Polymerization of α_{s1} - κ -Casein Complex by Calcium Ions

Shun'ichi DOSAKO, Toshiaki KIMURA, Shin'ichi TANEYA,
Toshimaro SONE, Shuichi KAMINOGAWA* and Kunio YAMAUCHI*

Technical Research Institute, Snow Brand Milk Products Co.,

1-2, Minamidai 1-chome, Kawagoe, Saitama 350, Japan

**Department of Agricultural Chemistry, The University of Tokyo,
Tokyo 113, Japan*

Received April 28, 1980

κ -Casein and α_{s1} - κ -casein complex with a weight ratio of unity were dissolved in 50 mM cacodylate-HCl-70 mM KCl buffer containing 0.02% of sodium azide (pH 7.1), and their size and shape in the absence and/or presence of calcium ions were observed with the electron microscope. In the absence of calcium ions, both κ -casein and α_{s1} - κ -casein complex were spherical particles. However, the mean length of α_{s1} - κ -casein complex (12 nm) was smaller than that of κ -casein (17 nm), which suggested that complex formation led to dissociation of the κ -casein polymer. The addition of calcium ions to the complex led to the formation of bent chains, though micelle-like aggregates were not observed even at 20 mM calcium. Comparison of the frequency distributions of α_{s1} - κ -casein complex at 0, 5, 10, 15 and 20 mM of calcium with the calculated probability distributions suggested that most α_{s1} - κ -casein complexes had two binding sites above 10 mM of calcium, which seemed to be essential for the stability of casein micelle.

Interaction between α_{s1} - and κ -caseins leads to complex formation with weight ratio of unity in the absence of calcium.¹⁾ This complex formation is the first and most important interaction leading to micelle formation.²⁾ Physicochemical studies on the complex formation in the absence of calcium have revealed that the interaction between α_{s1} - and κ -caseins leads to dissociation of polydispersed κ -casein polymer^{3~5)} and that a typical complex with a molecular weight of approximately 40×10^4 ^{4,6,7)} is formed from two α_{s1} -caseins and a quarter of κ -casein polymer on an average^{4,5)} through hydrophobic interaction.^{8,9)}

On the other hand, the interaction between α_{s1} - κ -casein complex and calcium ions is little known, though the sedimentation coefficient of α_{s1} - κ -casein complex increases with the increase in calcium concentration.¹⁰⁾

In this paper, polymerizations of α_{s1} - κ -casein complex by calcium ions were observed by electron microscope and the number frequency distributions at various calcium concentrations were compared to the probability

distributions calculated under simple assumptions.

MATERIALS AND METHODS

Preparation of α_{s1} - κ -casein complex. α_{s1} -Casein and κ -casein were prepared by the method of Zittle *et al.*^{11,12)} α_{s1} - κ -Casein complex was prepared according to the previous paper.⁴⁾ Buffer used in this experiments was 50 mM cacodylate-HCl-70 mM KCl (pH 7.1) buffer containing 0.02% of sodium azide (Standard buffer). A main fraction of α_{s1} - κ -casein complex was collected by gel filtration with Sepharose 4B (Pharmacia) as described before.⁴⁾ Protein concentration was 1.2 mg/ml. Calcium concentrations were adjusted by mixing the calcium-free standard buffer and the one containing 40 mM of calcium. The sedimentation coefficient of α_{s1} - κ -casein complex in the standard buffer was the same as the previous paper.⁴⁾

Electron microscopic observations. Complexes at various calcium concentrations were negatively stained with saturated uranyl acetate according to the preceding paper.¹³⁾ Electron micrographs were taken with a Hitachi HU-12A electron microscope at a magnification of 70,000 times.

Size frequency distribution. Particle size was measured by Shimadzu Size Frequency Analyzer SF-20. Calculations of size frequency and mean length were

performed with the same methods as described in the preceding paper.¹³⁾

RESULTS

Figure 1 shows the electron micrograph of κ -casein in the standard buffer. Although some of the particles were polymerized irregularly, most particles were spherical and similar to the results obtained by Schmidt and Buchheim.¹⁴⁾

The typical electron micrographs of α_{s1} - κ -casein complexes at calcium 0(a), 10(b) and 20(c) mM are given in Fig. 2. Most complexes in the absence of calcium were spherical. They were polymerized into bent chains as the concentration of calcium increased. Micelle-like aggregates were hardly observed even at 20 mM of calcium.

Size frequency distributions of κ -casein are shown in Fig. 3 and those of α_{s1} - κ -casein complexes at 0, 5, 10, 15 and 20 mM of calcium are in Fig. 4. Large polymers of α_{s1} - κ -casein

FIG. 1. Electron Micrograph of κ -Casein.

FIG. 2. Electron Micrographs of α_{s1} - κ -Casein Complex.
(a) Ca 0 mM; (b) Ca 10 mM; (c) Ca 20 mM.

