual ratio respectively). Increasing amounts of clathrin were cleaved. Significant quantities of tubulin- and actin-like proteins remained.

(Plate 2*b*). The newly formed 15000-mol.wt. doublet remained bound to clathrin after Sepharose 4B column chromatography. This preparation eluted as a broad plateau from the column.

Topography of proteins on vesicles and clathrin baskets

The ability of clathrin to withstand enzymic treatment enabled a preliminary topographical analysis of proteins bound to coated and plain synaptic vesicles. Coated vesicles showed cleavage of the CAPs and the appearance of the 15000-mol.wt. doublet (Fig. 4, lane 7). It should be noted that other proteins remained essentially intact. At higher enzyme ratios, increasing amounts of clathrin were proteolysed. Nevertheless, the 15000-mol.wt. doublet remained throughout the various enzyme concentrations (Fig. 4, lanes 8–10).

Triton X-100 combined with limited proteolysis was used to disrupt the vesicle membrane and to expose proteins. Increasing concentrations of Triton X-100 (0.1–0.75%) and constant amounts of enzyme cleaved increased amounts of clathrin. The 43000-mol.wt. band was not affected, although bands co-migrating with tubulin were diminished (Fig. 5, lanes 3–7).

Latex-immobilized chymotrypsin, employed to reach proteins only on the exterior of reconstituted clathrin baskets, removed bands migrating like actin, tubulin and the CAPs (Fig. 4, lane 5). In contrast, when the latex-immobilized enzyme treatment was applied to coated vesicles the CAPs decreased, whereas the other proteins remained relatively constant (Fig. 6, lane 1).

Plain synaptic vesicles were utilized to observe the accessibility of tubulin- and actin-like polypeptides to proteolysis. Free chymotrypsin decreased the content of actin-like bands and removed most of the tubulin-like bands (Fig. 6, lane 3). Interestingly, the 15000-mol.wt. doublet did not appear. However, latex-immobilized chymotrypsin left amounts of the actin- and tubulin-like bands intact (Fig. 6, lane 4).

Cross-linking determinations with lipophilic reagents were performed to examine whether proteins inaccessible to enzymic digestion were in close proximity to one other in the coated-vesicle lipid bilayer. Consistently, only one of the tubulin-like polypeptides diminished its staining intensity (Fig. 6, lanes 5 and 6), indicating that clathrin is not an integral part of the vesicle membrane.

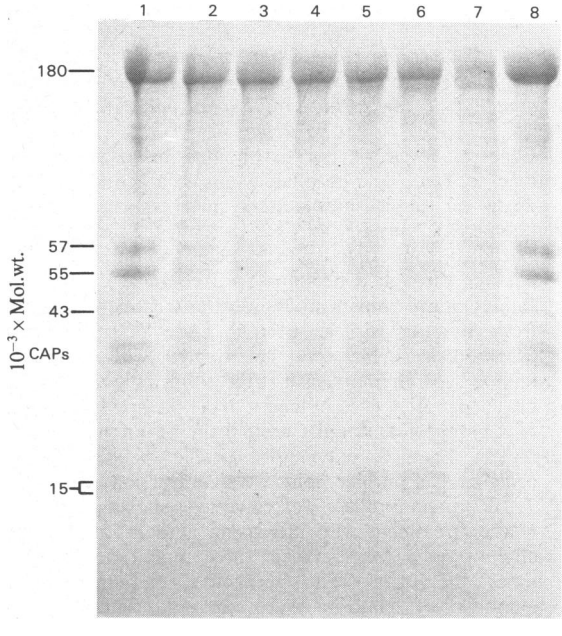


Fig. 5. *Polypeptide profile of coated vesicles after Triton X-100 and enzyme treatments*

Lane 1, 100 μg of total protein of purified coated vesicles (control). Lane 2, same sample as lane 1 after free chymotrypsin treatment. Lanes 3–7, coated vesicles treated with increasing amounts of Triton X-100 and constant amounts of enzyme (lane 3, 0.1%; lane 4, 0.2%; lane 5, 0.3%; lane 6, 0.5%; lane 7, 0.75% Triton X-100). Note the increased proteolysis of clathrin molecules as the concentration of Triton X-100 increases. Lane 8, coated vesicles treated with 1% Triton X-100 in the absence of chymotrypsin (control) (100 μg).

