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Physicochemical Properties of Calcium-Binding Protein Isolated from Rat Liver Cytosol: Ca^{2+}-Induced Conformational Changes

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The physicochemical properties of a calcium-binding protein (CaBP) isolated from rat liver cytosol was investigated. Isoelectric focusing in a polyacrylamide gel plate using the Broad pI Calibration Kit showed that the isoelectric point is 5.20. The ultraviolet (UV) absorption spectrum of CaBP showed a maximum at 278 nm. The conformational changes induced by Ca^{2+}-binding to CaBP were examined by measuring the UV difference, fluorescence emission, and circular dichroism (CD) spectra. These spectra were altered by titration of CaBP with 1.0 mM Ca^{2+}. The alterations could be attributed to an increased exposure of tyrosine and tryptophan residues to a more aqueous environment, resulting in an increased hydrophobicity of CaBP. From the CD spectrum, the apparent α-helical content of CaBP in Ca^{2+}-free buffer was estimated to be 34%. This value was decreased by 1.0 mM Ca^{2+} addition. The results suggest that Ca^{2+}-binding induces conformational changes of CaBP, and that the protein contains distinct and specific ligand-binding sites for Ca^{2+}.

Keywords——calcium-binding protein; calcium ion; physicochemical property; calcium ion-induced conformational change; rat liver cytosol

Introduction

In recent years, it has been demonstrated that calcium ion (Ca^{2+}) plays an important role in the regulation of cell function.[1,2] Calmodulin, a kind of calcium-binding protein, can amplify many effects of Ca^{2+} on cell function.[1,2] Recently it has been found that a calcium-binding protein (CaBP) isolated from rat liver cytosol can reverse the activation of several enzymes in liver cells by Ca^{2+}.[3-5] This effect results from Ca^{2+}-binding to CaBP.[3-5] The role of CaBP in regulation of cell functions may differ from that of calmodulin.

The molecular weight of CaBP was previously estimated to be 28800, and the calcium binding constant was found to be 4.19×10^5 M^{-1} by equilibrium dialysis.[6] These properties of CaBP are distinctly different from those of calmodulin.[2] The other physicochemical properties of CaBP, however, remain to be elucidated. The present study was undertaken to characterize in detail the properties of Ca^{2+}-binding to CaBP. This report presents the results of isoelectric focusing and spectroscopic studies on CaBP. It was found that Ca^{2+}-binding induces conformational changes in CaBP.

Materials and Methods

Animals——Male Wistar rats, weighing 130—150 g, were used. They were obtained commercially (Nippon Bio Supply Center, Tokyo, Japan). The animals were given commercial laboratory chow containing 1.1% Ca, 1.1% P and 57.4% carbohydrate (Oriental Test Diet, Tokyo, Japan) and tap water freely.

Chemicals——Calcium chloride and all other reagents were purchased from Wako Pure Chemical Co. (Osaka, Japan).

Isolation of CaBP——The livers of rats were perfused with ice-cold 0.25 M sucrose solution, frozen immediately,

and then cut into small pieces, which were suspended 1 : 4 in an ice cold 0.25 M sucrose solution and homogenized in a Potter–Elvehjem homogenizer with a Teflon pestle. The homogenate was spun at $105000 \times g$ in a refrigerated centrifuge for 60 min and the supernatant fluid was collected to obtain the cytosol. CaBP in the cytosol 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 diethylaminoethyl (DEAE)-cellulose, as reported previously.[7]

Isoelectric Focusing of CaBP——Purified CaBP (10 μg) was submitted to isoelectric focusing in a polyacrylamide gel plate using the Broad pI Calibration Kit (Pharmacia Fine Chemicals: Piscataway, New Jersey, U.S.A.). The polyacrylamide gels consisted of 5% acrylamide and 2.5% Ampholine, pH 3.5 to 9.5 (LKB, Pleasant Hill, California, U.S.A.). The samples were layered on the center of the gels in a volume of 10 μl. Isoelectric focusing was carried out at 25 W for 90 min with cooling to maintain the temperature at 5 °C. At the end of the isoelectric focusing, the gels were rapidly removed and stained for protein using Coomassie Brilliant Blue R-250 (Bio-Rad, Richmond, California, U.S.A.), then destained in 25% ethanol and 8% acetic acid. The gels were photographed.

