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Purification and properties of 100-kd proteins from coated vesicles and their reconstitution with clathrin

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Bullock brain coated vesicles contain a family of at least six 100-kd polypeptides which have the property of promoting clathrin assembly. These proteins have been purified from Triton X-100-extracted coated vesicles by a combination of gel filtration and chromatography on hydroxylapatite and DE-52 cellulose. Three major 100-kd species occur as complexes with a stoichiometric amount of a 50-kd polypeptide. On cross-linking these complexes, the chief products appear to contain two polypeptides of 100 kd and two of 50 kd. These 100-kd/50-kd complexes will polymerise with low concentrations of clathrin to give a relatively homogeneous population of coats predominantly of the 'barrel' size. In contrast, three other polypeptides of 100 kd lack the 50-kd protein but polymerise with clathrin under the same conditions to yield coats of a wide range of sizes including 'barrels', truncated icosahedra and particles of >100 nm diameter. When clathrin cages are reassembled with a saturating amount of 100-kd/50-kd complexes and studied by electron microscopy, the additional proteins appear to follow the underlying geometry of the clathrin polyhedra, partially filling in the polygonal faces of the cage structures. Saturation appears to require ~3 molecules of 100-kd polypeptide per clathrin trimer.

Key words: coated vesicles/clathrin/100-kd coat proteins/ reconstitution

Introduction

Clathrin forms the polyhedral cage of coated vesicles which mediate the transfer of membrane within cells (Pearse, 1975, 1976; Pearse and Bretscher, 1981). However, families of 100-kd proteins (which include polypeptides of 100 – 110 mol. wt.) were observed to co-purify with the clathrin of coated vesicles in early preparations from several different tissues and sources (Pearse, 1978) including pig brain, bullock brain, adrenal medulla and parotid salivary gland, human placenta and chicken oocytes. Keen *et al.* (1979) and Zaremba and Keen (1983) extracted the coat proteins from bullock brain coated vesicles in 0.5 M Tris-Cl pH 7.0, separated the clathrin and reported that the 100-kd protein fraction promoted clathrin assembly into coats with a sedimentation coefficient of 250 S. These same 100-kd proteins have also been implicated in the binding of clathrin to clathrin-depleted coated vesicles (Unanue *et al.*, 1981).

We describe a procedure for the purification of these 100-kd proteins and some of their properties. We reconstituted them with clathrin and attempted to maximise the binding of the 100-kd polypeptides (in association with 50-kd polypeptides) to clathrin cages to see what contribution they made to the structure.

Results

Purification of 100-kd proteins from bullock brain coated vesicles

Crude coated vesicles are rapidly prepared from bullock brain extract by treatment with 1% Triton X-100 followed by centrifugation for 1 h on a 5 – 25% sucrose gradient. The coat proteins are solubilised in buffer B and separated by gel filtration on Sepharose CL-4B. At this stage the clathrin is almost pure but the 100-kd coat proteins which behave as complexes, with a Stokes radius of 60 Å, are contaminated with residual clathrin and many other proteins present in small amounts. These are removed by a combination of chromatography on hydroxylapatite and DE-52 cellulose.

A typical elution profile from the hydroxylapatite column is shown in Figure 1 combined with a 7.5% SDS polyacrylamide gel analysis of samples of eluant fractions. Members of the 100-kd family of polypeptides appear across the elution profile essentially separating into two groups. (i) 100-kd polypeptides (HA-I, Hydroxyl Apatite-group I) which co-elute with residual clathrin, endogenous ferritin and many other proteins; (ii) 100-kd polypeptides (HA-II), which unlike the first group co-elute with a stoichiometric amount of 50-kd polypeptide.

Figure 2 shows samples of purified material after chromatography on DE-52 cellulose subjected to electrophoresis on 7.5% SDS polyacrylamide gels. The HA-I group of 100-kd polypeptides appears to separate into at least three bands: a faint, slow band, the slowest of all the 100-kd polypeptides observed, plus a doublet of slightly faster migrating bands. The HA-II group of 100-kd polpeptides, associated with the 50-kd protein, also separates into at least three bands when the gels are lightly loaded as in Figure 2c. The slowest of these bands appears to migrate behind the doublet of the HA-I set of 100-kd polypeptides whereas the faster bands migrate ahead of them. The 50-kd polypeptide appears to occur in a 1:1 molar ratio with the total of the 100-kd (HA-II) polypeptides.

Twelve bullock brains at a time were taken routinely through this procedure. Typical yields were 30 mg of pure clathrin (heavy chains plus light chains), 150 μg of the HA-I set of 100-kd polypeptides and 2 mg of the HA-II group of 100-kd proteins plus the 50-kd protein. All these proteins can be stored at 0 – 4°C in solution in buffer B for up to 2 weeks or more and still retain their ability to reconstitute into coat structures.

Reversible cross-linking of the 100-kd and 50-kd polypeptides

Consistently, the HA-II group of 100-kd polypeptides co-elutes in 1 to 1 stoichiometry with a 50-kd polypeptide during gel filtration and chromatography on hydroxylapatite and DE-52 cellulose. This suggests that these polypeptides exist as complexes.