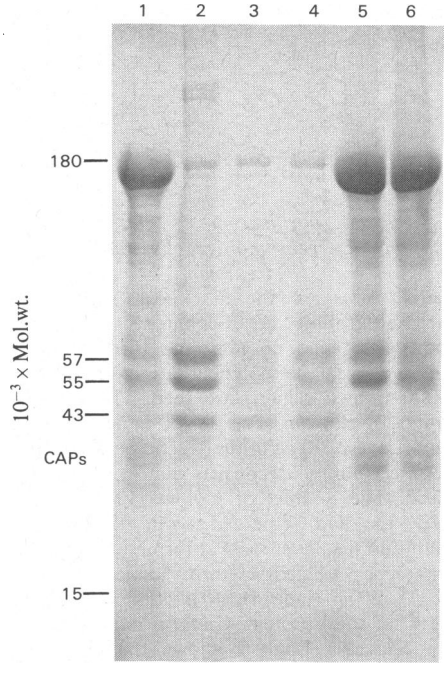


Fig. 6. *Polypeptide profile showing the effect of free and bound chymotrypsin treatment and photochemical cross-linking on proteins of coated vesicles*

Lane 1, 90 μg of purified coated vesicles after incubation with immobilized chymotrypsin at three times the usual ratio. Lane 2, polypeptide composition present in 65 μg of purified plain synaptic vesicles. Lane 3, same sample shown in lane 2 after treatment with free chymotrypsin. Lane 4, same sample shown in lane 2 after treatment with latex-immobilized chymotrypsin at three times the usual enzyme/protein ratio. Lane 5, 100 μg of purified coated vesicles before cross-linking, but containing 5% ethanol in the absence of 2-mercapto-ethanol. Lane 6, as lane 5, but after photochemical cross-linking with 4,4′-dithiobisphenylazide. Note the decrease in the α-tubulin-like subunit migrating in the 57000-mol.wt. region.

Discussion

We report here that a polypeptide doublet migrating in the 30000-mol.wt. region accompanies clathrin and is required for assembly of clathrin baskets *in vitro*. Gel electrophoresis and electron microscopy indicate that clathrin molecules and basket morphology were unaffected after controlled chymotrypsin treatment. However, when these basket structures were depolymerized by re-adjustment of pH, the capacity for re-assembly or closure into baskets was lost. Addition of purified CAPs to enzyme-treated clathrin did not promote re-assembly into baskets, possibly indicating that fragments of the CAPs (perhaps of mol.wt. 15000) remain attached to the clathrin polypeptides, preventing re-association of the native complex (M. P. Lisanti, W. Schook, N. Moskowitz, C. Ores & S. Puszkin, unpublished work).

Actin- and tubulin-like proteins, as well as other co-purifying polypeptides, were removed from clathrin by re-chromatography on Sepharose 4B; only the 30000-mol.wt doublet remained. Inasmuch as clathrin of this purity assembled into baskets, involvement of other polypeptides was eliminated. Attempts to separate these CAPs by DEAE-cellulose chromatography were unsuccessful. In all instances, clathrin precipitated with various concentrations of $(NH_4)_2SO_4$ or purified further by several chromatographic steps, i.e. DEAE-cellulose and hydroxyapatite, showed the presence of the CAPs. In contrast, when clathrin was extracted and purified from enzyme-treated crude coated vesicles the 30000-mol.wt. doublet did not co-purify.

These observations suggest that this doublet is bound natively to clathrin on coated vesicles *in vivo*, thus greatly diminishing the possibility of its spontaneous non-specific association with clathrin as a result of purification. In a recent report, a similar doublet was observed associated with clathrin (Ungewickell & Branton, 1981). It was suggested that these polypeptides represent the light chains of a clathrin complex.

A third identity was proposed for a similar doublet by Keen & Willingham (1980). They observed that a polypeptide doublet of slightly higher molecular weights, i.e. 38000 and 33000, antigenically reacted with clathrin antibodies, implying that these polypeptides originated from clathrin's cleavage during or after its purification. Therefore, we tried removing the CAPs at an early step of clathrin's purification procedure, before they were separated from coated vesicles and solubilized. This was attempted because all our preparations of coated vesicles showed the presence of a 30000-mol.wt. doublet as part of the vesicle's protein population. Clathrin prepared from enzyme-treated crude coated vesicles emerged free of polypeptides migrating in the 30000-mol.wt. region of the gel. Our data suggest that the CAPs are native to clathrin and unlikely to originate from clathrin's degradation during or after purification.