Spectroscopic Methods——Ultraviolet (UV) difference spectroscopy was performed from 250—320 nm using matched tandem cuvettes in a spectrophotometer. CaBP was dialyzed against 50 mM Tris–HCl (pH 7.3), 0.1 mM ethylene glycol bis(2-aminoethylether) *N,N,N',N'*-tetraacetic acid (EGTA) containing 1.0 mM $CaCl_2$ or none, and adjusted to 1.20 mg/ml. The CaBP sample (0.75 ml) was placed on one side of each cuvette. The opposite side of the reference cell was filled with 0.75 ml of CaBP sample plus EGTA. After reading the baseline spectrum, the sample cuvette was mixed by inversion and the difference spectrum was recorded.

The fluorescence measurements were made on a fluorescence spectrophotometer (Hitachi 650-60). Emission spectra were scanned from 300 to 460 nm using an excitation wavelength of 280 nm. Dialyzed CaBP was adjusted to a concentration of 0.90 mg/ml with 50 mM Tris–HCl (pH 7.3) containing 0.1 mM EGTA with or without 1.0 mM $CaCl_2$ solution.

Circular dichroism (CD) was measured on a Jasco J-20A spectropolarimeter over the range from 300 to 195 nm. CaBP was prepared by exhaustive dialysis against 50 mM Tris–HCl (pH 7.3), and was used at a concentration of 0.195 mg/ml in a 1 cm path-length cell. Poly-L-lysine ($[\theta] = -33000$ at 207 nm) was used to calibrate the instrument.[8]

Analytical Method——The protein concentration was determined by the method of Lowry *et al.*[9] with a standard solution of bovine serum albumin.

Results

The result of isoelectric focusing in a polyacrylamide gel plate using the Broad pI Calibration Kit is shown in Fig. 1. The isoelectric point of calcium-binding protein (CaBP) isolated from rat liver cytosol was 5.20, which indicates that the molecule is highly acidic. The amino acid composition of CaBP was reported previously.[6] The protein contains (mol%) about 20% glutamic and aspartic acids and about 17% amide residues (lysine, histidine, and arginine) and, therefore, contains a high proportion of charged residues.[6]

The ultraviolet (UV) absorption spectrum of CaBP is shown in Fig. 2. The UV absorption spectrum showed a maximum at 278 nm in the range from 240 to 330 nm. The absorption of $A_{278}^{1\%}$ was 6.848. In the presence of Ca^{2+}, a decrease in absorbance at 278 nm was observed. The spectrum can be attributed to changes in both tyrosine and tryptophan residues. Changes in the environment of both aromatic amino acids may occur upon Ca^{2+}-binding.

The conformational changes induced by Ca^{2+}-binding to CaBP were investigated by means of UV difference spectroscopy. In the presence of Ca^{2+} (0.1 and 1.0 mM), a decrease in absorption between 260 and 300 nm was observed (Fig. 3). Such a negative UV difference is similar to the Ca^{2+}-induced absorption changes in calmodulin that are thought to indicate an increased exposure of the aromatic amino acid tyrosine to the aqueous solvent in the presence of ligand.[9] For CaBP, the spectrum can also be attributed to a change in tyrosine residues. Ca^{2+} titration experiments revealed distinct UV difference effects. The first effect reached completion below a free Ca^{2+} concentration of 0.1 mM. It consisted of a broad negative peak ranging from 260 to 300 nm. A further change in the UV difference spectrum was observed if the Ca^{2+} concentration exceeded 0.1 mM. This change was completed at 1.0 mM Ca^{2+} and was characterized by a distinct negative peak between 260 and 300 nm. Separate gel filtration experiments showed the absence of any aggregation of CaBP under these conditions.

