Chem. Pharm. Bull. 31(3) 966—970 (1983)

A Conformation Change of the Porcine Intestinal Calcium Binding Protein on Binding of Ca^{2+}

KENZO CHIBA,* MASAYOSHI TAKEUCHI, TETSURO MOHRI and TAKAO OHYASHIKI

Department of Physiological Chemistry, School of Pharmacy, Hokuriku University, Kanagawa-machi, Kanazawa, Ishikawa 920-11, Japan

(Received July 24, 1982)

The intrinsic tyrosine fluorescence of the porcine intestinal calcium binding protein (CaBP, 7 μM) was quenched by the addition of ~170 μM ethyleneglycol-bis(2-amino ethyl-ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA), returning progressively to its original level with increasing concentration of subsequently added Ca^{2+} up to 117 μM, in a concentration-dependent manner. In the presence of an excess of EGTA, the intrinsic fluorescence of the CaBP was further quenched by 1 M or less of guanidine-HCl, while it was enhanced by 2—4 M guanidine. In the presence of an excess of Ca^{2+}, the fluorescence intensity increased monotonically with increasing concentration of guanidine (~4 M). Quenching of the intrinsic fluorescence of the CaBP by alkaline pH's (above 8) was moderated by addition of EGTA compared to that measured in the presence of Ca^{2+}. KCl (~100 mM) showed a quenching effect on the fluorescence in the presence of 83 μM EGTA, an enhancing effect in the presence of 1 mM EGTA, and no effect in the presence of Ca^{2+} at a concentration sufficient to saturate the CaBP. These experimental results suggest that Ca^{2+} binding to the CaBP induces microenvironmental and also significant conformational changes in the tyrosine-containing region of the protein.

Keywords——calcium binding protein; conformation change; tyrosine fluorescence; pH effect; potassium chloride effect; guanidine-hydrochloride effect; porcine intestine

Intestinal calcium binding protein (CaBP) was first reported by Wasserman and Taylor[1] in chick intestinal mucosa as a vitamin D-dependent calcium binding protein. Many lines of evidence show that the mucosal CaBP probably plays an essential role in intestinal calcium absorption.[2] Porcine intestinal CaBP was first purified and characterized by Hitchman *et al.*[3,4] and Dorrington *et al.*[5,6] and was found to have one tyrosine and five phenylalanine residues, but no tryptophan in the molecule. They found that the ultraviolet (UV) absorption spectrum of this protein showed conspicuous tyrosine and phenylalanine peaks, and the UV absorption difference spectrum and circular dichroism (CD) difference spectrum of calcium-bound and -free forms of this protein reflect a change of the physical state of the tyrosine residue upon binding of Ca^{2+} to this protein. They proposed that these phenomena can be ascribed to the difference of microenvironment around tyrosine between the two forms.

In the present work, we studied the interaction of the CaBP and Ca^{2+} in terms of the intrinsic tyrosine fluorescence of this protein, and we discuss the possible mechanisms of the change of the fluorescence intensity of the CaBP upon binding Ca^{2+}.

Materials and Methods

Preparation of CaBP——The porcine intestinal CaBP was prepared from the porcine small intestine according to the method described by Hitchman *et al.*[4] The purity of this preparation was checked by 10% polyacrylamide gel disc electrophoresis (pH 8.3). The purified CaBP migrated as a single band and the mobility of this protein band was much lower with addition of 100 μM $CaCl_2$ than without addition of $CaCl_2$ or with 100 μM EGTA, as previously reported by Hitchman *et al.*[3] Determination of protein was carried out by the method of Lowry *et al.*[7] with bovine serum albumin as a standard. Molar concentration of the protein was calculated using a molecular weight of 9000.[5]

Fluorescence Measurement——Fluorescence was measured at 25°C for a 2.5 ml sample in 10 mM Tris-HCl buffer, pH 7.4, containing a range of protein concentrations (6.9—14 μM) of the CaBP and reagents as specified in "Results," with a Hitachi MPF-4 fluorescence spectrophotometer at excitation and emission wavelengths

of 275 and 305 nm, respectively, unless otherwise specified. The assay medium was checked for pH with a microelectrode pH meter (Radiometer Co., PHM-62 standard pH meter with G297/G2 capillary electrode) after fluorescence measurement when the pH dependence of fluorescence was examined by stepwise addition of NaOH. Arbitrary units or percentages were used to express relative fluorescence intensity and its change.

