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Clathrin heavy chain, light chain interactions

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Purified pig brain clathrin can be reversibly dissociated and separated into heavy chain trimers and light chains in the presence of non-denaturing concentrations of the chaotrope thiocyanate. The isolated heavy chain trimers reassemble into regular polygonal cage structures in the absence of light chains. The light chain fraction can be further resolved into its two components Lα and Lβ which give different one-dimensional peptide maps. Radiolabelled light chains bind with high affinity (K_D $<10^{-10}$ M) to heavy chain trimers, to heavy chain cages and to a 110 000 mol. wt. tryptic fragment of the heavy chain. Both light chains compete with each other and with light chains from other sources for the same binding sites on heavy chains and c.d. spectroscopy shows that the two pig brain light chains possess very similar structures. We conclude that light chains from different sources, despite some heterogeneity, have a highly conserved, high affinity binding site on the heavy chain but are not essential for the formation of regular cage structures.

Key words: chaotropes/circular dichroism/clathrin/dissociation constant/peptide mapping

Introduction

Clathrin coats are an important part of the apparatus needed for membrane transport in eukaryotic cells (for a review, see Pearse and Bretscher, 1981). *In vitro* these coats can be reversibly dissociated into units which have been termed triskelions due to their characteristic three-legged structure seen in rotary shadowed preparations (Ungewickell and Branton, 1981). Triskelions consist of three polypeptide chains of mol. wt. 180 000, originally named clathrin by Pearse (1975), each of which is associated with one copy of a polypeptide of lower mol. wt. (Ungewickell and Branton, 1981; Kirchhausen and Harrison, 1981). Following the proposal made by Ungewickell and Branton (1982), we will use the term clathrin to describe the entire triskelion-shaped molecule containing three heavy chains each associated with one light chain. Two sizes of light chains with mol. wts. between 30 000 and 40 000 have been found in clathrin from different species and tissues. In common sources like calf, bovine or pig brain, their apparent mol. wts., all slightly different, are ~36 000 (Lα) and 33 000 (Lβ) and their molar ratio is ~1:2. Based on studies with clathrin, whose light chains were selectively degraded by mild proteolysis, it has been suggested that they are needed to maintain the regular triskelion structure (Schmid *et al.*, 1982) which could explain why clathrin after such treatment no longer forms cages but large irregular aggregates, as first observed by Kirchhausen and Harrison (1981). However, Schmid *et al.* (1982) also reported that prolonged proteolysis, which not only degrades the light chains but also removes part of the heavy chain, did not impair subsequent reassembly into cages. The role of the light chains in mediating proper assembly therefore remained doubtful. We have been able to remove the light chains under non-denaturing conditions without resorting to selective proteolysis. Here we report studies on clathrin heavy and light chain interactions carried out on this material.

Results

Separation of clathrin heavy chains and light chains

Dissociation of clathrin under a variety of denaturing conditions permits isolation of soluble light chains while heavy chains invariably aggregate during or after such treatment (Lisanti *et al.*, 1982b). The potential of chaotropic ions like thiocyanate, perchlorate, etc., in destabilizing hydrophobic interactions at concentrations which usually do not unfold proteins, is well documented (Hatefi and Hanstein, 1974;

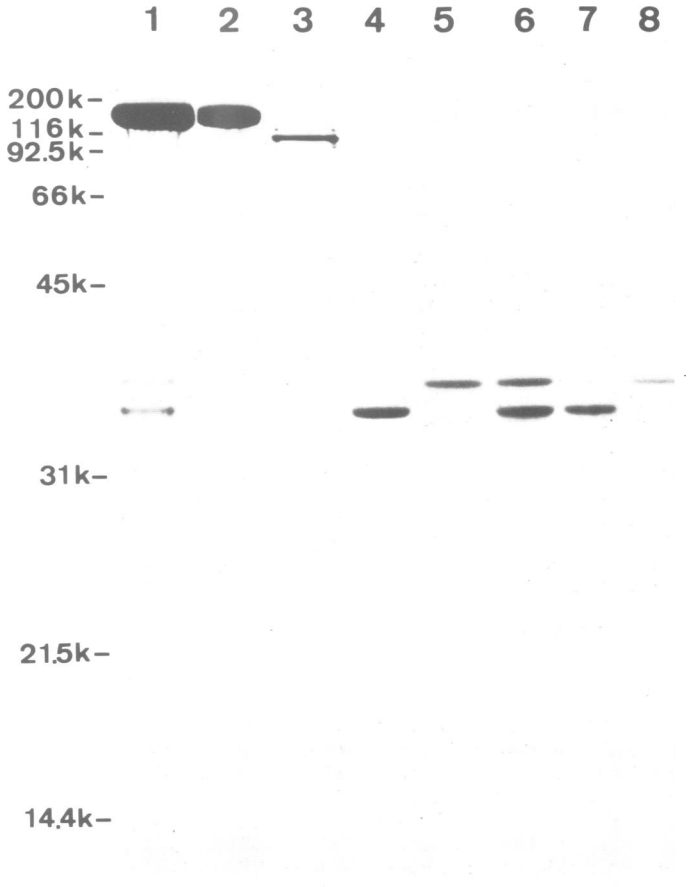

Fig. 1. SDS-PAGE of pig brain clathrin and its purified components (12.5% gel; Maizel, 1969). **Lane 1**, purified clathrin (40 μg); **lane 2**, heavy chains (20 μg) after separation in 1.3 M NaSCN; **lane 3**, tryptic heavy chain fragment (5 μg); **lane 4**, Lβ after DEAE-Sephacel chromatography (5 μg); **lane 5**, Lα after DEAE-Sephacel chromatography (4 μg); **lane 6**, light chains after separation in 1.3 M NaSCN (8 μg); **lane 7**, Lβ from preparative SDS-PAGE (5 μg); **lane 8**, Lα from preparative SDS-PAGE (3 μg). Mol. wts. are shown on the left.