To examine the nature of these complexes further, samples of the 100-kd and 50-kd proteins, co-eluted from the hydroxylapatite column, were treated with the reversible

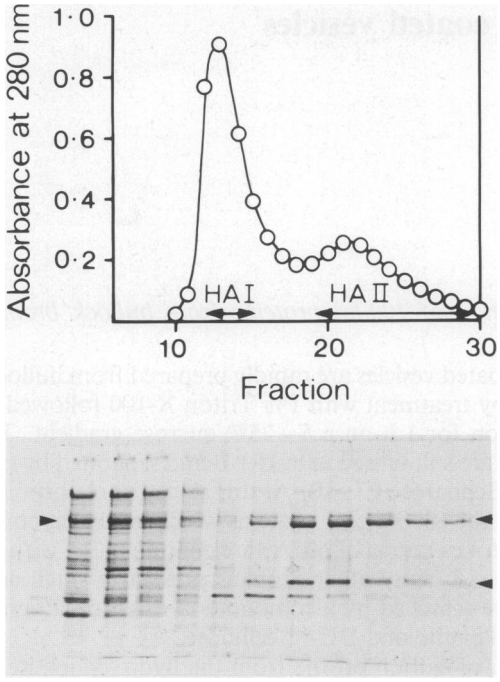

Fig. 1. (upper) Typical elution profile of 100-kd polypeptides after chromatography on hydroxylapatite. The bar lines indicate the fractions pooled to give two sets of 100-kd polypeptides HA-I and HA-II respectively. **(lower)** SDS polyacrylamide gel electrophoresis of samples across the profile (the first five tracks are fractions 11 – 15 and the rest are alternate fractions from 17 to 29). The arrows indicate the polypeptides of 100 kd and 50 kd.

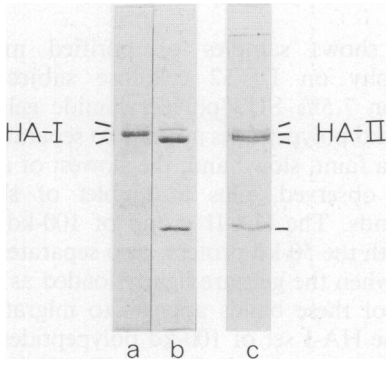

Fig. 2. SDS polyacrylamide gel eletrophoresis of the two groups of 100-kd polypeptides: **(a)** HA-I consisting of three distinguishable bands of different mobility as indicated by the markers; **(b)** HA-II run on the same gel as HA-I; **(c)** HA-II at a lower loading on a 0.4 mm thickness gel showing three distinguishable bands of different mobility as indicated by the markers.

cross-linker, ditho-bis (succinimidyl propionate). As shown in Figure 3a, during electrophoresis on a 5% SDS polyacrylamide gel, the major product migrated as a single broad band which ran more slowly than uncross-linked 100-kd polypeptides. The 100-kd polypeptides and the 50-kd protein are consumed to form the cross-linked species.

To check the composition of the cross-linked complexes, the cross-links were cleaved and the constituents of the complexes were separated by electrophoresis in a second dimension on 7.5% SDS polyacrylamide gels (Figure 3b). This shows that the major cross-linked complexes are indeed composed of the 100-kd and 50-kd polypeptides. As well as the major species, higher aggregates are also present. Intermediate species due to less complete cross-linking (Figure 3c) include single 100-kd polypeptides associated with the 50-kd protein and presumably dimers of 100-kd polypeptides lacking 50-kd protein. Thus the major products of cross-linking shown in Figure 3a and b probably contain two polypeptides of 100-kd and two of 50-kd. Complexes containing the slower 100-kd polypeptides migrate more slowly than those exclusively composed of the faster 100-kd polypeptides, as might be expected. It has not been possible to resolve which dimers exist out of the various possibilities.

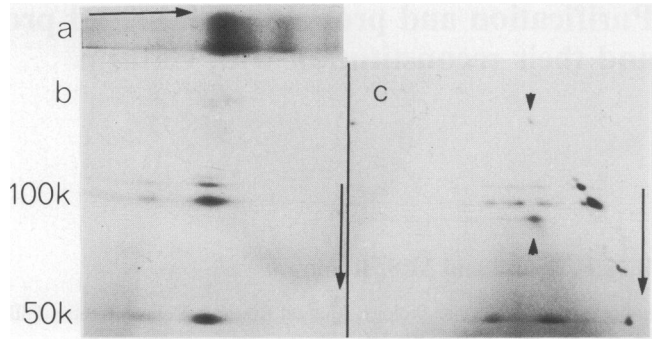

Fig. 3. Analysis of cross-linked species of 100-kd/50-kd complex on silver stained SDS polyacrylamide gels. The arrows indicate the direction of electrophoresis. **(a)** Samples of group HA-II containing 100- kd and 50-kd polypeptides moderately cross-linked with dithio-bis (succinimidyl propionate) and analysed on a 5% gel. **(b)** Example of a 2nd dimension (7.5% gel) analysis of maximally cross-linked sample. The major band observed in the 1st dimension is shown to contain complexes containing both 100-kd and 50-kd polypeptides, probably two of each. Some of the material is in the form of higher aggregates. **(c)** 2nd dimension (7.5% gel) analysis of group HA-II polypeptides after restricted cross-linking. Intermediate complexes are present including those containing one 100-kd polypeptide plus the 50-kd protein and dimers of the 100-kd polypeptide lacking 50-kd polypeptide. Marker spots (indicated by arrow heads) are clathrin (180 K) and transferrin receptor (180 K in the first dimension, 90 K in the second dimension). That the 50-kd polypeptides apparently stain more strongly than the 100-kd polypeptides is an anomaly of the silver stain.

Various markers of known mol. wt. were also applied to the first dimension gels including MAPS (brain microtubule-associated protein), dynein, thyroglobulin and clathrin. Clathrin trimers cross-linked with dithio-bis (succinimidyl propionate) and bovine serum albumin cross-linked with glutaraldehyde (Payne, 1973) were also used to calibrate the gels. All the cross-linked complexes migrate anomalously on different percentage gels according to Ferguson plots (Ferguson, 1964; Weber and Osborn, 1975); however, limits can be established for the apparent mol. wt. of the major 100-kd/50-kd complexes of 300 ± 50 kd. This is compatible with their consisting of two 100-kd proteins cross-linked to two 50-kd polypeptides.

The first set (HA-I) of 100-kd polypeptides which eluted from the hydroxylapatite column did not appear to cross-link efficiently with dithio-bis (succinimidyl propionate), and so their state of association is unknown.

