Interaction of Calcium Ion with Bovine Caseins

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In order to clarify the interaction of calcium ion with casein, the volume change associated with the interaction was measured by dilatometric procedures. When CaCl_2 was added to the casein solutions at neutral pH, a volume increase occurred and reached a constant saturated value of about 700 ml per 10^5 g protein with increasing CaCl_2 concentrations for whole-, α_s - and β -casein solutions, but there was no volume change for κ -casein solution. On the other hand, the binding of calcium ion to the casein fractions was determined by a gel filtration procedure at pH 6.0 to 9.0. The number of Ca^{2+} ions bound to the caseins increased with the CaCl_2 concentration and pH value, and the relative order of binding capacities for the caseins was: α_s -casein > whole-casein > β -casein > κ -casein.

It was found that the volume changes obtained by the dilatometry were smaller than the calculated volume increases based on the assumption that these are caused by the binding of Ca^{2+} ion to the caseins. Therefore it is necessary to introduce another factor which reduces the volume increase due to the Ca^{2+} ion binding in order to reasonably explain the measured volume changes. At present it is presumed that there occurs the unfolding of peptide chain of casein molecule on Ca^{2+} ion binding, which has been known to decrease the volume of the protein solution.

The interactions of caseins with mono- and di-valent cations have been extensively studied for several decades. Especially the calcium binding to casein molecules has been elucidated by many investigators as it is closely connected with the formation of casein micelles. Studies on the formation and structure of casein micelles have been comprehensively reviewed by Waugh.¹⁾ However, the relation between the ion binding to caseins and the micelle formation of caseins does not yet seem to have been completely solved.

One of the authors pursued the globular protein to fibrous protein transition (G-F transition) of actin and flagellin molecules by dilatometry and analyzed the polymerization phenomenon from the viewpoint of the formation of intermolecular hydrophobic bonding between them.^{2,3)} This procedure is very valid for study of ion-protein or protein-protein interactions as a volume change as-

sociated with the interactions always takes place and it concerns the characteristic property of the interaction.

A simple and precise gel filtration method was introduced by Hummel and Dreyer⁴⁾ to determine the amount of ion bound to protein molecule, and has been extensively applied for the study of the binding of small molecules to protein.⁵⁾ We also applied this method to determine the amount of Ca^{2+} ion bound to soybean 11S protein and a satisfactory result was obtained;⁶⁾ then the procedure was again used to detect the degree of Ca^{2+} ion binding to the casein molecules.

MATERIALS AND METHODS

Preparation of materials. Whole-casein was prepared from unpasteurized skim milk by acidification as described by Hipp *et al.*,⁷⁾ α_s -casein and κ -casein were prepared by the procedure described by Tsugo and Yamauchi,⁸⁾ which is essentially the same as the procedure of Long *et al.*⁹⁾ except for the additional treatment of α_s -casein at a higher alkalinity prior to the addition of calcium chloride, and β -casein was

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prepared by the method of Hipp *et al.*¹⁰⁾

The purified water passed through a mixed-bed ion-exchange resin was distilled with an all pyrex glass distillatory apparatus and then used throughout the preparation of caseins and the measurements. As we intended to clarify the interactions of caseins with calcium ion, it was necessary to check the content of the ion, which was expected to exist in the casein molecules, in advance of the measurements. Calcium determination was made by a flame analysis using an atomic absorption spectrophotometer (Hitachi Model 208). The result indicated that the contents of calcium existing in the casein molecules were negligibly little (see Table I).

TABLE I. Ca^{2+} ION EXISTING IN PREPARED CASEIN MOLECULES

Casein	Ca^{2+} mole/ 10^6 g casein
Whole-casein	0.56
α_s -Casein	0.70
β -Casein	0.65
κ -Casein	1.57

Dilatometry. The volume change that accompanied the interaction of calcium ion and casein molecule was measured with a Carlsberg type dilatometer described in the previous paper.¹¹⁾ All measurements were carried out at 25.00°C in a thermostated water bath with the allowance of $\pm 0.0002^\circ\text{C}$. One arm of the V tube was filled with 5 ml of 0.5% casein solution at pH 6.9 and 0.07 M KCl, and the other with 5 ml of 0.07 M KCl solution, containing a varying amount of CaCl_2 , at pH 6.9. Details of the measurements were described in the previous paper.^{6,11)}

By dilatometry, the volume change upon mixing of casein and CaCl_2 solutions can be directly measured even if there appears precipitation or aggregation in the mixing solution. On the other hand, if we measure the densities of casein, CaCl_2 and the mixing solutions separately, the volume change on mixing casein and CaCl_2 solutions can be easily calculated. Now we can use a very precise densitometer (Anton Parr, DMA-02C) to measure the density of solution with an accuracy of $\pm 2 \times 10^{-6}$ g/ml. Therefore, the volume change associated with the interaction of casein and calcium ion was also determined with the densitometer, but we cannot apply the apparatus to measure a solution density if there is precipitation or aggregation.

