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Tropomyosin-like Properties of Clathrin Light Chains Allow a Rapid, High-yield Purification

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ABSTRACT The light chains (LC_a and LC_b) of bovine brain clathrin are resistant to heat denaturation by boiling, a property shared by tropomyosin (Bailey, K., 1948, *Biochem. J.*, 43:271–281). Light chains were partially purified by boiling and centrifugation of a Tris-extract of crude membranes prepared from bovine brains (Keen, J. H., M. C. Willingham, and I. H. Pastan, 1979, *Cell.*, 16:303–312). Contaminant polypeptides were then removed by size-exclusion high-pressure liquid chromatography. The purified light chains were separated from each other by using an immunoaffinity column prepared from a monoclonal antibody CVC.7 specific for LC_a and not LC_b.

Clathrin is the major structural component of the protein coat of coated vesicles (1, 2, 3). It consists of a heavy chain of 180,000 mol wt and two light chains of 30,000–40,000 mol wt (4, 5, 6). A limited tissue polymorphism of clathrin light chains has been observed (7) (Brodsky, F. M., and P. Parham, manuscript submitted for publication). The light chains of clathrin called LC_a and LC_b are respectively of molecular weights 38,000 and 35,000 when isolated from bovine brain and those from other tissues are of 34,000 and 32,000 mol wt. Clathrin has been obtained by initial purification of coated vesicles and dissociation of the protein coat from the lipid by treatment with high pH, urea, Tris, or other amines (8–11). The polyhedral, clathrin lattice is thereby dissociated into trimeric structures called triskelions that have a characteristic morphology when visualized in the electron microscope (4–6). Each triskelion consists of three heavy chains and three light chains. Cross-linking studies suggest that each heavy chain is associated with a single light chain and comprises one arm of the triskelion (5). The overall ratio of the light chains in a pool of triskelions is approximately one LC_a to two LC_b. Three monoclonal antibodies (CVC.1, CVC.6, CVC.7) with specificity for LC_a but not LC_b have been produced. They all identify a similar antigenic site. One of them (CVC.7) has been used in immunoprecipitation studies to show that for individual triskelions the light chain composition is closer to a statistical rather than fixed distribution and both light chains can occur in the same triskelion (12).

The similarity in both molecular weight and isoelectric point of tropomyosin to clathrin light chains has been noted (13). Some antigenic similarity has also been observed (14). Tropomyosin is composed of two alpha helical chains of ~35,000 mol wt which form a coiled coil ≃400 Å in length (15). Experiments to compare brain clathrin light chains and brain and muscle tropomyosin showed that they were not identical (4, 13). There are, however, chemical differences between tropomyosins from brain and muscle (16), so the significance one can give to those conclusions is uncertain. A characteristic of tropomyosin, attributed to the stability of its coiled coil structure, is its resistance to heat denaturation (17). In fact, a highly selective step in the purification of tropomyosin is to boil a tissue extract for 10 min and separate denatured, precipitated proteins from soluble, native tropomyosin by centrifugation. To determine whether clathrin light chains share this structural property with tropomyosin, we examined the effect of boiling purified bovine brain clathrin preparations. The clathrin light chains remained stable after this treatment and were easily separable from the heavy chain which precipitated. A protocol for high-yield purification and subsequent separation of the two light chains was then developed, taking advantage of their resistance to boiling and the antigenic difference between LC_a and LC_b defined by monoclonal antibody, CVC.7.

MATERIALS AND METHODS

Gel Electrophoresis: SDS PAGE was done as described by Laemmli (18). Low molecular weight marker proteins (Pharmacia Fine Chemicals, Piscataway, NJ) were phosphorylase b (94 kdalton), bovine serum albumin (67 kdalton), ovalbumin (43 kdalton), carbonic anhydrase (30 kdalton), soybean trypsin inhibitor (20.1 kdalton), and α-lactalbumin (14.4 kdalton). Electrophoretic blotting was done according to the method of Towbin et al. (19, 20).