Conditions for reconstitution with clathrin

The group of 100-kd polypeptides plus 50-kd protein which elutes from the Sepharose CL-4B gel filtration column corresponds to the 'assembly polypeptide' fraction described by Zaremba and Keen (1983). 0.33 ml of a peak fraction (OD 280 of 0.5) of these polypeptides was combined with 0.04 mg pure clathrin in a total of 1 ml of buffer B. The coat proteins were reconstituted by overnight dialysis, at 0 – 4°C, against reconstitution buffer A. Aggregated material, which did not

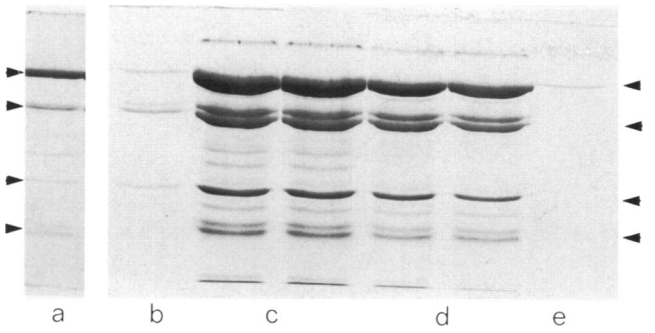

Fig. 4. SDS polyacrylamide gel electrophoresis of samples of re-assembled coats. The arrow heads indicate the major coat proteins, clathrin heavy chain (180 kd), 100-kd and 50-kd polypeptides and clathrin light chains (30–35 kd). **(a)** Coats reassembled from placental coat proteins. **(b)** 100-kd and 50-kd proteins pelleted in the absence of added clathrin under the same conditions as reassembled coats (compare **d**). **(c)** Duplicate samples of coats reassembled from a 1-ml starting mixture containing 0.08 mg/ml of clathrin and 0.5 ml of a peak fraction (with an OD of 0.5 at 280 nm) from the Sepharose CL-4B column containing 100-kd and 50-kd polypeptides. **(d)** Duplicate samples of coats reassembled from a 1-ml starting mixture containing 0.04 mg/ml of clathrin and 0.33 ml of the same fraction used in **(c)**. **(e)** Clathrin pelleted in the absence of 100-kd and 50-kd proteins under the same conditions as reassembled coats (compare **c**). Apparent molar ratios of 180 kd:100 kd:50 kd:35 kd in the coats were **(c)** 3:3.2:2.7:3.2 and **(d)** 3:3.4:2.8:3.1.

include coat proteins, was removed by centrifugation (20 000 *g*, 10 min) and the coats were then pelleted (100 000 *g*, 1 h at 5°C). The pellet was resuspended in a small volume of reconstitution buffer. In these conditions, neither clathrin nor the 100-kd/50-kd complexes appear to sediment substantially in the absence of the other. These conditions were therefore adopted for further assembly experiments.

Figure 4 shows the protein composition of pelleted coats from an experiment analysed by SDS polyacrylamide gel electrophoresis. The ratio of clathrin heavy chain:100-kd proteins:50-kd polypeptide:total light chains was estimated in this experiment to be 3:3.4:2.8:3.1.

Binding of 100-kd/50-kd complexes in reassembled coats

Two series of reassembly experiments were set up where the ratio of the 100-kd/50-kd complex (HA-II from the hydroxylapatite separation) to clathrin varied. In the first series the clathrin concentration was in the range 0.03–0.2 mg/ml in the presence of the 100-kd/50-kd complex at a constant concentration of 0.05 mg/ml or in its absence. Samples from the supernatants and pellets resulting from centrifugation of these reassembly mixes were analysed on SDS polyacrylamide gels (Figure 5). At each clathrin concentration, more clathrin is assembled into cages in the presence of the 100-kd/50-kd complex than in its absence. However, in the absence of the 100-kd/50-kd complex ~20% of the clathrin sediments (after overnight dialysis into polymerisation buffer A from buffer B) irrespective of its initial concentration. This is unlike the assembly of placental clathrin described previously (Crowther and Pearse, 1981) which behaved like a condensation process with a critical concentration of 0.05 mg/ml when clathrin was induced to polymerise from a low ionic strength solution by the addition of 0.1 volume of 1 M MES pH 6.2 containing 20 mM $MgCl_2$.

In the second series the clathrin was at the constant concentration of 0.16 mg/ml and the 100-kd/50-kd complex was varied between 0.02 and 0.1 mg/ml. (Above this concentration of 100-kd/50-kd complex, some precipitation of coat

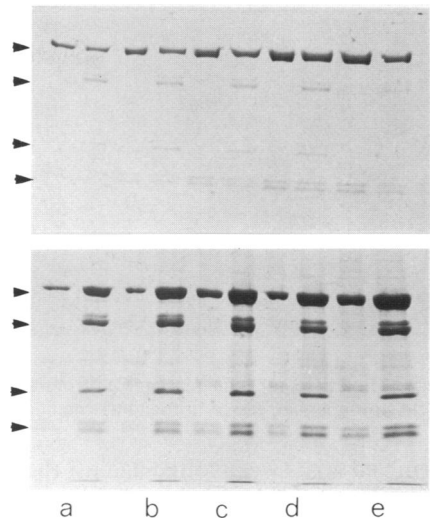

Fig. 5. Analysis of reconstitution experiments by SDS polyacrylamide gel electrophoresis. Arrow heads indicate the major coat proteins, as in Figure 4. The initial concentration of 100-kd/50-kd complex was constant in these experiments at 0.05 mg/ml and the initial clathrin concentration was varied. The top gel shows pairs of supernatants (with and without 100-kd/50-kd complexes) and the bottom gel shows corresponding pairs of pellets (concentrated 10-fold) from experiments at different clathrin concentrations **(a)** 0.05 mg/ml, **(b)** 0.08 mg/ml, **(c)** 0.12 mg/ml, **(d)** 0.16 mg/ml, and **(e)** 0.2 mg/ml.