Gel filtration. Gel filtration was carried out on a Sephadex G-25 coarse column (0.9 $\times$ 60 cm bed) equilibrated with a buffer containing a desired CaCl_2 concentration. When the protein binds calcium ion, the solvent of this sample will be depleted with respect to calcium ion. The resulting elution profile exhibits a peak in calcium ion concentration above the calcium

ion-saturated base-line. This peak represents the excess calcium ion bound, and a corresponding trough, which represents the depletion that resulted when the bound calcium ion was carried ahead with the casein, will follow. In principle, the areas of trough and peak are equal. From the area and the known concentration of casein applied, the binding ratio is determined corresponding to the calcium ion saturated level used. Casein concentration was determined by a dry method, and calcium concentration was measured by flame spectrophotometry. The concentration of 1 mM Ca^{2+} ion was determined with an error of about 1% that is, the error was about ± 1 mole $\text{Ca}^{2+}/10^6$ g casein for the elute protein solution of 1 mg/ml.

RESULTS AND DISCUSSION

Figure 1 shows the volume changes, ΔV , observed during the addition of CaCl_2 to whole-casein solution at pH 6.9 and 0.07 M KCl, and

FIG. 1. Volume Changes of Whole-casein and κ -Casein during Titration with CaCl_2 at pH 6.9 and 25°C.

○, obtained by dilatometer; ●, obtained by densitometer; s, saturation point.

the calculated volume changes of the same system obtained with the densitometer. The value of volume changes is recognized to be completely reliable as these two sets of measured values coincide very well with each other. The volume increment accompanying the interaction of CaCl_2 with casein molecules is saturated at 0.02 M CaCl_2 . Similar volume changes were observed for α_s - and β -casein plus CaCl_2 systems. In the case of these systems, there appeared white coagulations upon addition of CaCl_2 , and then it was im-

possible to measure the ΔV with the Anton Parr densitometer. When CaCl_2 was added to κ -casein solution there was no volume change as indicated in Fig. 1; it therefore seems that there is no interaction between CaCl_2 and κ -casein.

As described in another paper,⁶⁾ it has been recognized that the volume change upon mixing of protein solution with another containing some chemical is caused by the following factors, (i) neutralization of ionized groups on protein molecule with counterions, which accompanies the volume increase, (ii) polymerization or aggregation of protein molecules that causes a positive volume change of a few hundred milliliters per mole of protein,^{2,3,6)} and (iii) protein denaturation associated with a volume decrease of several hundred milliliters per 10^5 g of protein.^{12,13)}

Volume changes due to the interaction of CaCl_2 with casein molecules, except for κ -casein, reach a finite saturated value at a given concentration of CaCl_2 . If it is assumed that the volume changes are only due to the charge neutralization of Ca^{2+} ion bound to the protein, we can calculate the maximum amount of CaCl_2 interacting with casein molecules from these saturated CaCl_2 concentrations, and at the same time the volume increase per mole of casein molecule can be determined. In Table II the results are given.

The table indicates that (i) the maximum moles of bound Ca^{2+} ion per mole of casein molecule are much larger than the values reported by several authors¹⁴⁻¹⁶⁾ or the values obtained by our gel filtration, (ii) the volume increments, about 2 ml/mole of bound Ca^{2+} , due to the neutralization reaction are much

smaller than the values obtained for the reaction of Ca^{2+} ion with carboxylate or phosphate ions (see later discussion), and (iii) the result that the volume increase for α_s - and β -casein are almost the same cannot be explained only by the charge neutralization of Ca^{2+} ion with the negative charges on the protein, as it was reported that there was a big difference in moles of negative charge per mole of α_s - and β -casein molecules.¹⁶⁾

From the above discussion, it is clear that these volume increases are not caused only by the Ca^{2+} ion binding to the negative charges on the casein molecules. Though the Ca^{2+} ion binding to casein molecules has been studied extensively, there is a considerable discrepancy between the reported number of Ca^{2+} ions and the casein molecules. Then we tried to precisely determine the amount of bound Ca^{2+} ion to the molecules by the gel filtration method. Figure 2 shows the result of binding measurement on a 0.5 ml solution containing 10 mg whole-casein, 1 mM

FIG. 2. Elution Profile of Ca^{2+} Ion Compared with That of Whole-casein at pH 7.0.