FIG. 3. Size Frequency Distribution of κ -Casein. FIG. 4. Size Frequency Distributions of α_{s1} - κ -Casein Complex.

(a) Ca 0 mM; (b) Ca 5 mM (solid) and 10 mM (broken); (c) Ca 15 mM (solid) and 20 mM (broken).

complex with the length greater than 80 nm appeared above 10 mM of calcium. Since Kaminogawa *et al.*¹⁰ reported that a new peak

FIG. 5. Effect of Calcium Concentration on the Mean Length of α_{s1} - κ -Casein Complex.

Black circle represents the mean length of κ -casein.

with a high sedimentation coefficient appeared above 11 mM of calcium, it seemed that these polymers corresponded to the fast peak in the ultracentrifugal pattern.

Figure 5 shows the effect of calcium concentration on the mean length of α_{s1} - κ -casein complex. The mean length of κ -casein was 17.25 nm which was in good agreement with Parry and Carroll,¹⁵ however, a little smaller than the results obtained by gel filtration (22 nm,⁴ 24 nm³). The mean length of α_{s1} - κ -casein complex in the absence of calcium was approximately 12 nm which was also smaller than the previous result (19 nm⁴). The higher values previously obtained for the length of both κ -casein and α_{s1} - κ -casein complex may supports Buchheim and Welsch's hypothesis that a water-rich layer surrounds the particles.¹⁶ Smaller mean length of α_{s1} - κ -casein complex than that of κ -casein indicates that the complex formation leads to dissociation of polymerized κ -casein as suggested previously.^{3-5,17} The mean length of α_{s1} - κ -casein complex increased with the increase in calcium concentration, which agreed with the result of ultracentrifugal analysis.¹⁰

DISCUSSION

Most α_{s1} -casein (Unit denoted in the preceding paper¹³) have one binding site in the absence of calcium,¹³ whereas they have three

binding sites above 8 mM of calcium.^{18,19)} If we assume that α_{s1} -casein forms a complex with κ -casein through one of these binding sites and that κ -casein has no binding site on its surface, a typical complex in the absence of calcium may have no binding site. Since a typical α_{s1} - κ -casein complex has two α_{s1} -caseins,^{4,5)} on the other hand, it should have at least four binding sites and form micelles at the calcium concentration above 8 mM. Moreover, polymerization into bent chains above 10 mM of calcium (Fig. 2) suggests that a complex has at most two binding sites even at 20 mM of calcium.

Then, the probability that i -mers of complex are formed can be calculated on the basis of three assumptions which are similar to the preceding paper.¹³⁾

ASSUMPTIONS:

1) There are three kinds of complexes, which have no binding site (C0), one binding site (C1) and two binding sites (C2). The ratios of C0, C1 and C2 are Q_0 , Q_1 and Q_2 , respectively.

$$(Q_0 + Q_1 + Q_2 = 1)$$

2) The probability that C1 and C2 associate with other complexes is p , though C0 can not associate with any other complexes.

3) To make simple, sampling without replacement is assumed.

Possible cases to form i -mers of complexes are schematized in Fig. 6. If the number of total particles is N , the calculations for the probability to form i -mers are similar to the preceding paper.¹³⁾ The probability to form monomer ($P(1)$) is given by

$$P(1) = 1 - p(Q_1 + Q_2)(NQ_1 + NQ_2 - 1)/(N - 1). \quad (1)$$

If i is $2 \leq i \leq NQ_2 + 2$, the probability to form i -mer ($P(i)$) is calculated as

$$P(i) = \frac{(NQ_2)!(N - i - 1)!}{N!(NQ_2 - i + 2)!} p^{i-1} (NQ_1 + NQ_2 - i + 1) \times [NQ_1(N - 1) + (NQ_2 - i + 2)] \times \{N - i - p(NQ_1 + NQ_2 - i)\}. \quad (2)$$

FIG. 6. Possible Cases to Form i -Mers of α_{s1} - κ -Casein Complex.

Complexes having no binding site (C0), one binding site (C1) and two binding sites (C2) are represented by $\circ$, $\odot$ and $\bullet$, respectively. Probability that C1 or C2 associates with complexes is p .

Here, Q_1 , Q_2 and p are the parameters. The computed probability distributions were compared to the number frequency distributions obtained from the electron micrographs at 0, 5, 10, 15 and 20 mM of calcium. The best fit histograms at $N=1000$ are shown in Fig. 7. Values of parameters when the best fit distributions were obtained were $p=0.6$, $Q_1=0.25$, $Q_2=0.1$ at 0 mM of calcium, $p=0.6$, $Q_1=0.25$, $Q_2=0.22$ at 5 mM of calcium, $p=0.6$, $Q_1=0.08$, $Q_2=0.54$ at 10 mM of calcium, $p=0.6$, $Q_1=0.05$, $Q_2=0.60$ at 15 mM of calcium, and $p=0.6$, $Q_1=0.04$, $Q_2=0.70$ at 20 mM of calcium. In each calcium concentration, the computed distributions coincide with the experimental ones.