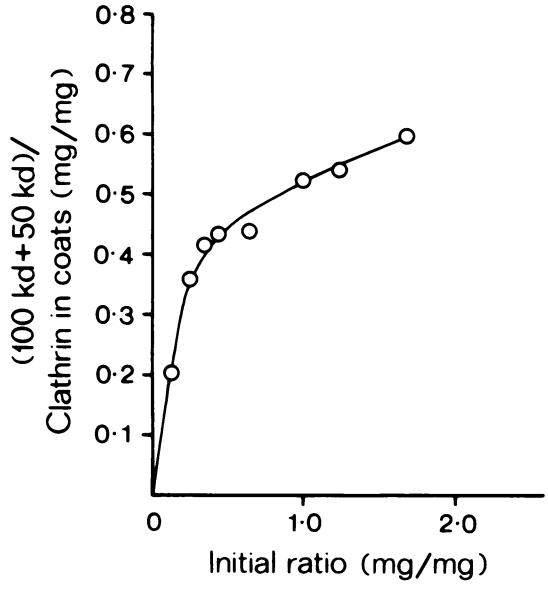

Fig. 6. Graph of the ratio of 100-kd/50-kd complex to clathrin in reconstituted coats *versus* the approximate corresponding ratios in the initial reassembly mixtures from which they were derived. Reconstitutions at high initial ratios were at low clathrin concentration (0.03–0.05 mg/ml).

proteins began to occur). Increasing the amount of 100-kd/50-kd complex up to 0.1 mg/ml resulted in a corresponding increase in the proportion of the total clathrin which reassembled into coats. Samples of the reassembled coats from both series of experiments (separated from the unassembled material by centrifugation, and concentrated) were analysed by SDS polyacrylamide gel electrophoresis (as in Figure 4). The polypeptide bands from such gels, stained in PAGE blue 83 (BDH), were cut out and the molar ratios of the coat proteins were estimated as described in Materials and methods. The ratio of the 100-kd/50-kd complexes to clathrin

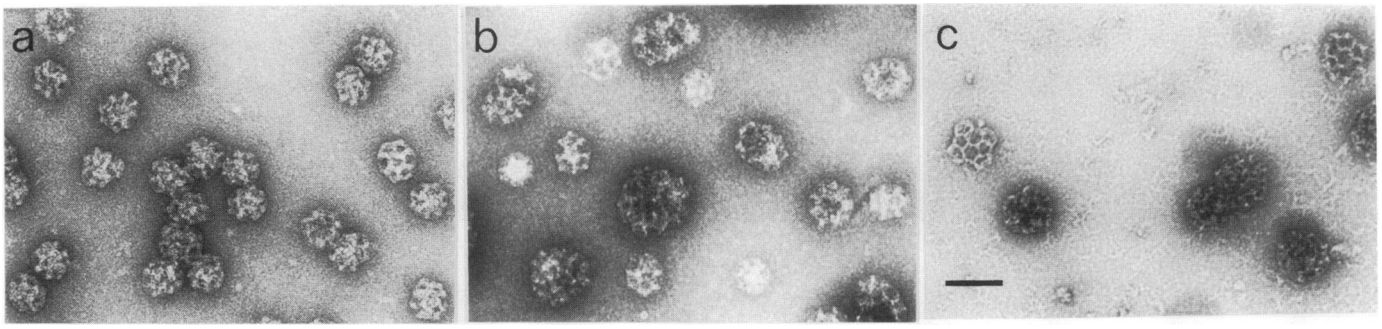

Fig. 7. Reconstituted particles (**a**) coats reassembled from clathrin and 100-kd/50-kd complexes (HA-II), (**b**) coats reassembled from clathrin and 100-kd proteins (HA-I), (**c**) clathrin alone pelleted in the same conditions as reassembled coats. The bar line represents 1000 Å.

in the reassembled coats was plotted against the ratio of proteins added to the assembly mixture. The result is shown in Figure 6. Increasing the relative amount of 100-kd/50-kd complex in the starting mixture results in the formation of coats with a higher proportion of these proteins to clathrin, approaching a composition of one polypeptide of 100 000 daltons and one of 50 000 daltons per clathrin heavy chain, (corresponding to a ratio of 0.7 mg/mg for 100-kd/50-kd complex per clathrin heavy plus light chain).

Structure of the reassembled coats

When freshly disassembled clathrin (0.05 – 0.20 mg/ml in buffer B) is dialyzed overnight at 0 – 4°C against reconstitution buffer A pH 7.0, and centrifuged for 1 h at 5°C at 100 000 *g*, ~20% of the protein is pelleted. After resuspension in a small volume of reconstitution buffer this material appears as a mixture of cages, cage fragments and free triskelions by electron microscopy (Figure 7c).

In contrast, when 0.03 mg/ml clathrin is mixed with an excess of 100-kd/50-kd complexes up to a concentration of 0.05 mg/ml, after dialysis against reconstitution buffer A pH 7.0, ~80% of the clathrin will sediment in the form of coats (Figure 7a). The particles are relatively homogeneous in size as described previously (Zaremba and Keen, 1983). Addition of the 100-kd/50-kd complexes to the structures seems to thicken the vertices and the edges of the polyhedra formed by the clathrin, giving the particles a characteristic 'chunky' appearance. Most of the polyhedra seem to maintain their structures in three dimensions in the stain whereas many of the cages formed from clathrin alone appear to be flattened.

In general, the size distribution amongst the reconstituted coats containing the 100-kd/50-kd complexes is reminiscent of the fields of particles originally purified from brain extracts (Pearse, 1975). The same types of particles can be identified amongst the reconstituted polyhedra as were observed in the purified preparations (Crowther *et al.*, 1976). To obtain a clearer view of the contribution made by the 100-kd/50-kd complexes to the structures, particular views of these three types of polyhedra were identified. Selected specimens are presented in Figure 8.

