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IDENTIFICATION OF A NOVEL HEPATIC CALCIUM BINDING PROTEIN

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Summary: Calcium binding activity in the 100,000 x g supernatant of bovine liver has been isolated by a procedure involving DEAE cellulose and Sephadex G-100 chromatography. In addition to calmodulin, two new high affinity calcium binding proteins have been identified. On gel filtration chromatography these proteins migrate with apparent molecular weights of 83,700 and 51,400; whereas by sodium dodecyl sulfate polyacrylamide gel electrophoresis, the two proteins migrate identically with M_r 63,000. In the presence of millimolar Mg^{2+}, both proteins bind up to one mol Ca^{2+}/mol protein. Half-maximal binding occurs at approximately 0.1 μM Ca^{2+}. Amino acid compositional analysis reveals that both proteins are acidic, and contain about 40% glx and asx. Peptide mapping procedures suggest that these proteins may be highly homologous or multiple forms of a single protein. The results show the existence of calcium binding protein(s) other than calmodulin in hepatic cytosol.

Free cytosolic calcium concentrations normally range from 50 to 200 nM in resting cells (1). However, the total cytosolic calcium content has been estimated to be in the range of 70 to 100 μM (1,2). This means that most of the cytosolic calcium is bound, presumably by ligands with a high affinity for calcium. Of the known calcium binding proteins, calmodulin has received the most attention (3-8). It is a small heat and acid stable protein which has been shown to mediate the calcium dependent regulation of many enzymes. It is found in many, if not all, eukaryotic cells (4) and its broad phylogenetic distribution and regulatory activities have led many investigators to propose that calmodulin is the primary intracellular receptor for calcium (8). It is probably because of this suggestion that no systematic search for other soluble calcium binding proteins has been carried out in mammalian tissues.

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In the present study, the calcium binding activity of the 100,000 x g supernatant of bovine liver has been analyzed. Two new, closely related calcium binding proteins have been identified and partially characterized. The results of this study suggest that in liver, calmodulin is not the only soluble calcium binding protein.

MATERIALS AND METHODS

$^{45}CaCl_2$ was purchased from Amersham, Chelex 100 was purchased from BioRad. All chemicals were of reagent grade or better.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed by the method of Laemmli (9) in 10% gel. Calcium binding activity was measured by the chelex competitive Ca^{2+} binding assay (10-11) with modifications as outlined in reference 12.

Fresh bovine livers were obtained from a local slaughterhouse. They were rinsed in distilled water, and frozen immediately. Five hundred grams of frozen liver were chopped and then minced in a meat grinder. The mince was mixed with three volumes (weight/volume) of ice cold 40 mM Tris/HCl (pH 7.5) containing phenylmethylsulfonyl chloride (1.0 mM), 0.2 mM EDTA, and homogenized in a Waring blender. The resultant crude fraction was centrifuged at 10,000 x g for twenty minutes and the supernatant centrifuged for sixty minutes at 100,000 x g. All experimental manipulations were performed at 4°C.

Calcium binding was determined by equilibrium dialysis. The buffer contained 50 mM KCl, 50 mM HEPES (pH 7.1). 3 mM $MgCl_2$, 1 mM EGTA and various amounts of $CaCl_2$, (45 Ca) $CaCl_2$ were added to Ca/EGTA buffers with a constant ratio of (45 Ca)/(40 Ca) to prevent isotope effects (13). Dialysis tubing was incubated in 0.1 mM EGTA then rinsed extensively with chelex-treated water before use. To calculate free Ca^{2+} concentration, an absolute association constant for Ca/EGTA of pKa = 10.95 (14) was used.

Amino acid analysis was performed on a Durrum D-500 Amino Acid Analyser as outlined in the manual. Tryptic peptide maps were prepared according to Luna et al (15). Calmodulin was assayed on the basis of its ability to activate the calcium activatable phosphodiesterase of bovine brain (16). Routinely, aliquots were incubated at 98°C for two minutes before the assay. Protein concentration was determined according to Bradford (17) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Bovine liver 100,000 x g supernatant was fractionated on DEAE cellulose. Results are presented in Figure 1. The total calcium binding activity eluted as two broad peaks. Peak I eluted between 0.025 and 0.11 M NaCl and peak II eluted at 0.22 M NaCl. The fractions were reassayed after incubation at 98°C for two minutes, to assess heat labile Ca^{2+} binding activity. This treatment resulted in the complete loss of calcium binding in peak I and a reduction of the activity in peak II by 80%. Calmodulin, as assayed by phosphodiesterase activation, eluted as a later peak distinct from the two major peaks of calcium binding activity. It is unlikely that a bound form of calmodulin, not accessible to phosphodiesterase, was present in either peak I or II since enzyme activation was measured after heat treatment, a maneuver which presumably disrupted such subunit associations (20).

