Calcium-Binding Proteins in Bovine Milk: Calcium-Binding Properties and Amino Acid Composition

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Summary The Ca^{2+} -binding properties and amino acid compositions of two calcium-binding proteins (mCaBP-3 and mCaBP-4) purified from bovine milk were studied. mCaBP-3 was identified as a Ca^{2+} -bound type and mCaBP-4 as a Ca^{2+} -free type by means of ion-exchange chromatography on a DEAE-Sephadex A-25 column. In polyacrylamide gel disc electrophoresis, both mCaBP-3 and mCaBP-4 had the same mobility of $R_f=0.73$ and the addition of 5 mM CaCl_2 to the electrode buffer decreased the mobility from $R_f=0.73$ to $R_f=0.49$. mCaBP-3 and mCaBP-4 consisted of 120 and 122 amino acid residues, respectively. The molecular weights were 13,758 and 13,967, respectively. The amino acid compositions of the two milk CaBPs very closely resembled each other. Both milk CaBPs were rich in aspartic acid, glutamic acid, leucine and lysine, but did not contain trimethylated lysine and amino sugar. An interesting feature is that each milk CaBP contained eight cysteine sulfone and three tryptophan residues per molecule. From these results, it is suggested that mCaBP-3 and mCaBP-4 are identical protein and that mCaBP-3 is formed from mCaBP-4 by means of a conformational change by binding of Ca^{2+} . Thus, mCaBP-3 is a holoprotein and mCaBP-4 is an apoprotein. Furthermore, it is suggested that milk CaBP is different from calmodulin, troponin C and vitamin D-dependent calcium-binding protein.

Key Words calcium-binding protein, bovine milk, Ca^{2+} -binding properties, amino acid composition

Calcium is involved in the regulation of a variety of cellular enzyme systems and in most types of cell motility (1, 2). It is possible that Ca^{2+} might not act in its free ionic form but rather requires the presence of a binding protein. Calcium-binding proteins such as calmodulin and vitamin D-dependent calcium-binding protein have been commonly identified in many tissues of many avian and mammalian species (3, 4).

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Previously, we demonstrated that two different calcium-binding proteins (mCaBP-3 and mCaBP-4) which are clearly different from casein can be purified from bovine milk (5). The physicochemical properties of the two CaBPs resembled each other very closely. Both milk CaBPs had a molecular weight of approximately 15,000 and two kinds of binding sites for Ca^{2+} ($K_d = 1.7 \times 10^{-6} \text{ M}$ and $K_d = 5.1 \times 10^{-4} \text{ M}$), and were heat-stable. Furthermore, these milk CaBPs modulated galactosyltransferase activity in a Ca^{2+} -dependent manner, as well as calmodulin (6).

On the other hand, calcium-binding proteins such as calmodulin (7,8), tropomyosin C (8) and vitamin D-dependent calcium-binding protein (9) elicited a large conformational change by the binding of Ca^{2+} to the affinity sites. Although the physicochemical properties of mCaBP-3 and mCaBP-4 very closely resembled each other, the fact that they were separated by ion-exchange column chromatography on DEAE-Sephadex A-25 seems to suggest that either mCaBP-3 or mCaBP-4 causes a Ca^{2+} -dependent conformational change.

The current research was designed to further characterize the milk CaBPs in the absence and presence of Ca^{2+} and to analyze the amino acid composition.

MATERIALS AND METHODS

1. *Isolation and purification of bovine milk CaBPs.* Two milk CaBPs were purified from unpasteurized bovine skim milk as previously described (5). The skim milk (about 20 ml) was fractionated using a Sephadex G-100 column ($2.6 \times 64 \text{ cm}$) equilibrated with 10 mM imidazole buffer (pH 6.9 at 5°C) containing 55 mM KCl and 25 mM NaCl (IKN buffer). The main peak with ^{45}Ca -binding activity, using the Chelex-100 procedure, was collected and further purified by ion-exchange chromatography on a DEAE-Sephadex A-25 column ($1.5 \times 36 \text{ cm}$) with a linear gradient of 25 to 400 mM NaCl in IKN buffer. The two major peaks eluted at the concentrations of 0.17 M and 0.21 M NaCl were respectively collected, and then subjected to the final step of separate gel filtration on Sephadex G-75 column ($1.8 \times 90 \text{ cm}$) chromatography. The milk CaBPs (mCaBP-3 and mCaBP-4) obtained appeared as a single homogeneous band on polyacrylamide gel disc electrophoresis.