Particularly striking are the views of the 'barrel' structure [Figure 8a(i)] where the central hexagon is surrounded by six outer polygons, two pentagons top and bottom and a hexagon at each side. The presence of the 100-kd/50-kd complexes in the structure thickens the vertices and the edges of the clathrin lattice giving rise to a 'Star of David' like appearance around the central hexagon most clearly seen on the lower right hand image. The 'faces' of the polygons are partially filled in, the sharp corners at their vertices being largely obscured to give just roundish 'holes' in the centres of the polygons. The central hexagon appears to contain extra material.

Also remarkable are two examples of another view of the 'barrel' structure related to the first one by a 30° turn around the 6-fold axis [Figure 8a(ii)]. This view has a pair of hexagons spanning the central region with a pentagon above and below plus a 'handle' at each corner formed by pentagons. The presence of the 100-kd/50-kd complex gives the view the appearance of a 'double bow' of thick ribbon. The hexagons and pentagons are almost obscured.

Figure 8b shows a view of structure B where again the hexagons and pentagons of the clathrin polyhedron are obscured by the additional 100-kd and 50-kd polypeptides.

The next set of images (Figure 8c) represents the view of structure C with a central hexagon surrounded by five pentagons and one hexagon. The particles show a 'star' like pattern around the central hexagon similar to the first set of views of the 'barrel'. The central hexagon also appears to contain extra material as in the 'barrel'.

Thus, in preparations where there are apparently up to three 100-kd and three 50-kd polypeptides per triskelion the extra material seems to follow the geometry of the polyhedra. This suggests that the 100-kd/50-kd complexes have unique binding sites on the clathrin lattice which are saturated when this amount of complex is bound.

Coats reconstituted using the two separated groups of 100-kd polypeptides, those with (HA-I) and those without the 50-kd protein (HA-II) were compared. 1-ml samples of solutions containing 0.04 mg/ml clathrin and 0.06 mg/ml of either 100-kd proteins or 100-kd/50-kd complexes in buffer B were dialysed against reconstitution buffer A overnight at 0 – 4°C. The resulting coats were harvested by centrifugation for 1 h at 100 000 *g*. The 100-kd proteins, like the 100-kd/50-kd complexes, promoted clathrin assembly dramatically over the small amount of clathrin which assembled and pelleted in their absence. The coats obtained with the 100-kd/50-kd complexes (see Figure 7a) look indistinguishable from those obtained with total assembly polypeptides, a high proportion being 'barrels' (~65 nm in diameter). In contrast, the coats obtained with the other (HA-I) 100-kd polypeptides (Figure 7b) look much more heterogeneous in size (Figure 9) including many larger polyhedra. One fairly frequent member of this group is the truncated icosahedron (~80 nm in diameter) which is made up of 60 triskelions (Figure 8D).

Reconstitution of coats from proteins of placental coated vesicles

Freshly extracted human placental coated vesicles contain

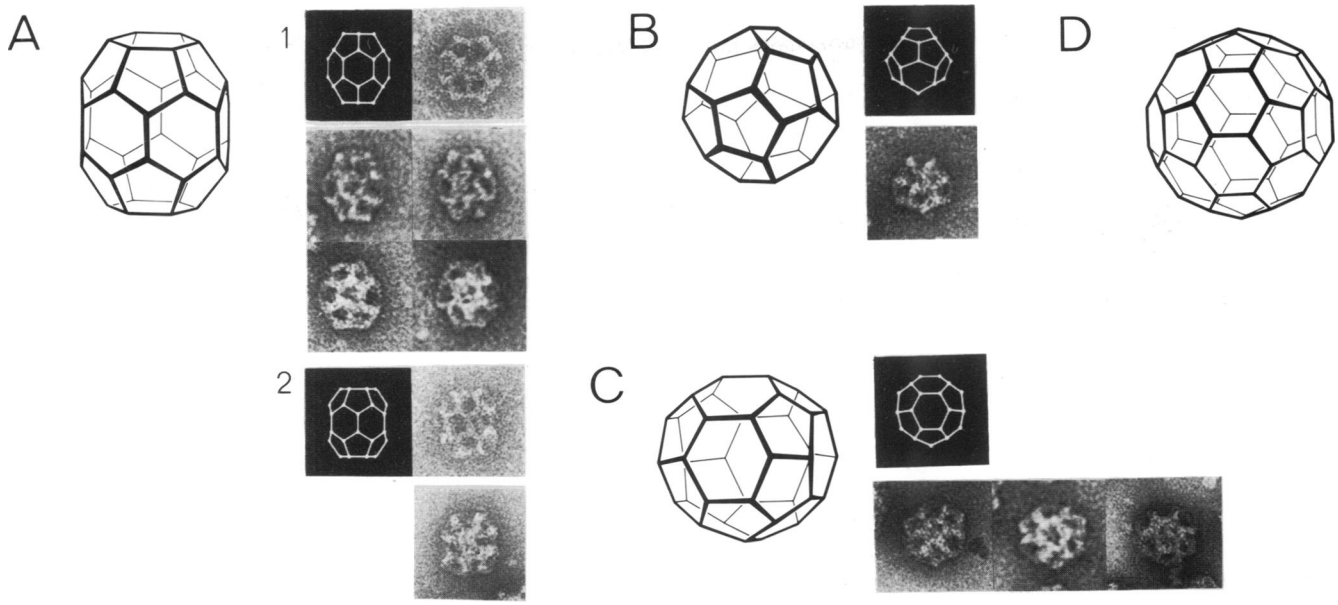

Fig. 8. Selected views of particles of known geometry showing the distribution of the 100-kd/50-kd complex over the clathrin lattice. Structure **A**, the hexagonal barrel, is represented by views (**1**) and (**2**). View (**2**) can be generated from view (**1**) by a rotation of 30° around the 6-fold axis passing through the centres of the top and bottom hexagons. The structures adjacent to the computer-simulated views contain clathrin alone. Underneath are shown corresponding views of particles from populations where apparently the clathrin cages were nearly saturated with 100-kd/50-kd complexes. Four examples are shown of view (**1**) and one example of view (**2**) where the difference from the cage constructed from clathrin alone (above) is particularly striking. One view of particle **B** and three views of particle **C** from the same populations of coats are shown under their corresponding simulated views of the clathrin geometry of these particles. Structure **D** is a truncated icosahedron, a frequent particle amongst coats constructed from clathrin and group HA-I 100-kd polypeptides.