Clathrin Purification: A crude coated vesicle fraction was prepared from six bovine brains (stored at −70°C and thawed) (8). The coated vesicles were homogenized in 240 ml. 0.75 M Tris, 25 mM 2,(*N*-morpholino)ethane sulfonic acid (MES), pH 6.2, 0.25 mM EGTA, 0.12 mM $MgCl_2$ with 0.02% NaN_3 and 0.005% phenylmethylsulfonyl fluoride (PMSF) (buffer B) left at 4°C for 60 min, then spun at 125,000 *g*, 4°C, 60 min. The resulting supernatant (Tris-extract) was made up to 50% saturated ammonium sulfate in buffer B, overnight at 4°C, then spun, 12,000 *g*, 20 min, 4°C. Pellets were resuspended in 9 ml. of 0.5 M Tris, 50 mM MES, pH 6.2, 0.5 mM EGTA, 0.25 mM $MgCl_2$ with 0.02% NaN_3, and

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0.005% PMSF (buffer C) and loaded on an AcA22 column (1.6 × 100 cm), equilibrated in buffer C. 2.5 ml fractions were collected. Analysis by SDS PAGE (10%) showed clathrin eluted in the void volume and in fractions 33–37 which were just included. Lower molecular weight proteins (fraction II) appeared in fractions 38–70.

Tropomyosin Purification: Bovine brain tropomyosin was prepared by the methods of Fine and Blitz (16).

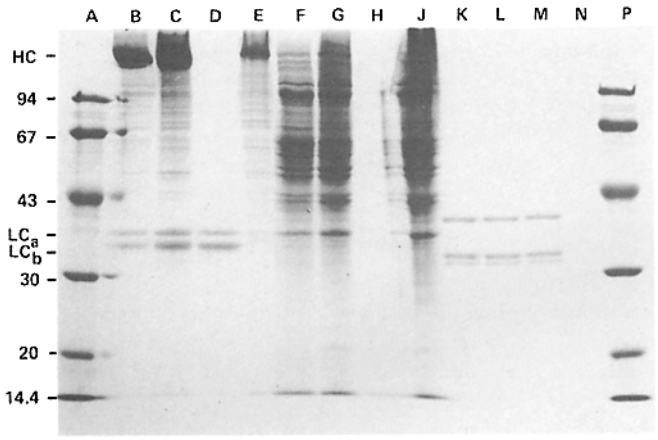

FIGURE 1 Clathrin light chains are resistant to heat denaturation. A sample of bovine brain clathrin was incubated for 10 min in a boiling water bath, centrifuged (60 min at 148,000 *g* in a Beckman airfuge), and then analyzed by SDS PAGE (10%). Lane *B* is clathrin before boiling, lane *C* after boiling before centrifugation, lane *D* the supernatant, and lane *E* the pellet after centrifugation. Bovine brain tropomyosin was treated identically. Lane *K* is tropomyosin before boiling, *L* after boiling before centrifugation, *M* is the supernatant, and *N* the pellet after centrifugation. The low molecular weight fraction (II) from the final size-exclusion chromatography step of the clathrin purification was analyzed in the same way. Lane *F* is fraction II before boiling, lane *G* after boiling and before centrifugation, *H* is the supernatant, and *J* the pellet after centrifugation. Lanes *A* and *P* are low molecular weight marker proteins. *HC* is the clathrin heavy chain and LC_a and LC_b, the clathrin light chains.