When purified clathrin from untreated vesicles was assembled as baskets and subjected to enzyme treatment, a smaller 15000-mol.wt. doublet was generated. Similarly, when crude coated vesicles were treated with chymotrypsin before solubilization of clathrin, a 15000-mol.wt. doublet co-purified. It appears that this smaller fragment was resistant to further cleavage under these conditions and remained tightly bound to clathrin. It is tempting to assume that the 15000-mol.wt. doublet originates from the CAPs. Clathrin, with the 15000-mol.wt. doublet attached to it, was adequate for assembly of lattices but lacked capacity for closure into baskets.

Since clathrin assembled as baskets was resistant to enzymic proteolysis at pH 6.5 under controlled conditions, a comparison of polypeptides accessible to proteolytic cleavage was carried out utilizing coated and plain vesicles. Free chymotrypsin in solution and immobilized chymotrypsin bound to the surfaces of Lytron polystyrene particles were employed to study the apparent location of CAPs in coated vesicles. Both enzyme preparations, bound and free, were capable of removing the CAPs from coated vesicles. Significant quantities of actin- and tubulin-like bands as well as other proteins remained. This implied that the CAPs protrude externally with respect to the clathrin lattice, and that the other proteins either were internal with respect to clathrin or on the vesicle membrane surface between clathrin's lattice and the lipid bilayer.

To determine whether these polypeptides were membrane proteins *per se*, and thus inaccessible to the enzyme's activity, various enzyme/protein ratios and concentrations of Triton X-100 were used. Increasing concentrations of free chymotrypsin proteolysed clathrin on coated vesicles, whereas other proteins remained constant. Treatment of coated vesicles with 0.75% Triton X-100 and constant amounts of enzyme gave identical results. Interestingly, when clathrin was cleaved an initial amount of the 30000-mol.wt. doublet did not increase, an indication that it did not arise from clathrin's proteolysis. In addition, these results precluded the possibility that inaccessible proteins were sandwiched between clathrin and the vesicle bilayer, because when clathrin was cleaved the actin- and tubulin-like bands remained constant. This enhanced the likelihood that these proteins were anchored to the lipid bilayer. Alternatively, when plain synaptic vesicles were treated with free chymotrypsin, actin- and tubulin-like proteins were cleaved. In contrast, polystyrene-bound chymotrypsin left most actin- and tubulin-like proteins intact, indicating that bound enzyme did not dissociate in the presence of vesicles.

Further information on the properties of proteins in vesicles was provided by a photochemical reaction using a lipophillic cross-linking agent. Results showed that in coated vesicles one of the tubulin-like subunits could be cross-linked, A marked decrease in the intensity of this band was noted, whereas all other protein bands remained constant, implying that both clathrin and the CAPs are located at some distance from the vesicle bilayer.

Brain coated vesicles were reported to contain polypeptides similar to those present in sarcoplasmic-reticulum vesicles. These brain coated vesicles have the ability to sequester Ca^{2+} and contain a Ca^{2+}-dependent ATPase similar antigenically to the one found in muscle microsomal fractions. Furthermore, clathrin extractions from coated vesicles did not affect Ca^{2+} uptake (Blitz *et al.*, 1977). In our hands, coated vesicles, incubated with up to three times the usual ratio of free chymotrypsin/protein at pH 6.5 for 60 min, retained the ability to sequester Ca^{2+}. This implied that the vesicle lipid bilayer remained intact after enzyme treatment and that the 30000-mol.wt. doublet did not play a role in Ca^{2+} uptake. In contrast, coated vesicles treated with Triton X-100 (1%) at pH 6.5, or with free chymotrypsin at pH 7.5, were unable to perform uptake (M. P. Lisanti, W. Schook, N. Moskowitz, C. Ores & S. Puszkin, unpublished work). Thus it seems Ca^{2+} sequestration depends on a relatively intact vesicle as well as the presence of various proteins migrating with electrophoretic mobilities between the 180000- and 30000-mol.wt. regions.

In summary, an intact polypeptide doublet of mol.wt. 30000 is needed for optimal assembly of clathrin into basket structures. Utilizing limited proteolysis and photochemical cross-linking of proteins tightly associated with clathrin on coated vesicles, we have provided data on the topographical location of CAPs in these ubiquitous organelles.

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