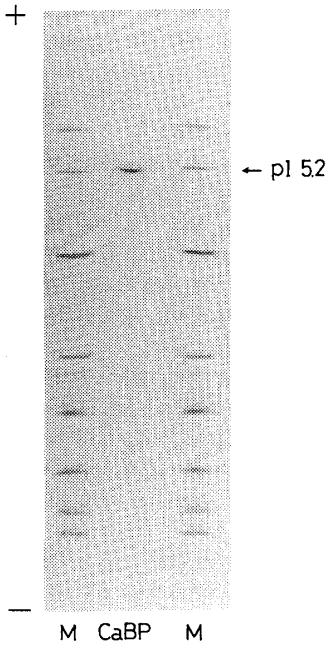

Fig. 1. Isoelectric Focusing of CaBP Isolated from Rat Liver Cytosol

The arrow indicates the pI value (5.20) of CaBP. Purified CaBP (10 μg) was applied to a polyacrylamide gel plate. M indicates pI markers.

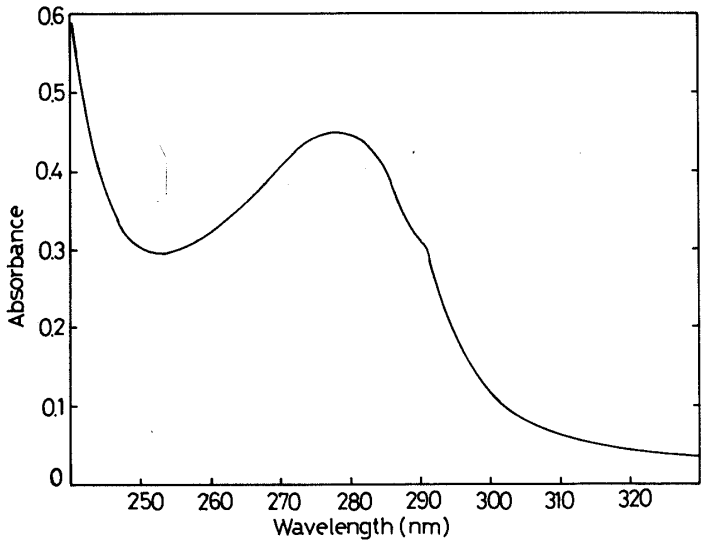

Fig. 2. UV Absorption Spectrum of CaBP Isolated from Rat Liver Cytosol

CaBP was used at a concentration of 0.20 mg/ml in 50 mM Tris–HCl (pH 7.3) containing 0.1 mM EGTA.

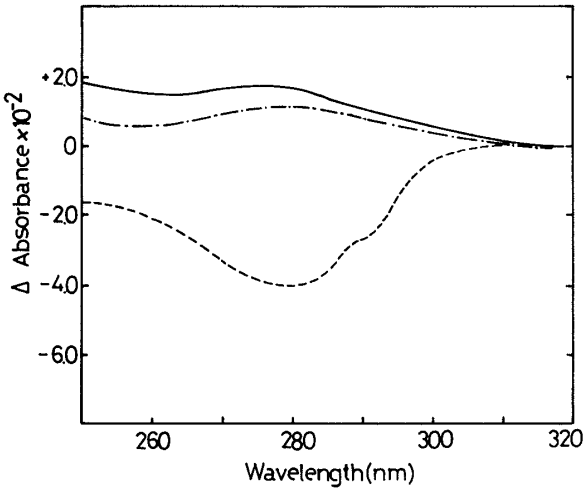

Fig. 3. Ca^{2+}-Induced UV Difference Spectra of CaBP Isolated from Rat Liver Cytosol

UV difference spectra were read at 0.1 and 1.0 mM final free Ca^{2+} concentrations. CaBP was used at a concentration of 0.90 mg/ml in 50 mM Tris–HCl (pH 7.3) containing 0.1 mM EGTA. After recording the base-line (solid line), $CaCl_2$ was added, and the difference spectrum was read.