Reagents——Chelex 100 (100—200 mesh, sodium form) was purchased from Bio-Rad Co. Possibly contaminating Ca^{2+} in Tris-HCl buffer was removed by passing it through a Chelex 100 column equilibrated with the same buffer. KCl and guanidine-HCl solutions were treated with Chelex 100 by the batch method to remove contaminating Ca^{2+}. EGTA (acid form), guanidine-HCl and all other chemicals used were of the purest grade commercially available.

Results and Discussion

Ca^{2+}-Dependence of the Tyrosine Fluorescence of the CaBP

As shown in Fig. 1 the intrinsic fluorescence spectra of the porcine intestinal CaBP showed a typical tyrosine band, with excitation maximum at 277 nm and emission maximum at 305 nm, and the fluorescence intensity was greatly quenched by the addition of EGTA without any other spectral change.

Figure 2 shows the quenching of the tyrosine fluorescence of 7 μM CaBP by a stepwise addition of EGTA and an almost complete recovery on subsequent addition of $CaCl_2$.

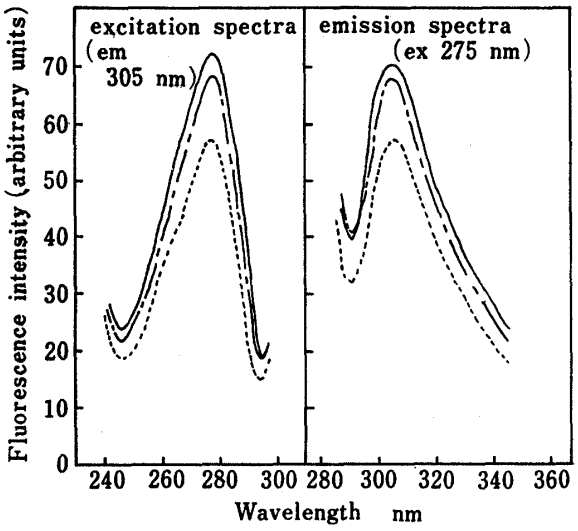

Fig. 1. Spectral Profiles of the Intrinsic Fluorescence of the CaBP and the Effect of Addition of EGTA

Protein concentration of CaBP was 6.7 μM. ——, CaBP alone; – – –, plus 100 μM EGTA; —·—, plus 100 μM EGTA and 100 μM $CaCl_2$. Other conditions are described under "Materials and Methods."

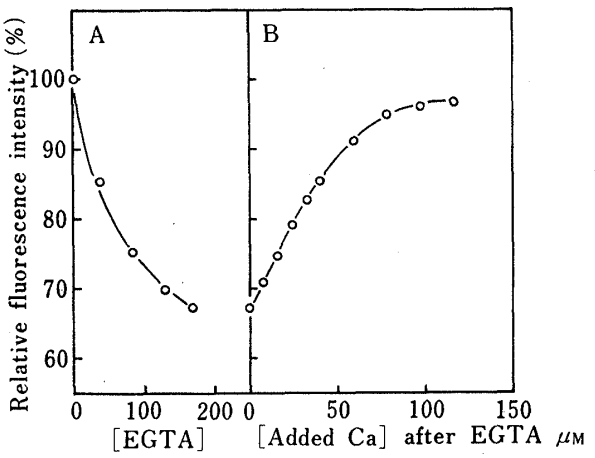

Fig. 2. Titration Profiles of the CaBP with EGTA and Ca^{2+}

Protein concentration was 7 μM. Fluorescence intensity is expressed relative to the value at no addition of EGTA. EGTA was added stepwise up to 170 μM (graph A) as measuring fluorescence and then $CaCl_2$ was added up to 117 μM (graph B). Other conditions are described under "Materials and Methods."