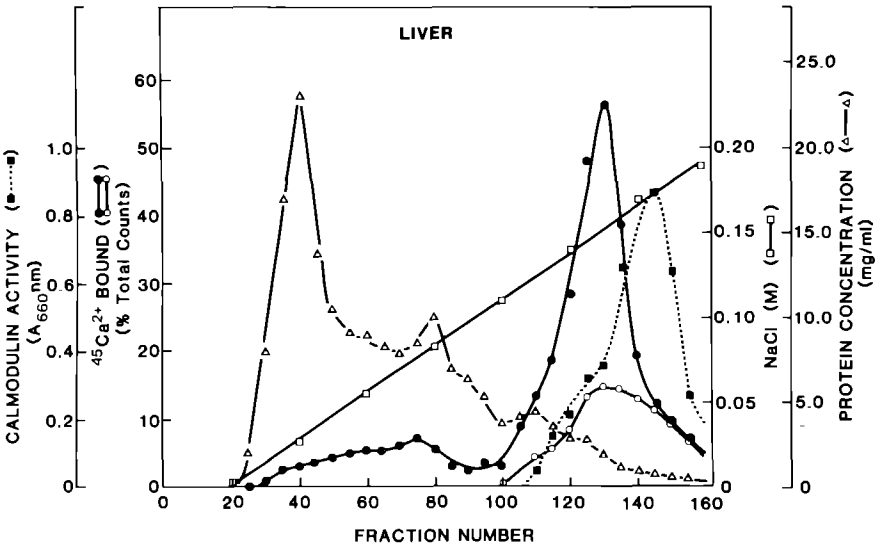

Figure 1. Chromatography of Liver 100,000 x g Supernatant on DEAE Cellulose. The 100,000 x g supernatant of bovine liver (see Methods and Materials) was diluted ten-fold into 10 mM Tris (pH 7.5), and 400 ml of DEAE cellulose (1/1 suspension in 10 mM Tris, pH 7.5) was added batchwise. The resultant slurry was stirred for sixty minutes at 4°C, filtered through a scintered glass funnel, and the DEAE cellulose resin was poured into a 5.0 cm x 30 cm column. The column was washed extensively with 10 mM Tris (pH 7.5) and then with a linear salt gradient from 0.0-0.3 M NaCl in 10 mM Tris (pH 7.5). $^{45}Ca^{2+}$ bound is expressed before (●—●) or after (○—○) incubation of aliquots at 100°C for two minutes.

The pooled peak II fraction (Figure 1) was concentrated by ultrafiltration with a PM 10 membrane (Amicon), and applied to a calibrated Sephadex G-100 column. Two peaks of calcium binding activity were eluted with apparent molecular weights of 83,700 (protein I) and 51,400 (protein II) (data not shown).

Protein I (83,700) and protein II (51,400) eluted from Sephadex G-100 were individually pooled, concentrated and each reapplied to Sephadex G-100. The Ca^{2+} binding activity was pooled and reapplied. After the third chromatography on Sephadex G-100, both calcium binding proteins appeared homogeneous. Figure 2 presents a typical elution profile of protein II after three cycles of purification on Sephadex G-100. The correlation between protein concentration and calcium binding suggests homogeneity. Similar results were obtained for protein I. In contrast to their behavior on gel filtration, the two proteins migrated with identical relative mobility on sodium dodecyl sulfate polyacrylamide gel electrophoresis. The molecular weight estimated from this procedure was 63,000. Furthermore, polyacrylamide gel electrophoresis of protein I and protein II (Figure 2, inset) in sodium dodecyl

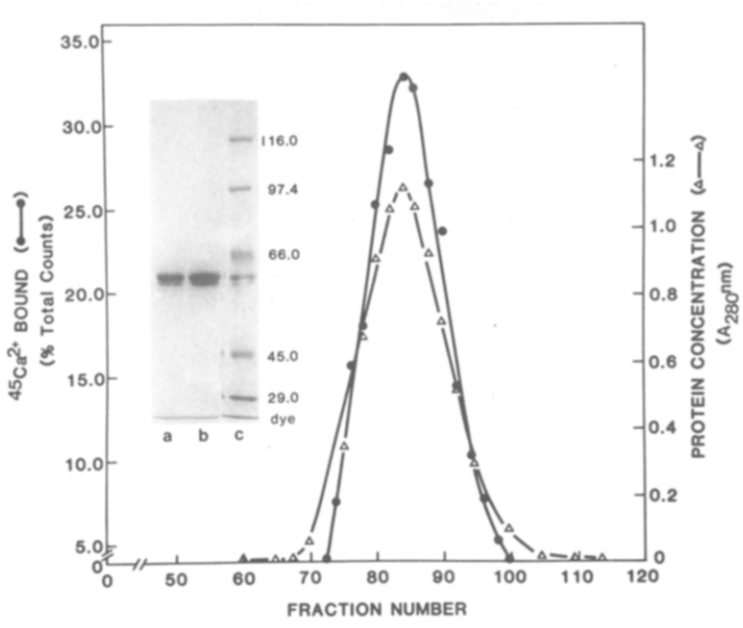

Figure 2. Correlation of Calcium Binding Activity and Protein Concentration. Purified protein II was subjected to chromatography on Sephadex G-100 and protein concentration (A_{280} nm) and calcium binding activity determined. A molecular weight of 51,400 was estimated by this procedure. Insert, 10% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate for protein I (a), and protein II (b). C, protein II was electrophoresed in the presence of β-galactosidase (116,000), phosphorylase b (97,400), bovine serum albumin (66,000), ovalbumin (45,000), and carbonic anhydrase (29,000).

sulfate reveals a higher molecular weight major band and a lower weight minor band in both the protein I and protein II fractions. Approximately 60 mg of protein I and 50 mg of protein II were purified from 1 kg liver.