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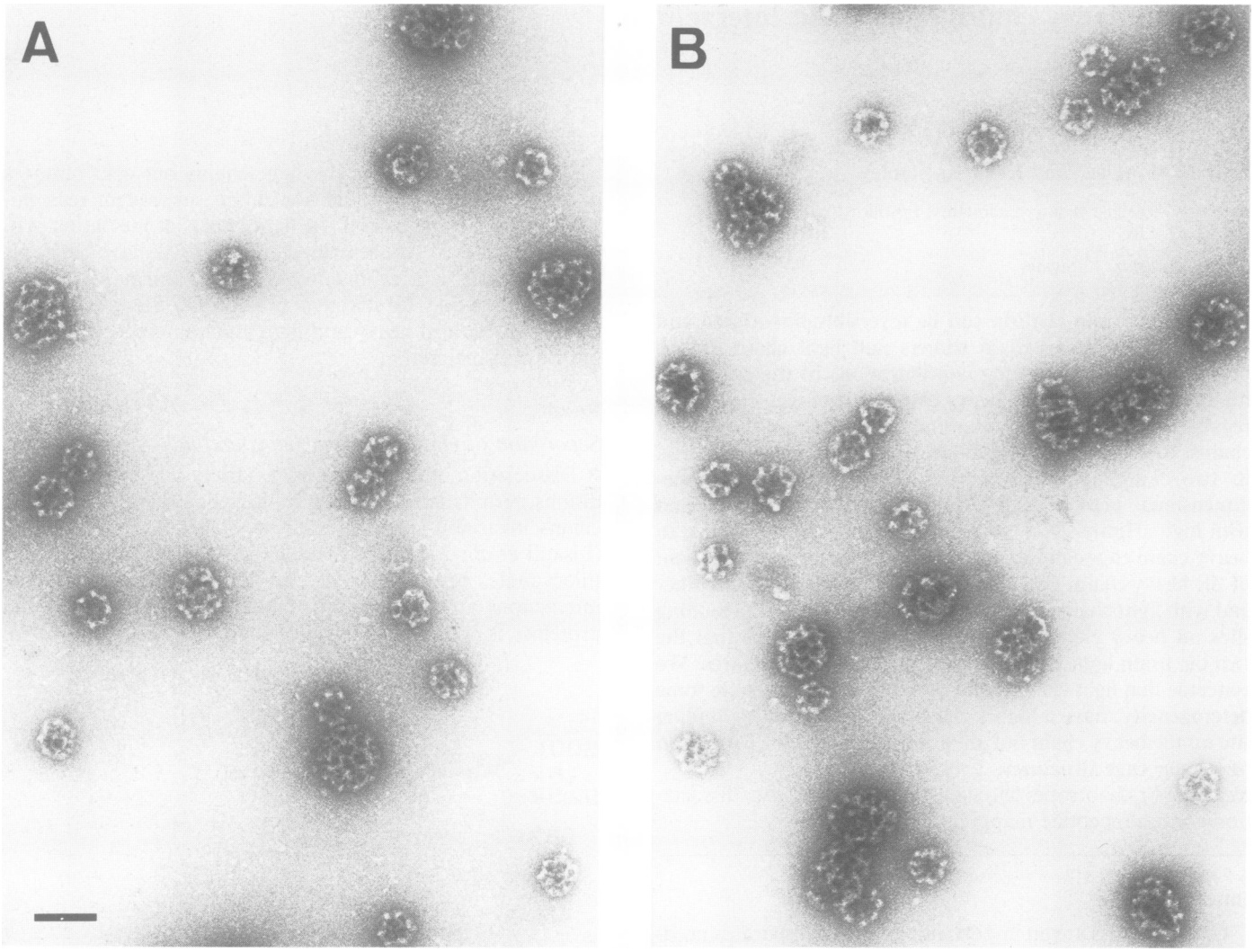

Fig. 2. Electron micrographs of negatively stained cages reassembled from clathrin (**A**) and from heavy chains (**B**). Scale bar = 100 nm.

Table I. Percentage of regular triskelions found in rotary shadowed preparations

Clathrin	Clathrin heavy chains	Clathrin heavy chains + light chains
38 ± 4[a]	32 ± 5	41 ± 5

[a]Mean ± standard error of the mean.

Burns and Schachman, 1982). Samples of pig brain clathrin, prepared as described in Materials and methods, were therefore exposed to increasing concentrations of thiocyanate, assayed for dissociation and, after removal of the chaotrope by dialysis, tested for reassembly into cages. The large difference in mol. wt. between free and bound light chains made it easy to follow dissociation by velocity sedimentation or gel filtration. Concentrations >1 M thiocyanate were found to be effective in dissociation, but at >1.5 M the heavy chains started to aggregate irreversibly, most likely because they unfolded. Below this limit, dissociation was reversible as judged by the fact that clathrin, after exposure to such conditions, still reassembled into cages on dialysis against our standard reassembly buffer [100 mM (2-N-morpholino)ethane sulfonic acid (MES) pH 6.3, 5 mM $MgCl_2$]. In the following, soluble clathrin heavy and light chains (Figure 1, lanes 2 and 6) were routinely prepared by gel filtration in a buffer containing 50 mM Tris-HCl pH 8.0, 1.2–1.4 M sodium thiocyanate, 2 mM EDTA and 2 mM dithiothreitol (DTT). The presence of EDTA prevents aggregation of the heavy chains during removal of thiocyanate. The thiol reagent is necessary to obtain reproducible yields of light chains and in particular of Lα.

Light chains separated from heavy chains as described above, or more conveniently by the heat treatment described by Lisanti *et al.* (1982b), have been separated into Lα and Lβ by two procedures. In the first, the mixture of light chains is fractionated by ion-exchange chromatography on DEAE-Sephacel as described in Materials and methods. As shown in Figure 1 (lane 4), pure Lβ is obtained while Lα (lane 5) co-elutes with a polypeptide running slightly ahead of Lβ on SDS-PAGE. The fact that its relative amount varies and that a band in the same position reacts strongly with an anti-Lα monoclonal antibody (Kirchhausen *et al.*, 1983) suggests that it derives from Lα. In the second procedure, Lα and Lβ were obtained by separation on SDS-PAGE, followed by elution from their gel slices and renaturation *via* acetone precipitation (Hager and Burgess, 1980). While pure Lα is obtained with