The fluorescence intensity was decreased to less than 70% by 170 μM EGTA, but returned to its original level on subsequent addition of a total of 117 μM $CaCl_2$.

This finding suggests that the physical state of the single tyrosine residue of the CaBP molecule is different on release from and binding to the molecule of Ca^{2+}.

Effects of Guanidine on the Intrinsic Fluorescence in the Presence of Ca^{2+} or EGTA

Figure 3 shows the concentration-dependent variation of the effect of guanidine-HCl on the tyrosine fluorescence of the CaBP in the presence of $CaCl_2$ or EGTA.

In the presence of EGTA (240 μM), the fluorescence intensity was further quenched by the addition of about 1 M or less of guanidine, but it was greatly enhanced at 2 M or more of guanidine. In the presence of Ca^{2+} (145 μM), in contrast, the fluorescence intensity increased

monotonically with increasing concentration of guanidine up to 4 M. The fluorescence intensity of L-tyrosine decreased monotonically with increasing guanidine concentration equally in the presence of corresponding concentrations of Ca^{2+} and in the presence of EGTA (data not shown).

It is thus postulated that the tyrosine residue in the CaBP is in effectively closer contact with solvent in the absence of Ca^{2+} than in the presence of sufficient Ca^{2+}, as has been suggested by Dorrington *et al.* for this protein.[5] The quenching effect observed at lower concentrations of guanidine in the presence of excessive EGTA may be a solvent effect on the tyrosine residue. According to our preliminary CD experiments, the secondary structure of the CaBP is destroyed at 4.5 M guanidine in the presence of an excess of EGTA, but not at 1 M guanidine (unpublished). It seems that the CaBP molecules can only be denatured[8] by strong base in the presence of excess Ca^{2+}.

Effect of pH on the Intrinsic Fluorescence in the Presence of Ca^{2+} or EGTA

As shown in Fig. 4, quenching of the tyrosine fluorescence of the CaBP by alkaline pH's was moderated by addition of 100 μM EGTA compared to that observed in the presence of 100 μM Ca^{2+}.

The fluorescence intensity was progressively quenched with increasing pH above 7.4, where dissociation of the phenolic hydroxyl group should occur.[9] The apparent dissociation constant (pK_a) for the phenolichydroxyl group in the protein was estimated from the data shown in Fig. 4 (as the pH at 50% quenching of the fluorescence intensity) at 10.9 and 11.8 for Ca^{2+}-bound and -depleted forms of the CaBP, respectively, compared to 10.4 for L-tyrosine under similar conditions.

These results support the possibility of the disruption of a hydrogen bond on binding of Ca^{2+} to the CaBP, as has been reported for several proteins.[10] Enhancement of tyrosine fluorescence of the CaBP on binding of Ca^{2+} can be interpreted as a result of disruption of a hydrogen bond linking any internal quencher, *e.g.* internal carboxylate or carbonyl group,