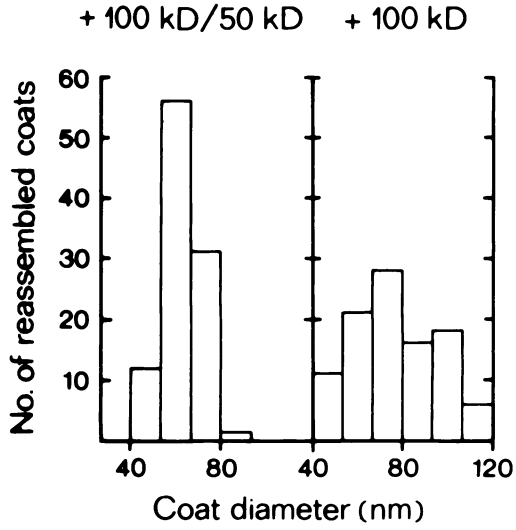

Fig. 9. Histogram of diameters of coats reconstituted from clathrin with an equivalent amount of either group HA-II 100-kd polypeptides complexed to the 50-kd protein or group HA-1 100-kd polypeptides which lack the 50-kd protein.

principally clathrin heavy chain, a tight doublet of 100-kd proteins, a 50-kd protein and a doublet of light chains (32 kd) which migrate faster than those of brain (35 kd). These polypeptides (180 kd:100 kd:50 kd:32 kd) occur in the apparent molar ratio of 3:2:1:3. In contrast to the bullock brain coat proteins, those of placental coated vesicles do not extract efficiently in buffer B. Instead, buffer E (containing 1.2 M Na thiocyanate) is used to solubilise both clathrin and the 100-kd and 50-kd proteins. After removal of the vesicles by centrifugation, the coat proteins (dialysed against buffer B to remove the thiocyanate) can be polymerised to form coats in the same way as the bullock brain coat proteins. A sample of such coats was analysed by SDS polyacrylamide gel electrophoresis as shown in Figure 4a. The doublet of 100-kd placental coat proteins migrates faster than the first group (HA-I) of bullock brain 100-kd polypeptides and in between the fastest and the slowest band of the second group (HA-II) of 100-kd bullock brain polypeptides.

Discussion

A bulk procedure is described here for the purification of coated vesicle coat proteins from 12 bullock brains at a time. The starting material for the separation of the coat proteins is Triton X-100-extracted coated vesicles (TCVs) prepared by a shorter route than that described previously (Pearse, 1982). Thus this preparation scheme involves the minimum of time-consuming centrifugation.

Coated vesicles contain a family of polypeptides which migrate on SDS polyacrylamide gels with apparent mol. wts. of ~100 000 daltons. Bullock brain coated vesicles contain at least six such 100-kd proteins with slightly different mobilities. These fall into two groups of three, those of HA-I which elute first during chromatography on hydroxylapatite and those of HA-II which are complexed with a 50-kd polypeptide and elute from hydroxylapatite at higher phosphate concentrations. Human placental coated vesicles contain two or more 100-kd coat proteins and a 50-kd protein, which are not so readily solubilised from coated vesicles as their counterparts from brain. While the 50-kd placental coat protein is similar in electrophoretic mobility to that from bullock brain, the 100-kd placental coat proteins do not clearly co-migrate with either group of brain 100-kd polypeptides. Thus the pattern of 100-kd polypeptides from a given tissue is complex and is variable from one tissue to another.

The total mixture of 100-kd polypeptides plus 50-kd protein (as eluted from the gel filtration column in buffer B) has the striking property of promoting clathrin assembly as

originally described by Keen *et al.* (1979) and more recently by Zaremba and Keen (1983). Both groups of 100-kd polypeptides, those with 50-kd protein (HA-II) and those without (HA-I), have the property separately to promote clathrin polymerisation. Thus there are probably unique binding sites for the 100-kd proteins on the clathrin lattice. When coats are formed with a saturating amount of 100-kd/50-kd complex, the extra material thickens the clathrin lattice generally. Judging from the selected views of identified structures amongst the clathrin cages, the addition of the 100-kd/50-kd complexes appears to follow the underlying geometry of the polyhedra. This results in a partial filling in of the polygonal faces of the structures. At present, it is not clear precisely what interactions the 100-kd proteins make with themselves or with clathrin in the coat structure.

Generally, rather less than this saturating amount of 100-kd protein relative to clathrin (about three per clathrin trimer) is found in coats isolated from tissues. Thus bullock brain TCVs appear to contain about one 100-kd polypeptide per clathrin trimer whereas coated vesicles isolated from human placenta seem to contain about two 100-kd polypeptides per clathrin trimer. These figures may not reflect the composition of coated vesicles *in vivo* and may be the result of the nature of the purification procedure. Many of the coated particles purified from human placenta, especially the smaller ones, appear to lack vesicles. It has been observed that when tissues are starved of oxygen or their ATP supplies are depleted, there is an apparent increase of empty clathrin cages in the cytoplasm (Palade and Fletcher, 1977). These are likely to be amongst the purified coated particles.

