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Properties of Calcium-Binding Protein isolated from the Soluble Fraction of Normal Rat Liver

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The molecular weight of calcium-binding protein (CaBP) purified from the soluble fraction of normal rat liver was estimated to be 28800 by calibrated gel filtration on Sephadex G-100.

Amino acid analysis of the CaBP showed glycine and glutamic acid to be the predominant amino acids. The calcium binding constant was found to be $4.19 \times 10^5 \text{M}^{-1}$ by equilibrium dialysis, and there appear to be 6—7 high affinity binding sites for calcium per molecule of protein.

Keywords——liver calcium; calcium-binding protein in liver; properties of calcium-binding protein; calcium metabolism; normal rat liver

Introduction

Calcium-binding protein (CaBP) has been identified in the intestine,[1] kidneys[2] and parathyroid glands[3] in mammals, and in the eggshell gland of laying hens[4] where there is a large flux of calcium. The physiological function of CaBP remains unknown, but its high concentration in the above tissues suggests that it may be involved in transcellular transport of calcium.[5]

The liver also participates in the regulation of calcium metabolism in mammals.[6–8] We found a calcium-binding activity in the soluble fraction from normal rat liver,[9] and isolated a CaBP from that tissue.[10] This paper describes the molecular weight, amino acid composition and calcium binding constant of a CaBP in the soluble fraction from normal rat liver.

Materials and Methods

Isolation of Calcium-Binding Protein——CaBP in the soluble fraction of rat liver was purified to electrophoretic homogeneity by gel filtration on Sephadex G-75 and G-50 followed by ion-exchange chromatography on diethylamino-ethyl-cellulose, as reported previously.[10] The protein was determined by the method of Lowry *et al.*[11]

Molecular Weight Determination by Gel Filtration——Determination of the molecular weight of the protein was performed by a modification of the gel filtration methods of Roubal and Tappel[12] and Andrews[13] on a column (2.5×90 cm) of Sephadex G-100. A solution containing 3.0 mg of Blue Dextran (mol wt, 2000000), 4.5 mg of bovine serum albumin (mol wt, 65000), 4.5 mg of cytochrome C (mol wt, 13000), and 3.0 mg of α-chymotrypsin (mol wt, 22500) in a volume of 2 ml was placed on the column and eluted with Tris-HCl buffer (0.1 M, pH 7.3). The molecular weight of CaBP was determined by reference to a calibration curve obtained with standard proteins.

Amino Acid Analysis——CaBP purified by DEAE-cellulose chromatography was hydrolyzed with 6 N HCl *in vacuo* for 48 hr at 100° and the hydrolysate was analyzed with a Beckman 120 C amino acid analyzer. Duplicate samples were oxidized with perdormic acid for 4 hr prior to hydrolysis for the determination of cysteine and methionine as cysteic acid and methionine sulfone, respectively. Corrections were made for recovery of norleucine.

Calcium Binding Assay——Calcium-binding studies were carried out in the bags made from EDTA-washed 55-mm dialysis tubing, essentially by the procedure described by Ingersoll and Wasserman.[14] For experiments in which 0.5 ml of protein solution was used, the thin-film method of Craig[15] was used. A range of calcium concentrations of $1.0 \times 10^{-6} - 5.0 \times 10^{-5}$ M was used with ^{45}Ca of constant specific activity (40 μCi/mol of calcium) in the dialysate. Samples of the inner and outer solutions were obtained at hourly intervals to determine when equilibrium had been reached. The number of moles of calcium bound to the protein was calculated from the total radioactivity bound and the specific activity of the ^{45}Ca in the dialysate.

Results and Discussion

The gel filtration of CaBP from rat liver on Sephadex G-100 yielded an estimated molecular weight of 2.88×10^4 (Fig. 1), which is close to that of CaBP isolated from the chick intestine (28000),[16] and larger than those of CaBPs from rat, pig, or cow intestine[17-20] and porcine parathyroid glands,[3] which were estimated to be 8000—15500.