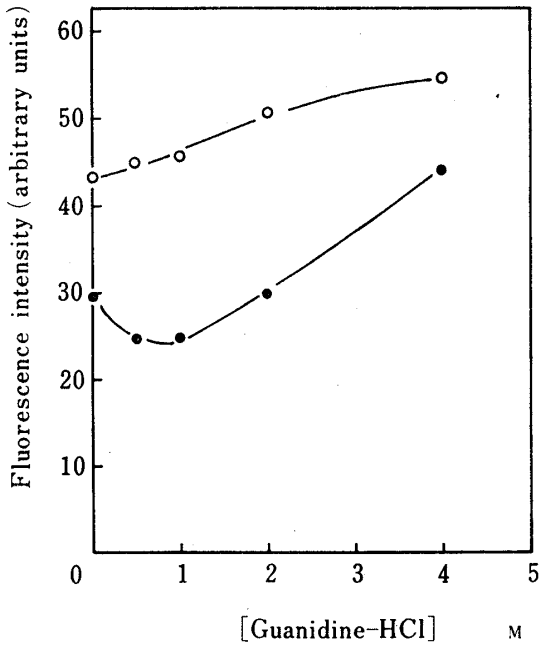

Fig. 3. Effect of Guanidine on the Fluorescence Intensity of the CaBP in the Presence of Ca^{2+} or EGTA

Protein concentration was 14 μM. —○—, 145 μM $CaCl_2$; —●—, 240 μM EGTA. Fluorescence intensity is expressed in arbitrary units.

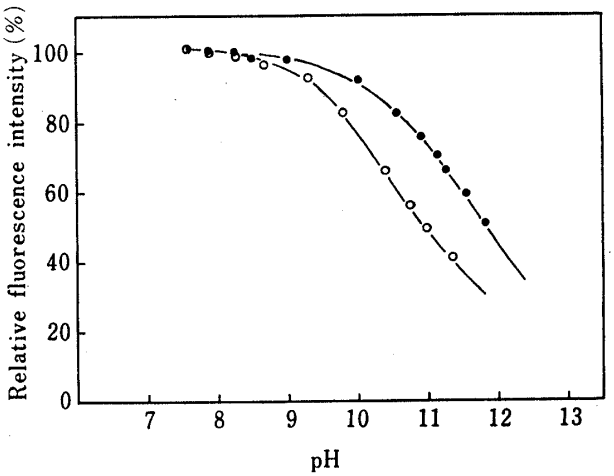

Fig. 4. pH Dependence of the Fluorescence Intensity of the CaBP in the Presence of Ca^{2+} or EGTA

Protein concentration was 8.1 μM. —○—, 100 μM $CaCl_2$; —●—, 100 μM EGTA. The assay medium was titrated with portions of NaOH solution and the pH was checked after fluorescence measurement. Fluorescence intensity is expressed relative to the value at pH 7.57 in each of the curves.

and the phenol group of the tyrosine residue,[11] or as a result of dehydration of water bound to the phenolic hydroxyl group.[5]

Effect of KCl on the Intrinsic Fluorescence in the Presence of Ca^{2+} or EGTA

The effect of various concentrations of alkali metal cation on the tyrosine fluorescence of the CaBP was examined in the presence of an appropriate concentration of Ca^{2+} or various concentrations of EGTA with 6.7—12.7 μM of the protein (Fig. 5). KCl had a considerable quenching effect over a wide range of concentrations up to 100 mM on the fluorescence in the presence of 83 μM EGTA, whereas it showed an enhancing effect at similar concentrations in the presence of 1 mM EGTA. It had no effect on the fluorescence in the presence of 25 μM added Ca^{2+} (Fig. 5A). This concentration of added Ca^{2+} was considered to be enough to saturate the CaBP (12.7 μM) at the binding site(s), since the fluorescence intensity reached the maximum at around 25 μM of added Ca^{2+} and was substantially unchanged above that up to 38 μM (data not shown). The results shown in Fig. 5B confirm the differential effects of KCl mentioned above on the fluorescence of the CaBP in the presence of various concentrations of EGTA. The quenching effect of EGTA on the fluorescence was intensified by the addition of KCl in the range of concentration of EGTA below 150 μM.