Apparently the 100-kd/50-kd complexes at clathrin concentrations of <0.2 mg/ml promote the formation of coats which predominantly contain 36 clathrin trimers. A similar range of coat sizes is obtained when bullock brain clathrin alone is polymerised in buffer A at a concentration of 0.5 mg/ml as described previously for placental clathrin (Crowther and Pearse, 1981). Under the same conditions as those used for the 100-kd/50-kd complexes, the HA-I group of 100-kd polypeptides (which lack the 50-kd protein) promotes the assembly of a greater variety of coats: these include truncated icosahedra made of 60 triskelions, and still larger species above 100 nm in diameter. This size distribution is similar to that observed when clathrin alone is polymerised at a 10-fold higher concentration (i.e., 5 mg/ml). However, many other factors such as pH, ionic strength and route of polymerisation have also been observed to affect the size of reassembled coats (Irace *et al.*, 1982). Whether the 50-kd protein plays a role in this size modulation is not clear.

In terms of the function of the 100-kd proteins *in vivo*, a great deal has still to be discovered. Intact 100-kd proteins left on the surface of clathrin-stripped vesicles are able to promote clathrin assembly on the vesicles, whereas after mild treatment of the vesicles with elastase, which cleaves the 100-kd polypeptides fairly specifically, clathrin binding is markedly reduced (Unanue *et al.*, 1981). Presumably therefore the assembly of clathrin in a forming coated pit is promoted by 100-kd polypeptides bound to the membrane. It is not known to what membrane components the 100-kd polypeptides bind. It is possible that they interact with the cytoplasmic tails of receptors in the membrane and thereby help to select the correct group of molecules into coated pits as they form. If there were three receptor molecules (perhaps bound via the 100-kd proteins) per clathrin trimer, the receptors might be fairly close-packed on the non-cytoplasmic surface of the coated pit. There is about one clathrin monomer per 100 nm^2 of membrane surface when it is in the form of a flat hexagonal network (Heuser, 1980). This is reasonably close to the cross-sectional area of a large globular protein of ~100 000 or 200 000 mol. wt., which is the size of many receptors which are concentrated in coated pits. Thus, this close packing, if it occurred, would tend to push out unbound proteins, especially when the coated pits bud inwards and the space available on the non-cytoplasmic surface becomes reduced.

As there is a variety of 100-kd polypeptides, the possibility exists that the different molecules tend to occur on different membranes in cells and that individual ones bind to specific groups of membrane proteins in their respective membranes. It is possible that these proteins with or without the 50-kd polypeptide play a role in determining the size of the coated vesicles which bud from a particular membrane.

Materials and methods

Materials

Bullock brains were obtained from British Beef, Bury St. Edmunds, UK and processed within ~2 h of slaughter. Human placentae, delivered normally at term, were obtained from the Rosie Maternity Hospital, Robinson Way, Cambridge, UK and coated vesicles were prepared as described previously using an isotonic density gradient (Pearse, 1982). Sepharose CL-4B was obtained from Pharmacia, hydroxylapatite from Bio-Rad Laboratories and DE-52 cellulose from Whatman. Dithio-bis (succinimidyl propionate) was obtained from Pierce Chemical Co. MAPS and dynein were kindly provided by Dr. J.V. Kilmartin and bovine thyroglobulin, ferritin and aldolase were from Pharmacia Fine Chemicals, Uppsala, Sweden. TMV was kindly provided by Dr. P.J.G. Butler.

Buffers

Buffer A - reconstitution buffer: 0.1 M MES-NaOH (pH 6.5 or pH 7.0 as specified) 0.2 mM EGTA, 0.5 mM $MgCl_2$, 0.02% NaN_3 and 0.1 mM phenylmethylsulfonylfluoride (PMSF).

Buffer B - Tris extraction buffer: 1 M Tris-Cl pH 7.0, 1 mM EDTA, 0.1% 2-mercaptoethanol, 0.02% NaN_3 and 0.2 mM PMSF.

Buffer C: 10 mM phosphate-NaOH (pH 7.0), 0.1 M NaCl, 0.1% 2-mercaptoethanol, 0.02% NaN_3 and 0.2 mM PMSF.

Buffer D - isolation buffer: 10 mM Hepes-NaOH (pH 7.2), 0.15 M NaCl, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.02% NaN_3 and 0.2 mM PMSF.

Buffer E: 50 mM Tris-Cl pH 8.0, 0.1% 2-mercaptoethanol, 2 mM EDTA, 1.2 M Na thiocyanate and 0.2 mM PMSF.

Protein concentrations were estimated spectrophotometrically assuming an absorbance at 280 nm of $E_{1\ cm}^{1\%} = 10.0$. Extinction coefficients for clathrin have been determined by μ-Kjedahl analysis to be 11.0 ± 0.6 (Winkler and Stanley, 1983) or 11.9 (Unanue *et al.*, 1981).

Purification of clathrin and associated coat proteins from bullock brains

Step I. Bullock brains are homogenised in buffer D as previously described (Pearse, 1976). The supernatant, resulting from centrifugation for 0.5 h at 20 000 *g*, is clarified at room temperature by the addition of a solution of 20% Triton X-100 to a final level of 1% Triton X-100. The clarified extract is centrifuged for 1 h at 100 000 *g* to yield pellets of crude coated vesicles. The pellets are resuspended in the minimum volume of isolation buffer D containing 1% Triton X-100, applied to 50 ml 5–25% sucrose gradients in the same buffer and then centrifuged for 1 h at 45 000 *g* (Pearse, 1982). The top 10 ml of the gradients are discarded and the next 40 ml are collected, diluted 3-fold with extraction buffer and the coats are pelleted by centrifugation for 1 h at 100 000 *g*.

Step II. The coat pellets are resuspended in a small volume of isolation buffer D and the coat proteins, including clathrin and the 100-kd and 50-kd polypeptides, are solubilized by the addition of an equal volume of twice strength buffer B. The resulting solution is centrifuged for 1.5 h at 50 000 r.p.m. in an MSE 10 x 10 rotor and the pellet is discarded. The bulk of the clathrin is separated from the 100-kd polypeptides by gel filtration of this solution on a 4 x 100 cm column of Sepharose CL-4B essentially as first described by Keen *et al.*, 1979.

