Clathrin Light Chains Are Calcium-binding Proteins*

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Clathrin light chains have been purified to near homogeneity. When analyzed by sodium dodecyl sulfate gel electrophoresis followed by silver stain for proteins, no bands corresponding to light chains were detected. As calmodulin and tropinin C are known to behave in the same manner on silver staining, the possibility that clathrin light chains were Ca\(^{2+}\)-binding proteins was investigated. Light chains fixed to nitrocellulose filters were found to bind \( ^{45}\text{Ca}^{2+}\) in the presence of 5 mM Mg\(^{2+}\). The Ca\(^{2+}\)-binding capacity of the light chains was further investigated, using gel filtration and equilibrium dialysis. The light chains were shown to bind, in the presence of 3 mM Mg\(^{2+}\), 1 mol of Ca\(^{2+}\) per mol of light chain with a \(K_d\) of 25-55 \(\mu\)M. Nitrocellulose binding and gel filtration studies showed that light chains present in triskelions are still capable of binding Ca\(^{2+}\), in this case with a calculated \(K_d\) of 45 \(\mu\)M.

Coated vesicles, which are cellular organelles containing an outer protein lattice, are involved in intracellular protein transport. The vesicle coat can be dissociated into basic structural units termed triskelions which are composed of three clathrin heavy chains and three clathrin light chains. There are two types of light chains, a and b, which in bovine brain have molecular weights of 36,000 and 33,000, respectively.

Cellular Ca\(^{2+}\) is a general second messenger involved in multitudes of cellular functions (1). Evidence suggests that receptor-mediated endocytosis may be regulated by calcium (2, 3). Because of this, a number of investigators have studied the possible involvement of calmodulin in this system. Clathrin light chains, along with a number of other coated vesicle associated proteins, have been shown to bind calmodulin (4), and it has been suggested that calmodulin may facilitate the recruitment of clathrin components to the plasma membrane for the assembly of coated pits (5). In this paper we report that clathrin light chains are Ca\(^{2+}\)-binding proteins. This binding has a dissociation constant in the micromolar range and can be demonstrated in the presence of high concentrations of Mg\(^{2+}\).

**MATERIALS AND METHODS**

**Purification of Clathrin Light Chains**—Clathrin light chains were purified from fresh bovine brain according to a modified procedure of Lisanti et al. (6). Fresh tissue was homogenized in 0.1 M MES\(^{1}\) (pH 6.5), 1 mM EGTA, 0.5 mM MgCl\(_2\), 0.02% NaN\(_3\), 100 mg/liter crude soybean trypsin inhibitor, 100 mg/liter phenylmethylsulfonyl fluoride, and 200 mg/liter benzamidine and centrifuged at 16,000 \(\times\) g for 1 h. The supernatant was then centrifuged at 48,000 \(\times\) g for 2 h. The resultant pellet was resuspended in 50 mM Tris (pH 8.0) containing 1 mM EDTA and fresh protease inhibitor and placed in a boiling water bath for 5 min. The boiled sample was centrifuged at 100,000 \(\times\) g for 1 h. CaCl\(_2\) was added to the supernatant to a final concentration of 1.25 mM, and the sample was applied to a calmodulin-Sepharose affinity column equilibrated in 20 mM Tris (pH 7.5), 0.5 M NaCl, 20 mM 2-mercaptoethanol, and 0.25 mM CaCl\(_2\). The column was washed with the same buffer until all unbound protein was eluted, and a 0.25 mM CaCl\(_2\) to 0.1 mM EGTA gradient was applied. The clathrin light chains were eluted in 0.1 M EGTA. The fractions containing the light chains were pooled and applied to a Sephadex G-100 column equilibrated in 20 mM Tris (pH 7.5), 0.5 M NaCl, 20 mM 2-mercaptoethanol, and 0.1 M EGTA. The peak fractions containing the light chains were pooled, and the light chains were stored at -20 \(^\circ\)C.

**Purification of Triskelions**—Triskelions were purified from fresh bovine brain essentially by the method of Pearse and Robinson (7). Nitrocellulose Binding—Calcium-binding proteins were detected on nitrocellulose blots by \( ^{45}\)Ca\(^{2+}\) autoradiography. Purified light chains (10-20 \(\mu\)g) or triskelions (20-40 \(\mu\)g) were electrophoresed on SDS-polyacrylamide gels according to the method of Laemmli (9). Electrophoretic transfer of the proteins from the polyacrylamide gels to nitrocellulose membranes was performed according to the method of Towbin et al. (10). Transfer was carried out at a constant current of 55 mA for 2 h at 4 \(^\circ\)C in buffer containing 25 mM Tris (pH 8.5), 129 mM glycine, and 20% methanol. The nitrocellulose membrane was then labeled with \( ^{45}\)Ca\(^{2+}\), as outlined by Maruyama et al. (8), and autoradiographed. Following autoradiography, the membrane was stained with Amido Black stain.