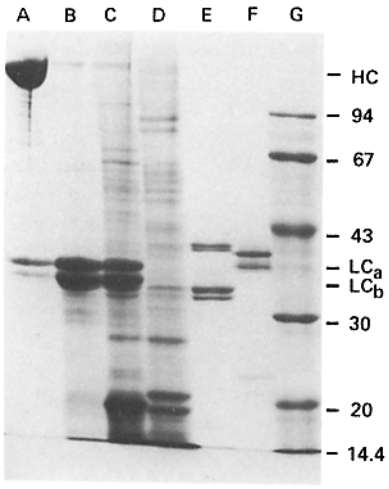

FIGURE 2 Partial purification of clathrin light chains by boiling a Tris-extract of crude membranes. A Tris-extract of the crude membrane fraction from bovine brains was boiled and centrifuged. The cytoplasmic fraction from the same membrane preparation was treated identically. Proteins from the post-boiling supernatants were concentrated by addition of 60% ammonium sulfate and resuspended in PBS, pH 7.4. Samples were analyzed by SDS PAGE (10%) as follows: lane *A*, AcA22 column purified bovine brain clathrin; lane *B*, clathrin light chains purified by boiling and centrifugation of AcA22 column-purified bovine brain clathrin; lane *C*, protein from supernatant after boiling and centrifugation of Tris extract; lane *D*, protein from supernatant after boiling and centrifugation of the cytoplasmic fraction; lane *E*, bovine brain tropomyosin; lane *F*, bovine muscle tropomyosin; lane *G*, low molecular weight protein markers. *HC*, LC_a, and LC_b are the clathrin heavy and light chains.

Partial Purification of Clathrin Light Chains by Boiling: A Tris-extract of a crude coated vesicle fraction was prepared as described for purification of clathrin. 25 ml of buffer B was used to extract membranes from one bovine brain. This extract was incubated for 10 min in a boiling water bath and centrifuged at 135,000 *g*, 1 h, 4°C.

High Pressure Liquid Chromatography: Size exclusion chromatography was performed with a Beckman 324 MP liquid chromatograph (Beckman Instruments, Inc., Palo Alto, CA) with a 60 × 0.75 cm Spherogel TSK 3000SW column. The mobile phase was 100 mM phosphate buffer, pH 7.0, and was filtered (0.45 μm, Millipore Corp., Bedford, MA) and degassed by the passage of helium before use. Eluted proteins were monitored by absorbance at 280 nm.

Immunoaffinity Column: Purified CVC.7 (anti-LC_a) immunoglobulin was coupled to sepharose 4B-CL at a ratio of 1–2 mg protein/ml of sepharose by the carbonate buffer procedure (21). The light chain containing fractions from the Spherogel TSK 3000SW column were pooled applied to a 5-ml column of CVC.7-Sepharose and washed through with PBS, pH 7.4 (Gibco Laboratories,