2. *Identification of mCaBP-3 and mCaBP-4 by ion-exchange chromatography.* The ion-exchange chromatography was carried out on a DEAE-Sephadex A-25 column ($1.5 \times 36 \text{ cm}$) to identify mCaBP-3 and mCaBP-4. The column was eluted with a linear gradient of 25 to 400 mM NaCl in the IKN buffer with either 1 mM CaCl_2 or 1 mM ethyleneglycol-bis(β -aminoethyl ether)*N,N'*-tetraacetic acid (EGTA). The flow rate was 10 ml per hr. The elution profile was monitored by absorbance at 280 nm.

3. *Amino acid analysis.* The amino acid analysis was performed by the method of Inglis and Liu (10) with a slight modification. The purified CaBPs (exactly 1 mg protein each) were dialyzed exhaustively against distilled water, lyophilized and then hydrolyzed in 1 ml of 4N methane sulfonic acid in evacuated tubes at 115°C for

24, 48 and 72 hr. After the samples were neutralized with the same volume of 4 N NaOH, the mixtures were reduced by 4 μ moles dithiothreitol, and then sulfonated by 200 μ moles sodium tetrathionate for analysis of cystine and cysteine. After sulfonation, each hydrolysate was lyophilized again and dissolved in 0.2 M sodium citrate buffer (pH 2.2). The analysis of amino acid was carried out on a JEOL AH-6 amino acid analyzer (Japan Electric Co., Japan).

4. *Other methods.* Analytical disc electrophoresis was carried out essentially by the method of Davis (11) using 7.5% polyacrylamide gel. The Ca^{2+} -binding activity was measured by the competitive binding assay of Wasserman *et al.* (12) using 50% Chelex-100 chelating resin suspension. Protein concentration was determined by the method of Lowry *et al.* (13) with crystalline bovine serum albumin as a standard.

5. *Chemicals.* $^{45}\text{CaCl}_2$ (21.4 mCi/mgCa) and Omnifluor were purchased from New England Nuclear (Boston, MA). Sephadex G-100 and G-75, and DEAE-Sephadex A-25 were obtained from Pharmacia Fine Chem. (Uppsala, Sweden). Chelex-100 chelating resin was purchased from Bio-Rad Lab. (Richmond, CA). Other chemicals were of analytical grade.

RESULTS

1. *Interrelation of mCaBP-3 and mCaBP-4 on ion-exchange column chromatography*

Calcium-binding proteins such as calmodulin (7,8), troponin C (8) and vitamin D-dependent calcium-binding protein (9) are known to undergo a conformational change by binding of Ca^{2+} . Thus, a certain calcium-binding protein can be observed as two different proteins depending on the binding of Ca^{2+} .

When the purified mCaBP-3 was again chromatographed using on a DEAE-Sephadex A-25 ion-exchange column equilibrated with a regular IKN buffer, two protein peaks were detected at positions corresponding to mCaBP-3 and mCaBP-4, respectively (Fig. 1). However, when the IKN buffer containing 1 mM CaCl_2 was used instead of the regular IKN buffer, the re-chromatogram of mCaBP-3 was nearly the same as original chromatogram of mCaBP-3 (Fig. 2). Further, in the calcium-free buffer system containing 1 mM EGTA, the purified mCaBP-3 was eluted at the position corresponding to mCaBP-4 and was not detected at the position of mCaBP-3 (Fig. 2). On the other hand, when purified mCaBP-4 was re-chromatographed using the same ion-exchange column, the protein peak was detected at the position of mCaBP-3 in the IKN buffer system containing 1 mM CaCl_2 and at the position of mCaBP-4 in the Ca^{2+} -free buffer system containing 1 mM EGTA, respectively (data are not shown). These results strongly suggest that mCaBP-3 is a charge-isomer of mCaBP-4 which is induced by binding of Ca^{2+} . Thus, they indicate that mCaBP-3 and mCaBP-4 are a holoprotein and an apoprotein, respectively.