The major and minor bands of protein I and protein II were cut from gels and subjected to peptide mapping (Figure 3). The similar pattern for both proteins suggests that protein I and protein II are homologous proteins. Also presented in Figure 3 is a tryptic peptide map of the minor (lower molecular weight) band of protein II which produces an identical pattern to that of the major bands of protein I and protein II. An identical peptide map was also produced by the minor band of protein I. These results suggest that the major and minor bands of protein I along with the major and minor bands of protein II may be multiple forms of the same protein.

Table 1 presents the amino acid composition of the 24 hour acid hydrolysate of the two proteins. The amino acid composition of the two proteins is very similar: both proteins are highly acidic, containing about 40% asx and glx. Proteins I and

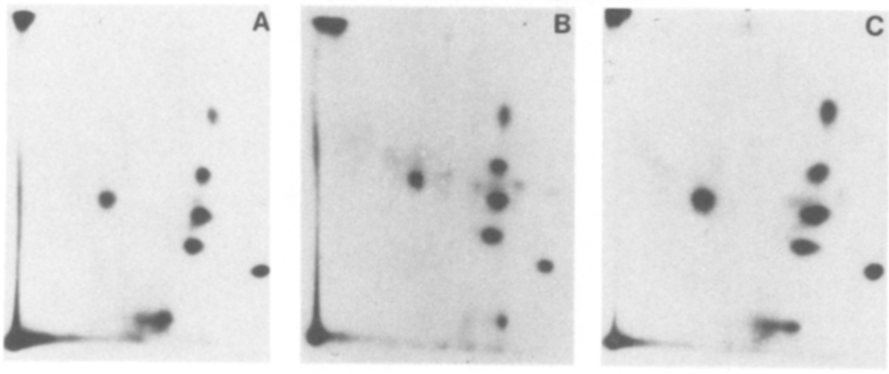

Figure 3. Tryptic Peptide Maps of A, Protein I (major band); B, Protein II (major band); C, Protein I (minor band).
Tryptic peptide maps were prepared as outlined in "Methods and Materials". In Figure 4, electrophoresis was performed in the horizontal plane (right to left) and chromatography in the vertical plane (bottom to top).

II each contain about 10% lysine and a total of about 14% basic residues. Neither protein contains γ-carboxyglutamic acid, and therefore these proteins do not appear to be a member of the vitamin K superfamily of calcium binding proteins (21).

TABLE 1. Amino acid composition of protein I and II.

Amino acid	Protein I residues/mol[a]	Protein II residues/mol[a]
Aspartic acid	96.5	102.2
Threonine	19.0	17.8
Serine	25.9	23.1
Glutamic acid	103.8	108.9
Proline	34.9	38.7
Glycine	41.8	40.7
Alanine	35.9	28.2
Valine	11.6	12.6
Methionine	4.9	5.0
Isoleucine	15.6	16.2
Leucine	29.4	22.0
Tyrosine	15.9	16.4
Phenylalanine	25.0	22.9
Histidine	10.5	9.7
Lysine	55.2	53.2
Arginine	12.7	11.6
Tryptophan	9.6[b]	13.4[b]
Half-cystine	5.4[c]	5.0[c]
γ-carboxyglutamic acid	<0.1[d]	<0.1[d]

[a] Residues per polypeptide chain assuming a monomeric molecular weight of 63,000 derived from SDS gels.

[b] Tryptophan was determined after hydrolysis in methane sulfonic acid at 110°C for 22 hours in vacuo according to Ref. 18.

[c] Half-cystine was determined as cysteic acid after performic acid oxidation. Values were determined at 24 hour hydrolysis according to Ref. 19.

[d] γ-carboxyglutamic acid was determined after alkaline hydrolysis according to Ref. 20.

TABLE 2. Calcium binding properties of proteins I and II.

Free Ca^{2+} (μM)	Protein I mol Ca^{2+}/mol protein*	Protein II mol Ca^{2+}/mol protein[a]
0.13	0.46	0.66
0.75	0.72	0.94
11.40	0.75	0.89
101.40	1.02	1.35

[a]The stoichiometry was calculated by assuming a M_r of 63,000 derived from SDS gels.

Table 2 presents a comparison of the calcium binding properties of proteins I and II. In the presence of 3 mM Mg^{2+}, both proteins bind up to one mol Ca^{2+}/mol protein, and half-maximal calcium binding occurs at about 0.1 μM Ca^{2+}. This result indicates that the calcium binding site of these proteins is a specific, high affinity calcium binding site.

The results presented in this paper suggest the presence, in the hepatic 100,000 x g supernatant, of calcium binding proteins other than calmodulin. Elucidation of the function of these proteins will be necessary to fully understand the role of calcium in hepatic function.

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