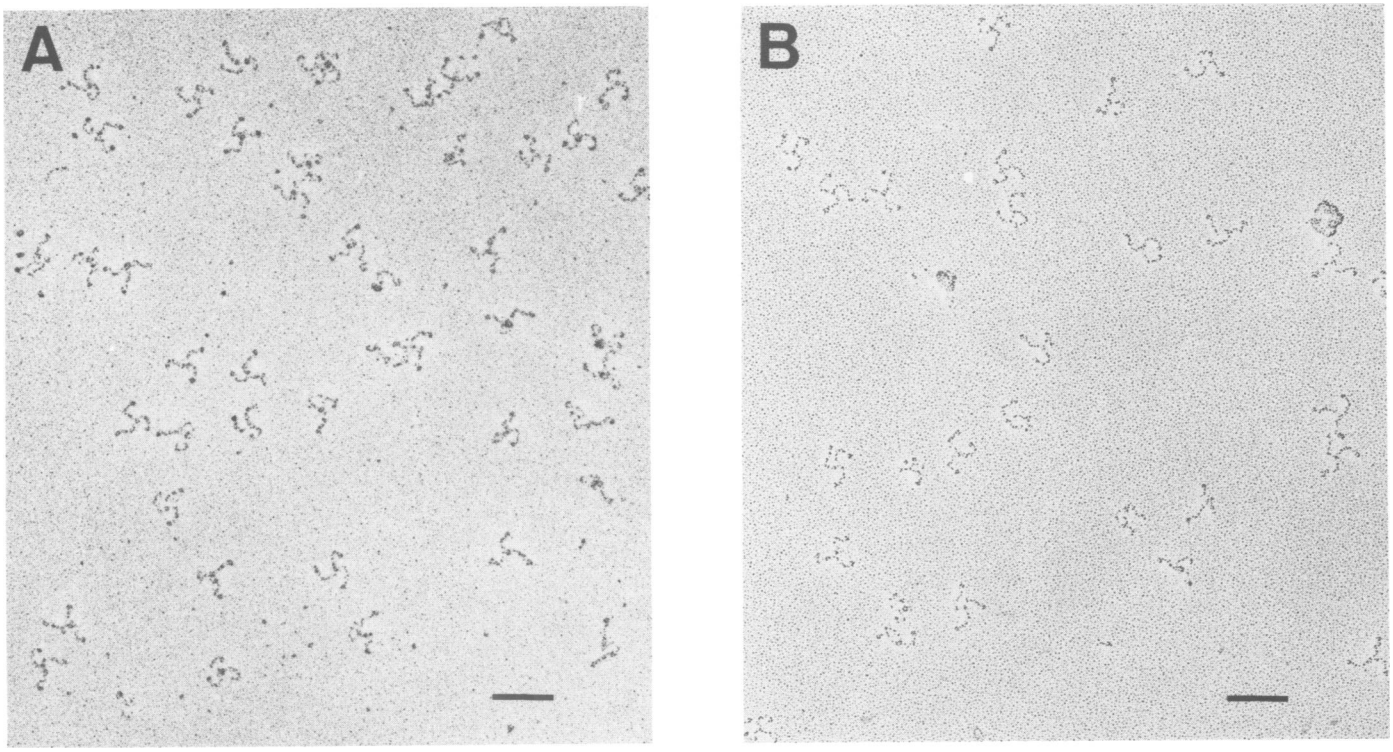

Fig. 3. Electron micrographs of rotary shadowed clathrin (**A**) and heavy chains (**B**). Scale bar = 100 nm.

this method, Lβ shows a small amount of the above-mentioned Lα fragment (Figure 1, lanes 7 and 8). Occasionally, light chains prepared by this second procedure showed reduced affinity in rebinding to heavy chains possibly due to incomplete removal of detergent.

Assembly and disassembly of clathrin heavy chains

As shown in Figure 2, negatively stained preparations of heavy chains after dialysis against reassembly buffer reveal cage structures with a morphology indistinguishable from that of clathrin cages. Van Jaarsfeld *et al.* (1981) found that the rate of polymerisation of clathrin depends on pH, ionic strength, temperature and protein concentration. To compare the rate and extent of polymerisation of heavy chains with that of clathrin, both materials were subjected to identical reassembly conditions at equal concentrations. We find that at 0.5 mg/ml in buffer C, both require 10–20 h of dialysis against reassembly buffer in the cold until they become turbid and until >50% can be pelleted under conditions where coated vesicles sediment. When both materials at the same concentrations (in 10 mM Tris HCl pH 8.0) are mixed with one tenth of their volume of a solution containing 1 M MES pH 6.3, 50 mM $MgCl_2$ at room temperature they both turn turbid within a few minutes and again 50–70% of the material can be pelleted. With some heavy chain preparations we have observed that reassembly in the presence of 2 mM $CaCl_2$ rather than 5 mM $MgCl_2$ resulted in heavy chain aggregation. Clathrin and heavy chain cages, resuspended in 20 mM sodium phosphate pH 6.5 after sedimentation, show an instant loss of absorbance at 320 nm after a pH shift to 7.5 indicating rapid depolymerisation.

Comparison of rotary shadowed clathrin and heavy chains

A characteristic property of soluble clathrin is its three-legged structure seen in rotary shadowed or negatively stained preparations (Ungewickell and Branton, 1981). As can be seen in Figure 3, many molecules display a handedness in that all three legs tend to bend in the same sense, thereby resembling the triskelion structure. The unique handedness observed implies that the trimeric clathrin molecules preferentially adsorb with one face to the support (Crowther and Pearse, 1981). Schmid *et al.* (1982) report that rotary shadowed preparations of elastase-treated clathrin show fewer regular triskelions (31%) than those of clathrin having intact light chains (61%). They suggest that this randomization in the orientation of triskelion legs may be the cause of failure for such preparations to reassemble into cages. We therefore examined what proportion of regular structures is present in rotary shadowed preparations of clathrin heavy chains which reassemble normally into cages. Fresh preparations of clathrin, of heavy chains and of heavy chains with light chains added back were shadowed in the same experiment. For each sample, 8–10 fields, each containing ~100 distinct three-legged molecules, were analysed and Table I lists the percentages of regular triskelions found. Despite the fact that all three preparations yield significantly smaller fractions of regular structures than those reported by Schmid *et al.* (1982) for native clathrin, they all reassembled into cages with normal efficiency. It therefore appears possible that the fraction of distorted molecules determined from such experiments reflects variable conditions during adsorption and drying of the samples rather than differences in the conformation of the molecules in solution. Indeed, analysis of some electron micrographs from earlier shadowing experiments with clathrin and heavy chains revealed variations in the percentage of regular structures between 25 and 60%. Figure 3 shows fields of rotary shadowed molecules of clathrin and of heavy chains taken from larger areas both of which show >50% regular triskelion structures.