Gel filtration was carried out at a flow rate of 15 ml per hour.

○, Ca^{2+} ion concentration; ●, casein concentration.

TABLE II. MAXIMUM MOLES OF CaCl_2 REACTED WITH CASEIN IN pH 6.9 AND 0.07 M KCl SOLUTION, AND VOLUME INCREMENT OBTAINED UPON MIXTURE OF CaCl_2 AND CASEIN AT 25°C

Casein	Reacted CaCl_2 moles/ 10^5 g of protein	Max ΔV ml/ 10^5 g of protein	ΔV ml/ Ca^{2+} mole
Whole-casein	800	680	2.3
α_s -Casein	1200	680	1.7
β -Casein	1200	700	1.8
κ -Casein	0	0	0

CaCl_2 , 0.01 M Tris-HCl and 0.07 M KCl at pH 7.0 by gel filtration. The elution profile exhibits a peak and trough representing the existence of Ca^{2+} ions bound to protein. From the area of the trough and the mole of the casein applied, the number of Ca^{2+} ions bound to protein was estimated to be 8 ± 1 moles of $\text{Ca}^{2+}/10^5$ g of whole-casein. The same elution measurements were done for all casein solutions containing various concentrations of CaCl_2 at pH 6.0 to 9.0. Summaries of the results are shown in Figs. 3, 4, 5 and 6

FIG. 3. Ca^{2+} Ion Bound to Whole-casein in 0.01 M Tris-HCl and 0.07 M KCl.

●, pH 6.0; ○, pH 6.5; ▲, pH 7.0; △, pH 7.5; ■, pH 8.0; □, pH 9.0.

FIG. 4. Ca^{2+} Ion Bound to α_s -Casein in 0.01 M Tris-HCl and 0.07 M KCl.

Notation for pH is the same as in Fig. 3.

for whole-casein, α_s -casein, β -casein and κ -casein, respectively. On the other hand we

FIG. 5. Ca^{2+} Ion Bound to β -Casein in 0.01 M Tris-HCl and 0.07 M KCl.

Notation for pH is the same as in Fig. 3.

FIG. 6. Ca^{2+} Ion Bound to κ -Casein in 0.01 M Tris-HCl and 0.07 M KCl.

Notation for pH is the same as in Fig. 3.

determined the amount of bound Ca^{2+} ion to caseins by this method for casein solutions at pH 6.7 to 7.0, 0.07 M KCl, and a CaCl_2 concentration less than 3 mM. The results indicated the binding number of Ca^{2+} ion to casein molecules was almost the same as in Tris-HCl buffer solutions within the experimental error.

Binding of Ca^{2+} to caseins increases as the free Ca^{2+} ion concentration is raised and the caseins are precipitated or aggregated at a higher concentration than 5 mM except for κ -casein; so the gel filtration procedure was applied to 5 mM CaCl_2 in Tris-HCl buffer solutions. The relative order of binding capacities for Ca^{2+} ion to casein fractions is: α_s -

casein > whole-casein > β -casein > κ -casein, which is the same as that determined by Dickson and Perkins.¹⁵⁾ The moles of bound Ca^{2+} per 10^5 g casein at pH 7.4 (Tris-HCl, $I=0.1$) determined by them using equilibrium dialysis for α_{s1} -casein, β -casein and κ -casein fairly well coincided with ours. The effect of pH on the binding of Ca^{2+} to casein is also seen from the figures. The result that the binding of Ca^{2+} ion increased with the pH is also similar to their finding.

Now we must interpret the mechanism of the interaction between Ca^{2+} ion and casein molecule based on the results of both the gel filtration and the volume change. The volume changes associated with the binding of Ca^{2+} , Mg^{2+} and Ba^{2+} ions to synthetic polyelectrolytes have been elucidated by several authors.¹⁷⁻²⁰⁾ According to Strauss and Leung,¹⁷⁾ the binding of Mg^{2+} ion to carboxylate ion causes a volume increase of 26 ml per mole of Mg^{2+} ion. The volume increase due to Ca^{2+} ion binding to carboxylate and phosphate ions was estimated to be nearly the same. Therefore, the volume increase caused by the Ca^{2+} ion bound to protein at pH 6.9 and 0.07 M KCl can be readily calculated for each casein fraction, and the values and observed ones in 5 mM CaCl_2 solution at pH 6.9 and 0.07 M KCl are given in Table III. The amounts of Ca^{2+} ion bound to protein in the solution are also given.

From the table it is clear that the observed volume changes are much smaller than the calculated ones. Therefore, it is impossible to attribute the volume changes to only the charge neutralization reaction of bound Ca^{2+} ion with the negative charges on the protein molecules.