**Sephadex G-25 Column Chromatography**—Calcium binding was measured by the technique of Hummel and Dreyer (11). Clathrin light chains (1 mg) or triskelions (1.5 mg) were dialyzed extensively against 50 mM Tris (pH 7.0) and 3 mM MgCl\(_2\) and then applied to a Sephadex G-50 column equilibrated in the same buffer to remove traces of EGTA. The peak fractions were pooled and applied to a 0.5 \(\times\) 20-cm Sephadex G-25 column equilibrated in 50 mM Tris (pH 7.0), 3 mM MgCl\(_2\), 1 mM CaCl\(_2\), and a trace amount of \( ^{45}\)!Ca\(^{2+}\) (60 cpn/ml).

**Equilibrium Dialysis**—Clathrin light chains (1 mg/ml) were dialyzed with three changes of buffer against 10 mM MOPS (pH 7.0), 1 mM dithiothreitol, and 3 mM MgCl\(_2\). A 0.3-ml sample of protein was then dialyzed for 24 h at 4 \(^\circ\)C in the same buffer containing \( ^{45}\)Ca\(^{2+}\) and varying amounts of CaCl\(_2\). Aliquots of the solution inside and outside the dialysis tubing were counted, and the protein concentration was determined.

Protein determinations were by the method of Bradford (12), and SDS-PAGE was performed according to the method of Laemmli (9) using 10 or 5-15% gradient slab gels as indicated.

**RESULTS**

Clathrin light chains were purified from fresh bovine brain using calmodulin affinity and gel filtration chromatographic.

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\(^{1}\)The abbreviations used are: MES, 4-morpholineethanesulfonic acid; EGTA, [ethylenbis(oxyethylenedinitrilo)tetraacetic acid; SDS, sodium dodecyl sulfate; MOPS, 4-morpholinepropanesulfonic acid.
judged by SDS gel electrophoresis and Coomassie Brilliant Blue protein techniques. Routinely, about 8–10% of the protein applied to containing buffer. This peak was pooled and applied to a calmodulin affinity column was eluted in the EGTA-peak consisting of proteins can be detected with silver stain. It appeared that could be detected (Fig. 1, protein (13, 14) no bands corresponding to the light chains and were homogeneous as contained clathrin light chains which were found to bind calcium. This result suggested that the binding of Ca\(^{2+}\) to the purified light chains was not an artifact of the purification procedure. Neither the clathrin heavy chain nor any of the other proteins in the triskelion preparation were found to bind calcium.

Although bovine brain light chains have been reported to contain tightly bound nucleotide (16), the ultraviolet spectrum of our purified light chains showed an absorbance maximum at 280 nm (data not shown). This information demonstrated that our purified light chains did not contain bound nucleotide, thus favoring the proposed direct binding of Ca\(^{2+}\) to the light chains and not to associated nucleotide.

To confirm further our finding that clathrin light chains were Ca\(^{2+}\)-binding proteins, the Hummel-Dreyer gel filtration method (11) was used. The typical gel filtration elution pattern obtained (Fig. 3A) was characteristic of Ca\(^{2+}\)-binding proteins. The peak of \(^{40}\text{Ca}\) coincided with the protein peak and was resolved from the resultant trough. A similar pattern was obtained when 10-fold higher concentrations of calcium were used. Assuming that 1 mol of Ca\(^{2+}\) binds per mol of light chain, a \(K_d\) of 28 μM was calculated at both Ca\(^{2+}\) concentrations. Intact triskelions also showed the characteristic gel filtration pattern (Fig. 3B). The calculated \(K_d\) was 45 μM, assuming that 3 mol of calcium bound per mol of triskelion. As the triskelion preparation was relatively impure (Fig. 2, light chains derived from triskelions in the absence of a boiling step also bound Ca\(^{2+}\). This result suggested that the binding of Ca\(^{2+}\) to the purified light chains was not an artifact of the purification procedure. Neither the clathrin heavy chain nor any of the other proteins in the triskelion preparation were found to bind calcium.

The behavior of the light chains on silver staining raised the possibility that the light chains were calcium-binding proteins. Calcium binding was initially investigated using the nitrocellulose binding method of Maruyama et al. (8). By this method both light chains were found to bind Ca\(^{2+}\) (Fig. 2) in a buffer containing 150 mM KCl, 3 mM Mg\(^{2+}\), and submicromolar Ca\(^{2+}\) concentrations. Analysis of triskelions by the nitrocellulose binding method (Fig. 2, lane B) showed that...
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![Graph](image)

**DISCUSSION**

We have purified the clathrin light chains from bovine brain to near homogeneity and have shown that both light chains are capable of binding Ca\(^{2+}\) with a \(K_d\) of 25-55 \(\mu M\). We have also shown that the light chains can bind Ca\(^{2+}\) either in the "free state" or when bound to the clathrin heavy chain in the form of triskelions. This finding suggests that Ca\(^{2+}\) binding by the light chains may occur when the triskelions are present in coat structures.