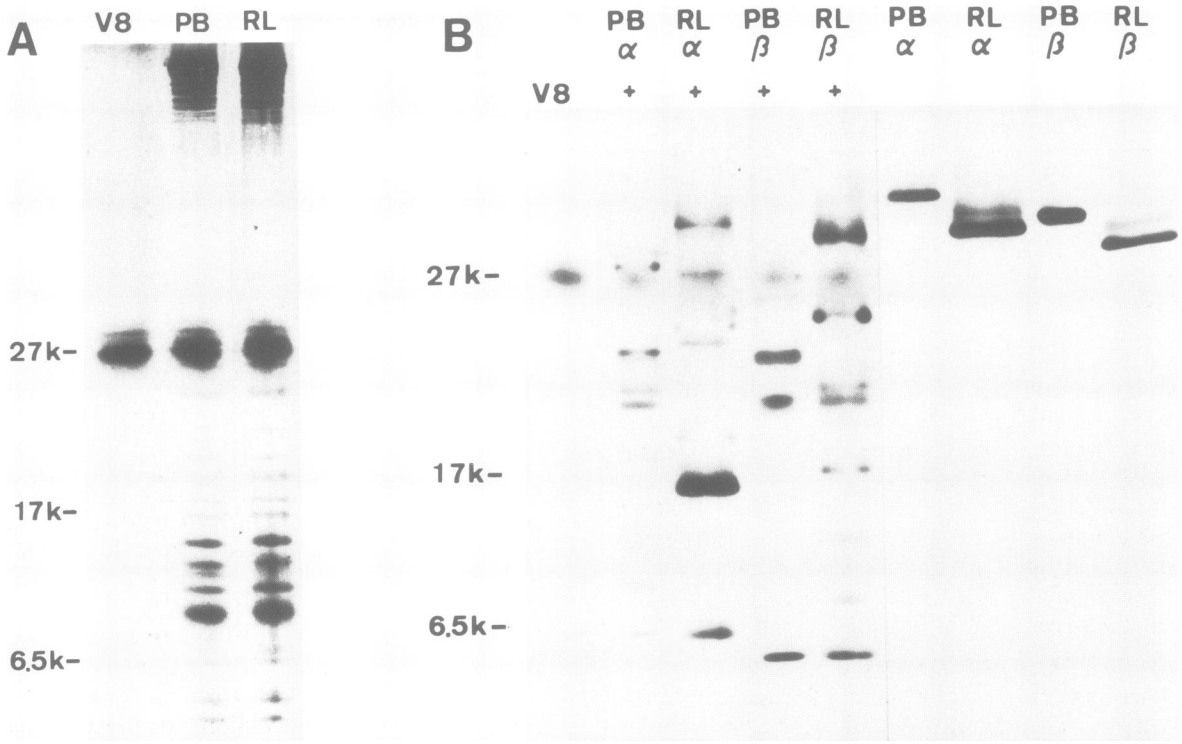

Fig. 4. One-dimensional peptide analysis of heavy chains (**A**) and light chains (**B**) from pig brain (PB) and rat liver (RL) performed according to the method of Cleveland *et al.* (1977). Polypeptides excised from a 12.5% polyacrylamide SDS-gel (~10 μg for heavy and 5 μg for light chains) were transferred to the sample slots of a second gel (16% for **A**, 20% for **B**) and digested *in situ* with *S. aureus* V8 protease (0.8 μg per slot for heavy chains and 0.06 μg for light chains). The small amounts of contaminants present in the control rat liver bands run without protease appear very resistant to proteolysis and do not interfere with the analysis. After separation, the fragments were visualized by silver staining as described by Ansorge (1983). Mol. wts. are shown on the left.

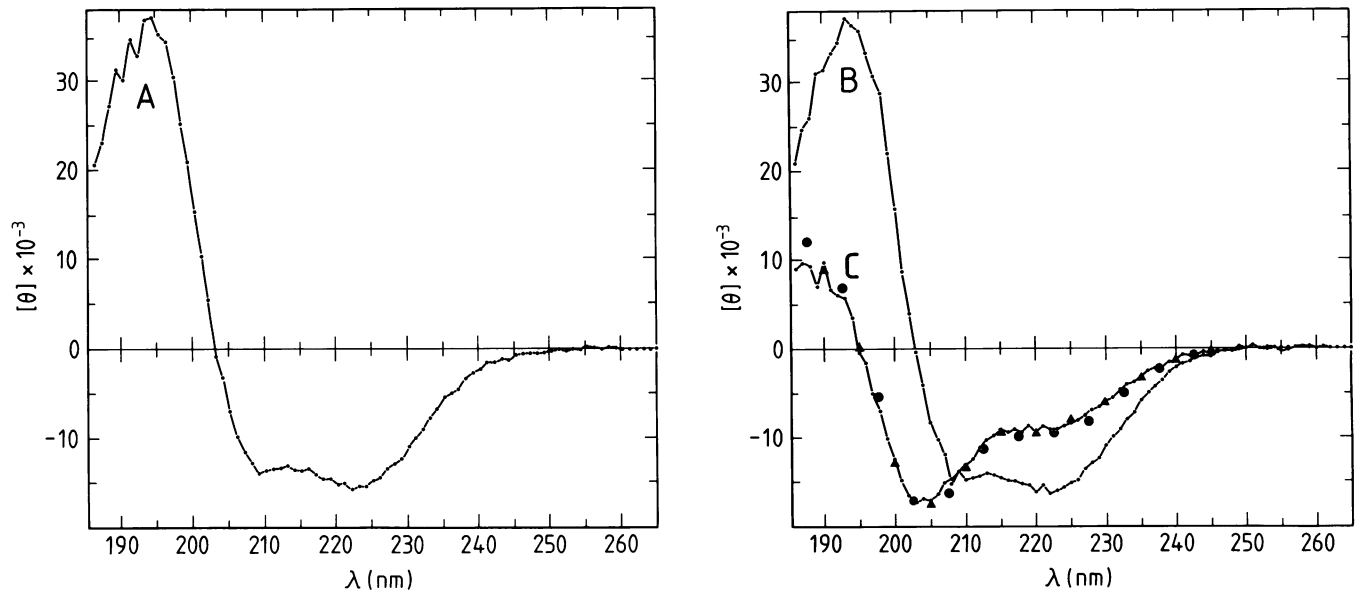

Fig. 5. Far ultraviolet c.d. of clathrin and its polypeptide components. (**A**) Clathrin, (**B**) clathrin heavy chains and (**C**) clathrin light chain mixture Lα and Lβ. The mean residue ellipticities were calculated from the digitized spectra as described in Materials and methods and are plotted in 1 nm intervals. Ellipticities derived from the spectra of Lα (▲) and Lβ (●) separated by DEAE-Sephacel chromatography are shown superimposed on spectrum **C** in 5 nm intervals.