Fig. 1. Re-chromatography of mCaBP-3 purified from bovine milk on a DEAE-Sephadex A-25 column. mCaBP-3 purified from bovine skim milk (about 38 mg) was applied again to a DEAE-Sephadex A-25 column (1.5×36 cm) equilibrated with IKN buffer (pH 6.9 at 5°C) and eluted with a linear gradient of NaCl (25–400 mM) in the same buffer. The flow rate was 10 ml per hr. Two ml of fractions were collected and monitored for protein concentration by absorbance at 280 nm. The arrows represent the positions of mCaBP-3 (open arrow) and mCaBP-4 (solid arrow) eluted with ion-exchange chromatography on the DEAE-Sephadex A-25 column, which was carried out under the same conditions as above, in the step of purification of milk CaBPs. mCaBP-3 and mCaBP-4 were eluted at the concentration of 0.17 M and 0.21 M NaCl, respectively, on a linear gradient of NaCl. See the METHOD for details.

2. Effect of Ca^{2+} on mobility of milk CaBP in polyacrylamide gel disc electrophoresis

One of the characteristics of calmodulin is that the migration rate in polyacrylamide gel disc electrophoresis changes dramatically by the binding of Ca^{2+} (14). When the purified mCaBP-3 and mCaBP-4 were analyzed by 7.5% polyacrylamide gel disc electrophoresis without the additional Ca^{2+} to the samples and the electrode buffer, the relative mobilities of the two mCaBPs were the same, the values being 0.73 (Fig. 3). In disc electrophoresis using the buffer containing 5 mM CaCl_2 , the relative mobilities of the two CaBPs decreased from $R_f = 0.73$ to $R_f = 0.49$. The difference of electrophoretic mobility between mCaBP-3 and mCaBP-4 was neither observed in the absence nor the presence of Ca^{2+} . These results indicate that the electric charge of milk CaBPs is greatly affected by the binding of Ca^{2+} .

Fig. 2. Re-chromatography of mCaBP-3 purified from bovine milk on a DEAE-Sephadex A-25 column in the presence and absence of Ca^{2+} . mCaBP-3 (about 15 mg each) purified from bovine milk was applied to DEAE-Sephadex A-25 columns (1.5×36 cm) equilibrated with IKN buffer containing either 1 mM CaCl_2 or 1 mM EGTA. Each column was eluted with a linear gradient of NaCl (25–400 mM) in IKN buffer containing either 1 mM CaCl_2 (○—○) or 1 mM EGTA (●---●). The flow rate was 10 ml per hr. Two ml of fractions were collected.

3. Amino acid compositions of mCaBP-3 and mCaBP-4

The above results suggest strongly that mCaBP-3 and mCaBP-4 are the same protein and therefore, the respective amino acid compositions were analyzed. The results are shown in Table 1. mCaBP-3 consisted of 120 amino acid residues and the calculated molecular weight was 13,758. mCaBP-4 consisted of 122 amino acid residues and the calculated molecular weight was 13,967. Both milk CaBPs were rich in aspartic acid, glutamic acid, leucine and lysine. Trimethylated lysine and amino sugar could not be detected. The amino acid compositions of mCaBP-3 and mCaBP-4 very closely resembled each other. An interesting feature was that each milk CaBP contained eight cysteine sulfone and three tryptophan residues per molecule.

DISCUSSION

In order to clarify the situation with regard to bovine milk CaBP, Ca^{2+} -binding properties and amino acid compositions of mCaBP-3 and mCaBP-4 were further investigated. When purified mCaBP-3 or mCaBP-4 was fractionated again by ion-exchange chromatography using a DEAE-Sephadex A-25 column equi-

Fig. 3. Change of electrophoretic mobilities of mCaBP-3 and mCaBP-4 by binding of Ca^{2+} . Disc electrophoresis was carried out essentially by the method of Davis (11) using 7.5% polyacrylamide gel. Group A: no addition of CaCl_2 to either sample and electrode buffer. Group B: addition of 5 mM CaCl_2 to both samples and electrode buffer. No. 1 and 3, mCaBP-3 (20 μg protein); No. 2 and 4, mCaBP-4 (20 μg protein).