The fractions containing clathrin are pooled and the clathrin precipitated by the additon of 1 volume of saturated ammonium sulphate. The precipitated clathrin is redissolved in a small column of buffer B and dialysed against 2 x 100 volumes of buffer B. The clathrin solution is then centrifuged for 1 h at 50 000 r.p.m. in an MSE 10 x 10 rotor to remove a trace of turbid material.

Before use in reconstitution experiments the clathrin is subjected to a cycle of polymerisation and depolymerisation essentially as described before (Crowther and Pearse, 1981) except using buffer B as disassembly buffer.

The peak of 100-kd polypeptides elutes from the Sepharose CL-4B column with marker horse spleen ferritin indicating that these proteins behave as complexes with a Stokes radius of 60 Å. This was confirmed by calibration of an equivalent 1 x 150 cm column using TMV and bovine thyroglobulin, ferritin and aldolase (Pharmacia Fine Chemicals, Uppsala, Sweden).

Step III. The fractions containing 100-kd proteins are pooled and dialysed aginst 2 x 20 volumes of buffer C and applied to a 5-ml hydroxylapatite column made in the same buffer. Proteins are eluted with an increasing linear phosphate gradient made from 30 ml of buffer C and 30 ml of 0.5 M Na phosphate pH 7.0 containing 0.1% β-mercaptoethanol and 0.2 mM PMSF. The 100-kd polypeptides separate into two groups: those (HA-I) which elute first with many other proteins and those (HA-II) which elute as a peak with a 50-kd polypeptide. Generally, these peak fractions containing both the 100-kd and 50-kd polypeptides are pooled at this stage and concentrated by precipitation with 50% saturated ammonium sulphate. The precipitate is harvested by centrifugation and redissolved in buffer B and the sample dialysed against buffer B to remove the ammonium sulphate. If further purification is necessary, before concentration, trace contaminants of clathrin and a 40-kd polypeptide may be removed by chromatography on DE52-cellulose as described in step IV.

Step IV. Peak fractions containing either group of 100-kd polypeptides are pooled and dialysed against 2 x 50 volumes of 50-fold diluted buffer B containing in addition 0.1 mM PMSF. The sample is applied to a 5-ml DE-52 cellulose column equilibrated in the same buffer. After washing with two column volumes of the equilibration buffer, the 100-kd polypeptides are eluted with an increasing linear Tris-Cl gradient made from 30 ml of 50-fold diluted buffer B and 30 ml of 5-fold diluted buffer B with 0.1 mM PMSF throughout. Fractions containing the 100-kd polypeptides are pooled and concentrated by ammonium sulphate precipitation as described in Step III.

Cross-linking

Dithio-bis (succinimidyl propionate) (Lomant and Fairbanks, 1976) was used as a cleavable cross-linking reagent. It was first dissolved in acetone (14 mg/ml), diluted 10 times with either 0.2 M thiethanolamine pH 8.0 or 0.02 M Na acetate (pH 6.2) and added to the protein sample (0.1 – 0.5 mg/ml) to a final concentration of ~0.25 mg/ml. Proteins were in 50 μl of either buffer A pH 6.5 containing in addition 0.1 M NaCl and 0.03% Triton X-100 (when transferrin receptor was included) or 0.2 M triethanolamine pH 8.0. The cross-linking reaction was carried out for 1 h at 0 – 4°C. 2.5 μl of 1 M ammonium acetate and 0.5 μl of 10% N-ethyl maleimide in the appropriate buffer were added and the samples incubated for at least another hour or overnight. 10 μl portions of the cross-linked proteins were then mixed with 5 μl of SDS gel sample buffer without dithiothreitol (DTT) or 2-mercaptoethanol, incubated at 37°C for 5 – 10 min, and subjected to electrophoresis as described below. To separate the components of the cross-linked species, sample lanes were excised from the first dimension gel and incubated in sample buffer containing 4% DTT and 5% 2-mercaptoethanol at 37°C for 30 min. These gels were then layered over a new (10 x 16 cm) gel and electrophoresis performed as before. The gels were stained with silver (Wray *et al.*, 1981).

SDS polyacrylamide gel electrophoresis

Electrophoresis was carried out on 8 x 4 cm slab gels based on the procedure of Laemmli (1970). Sample buffer contained 0.2 M CHES [2-(cyclohexylamino)ethane sulphonic acid] pH 9.5, 40% glycerol, 8% SDS and 4% DDT, 5% 2-mercaptoethanol and 0.004% bromophenol blue, the last three added just before use. 5 μl sample buffer was added to 10 μl of each sample and the mixture was incubated for 5 – 10 min at 37°C before application to the gel. After electrophoresis, the gels were stained with PAGE blue 83 (BDH Chemicals Ltd., Poole, England). The molar ratios of the coat proteins in the gels were estimated from the colour response of the dye (Fenner *et al.*, 1975). The blue bands were excised and eluted overnight in 0.8 ml of 20% pyridine. The absorptions of the eluates were measured in a spectrophotometer at 605 nm. The colour response for clathrin heavy and light chains has been calibrated and found to differ by <10% for the different polypeptides (Ungewickell, 1983). This also seems to hold approximately true for the total for the 100-kd/50-kd complex *versus* the total for the clathrin triskelion (heavy and light chains). The ratios of clathrin heavy chain to light chains and of 100-kd polypeptide to 50-kd polypeptides remained constant over the range of sample concentrations applied to the gels.

Electron microscopy

Samples were applied to carbon coated Formvar grids, negatively stained with 1% uranyl acetate (Huxley, 1963), air dried, and examined in a Philips EM301 microscope operating at 80 kV and at a magnification of 25 000. Micrographs of random fields were printed at a final magnification of 75 000 and coat diameters were measured on discrete particles.

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