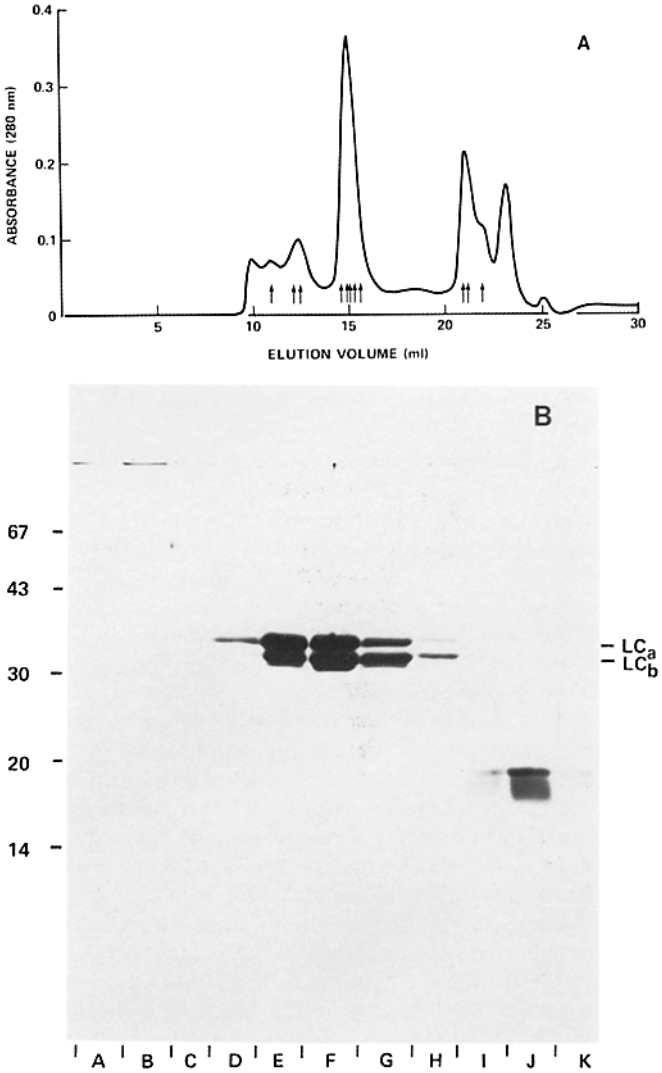

FIGURE 3 Purification of clathrin light chains by size exclusion HPLC after boiling of a Tris extract of crude membrane fraction. A Tris-extract of crude membranes from a single cow brain was incubated for 10 min in a boiling water bath and centrifuged. The supernatant was precipitated with saturated ammonium sulfate (30–40%), and the total protein precipitated (2–3 mg) was resuspended in 800 μl of PBS. 350 μl was applied to a 60-cm Spherogel TSK 3000SW column equilibrated with 100 mM phosphate buffer, pH 7.0. The flow rate was 0.2 ml/min, and 0.3-ml fractions were collected. Panel *A* shows the elution of protein as assessed by absorbance at 280 nm. Selected fractions as shown by the vertical arrows were analyzed in order by SDS PAGE (12.5%) as shown in panel *B*.

Grand Island Biological Co., Grand Island, NY). 1-ml fractions were collected and monitored for protein by absorbance at 280 nm. When the absorbance reached background levels, the column was eluted with 50 mM diethylamine-HCl, pH 11.5 (22). Fractions were neutralized by addition of an equal volume of 2X concentrated buffer C.

RESULTS

Effect of Boiling on Clathrin Light Chains

Purified bovine brain clathrin was incubated for 10 min in a boiling water bath and then centrifuged to yield a pellet and supernatant. The fractions were analyzed by SDS PAGE (Fig. 1). Boiling had no dramatic affect upon the migration pattern of the polypeptides in the preparations as shown in Fig. 1, lane *C*. However, after centrifugation all the proteins including clathrin heavy chain were found in the pellet (Fig. 1, lane *E*), except for the two light chains which were found quantitatively in the supernatant (Fig. 1, lane *D*). Fig. 1 shows the same experiment performed with purified bovine brain tropomyosin and with a low molecular weight fraction (II) from the final column in the clathrin purification. This fraction contains a protein co-migrating with LC_a, that we initially suspected to be free LC_a. Tropomyosin was completely stable to boiling in contrast to fraction II where all the material including the LC_a-like protein was precipitated. This result suggested that boiling might be a useful preparative method for separating clathrin heavy and light chains. Larger amounts of purified clathrin were boiled and centrifuged. The supernatant was analyzed by size-exclusion chromatography on a high-pressure Spherogel TSK 3000SW column. The light chains eluted in a major, homogeneous peak of apparent molecular weight 90,000. This high apparent molecular weight could be due to molecular asymmetry or specific oligomerisation of the light chains. That little nonspecific aggregation was observed provided evidence for the integrity of the boiled light-chain preparation. This is supported by the observation that light chains purified in this way will efficiently rebind with high affinity to clathrin cages from which the light chains have been removed by elastase digestion (Braell, B., and J. Rothman, personal communication).