Proteolytic cleavage patterns of clathrin heavy and light chains

Figure 4 shows the results of *in situ* digestion of the heavy and light chains from pig brain and rat liver with V8 *Staphylococcus aureus* protease according to the method of Cleveland *et al.* (1977). The nearly identical patterns consistently observed for the two heavy chains with a number of proteases (elastase, chymotrypsin, subtilisin, papain; data not shown) demand a highly conserved amino acid sequence. In contrast, each light chain pattern appears different, despite

Table II. Secondary structure estimates for clathrin and its components determined from their c.d. spectra (Figure 5) by the method of Provencher and Glöckner (1981)

	% α-helix	% β-sheet	% other
Clathrin	53 ± 4[a]	15 ± 3	32 ± 2
Heavy chains	59 ± 4	12 ± 5	29 ± 4
Light chains	30 ± 5	13 ± 7	56 ± 5

[a]Mean ± range of values estimated from uncertainties in concentration measurements.

one or two co-migrating bands. The main features of these patterns are very reproducible and were observed over a large range of initial protease concentration. The information provided by such different patterns is, however, very limited especially since the apparent mol. wts. of the four polypeptides differ by up to ~5000 daltons. However, the possibility that, apart from different N- or C-terminal extensions, their sequences are as highly conserved as those of the heavy chains, can almost certainly be excluded.

C.d. of clathrin and of its heavy and light chains

Figure 5 shows the far ultraviolet c.d. of clathrin and of its heavy and light chains. Most importantly, we find that Lα and Lβ display essentially the same dichroism. Table II lists the secondary structure estimates derived for clathrin and its heavy and light chain components by the method of Provencher and Glöckner (1981) which is based on 16 reference spectra from proteins of known structure. The uncertainties in these values are dominated by the uncertainties in the residue concentrations estimated to be ~5% for clathrin and for heavy chains and ~10% for light chains. Clathrin and heavy chains both have >50% α-helical residues but only little β-structure. Similar values have been reported for clathrin isolated from human brain (Pretorius *et al.*, 1981), calf brain (Ungewickell *et al.*, 1982) and rat liver (Steer *et al.*, 1982). In contrast, free light chains have only about half as much α-helical structure and clearly are not fibrous or tropomyosin-like molecules as suggested by Brodsky *et al.* (1983). One can ask whether the conformation of heavy and light chains is the same independent of whether they are free or associated with each other. If the estimates listed for heavy and light chains are combined in a 5:1 ratio as required by their relative mass in clathrin, estimates of 54% α and 12% β structure result for clathrin, which is reasonable in view of the given uncertainties. Although large secondary structure changes of the heavy chains on binding light chains can be excluded, the small contribution of the latter to the total mass of clathrin leads to large uncertainties in the secondary structure estimates of bound light chains. This c.d. analysis therefore cannot exclude major structural differences between free and bound light chains, a conclusion which is also reached when the spectra themselves are linearly combined.

Heavy chain, light chain binding studies

^{3}H-labelled light chains were produced by labelling clathrin with [^{3}H]$NaBH_4$ and subsequently isolating the light chains. In this way, light chains unmodified at the heavy chain binding site were obtained. Binding assays were performed by incubating unlabelled clathrin heavy chains with radiolabelled light chains and measuring the amount of unbound light chains after apparent equilibration. Separation of free and bound light chains was achieved by centrifugation in an airfuge in the presence of 0.5% dextran T-40 to reduce convection (Howlett *et al.*, 1978). Control experiments were carried out to assess the sedimentation behaviour of free and bound light chains. We find that <2% of clathrin or of heavy chains but ~36% of free light chains remain in the top half of samples of these materials after centrifugation for 1 h at 135 000 *g* in an airfuge. The same sedimentation conditions were used to measure binding to heavy chain cages, because partial cage disassembly was suspected to occur at the high dilutions required in the binding assay. However, the fact that >85% of the bindable label was recovered in the pellet when such assays were centrifuged for only 20 min at 70 000 *g*, indicates that the majority of cages remain intact. About 70% of the total label rebinds with high affinity to heavy chains and another 5–10% sediments in the presence of a large excess of heavy chains. The remaining 20–25% was non-dialysable, did not sediment under the conditions used and did not produce visible bands on fluorographed SDS-polyacrylamide gels. For each assay, the two extremes, i.e., no light chains bound and all light chains bound, were established by including two control samples with the same amount of label but one with no heavy chains and the other with a moderate excess of heavy chains. The relative distribution of label in these controls proved to be very reproducible and was used to correct for the unbound counts.

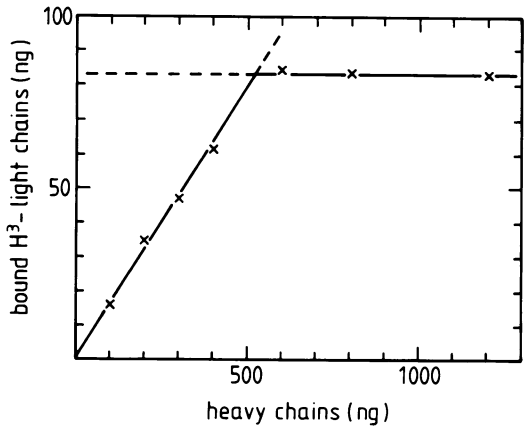

Fig. 6. Heavy chain, light chain binding curve. A constant amount of labelled light chains (83 ± 8 ng) was incubated for 3 h with increasing amounts of heavy chains in 100 μl buffer and the amount of bound light chains was determined by sedimentation as described in the text. Saturation occurs at ~515 ± 25 ng of heavy chains.

As shown in Figure 6, binding to heavy chain trimers is saturable at a stoichiometry close to one light chain per heavy chain. Only an upper limit could be determined for the dissociation constant because even at the minimum amount of label, allowing sufficiently accurate measurements, saturation of binding was observed. We estimate that at ~2×10^{-9} M concentrations of heavy and light chains, >90% of the light chains are bound after a 24 h incubation. This sets an upper limit of ~10^{-10} M for the equilibrium dissociation constant of a single site. This constant is also defined as the ratio of the rate constants of dissociation and association K_{off}/K_{on}. Most reported protein-protein association rates are ~10^5–10^6/M/s (Koren and Hammes, 1976). The expected K_{off} is therefore <10^{-4}/s which means that the expected half life of the complex is >2 h. To confirm this slow dissociation of the complex, we have incubated clathrin