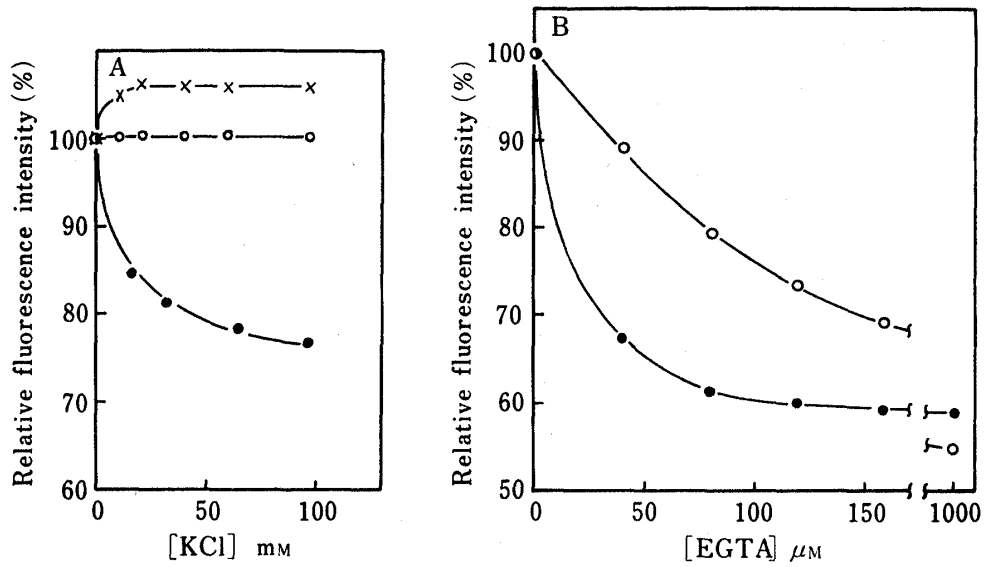

Fig. 5. Effects of K^+ on the Fluorescence Intensity of the CaBP in the Presence of Ca^{2+} or EGTA

A) KCl titration profile. Protein concentration was either 6.7 μM (—●—) or 12.8 μM (—○—, —×—). —○—, 25 μM $CaCl_2$; —●—, 83 μM EGTA; —×—, 1 mM EGTA. Fluorescence intensity is expressed as % of that at 0 mM KCl in each of the curves.

B) EGTA titration profile. Protein concentrations was 9.2 μM. —○—, no KCl; —●—, 100 mM KCl. Fluorescence intensity is expressed as % of that at 0 μM EGTA in each curve.

These results also indicate that the states of the tyrosine residue of the CaBP in the presence and absence of Ca^{2+} are different. K^+ may have access only to the Ca^{2+}-free or partially Ca^{2+}-deprived molecules of the CaBP, causing qualitatively different structural changes depending on the extent of Ca^{2+} occupation of the binding sites. It is reported that in proteins such as myosin light chain, troponin-C and parvalbumin that monovalent cations (H^+, K^+ or Na^+) compete with Ca^{2+} in binding to these proteins and induce conformational changes of the molecules, as does the Ca^{2+} ion.[12]

It has been found in our laboratory that energy transfer from the tyrosine residue to bound Tb^{3+} is not feasible with the porcine CaBP when terbium fluorescence is measured with excitation at 275 nm (unpublished), although the possibility of energy transfer from any

aromatic amino acid residue, including tyrosine, to bound Tb^{3+} was previously demonstrated with a rat intestinal mucosal membrane preparation possessing Ca^{2+} binding activity.[13] A competition between the bindings of Ca^{2+} and Tb^{3+} has been shown in the porcine intestinal CaBP (unpublished data). The Ca^{2+} binding site of the porcine intestinal CaBP is therefore not considered to be located so close to the tyrosine residue of the protein molecule that tyrosine can directly participate in Ca^{2+} binding.

Based on the above considerations, all the experimental results described in the present communication suggest that Ca^{2+} binding to the porcine intestinal CaBP induces substantial changes in the microenvironment and conformation of this protein molecule, especially in the region including the single tyrosine residue.

References and Notes

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