——, base line; —·—, addition of 0.2 mM Ca^{2+}; – – – –, addition of 1.0 mM Ca^{2+}.

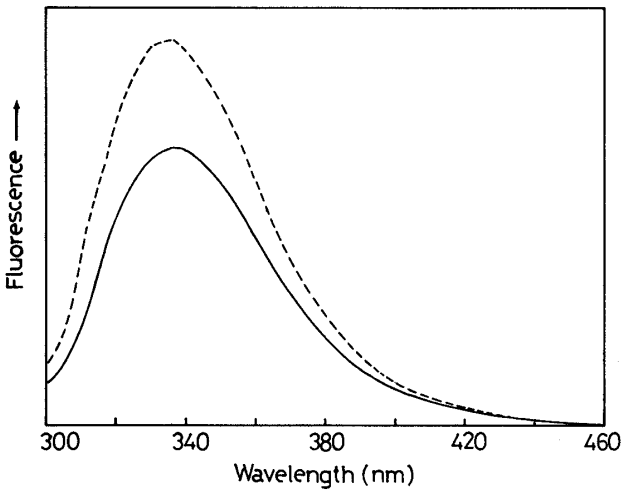

Fig. 4. Effect of Ca^{2+} on the Fluorescence Emission Spectrum of CaBP Isolated from Rat Liver Cytosol

CaBP was used at a concentration of 0.90 mg/ml in 50 mM Tris–HCl (pH 7.3) containing 0.1 mM EGTA with or without 1.0 mM $CaCl_2$. The emission spectra were recorded between 300 and 460 nm.

——, none; – – – –, addition of 1.0 mM Ca^{2+}.

The effect of Ca^{2+} on the conformation of CaBP was also studied by means of fluorescence spectroscopy. Using an excitation wavelength of 280 nm, the fluorescence emission was recorded in the absence or presence of 1.0 mM Ca^{2+}. As shown in Fig. 4, the spectra emission was quenched after the addition of Ca^{2+}. Known fluorescence emission

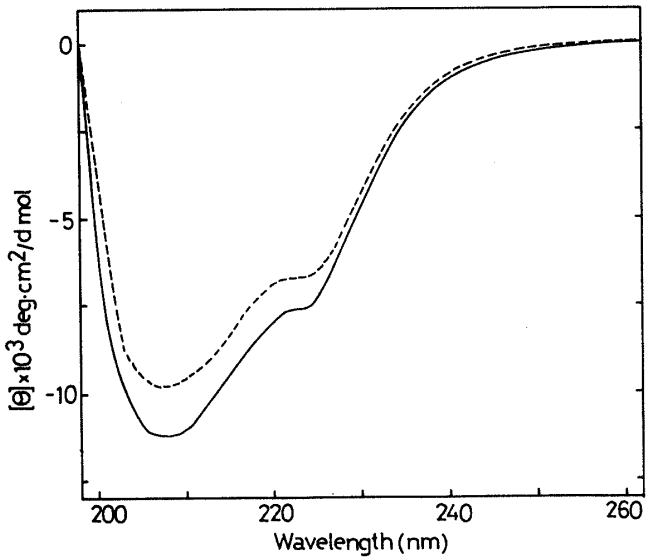

Fig. 5. CD of CaBP Isolated from Rat Liver Cytosol

Spectroscopy was performed as described in Materials and Methods. CaBP was used at a concentration of 0.20 mg/ml in 50 mM Tris–HCl (pH 7.3) containing 0.1 mM EGTA with or without 1.0 mM $CaCl_2$.
——, none; – – – –, addition of 1.0 mM Ca^{2+}.

properties of isolated tyrosine and tryptophan residues[11] suggest that, as indicated above, changes in the environment of both aromatic amino acids occur upon Ca^{2+}-binding.