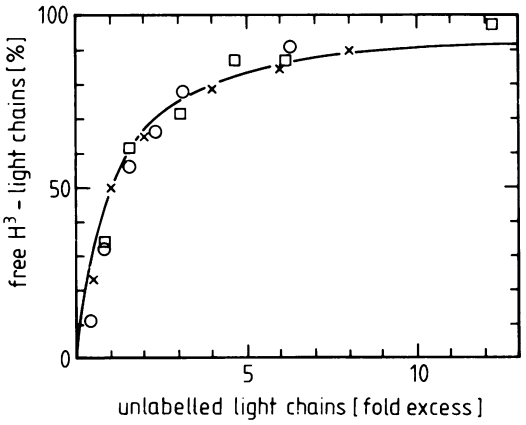

Fig. 7. Competition of unlabelled Lα and Lβ light chains with mixtures of labelled Lα + Lβ in rebinding to heavy chains. For each assay, ~150 ng of heavy chains in 50 μl buffer G were mixed with 50 μl of a solution containing approximately equimolar amounts of labelled Lα + Lβ (~25 ng) and variable amounts of unlabelled light chains in buffer F. After incubation for 24 h in the cold, the amount of free labelled light chains was determined as described in Materials and methods. The points of the graph show the percentage of free labelled light chains as a function of increasing amounts of unlabelled light chains; ○ – competition with Lα, □ – (Lβ) and x – (Lα + Lβ). The curve drawn is that expected if unlabelled and labelled light chains compete for binding with equal efficiency and if heavy chains and labelled light chains are present in equimolar amounts.

with an excess of labelled light chains and find that, after 1 week, <20% of the label has become bound. To make sure that this is not caused by a lower affinity of labelled light chains we have also followed the release of bound labelled light chains after adding excess cold light chains and find a similarly slow exchange. These data suggest that the dissociation constant is even below 10^{-11} M. Analogous binding and exchange experiments were carried out with heavy chain cages and with trimers and cages consisting of a 110 000 mol. wt. tryptic heavy chain fragment (Figure 1, lane 3). In none of these cases was binding detectably reduced and the tryptic fragment must therefore have an essentially intact light chain binding site. As shown in Figure 7, the preparations of Lα and Lβ obtained by DEAE-Sephacel chromatography compete with a labelled light chain mixture with equal efficiency for all binding sites on clathrin heavy chains. Preparations obtained by recovering Lα and Lβ from polyacrylamide gel slices occasionally proved inefficient in competition, perhaps due to residual bound SDS.

Competition of Lα and Lβ from pig brain with rat liver light chains in rebinding to rat liver heavy chains was studied with a slightly modified procedure. Samples of ^{3}H-labelled rat liver clathrin were made 1.2 M in NaSCN to achieve dissociation. After addition of an excess (~10-fold) of unlabelled Lα or Lβ from pig brain, the samples were diluted 6-fold and aliquots were spun in the airfuge. Samples of the upper and lower halves of the centrifuge tubes were then separated by SDS-PAGE and the distribution of rat liver light chains determined by fluorography of the gel. In the absence of competing pig brain light chains, most of the dissociated rat liver light chains rebind to heavy chains after dilution of the thiocyanate (Figure 8, lanes 7 and 8). Addition of either Lα or Lβ from pig brain, however, increases the relative amount of rat liver light chains in the upper half close to the ~40% expected for free light chains (lanes 3–6). It appears therefore that considerable homology must be conserved in the light

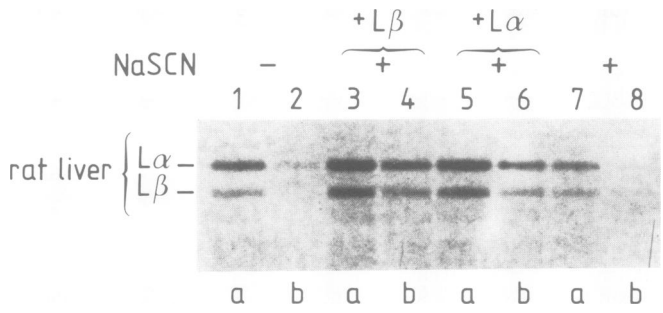

Fig. 8. Competition between pig brain and rat liver light chains. ^{3}H-labelled rat liver triskelions were incubated with purified pig brain light chains in the presence of 1.2 M NaSCN for 30 min at 20°C and then diluted 6 times with buffer C. After 15 min at 20°C the mixture was centrifuged at 135 000 *g* for 45 min in an airfuge. Upper **(b)** and lower **(a)** halves of the airfuge tube were analysed by fluorography of SDS-gels. **1,2** control – no NaSCN, no light chains.; **3,4** + Lβ; **5,6** + Lα; **7,8** control – NaSCN, no light chains.

chain binding sites of these two species.

Discussion

Dissection and reconstitution are frequently used to probe the function of the individual polypeptides that constitute multisubunit complexes. With this strategy in mind, we have established conditions under which clathrin dissociates reversibly into its heavy and light chains. A characteristic of native clathrin is its ability to assemble into polygonal cage structures morphologically identical to those observed on coated vesicles. In all previous studies with clathrin heavy chains, the light chains were selectively removed by mild proteolysis using elastase (Kirchhausen and Harrison, 1981; Ungewickell *et al.*, 1982; Schmid *et al.*, 1982) or chymotrypsin (Lisanti *et al.*, 1981, 1982a). Such heavy chains predominantly formed large irregular aggregates under standard reassembly conditions, suggesting that the light chains were required for the formation of proper cages. In support of such a conclusion, Schmid *et al.* (1982) noticed that elastase-treated clathrin had lost its characteristic handedness in rotary shadowed preparations. Curiously, however, more extensive proteolysis, which (in addition to the light chains) also removed the outermost third of each triskelion arm, did not impair proper reassembly (Ungewickell *et al.*, 1982; Schmid *et al.*, 1982). Rotary shadowing of clathrin heavy chains prepared by the method decribed here shows trimeric molecules, more than half of which can show the regular handed triskelion structure typical of native clathrin. However, we have observed that the fraction of regular handed structures in larger fields of molecules can vary considerably in such experiments for both clathrin and heavy chains, probably as a result of some variation of the disruptive forces that operate during adsorption and drying of the samples. It is therefore difficult to draw any conclusion on the solution structure from the frequency of distorted molecules.

Heavy chain trimers reassemble into polygonal cage structures indistinguishable in their size distribution and lattice morphology from those obtained from clathrin. Similarly, rate and efficiency of polymerisation and depolymerisation appear essentially unchanged. The specific bonds that direct proper assembly and the conformational flexibility needed to accommodate the large range of local curvature present in clathrin coats, therefore, must both reside in the clathrin heavy chain.