The binding affinity of the light chains for Ca\(^{2+}\) is less than that reported for other Ca\(^{2+}\)-binding proteins such as calmodulin or troponin C but is in the same range as those reported for other Ca\(^{2+}\)-binding, membrane-associated proteins. Calmodulin and myosin light chains belong to the Ca\(^{2+}\)-binding protein family characterized by an "EF hand structure" (17). This family of proteins are in the 10-20 kDa range and most of them are soluble cytosolic proteins. A new Ca\(^{2+}\)-binding family has been speculated to exist; this includes the calelectrins, endonexin, and the sarcoma virus tyrosine kinase substrate, p36 (18). These proteins share some sequence homology, have molecular masses predominantly in the 30-40 kDa range, and their affinity for Ca\(^{2+}\) is relatively low; a \(K_d\) = 100 \(\mu M\) for p36 (19) and a \(K_d\) = 70-80 \(\mu M\) for endonexin (20). These proteins have been suggested to possess similar cellular functions; all are thought to be involved in various aspects of intracellular transport. The molecular masses of bovine brain clathrin light chains (36 and 33 kDa) and their affinity for Ca\(^{2+}\) (45 \(\mu M\)) are well within the range of these proteins. Also, the light chains are involved in intracellular transport, predominantly in endocytosis. Thus, we suggest that clathrin light chains are also members of this family of novel Ca\(^{2+}\)-binding proteins.

The low affinities for Ca\(^{2+}\) characterized by this family may be related to the observation that most of these proteins are membrane-associated. The concentration of Ca\(^{2+}\) at the endoplasmic face of the plasma membrane may be significantly higher than the measured mean value for cytosolic Ca\(^{2+}\) (21). The lower affinities may then be a reflection of the fluctuations of Ca\(^{2+}\) concentration at the membrane and not in the cytosol. Tupper and Bodine (3) showed a decrease in the endocytosis of the epidermal growth factor receptor by normal W138 human fibroblasts when the extracellular Ca\(^{2+}\) concen-
trations were lowered. They suggested that this decrease was due to a change in surface membrane localized Ca\(^{2+}\). Thus, it may be the local membrane Ca\(^{2+}\) concentration which is the important regulator of clathrin light chain function.

The physiological significance of our observation that clathrin light chains can bind Ca\(^{2+}\) as well as calmodulin awaits further elucidation. The light chains have been proposed to play a regulatory role in coated vesicle function. They have a stimulatory effect on a coated vesicle-associated protein kinase activity (22) and are required for the activity of an "uncoating" ATPase (23). They have also been conjectured to modulate clathrin-membrane interaction (24). The effect of Ca\(^{2+}\) on light chain function in these reported studies is unclear; however, if the light chains also share functional similarities with the calelectrins and other members of this calcium-binding family, the light chains may be important in coated pit formation at the plasma membrane. In view of our current findings, the modulatory role of Ca\(^{2+}\) in clathrin light chain function deserves further investigation.

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REFERENCES
1. Rasmussen, H. (1970) Science 170, 404-412
2. Korc, M., Matrisian, L., and Magun, B. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 461-465
3. Tupper, J. T., and Bodine, P. V. (1983) J. Cell. Physiol. 115, 159-166
4. Linden, C. (1982) Biochem. Biophys. Res. Commun. 109, 186-193
5. Salisbury, J., Condeelis, N., and Satir, P. (1980) J. Cell Biol. 87, 132-141
6. Lisanti, M. P., Shapiro, L. S., Moskowitz, N., Hua, E. L., Puszkin, S., and Schook, W. (1982) Eur. J. Biochem. 125, 463-470
7. Pearce, B. M. F., and Robinson, M. S. (1984) EMBO J. 3, 1951-1967
8. Maruyama, K., Mikawa, T., and Ebashi, S. (1984) J. Biochem. (Tokyo) 86, 511-519
9. Laemmlli, U. K. (1970) Nature 227, 680-685
10. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350-4354
11. Hummel, J. F., and Dreyer, W. T. (1982) Biochim. Biophys. Acta 65, 539-552
12. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254
13. Oakley, B., Kirsch, D., and Morris, N. R. (1980) Anal. Biochem. 105, 361-363
14. Wray, W., Boulikas, T., Wray, V., and Hancock, R. (1981) Anal. Biochem. 118, 197-203
15. Schleicher, M., and Watterson, M. (1983) Anal. Biochem. 131, 312-317
16. Schook, W., Andres, A., and Puszkin, S. (1983) FEBS Lett. 164, 303-306
17. Kretsinger, R. H. (1980) CRC Crit. Rev. Biochem. 8, 119-174
18. Geisow, M., Fritsche, U., Hexham, J., Dash, B., and Johnson, T. (1986) Nature 320, 636-638
19. Gerke, V., and Weber, K. (1985) J. Biol. Chem. 260, 1688-1695
20. Geisow, M., and Burgoine, R. (1982) J. Neurochem. 38, 1735-1741
21. Rasmussen, H., Kojima, I., Kojima, K., Zawalich, W., and Apfeldorf, W. (1984) J. Cell. Physiol. 115, 159-166
22. Pauloin, A., and Jolles, P. (1984) Nature 311, 265-267
23. Schmid, S., Brasel, W., Schlossman, D., and Rothman, J. (1984) Nature 311, 228-231
24. Ungewickell, E. (1983) EMBO J. 2, 1401-1408
25. Scatchard, G. (1949) Ann. N. Y. Acad. Sci. 51, 660-672