Purification and Separation of Clathrin Light Chains

A major problem in studying clathrin light chains has been obtaining enough material. The standard clathrin purification (5) from isolated coated vesicles was monitored by an inhibition assay using the monoclonal antibody CVC.7. The result showed that the major losses of clathrin (60%) were in the sucrose gradient steps used to purify coated vesicles away from other membranes. These steps were therefore eliminated and the crude membrane fraction from one bovine brain was used

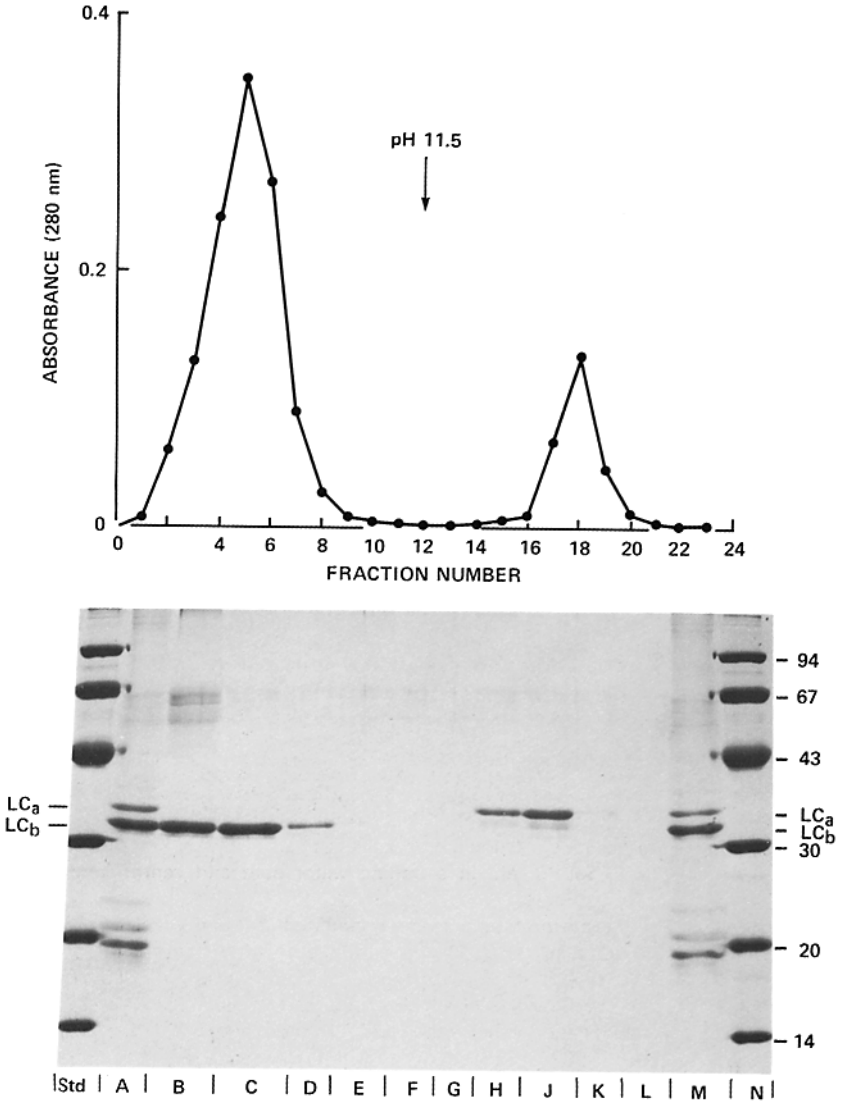

FIGURE 4 Separation of LC_a and LC_b on an anti-LC_a monoclonal antibody column. Fractions containing light chains, eluted from the Spherogel TSK 3000SW column (Fig. 3), were pooled and applied to a column of Sepharose coupled to CVC.7 (anti-LC_a monoclonal antibody). The *upper* panel shows the elution of the antibody column with 50 mM diethylamine, pH 11.5, as analyzed spectrophotometrically at 280 nm. The *lower* panel shows analysis of the column fractions by SDS PAGE (12%). Lanes *A* and *M* are samples containing both LC_a and LC_b at the ammonium sulfate stage of purification. Lanes *B–L* are fractions 4, 6, 8, 10, 13, 16, 18, 19, 20, and 21 from the CVC.7 column. Lanes *Std* and *N* consists of molecular weight markers as indicated. (Cultures of the CVC hybridoma cell lines are available from the American Type Culture Collection, Rockville, MD 20852.)