brated with the regular IKN buffer, the protein was detected at two positions corresponding to mCaBP-3 and mCaBP-4. However, when Ca^{2+} -free buffer (regular IKN buffer + EGTA) or Ca^{2+} -containing buffer (regular IKN buffer + CaCl_2) was used for the column chromatography, only one peak which corresponded to mCaBP-4 or mCaBP-3, respectively, was eluted. These facts indicate that a portion of the Ca^{2+} bounds to mCaBP-3 is removed by the ion-exchange resin and that mCaBP-4 binds the Ca^{2+} contaminating in the regular IKN buffer at trace level ($<10^{-6}\text{ M}$) during the ion-exchange column chromatography. Thus, these results suggest that mCaBP-3 is identical to mCaBP-4. The facts that the amino acid compositions of the two milk CaBPs closely resembled each other and that they crossreacted immunologically (5), strongly suggest that mCaBP-3 and mCaBP-4 are charge-isomers induced by the binding of Ca^{2+} . Further, it is implied that the binding of Ca^{2+} to the binding sites in the milk CaBP elicits a large conformational change. This conformational change by binding of Ca^{2+} is particularly characteristic in calcium-binding proteins such as calmodulin (7, 8), troponin C (8) and vitamin D-dependent calcium-binding protein (9).

The electrophoretic mobilities of calmodulin (14) and vitamin D-dependent

Table 1. Amino acid compositions of mCaBP-3 and mCaBP-4. mCaBP-3 and mCaBP-4 purified from bovine skim milk were hydrolyzed in 4N methane sulfonic acid at 115°C for 24, 48, and 78 hr. Values are obtained by extrapolation to zero hydrolysis time and are expressed as minimum residues per molecule. See text for details.

Amino acid	Integer residues/molecule	
	mCaBP-3	mCaBP-4
Aspartic acid	20	20
Threonine	7	7
Serine	6	6
Glutamic acid	13	13
Proline	2	2
Glycine	7	7
Alanine	3	4
Valine	6	6
Cysteine sulfone	8	8
Methionine	1	1
Isoleucine	7	7
Leucine	13	13
Tyrosine	4	4
Phenylalanine	4	4
Tryptophan	3	3
Lysine	12	12
Histidine	3	4
Arginine	1	1
Total residues	120	122
Molecular weight	13,758	13,967

calcium-binding protein (15) are delayed by the binding of Ca^{2+} . This change in mobilities, also, depends on a conformational change of the protein. In polyacrylamide gel disc electrophoresis, mCaBP-3 and mCaBP-4 had the same mobility of $R_f = 0.73$, in spite of the Ca^{2+} bound to mCaBP-3 not previously being removed from the protein. The Ca^{2+} bound to mCaBP-3 must be removed by the potential difference, and mCaBP-3 transferred to the Ca^{2+} -free form, mCaBP-4. On the other hand, the mobilities of both mCaBP-3 and mCaBP-4 were delayed from $R_f = 0.73$ to $R_f = 0.49$ by the addition of excess calcium to the electrode buffer. This change of mobility suggests the involvement of a large change of the electric charge dependent upon a conformational change of milk CaBP by binding of Ca^{2+} .

Calmodulin characteristically contains trimethylated lysine, but not cysteine and tryptophan (3). Vitamin D-dependent calcium-binding protein also did not contain cysteine and tryptophan (4). Calmodulin contains aspartic acid and glutamic acid at the level of approximately 34% of total amino acid residues present (3). Milk CaBP was rich in acidic amino acids such as aspartic acid and glutamic acid, and

further contains eight cystein sulfones and three tryptophans, but not trimethylated lysine. Therefore, milk CaBP is apparently different from calmodulin, troponin C and vitamin D-dependent calcium-binding protein, though the several physicochemical properties of milk CaBP are similar to those of other CaBPs.

Calmodulin is known to function in the intracellular system (1). One of its functions is to activate phosphodiesterase *via* the enzyme-calmodulin complex which is induced by the binding of Ca^{2+} (16). Milk CaBP can also stimulate the enzyme activity such as galactosyltransferase in the presence of Ca^{2+} , though it is abundant in secretions such as milk. This enzyme activation may involve the same mechanism as that of phosphodiesterase by calmodulin.

The amino acid composition of purified milk CaBP was closely similar to that of α -lactalbumin reported elsewhere (17). Further, rabbit anti-mCaBP-3 antiserum crossreacted with α -lactalbumin in micro-Ouchterlony double immunodiffusion (18). Therefore, milk CaBP may be identical to α -lactalbumin involved in lactose synthesis in mammary gland.

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