On the other hand, we measured the volume change when Mg^{2+} , Sr^{2+} and Ba^{2+} ions were added to whole-casein solution at pH 6.9 and 0.07 M KCl. The results are shown in Fig. 7.

FIG. 7. Volume Change of Whole-casein during Titration with MgCl_2 , SrCl_2 , CaCl_2 and BaCl_2 at pH 6.9, 0.07 M KCl and 25°C.

According to Dickson and Perkins there are differences in the amount of individual alkalin-earth-metal ions bound to α_{s1} -casein and β -casein fractions, and the relative order of binding capacities to either is $\text{Mg}^{2+} > \text{Ca}^{2+} > \text{Ba}^{2+} > \text{Sr}^{2+}$. As the volume increases owing to the binding of Mg^{2+} , Ca^{2+} and Ba^{2+} ions to negative charges on casein molecules are the same (we have no data on Sr^{2+}), the order of volume change for the individual cations must be the same as the above order, but the result is just the opposite. This is another definite evidence that the volume changes are not only due to the charge neutralization of the cations by the negative charges on casein molecules.

One of the authors measured the volume change associated with the G-F transition of flagellin and actin. In the flagellin solution the volume increment of 400 ml/ 10^5 g of

TABLE III. AMOUNTS OF Ca^{2+} ION BOUND TO CASEIN AND ΔV AT pH 6.9 AND 5 mM CaCl_2

Casein	Moles of $\text{Ca}^{2+}/10^5$ g casein	ΔV calculated by (mole of Ca^{2+} bound $\times 25$) ml/ 10^5 g of protein	ΔV obtained by dilatometry ml/ 10^5 g of protein
Whole-casein	16	400	300
α_s -Casein	25	625	250
β -Casein	14	350	130
κ -Casein	6	130	0

flagellin was observed at 25°C, and in the case of actin the volume increase of 690 ml/10⁵ g of actin was observed after correction for such factors as the volume increase due to the binding of ATP and Mg²⁺ ion. Recently, the volume increase of about 650 ml/10⁵ g of protein was obtained when CaCl₂ was added to soybean 11S protein solution at pH 6.8 where it is considered that Ca²⁺ ion does not bind to the protein.⁶⁾ From these results, the volume increase is recognized to take place when the aggregation or polymerization of protein molecules occurs.

In the case of caseins, except for κ -casein, since coagulation or precipitation occurs when alkaline-earth-metal cations are added to the protein solution, the excess volume increase in the order of several hundred milliliters must be added upon mixing of the protein with the cations. However, as the observed volume changes were much smaller than the expected values, it must be concluded that there exists another factor which reduces the volume increase. As explained before, it has been noticed that protein denaturation causes the volume decrease of several hundred milliliters per 10⁵ g of protein. This volume decrease is considered due to the transition of the secondary structure of the peptide chain to a coiled form,¹¹⁾ the rupture of intramolecular hydrophobic bonds and the disappearance of the void volume in the protein. It is therefore presumed that the unfolding of the polypeptide chain or the conformational change of casein molecules occurs when Ca²⁺ ions bind to them, resulting in a volume decrease of the protein solution. The hydrophobic groups on the peptide chain come out as a result of the unfolding of the protein molecule, and the unfolded protein molecules interact each other through the hydrophobic groups; that is, there occurs the intermolecular hydrophobic bonding and the coagulation or precipitation takes place. The presumption that the conformational change of casein molecules is induced by the bound Ca²⁺ ions is not absurd as it was reported that one subunit of troponin (Ca²⁺ binding

component) bound Ca²⁺ ion and a striking change in conformation of the component was introduced.²¹⁾

As a whole the neutralization reaction with negative charges on protein, the conformational change of the protein and the formation of intermolecular hydrophobic bonding take place when CaCl₂ is added to the casein solutions. These reactions are completed before a given CaCl₂ concentration is added, then the volume increase reaches a saturated constant value at this critical concentration of CaCl₂.

The purified casein components were reported to undergo endothermic associations. In the case of α_{s1} -casein,²²⁾ it was found that the average molecular weight rapidly increased with concentration and also with temperature at pH 6.4. On the other hand, β -casein exists in the monomeric state at 4°C, and it strongly polymerizes at a higher temperature.²³⁾ Therefore we have investigated the interaction of associated casein molecules and Ca²⁺ ions, but it is advisable to pursue the interaction of Ca²⁺ ion with monomeric casein molecule to clarify the mechanism of the micelle formation of casein molecules. Then, there still remain many difficulties to be overcome in order to reach a final conclusion on the mechanism of the micelle formation of casein molecules by the addition of CaCl₂.

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