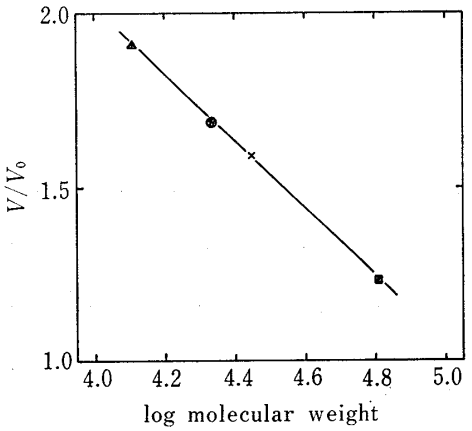

Fig. 1. Molecular Weight of Calcium-Binding Protein from the Soluble Fraction of Normal Rat Liver

The molecular weight was estimated by gel filtration on a 2.5×90 cm column of Sephadex G-100 superfine gel; flow rate, 0.21 ml min; fraction size, 3.1 ml. V_0; void volume for Blue Dextran 2000, V; elution volume. ▲; cytochrome C (mw=13000), ●; α-chymotrypsin (mw=22500), ■; bovine serum albumin (mw=65000), × ; calcium-binding protein (mw=28800).

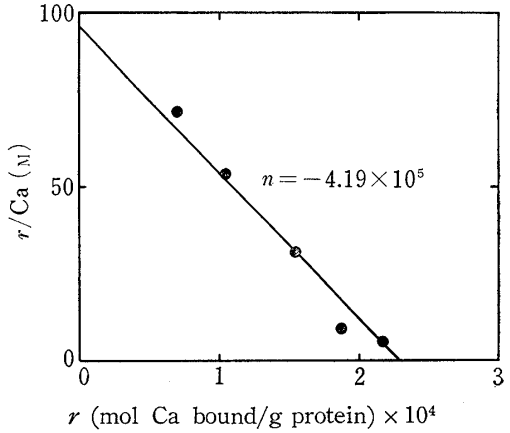

Fig. 2. Scatchard Plot for the Binding of ^{45}Ca by Calcium-Binding Protein

Protein was used at a concentration of 75 μg/ml. Dialysis conditions: 0.5 ml of protein solution was dialyzed against 225 ml of Tris-saline buffer containing ^{45}Ca (specific activity, 40 μCi/mol of calcium) for 8 hr at 22°. The line was plotted by linear regression analysis. From the slope of the line, n, the association constant (Kf) was estimated to be 4.19×10^5 M^{-1}.

Table I. Amino Acid Composition of Calcium-Binding Protein from the Soluble Fraction of Normal Rat Liver

Amino acid	Residues/mol of peptide (combined results)[a]	Mole integer
Aspartic acid	24.48	24
Threonine	15.34	15
Serine	16.02	16
Glutamic acid	28.02	28
Proline	10.62	11
Glycine	34.65	35
Alanine	20.29	20
Valine	26.25	26
Isoleucine	14.03	14
Leucine	18.17	18
Thyrosine	5.79	6
Phenylalanine	8.13	8
Lysine	20.36	20
Histidine	14.42	14
Arginine	10.11	10
Cysteine	0.32	0
Methionine	0.00	0
Tryptophan[b]		

a) Residues/mol of peptide were calculated from the mole fraction by best fit based on the recovery of all stable residues. The combined results represent the average of duplicate analyses on two separate preparations.
b) Not determined.

The amino acid composition of CaBP from the soluble fraction of rat liver is shown in Table I. The protein has a relatively high content of glycine and slightly lesser amounts of glutamic acid, valine, aspartic acid, and lysine. The minimum molecular weight calculated from this analysis was about 28600. Acidic amino acids comprise approximately one-fifth of the liver CaBP molecule. A prevalence of acidic amino acids appears to be characteristic of the composition of all CaBPs. The porcine parathyroid gland CaBP, and the chick and porcine intestinal CaBP are acidic proteins.[3,21,22] Acidic amino acids are also prevalent in CaBP of brain[23] and in the high-affinity CaBP associated with the contractile elements in mammalian and fish muscle.[24] The significance of the high dicarboxylic acid content of these proteins is suggested by the work of Kretsinger and Nockolds.[24]