The conformation of the polypeptide backbone of CaBP was studied by means of circular dichroism (CD) spectroscopy. The presence of Ca^{2+} caused clear alterations in the CD spectrum (Fig. 5). Using the approximation of Holzwarth and Doty,[8] the apparent α-helical content of CaBP in Ca^{2+}-free buffer was estimated to be 34%. The presence of 1.0 mM Ca^{2+} resulted in a decrease of 4.5%. Thus, conformational changes were induced by Ca^{2+}-binding to CaBP.

Discussion

Recently Waisman *et al.* have purified and characterized and M_r 63000 protein by Chelex-100 competitive calcium-binding assay of eluates from DEAE-cellulose chromatography of bovine liver $100000 \times g$ supernatant.[12–14] This protein is referred to as calregulin or CAB-63 and based on its physical, chemical, and calcium binding properties, is established as a novel calcium-binding protein, although its physiological function is unknown.[15] We isolated a calcium-binding protein (CaBP) from rat liver cytosol.[7] This CaBP, a M_r 28800 protein, clearly differed from the protein which was reported by Waisman *et al.*[6,15] Thus far, it has been demonstrated that CaBP can reverse the activation of several enzymes by Ca^{2+} and /or calmodulin, indicating that CaBP can regulate the Ca^{2+} effect on liver cell functions.[3–5] This reversible effect of CaBP is based on Ca^{2+} binding to the protein, because the Ca^{2+} binding constant of CaBP was found to be 4.19×10^5 M^{-1} by equilibrium dialysis, and this protein has 6—7 high-affinity binding sites per molecule of protein.[6]

To characterize fully the interaction of CaBP with Ca^{2+}, spectroscopical studies were performed, as has been described for proteins such as calmodulin.[10] UV difference spectroscopy revealed distinct absorbance spectra at different Ca^{2+} concentrations. Negative UV difference changes upon binding of calcium have been previously observed in calmodulin.[10] The spectra observed with CaBP indicate that tyrosine and tryptophan residues in the protein become exposed to a more aqueous environment in the presence of the ligand.[10] Intrinsic protein fluorescence has also been used to analyze the conformational changes in CaBP during interaction with Ca^{2+}. Ca^{2+} binding to CaBP results in a dramatic increase in its intrinsic fluorescence. It is known that fluorescence intensity is affected by movement of charged groups along with hydrophobic changes in the microenvironment.[16] These results

clearly demonstrate that Ca^{2+}-binding induces the conformational changes in CaBP. The Ca^{2+}-dependent conformational changes may result in the increase of the hydrophobicity of CaBP.

The conformation of the polypeptide backbone of CaBP was studied by means of CD spectroscopy. Ca^{2+}-binding to CaBP caused an alteration in the CD spectrum, again indicating Ca^{2+}-induced conformational changes. The apparent α-helical content of CaBP in Ca^{2+}-free buffer was estimated to be 34%. This value was decreased by Ca^{2+}-binding. This result suggests that Ca^{2+}-binding loosens the conformation of CaBP. It is known that the apparent α-helical content of calmodulin is 30% and this content is increased by Ca^{2+}-binding.[10] The Ca^{2+}-dependent increase in hydrophobicity represents the mechanism by which calmodulin activates its target proteins.[17] However, CaBP could reverse the activation of hepatic fructose 1,6-bisphosphatase by Ca^{2+}-calmodulin.[3] Thus, CaBP plays a role different from that of calmodulin in the regulation of cell functions by Ca^{2+}.

In conclusion, the present investigation clearly demonstrates that the conformational changes of CaBP are induced by binding of Ca^{2+}. This suggests that CaBP contains distinct and specific ligand-binding sites for Ca^{2+}.

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