Our binding studies with radiolabelled light chains show that the interaction with the heavy chain is very strong for both light chains and that they both compete for the one binding site that is present on each heavy chain. Based on the binding experiments, the dissociation constant K_D must be $<10^{-10}$ M. Estimates of the on and off rates indicate values even smaller than 10^{-11} M. Such a small off rate ($<10^{-6}$/s) means that binding is essentially irreversible on the time scale of our incubations. It also means that no re-equilibration occurs during the 1 h sedimentation time needed to separate unbound from bound light chains. Such re-equilibration, even if taking place, would not have greatly distorted our results since, to a first approximation, heavy chain trimers and their complexes with one to three light chains sediment with similar rates. Strong binding is observed to heavy chain trimers, to heavy chain cages and to trimers consisting of three 110 000 mol. wt. tryptic heavy chain fragments which appear to have intact light chain binding sites. This result is consistent with the light chains being located on the central part of the triskelion arms, as found in electron microscopic studies by Ungewickell *et al.* (1982) and Kirchhausen *et al.* (1983). The tight binding renders it unlikely that reversible dissociation of the light chains could be of any functional significance *in vivo*. It should also prevent redistribution of the light chains during isolation. The random distribution of Lα and Lβ found in clathrin using a monoclonal antibody against Lα (Kirchhausen *et al.*, 1983) is unlikely to be a consequence of redistribution between, for example, Golgi-derived and endocytotic-coated vesicles. Coated vesicles on different pathways of membrane transport do not therefore appear to be distinguished on the basis of their light chain content. In addition, since both light chains compete for the same sites on clathrin heavy chains, a special mechanism of synthesis and assembly would be required to generate a non-random distribution.

On the basis of one-dimensional peptide maps, clathrin heavy chains appear highly conserved among species and tissues, as previously reported by Pearse (1976) and Kartenbeck *et al.* (1981). Light chains, on the other hand, have different mol. wts. and dissimilar one-dimensional peptide maps. They appear less conserved than the heavy chains but this need not reflect grossly different structures. Competition of the pig brain light chains with rat liver light chains in binding to rat liver heavy chains indeed shows that they share a common site for binding on the heavy chain. Furthermore, the essentially identical c.d. displayed by the Lα and Lβ light chains from pig brain suggests that they possess very similar secondary and tertiary structures. These same c.d. data also rule out a predominantly α-helical, tropomyosin-like structure as proposed by Brodsky *et al.* (1983).

We are left with no function for the light chains and with no clue as to why there should be two kinds of them in any one tissue. A somewhat similar situation holds for skeletal muscle myosin where the essential light chains have become non-essential after more careful dissociation studies (Sivaramakrishnan and Burke, 1982). Obviously, *in vivo*, clathrin is expected to interact with as yet unknown cellular components that regulate or influence assembly of coated pits, transformation to coated vesicles and disassembly of the coats. Two characteristics of this machinery, cage formation and rebinding to stripped vesicles, both amenable to *in vitro* analysis, do not seem to depend on molecular interactions with the light chains or with the distal part of the heavy chains (Ungewickell *et al.*, 1982). As more components involved in the clathrin coating and uncoating process become better characterized, for example a cytosolic factor reported to uncoat coated vesicles in the presence of ATP (Patzer *et al.*, 1982), those parts of clathrin presently without a function might become important. In this respect, the preparation of purified undenatured clathrin heavy chains should be of value in the identification of light chain function.

Materials and methods

Isolation of clathrin

Clathrin was purified from whole pig brain following the procedure of Keen *et al.* (1979) with some modifications. Unless otherwise stated, all operations were performed at 0–4°C. 300–400 g of whole pig brain, frozen in liquid nitrogen after slaughter and stored at −80°C, were thawed overnight at 4°C, minced coarsely and placed in a Waring blender with an equal volume of buffer A [100 mM MES pH 6.5, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.02% NaN_3, 0.1 mM phenylmethylsulfonyl fluoride (PMSF)]. After homogenisation by three 10 s bursts at high speed, the volume was brought to 1 l with buffer A and cell debris was separated by centrifugation for 30 min at 20 000 *g*. A crude vesicle fraction was obtained from this supernatant by centrifugation for 60 min at 100 000 *g*. The pellets were resuspended with the aid of a Dounce homogeniser in a total of ~20 ml buffer A and applied to the top of six discontinuous sucrose gradients consisting of 4.9 ml steps of 5, 10, 20, 30, 40, 50 and 60% sucrose (w/v) in buffer A. These gradients were spun at 85 000 *g* for 90 min. The middle sections of each gradient comprising the 20 and 30% sucrose steps showing two broad turbid bands were carefully collected. The pool of ~60 ml was diluted with an equal volume of buffer A and centrifuged for 60 min at 100 000 *g*. The pellets were allowed to stand overnight in a total volume of 10 ml of 10-times diluted buffer B (0.5 M Tris-HCl pH 7.5, 1 mM EDTA, 1 mM DTT, 3 mM NaN_3, 0.1 mM PMSF). After addition of 2.5 ml of five times concentrated buffer B the sample was resuspended with gentle homogenisation and subjected to gel filtration on a CL-4B (Pharmacia) column (2.5 x 85 cm) equilibrated with buffer B. Clathrin eluted in a distinct peak shortly after the void volume and the pooled fractions were freed from some residual membranous vesicles by centrifugation for 60 min at 100 000 *g*. To concentrate clathrin, the supernatant was brought to 50% $(NH_4)_2SO_4$ and, after 1 h, the precipitate was collected by a 30 min centrifugation at 12 000 *g*, resuspended in ~1 ml of buffer C (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 2 mM EDTA, 1 mM DTT, 0.1 mM PMSF) and dialysed against 1 l of this buffer. Typical final yields of clathrin were ~10 mg based on a $E^{1\%}_{1\,cm}$ (280) = 11.0.

Rat liver clathrin was prepared analogously with the following modifications. About 50 g of liver from six rats starved for 16 h was washed in ice-cold 0.25 M sucrose, put into 90 ml of buffer A and cut into small pieces. After passing through a tissue press, the suspension was homogenised with a Teflon coated homogeniser using eight strokes at top speed. The homogenate was diluted with buffer A to ~900 ml and centrifuged at 1500 *g* for 10 min followed by 13 000 *g* for 10 min. The crude vesicle fraction was pelleted from this supernatant and processed as described above for pig brain clathrin, except for scaling down the gel filtration step in high Tris by a factor of three. About 1 mg of purified rat liver clathrin was obtained from one such preparation.

Separation of heavy and light chains

In a typical separation, concentrated pig brain clathrin, ~5 mg in 1 ml of buffer C, was brought to 1.3 M NaSCN by adding 170 μl of a solution 9 M in NaSCN and 14 mM in EDTA and subjected to gel filtration on a Ultrogel AcA 34 (LKB) column (1.6 x 30 cm) equilibrated with buffer D (50 mM Tris-HCl pH 8.0, 1.3 M NaSCN, 2 mM EDTA, 2 mM DTT). Heavy chains, eluting close to the void at ~0.35 column volumes, and light chains eluting at ~0.58 column volumes were pooled according to the OD_{280} profile or protein content (BioRad protein assay) of the fractions. After dialysis against two 1 l volumes of buffer C, some aggregated heavy chains were removed by centrifugation for 60 min at 100 000 *g*. From 5 mg clathrin, ~3 mg heavy chains and 0.3–0.5 mg of light chains were recovered.