The apparent association constant of the calcium-CaBP complex was determined by equilibrium dialysis. The experimental data, plotted according to the method of Scatchard,[25] are shown in Fig. 2. The apparent association constant (Kf) estimated from the slope of the line (n) was 4.19×10^5 M^{-1} with a correlation coefficient of 0.99. Extrapolation of the regression line to infinite calcium concentration indicates a maximal calcium binding of 2.28×10^{-4} mol of Ca/g of protein. In view of the molecular weight estimated from amino acid analysis, 28587, there are 6.52 high-affinity binding sites per molecule of protein. The calcium-binding constant of the liver CaBP, $Kf=4.19\times10^5$ M^{-1}, is similar to those reported for rat parathyroid gland CaBP[3] and rat,[26] pig[19] and chick[14] intestinal CaBPs, *i.e.*, 9.3×10^5 M^{-1}, 1.5×10^6 M^{-1}, 1.5×10^6 M^{-1}, and 2.0×10^6 M^{-1}, respectively.

On the other hand, a calcium-binding phosphodiesterase activator protein (PAP) has been found in the soluble fraction from rat liver.[27] The molecular weight of PAP estimated by gel filtration of Sephadex G-200 was higher than 50000.[27] Thus, our CaBP from rat liver apparently differs from PAP in rat liver.

CaBP in various tissues acts as a mediator of Ca^{2+} regulation. Because it modulates enzyme activities in a Ca^{2+}-dependent manner, this CaBP is now commonly known as calmodulin.[28] Calmodulin regulates a number of fundamental cellular activities such as cyclic nucleotide and glycogen metabolism, intracellular motility, and calcium transport, as well as less-defined Ca^{2+}-dependent protein kinases.[28] Calmodulin exists as a monomer of molecular weight 17000 and contains 4 calcium binding sites.[28,29] Our CaBP in rat liver has a molecular weight of 28800 and contains 6—7 calcium binding sites.

Recently, it has been reported that the liver participates in the regulation of calcium metabolism in rats.[6-8] Our CaBP may be involved in calcium transport in the liver cells of rats.

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Studies on the Metabolism of Unsaturated Fatty Acids. IV.[1] N-Ethylmaleimide Reducing Activity in *Escherichia coli* K-12

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An enzyme fraction which catalyzes the reduction of N-ethylmaleimide (NEM) to N-ethylsuccinimide in the presence of reduced nicotinamide adenine dinucleotide phosphate was partially purified from *Escherichia coli* extracts.

The apparent Michaelis-Menten constant for NEM was estimated to be 4 μM. The enzyme showed a rather broad pH optimum between 7 and 8.

The enzyme was 90% inhibited after being heated at 55° for 15 min, and was 94% inhibited by 1 mM *p*-hydroxymercuribenzoic acid.

Keywords——N-ethylmaleimide; N-ethylsuccinimide; N-ethylmaleimide reducing enzyme; *Escherichia coli*; NADPH

Among organic substances which bind to reactive side chains of amino acid residues in proteins, *p*-hydroxymercuribenzoic acid, iodoacetic acid, and N-ethylmaleimide (NEM) are commonly used as binding reagents for the thiol groups of cysteine residues. These compounds usually inhibit enzymatic reactions. On rare occasions, however, stimulatory effects of these compounds on certain enzymatic reactions have been reported. For example, increases of enzyme activities in the presence of NEM have been reported for enoyl-acyl-carrier-protein (enoyl-ACP) reductase[2] and for microsomal glutathione S-transferase.[3] In the case of the enoyl-ACP reductase, it was explained that NEM at a concentration of 1 mM interacted with the enzyme at a site other than the active thiol group and stimulated its activity. Microsomal glutathione S-transferase activity was stimulated strongly by treatment of the microsomes with NEM at a concentration of 1 mM, although the mechanism of its activation remains to be established.