directly for Tris extraction. After centrifugation the Tris-extract was boiled for 10 min, centrifuged, and the supernatant analyzed by SDS PAGE (Fig. 2). For comparison, the cytoplasmic fraction generated in the preparation of crude membranes was also boiled, centrifuged, and the supernatant analyzed. In the supernatant of the boiled Tris-extract of membranes, a limited number of proteins were seen and the major species were of molecular weight corresponding to clathrin light chains. Electrophoretic blotting (19, 20) with CVC.6 monoclonal antibody confirmed the presence of LC_a in these preparations. Another major species with apparent molecular weight of 20,000 was also present. No polypeptides corresponding to the clathrin light chains were visible in the boiled cytoplasmic fraction. Another Tris-extract of bovine brain membranes was boiled and centrifuged and the supernatant subjected to serial precipitation with 30, 35, 40, 45, 50, 55, 60, 65, and 70% ammonium sulphate. The light chains predominately precipitated between 30 and 40% saturated ammonium sulphate as did most of the contaminating species. This provides a way of removing tropomyosin which precipitates at concentrations of ammonium sulphate between 40 and 53% saturated (16). The 30–40% precipitates were redissolved in PBS, pH 7.4, pooled, and applied to a Spherogel TSK 3000SW column. As shown in Fig. 3, this column separated the clathrin light chains from both minor, high molecular weight contaminants and the major contaminants of lower molecular weight. A very slight separation of LC_a and LC_b is also seen. The pooled light chain fractions were then applied to an immunoaffinity column of CVC.7 monoclonal antibody coupled to cyanogen bromide-activated Sepharose 4B-Cl (21). The sample was washed through the column with PBS and then eluted with 50 mM diethylamine-HCl pH 11.5 (22). One-third to -half of the original protein, as assayed by absorbance at 280 nm, bound to the column and was eluted with base. SDS PAGE analysis of the column fractions showed that all the LC_b passed through the column and that all the LC_a bound to the column and was eluted with base (Fig. 4). Yields of ~0.5 mg of pure LC_a and ~1.0 mg of pure LC_b have been obtained from a single cow brain.

DISCUSSION

The methods described above provide a complete separation and purification of the clathrin light chains LC_a and LC_b from each other, from clathrin heavy chains, and from other coated vesicle proteins. This should facilitate analysis of both the functional and structural characteristics of these proteins and a study of their relationship to tropomyosin. In agreement with previous experiments (4, 13) we find that a comparison of clathrin light chains with brain and skeletal muscle tropomyosins by limited proteolysis and SDS PAGE (23) shows they are not identical at the level of primary sequences. Our results so far tend to suggest that clathrin light chains may, like tropomyosin, consist predominantly of α-helical secondary structure. The size range of clathrin light chains is 32,000–38,000 mol wt corresponding to ≃290–345 amino acid residues. An α-helix of this size would extend 435–517 Å which is similar to the length (≃430 Å) of the triskelion arm as measured by electron microscopy (4, 24). A predominantly α-helical structure for the clathrin light chains would not be surprising, considering the extended asymmetrical structure of the heavy chains with which they interact.

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Note Added in Proof: Lisanti et al. (Lisanti, M. P., L. S. Shapiro, N. Moskowitz, E. L. Hua, S. Puszkin, and W. Schook, 1982. Isolation and preliminary characterisation of clathrin-associated proteins, *Eur. J. Biochem.*, 125:463–470) have independently discovered the heat stability of clathrin light chains.

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