The tryptic 110 000 mol. wt. heavy chain fragment was prepared by incubation of clathrin heavy chain cages at 0.5 mg/ml in 0.1 M MES pH 6.4, 5 mM $MgCl_2$ with 2% of their weight of trypsin (treated with TPCK; Worthington) for 2 h at 37°C after which the reaction was stopped by adding excess PMSF. Lower mol. wt. fragments released from the cages together with trypsin were removed by sedimenting the trypsinised cages under standard conditions.

Separation of Lα and Lβ

DEAE-Sephacel chromatography. About 1.3 mg of light chains in 13 ml of buffer D were dialysed against 1 l of buffer E [50 mM MES pH 5.8, 50 mM NaCl, 2.5 mM DTT, 1 mM NaN_3, 0.1 mM PMSF, 10% glycerol (v/v)] and applied to a DEAE-Sephacel (Pharmacia) column (1 x 27 cm) equilibrated with buffer E. After washing with 20 ml of buffer E, the column was eluted with 220 ml of a linear salt gradient ranging from 0.05 to 0.55 M NaCl. Analysis by SDS-PAGE showed that the light chains eluted in the order Lβ, Lα between 0.2 and 0.3 M NaCl in slightly overlapping peaks. Fractions were frozen and stored at −20°C until further use.

Preparative SDS-gel electrophoresis. A concentrated light chain sample was prepared as described by Lisanti *et al.* (1982b) by incubating 1.5 ml of clathrin (9 mg/ml) at 95°C for 10 min. After removal of the heavy chain precipitate by a 5 min spin in an Eppendorf centrifuge, ~1.2 mg of light chains were recovered in the supernatant. These were subjected to SDS-PAGE, but heating and carboxymethylation of the sample were omitted and 2 mM DTT was present in the gel polymerisation and running buffer. After brief fixing and staining of the gel with dilute Coomassie Brilliant Blue (Sigma), the Lα and Lβ bands were cut out and processed as described by Hager and Burgess (1980). After removal of guanidine hydrochloride by dialysis against buffer C, Lα and Lβ samples were frozen and stored at −20°C. Recovery was ~30% of the material loaded.

Radiolabelling of clathrin

Clathrin (0.1−1.0 mg) was dialysed against 0.2 M sodium borate buffer pH 8.8 at 4°C and then labelled with [^{3}H]$NaBH_4$ by reductive methylation as previously described (Tack *et al.*, 1980). After the reaction, free [^{3}H]$NaBH_4$ was removed by gel filtration. About 4% of the lysine residues were modified giving a specific activity of ~0.07 μCi/μg of protein. After dialysis against buffer C, labelled light chains were obtained by heating clathrin for 5 min at 95°C and removing the heavy chain precipitate by a 5 min spin in an Eppendorf centrifuge followed by a high speed spin at 135 000 *g* in an airfuge for 30 min.

Binding assays

Stock solutions of labelled light chains (~0.04−0.05 mg/ml in buffer C) and of heavy chains (~0.5 mg/ml in buffer C) were diluted as required into buffer F [C + 0.4 mg/ml bovine serum albumin (BSA)] and buffer G (C + 0.4 mg/ml BSA, 1% dextran T-40) respectively. Equal volumes were mixed and, after incubation for the times indicated in the text, 100 μl samples were spun in tubes previously washed with buffer F in an airfuge at 135 000 *g* for 1 h. The upper half of the solution, carefully removed from the top with a 50 μl micropipette, the lower half and the pellet, resuspended in 100 μl 0.1% SDS, were then dispersed into 10 ml of a toluene based scintillation fluid and counted for 5 min in a Beckman LS 9000 counter.

Binding to heavy chain cages was carried out analogously but buffer C was replaced by 100 mM MES pH 6.3, 5 mM $MgCl_2$.

Protein concentrations

Whenever possible, protein concentrations were determined from the absorbance at 280 nm after small corrections for turbidity. Extinction coefficients were determined by μ-Kjeldahl analysis (Jaenicke, 1974) and are 11.0 ± 0.6 for clathrin and clathrin heavy chains and 9.5 ± 1.0 for light chains. Nitrogen contents of 15.8% for clathrin and heavy chains and of 16.9% for light chains were based on the amino acid composition determined for clathrin (Pearse, 1976) and for Lβ of pig brain (A. Tsugita, personal communication). In cases where only small amounts or very dilute solutions were available, the BioRad protein assay was used. Controls with solutions of known concentration showed that calibration of this assay with BSA gives concentration estimates for clathrin, heavy and light chains that are within 10% of those based on extinction coefficients.

C.d. spectroscopy

Ultraviolet c.d. spectra were recorded at room temperature on a Jasco model J-40 spectropolarimeter. The mean residue ellipticity, [θ], was calculated by the equation:

$$[\theta] = \bar{M}\theta_{obs}/10\,C\,l$$

where $\bar{M}$ is the mean residue mol. wt., θ_{obs} is the observed ellipticity in degrees, C is the concentration in g/ml and l is the path length in centimeters. Clathrin, at 0.060 mg/ml in 20 mM sodium phosphate buffer pH 7.5, was measured in a 0.1 cm cell, heavy chains at 0.036 mg/ml in 10 mM sodium phosphate buffer pH 7.5 in a 0.2 cm cell and light chains at 0.034 mg/ml for Lα + Lβ, 0.020 mg/ml for Lα and 0.018 mg/ml for Lβ in 10 mM sodium phosphate buffer pH 7.0 in 0.2 cm cells. Values of $\bar{M}$ used were 113 for clathrin (Pearse, 1976) and 114 and 106 for heavy and light chains respectively (A. Tsugita, personal communication).

Electron microscopy

For negative staining, preparations of clathrin cages and of heavy chain cages at ~25 μg/ml in 1:10 diluted reassembly buffer were stained with 2% uranyl acetate on freshly glow-discharged carbon-film coated grids. For rotary shadowing, solutions of 30−50 μg/ml protein in 50 mM Tris/HCl pH 8.0, 50 mM NaCl were diluted with two volumes of 87% glycerol and sprayed onto freshly cleaved mica. Shadowing with platinum was carried out as described by Ungewickell and Branton (1981). All grids were examined at 80 kV in a Philips 301 electron microscope at a nominal magnification of 25 000.

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