Figure 8 shows the effect of calcium concentration on Q_0 , Q_1 and Q_2 . Although p is independent on calcium concentration, Q_0 and Q_1 decrease with the increase in calcium concentration and Q_2 increases markedly in the vicinity of 10 mM calcium. These results indicate that a typical complex has no binding site in the absence of calcium, but it has two binding sites at the calcium concentration above 10 mM. Since a typical complex has two α_{s1} -caseins (Unit),^{4,5)} it seems that these two binding sites in the complex are newly pro-

FIG. 7. Comparisons between the Best Fit Probability and Number Frequency Distributions of α_{s1} - κ -Casein Complex.

Solid and broken lines represent the calculated and experimental distributions obtained from electron micrographs, respectively. Values of Q_1 , Q_2 and p were as follows: $Q_1=0.25$, $Q_2=0.1$, $p=0.6$ at Ca 0mM; $Q_1=0.25$, $Q_2=0.22$, $p=0.6$ at Ca 5mM; $Q_1=0.08$, $Q_2=0.54$, $p=0.6$ at Ca 10mM; $Q_1=0.05$, $Q_2=0.6$, $p=0.6$ at Ca 15mM; $Q_1=0.04$, $Q_2=0.7$, $p=0.6$ at Ca 20mM.

FIG. 8. Effect of Calcium Concentration on Q_0 , Q_1 and Q_2 .

Q_0 , Q_1 and Q_2 are the ratios of C0, C1 and C2, respectively. Q_0 , (○); Q_1 , (◐); Q_2 , (●).

duced binding sites in each α_{s1} -casein contained in the complex.

In the preceding paper,¹³⁾ it has been suggested that 46% of α_{s1} -casein (Unit) have two binding sites at 3 mM of calcium. According to Parker and Dalglish,^{18,19)} α_{s1} -casein has three binding sites at 8 mM of calcium. Moreover, we have already obtained a suggestion that the third binding site is produced in the vicinity of 10 mM of calcium.²⁰⁾ Therefore, the newly produced binding sites in complex may correspond to the third binding site in α_{s1} -casein. The production of new binding sites in complex may be due to the calcium-induced conformational changes, since the partial specific volume of the complex changes at the calcium concentration above 10 mM.¹⁰⁾

Although the second binding site is produced in α_{s1} -casein (Unit) at 3 mM of calcium,¹³⁾ Q_2 of the complex at 5 mM of calcium is only 0.22 (Fig. 8). This may be due to that the second binding site in α_{s1} -casein (Unit) is not formed in the α_{s1} -casein contained in the complex, or that the regions of the first and second binding sites in α_{s1} -casein (Unit) are near so that the region of the second binding site is hidden by the complex formation with κ -casein. However, details are not clear at the present time. Kaminogawa *et al.*²¹⁾ have reported that α_{s1} -I casein which is lacking for 23 amino acids in the *N*-terminal of α_{s1} -casein is stable against calcium, though the sedimentation coefficient increases at the calcium concentration above 10 mM. Such behavior in α_{s1} -I casein against calcium resembles to that in α_{s1} - κ -casein complex.¹⁰⁾

The number of total binding sites of complex at 5 mM of calcium (0.69 N) is approximately half of that at 10 mM of calcium (1.16 N) (Fig. 8). Therefore, complexes at 10 mM of calcium have an ability to combine free α_{s1} -caseins as twice as that at 5 mM of calcium. Since this assumption is consistent with the "dip" observed by Noble and Waugh,¹⁾ the formation of two binding sites in α_{s1} - κ -casein complex at the calcium concentration above 10 mM seems to be essential

to the stability of casein micelles.

Acknowledgment. The authors thank to Dr. C. W. Slattery of Loma Linda University, California, U.S.A., for his useful discussion and comments.

REFERENCES

- 1) R. W. Noble and D. F. Waugh, *J. Am. Chem. Soc.*, **87**, 2236 (1965).
- 2) D. F. Waugh, "Milk Proteins," Vol. II, ed. by H. A. McKenzie, Academic Press, New York and London, 1971, p. 23.
- 3) L. Pepper, *Biochim. Biophys. Acta*, **278**, 147 (1972).
- 4) S. Kaminogawa, S. Dosako and K. Yamauchi, *Agric. Biol. Chem.*, **38**, 2337 (1974).
- 5) S. Kaminogawa, S. Dosako, K. Yamauchi and K. Kinoshita, *ibid.*, **39**, 533 (1975).
- 6) S. Nakai and F. Voort, *J. Dairy Res.*, **46**, 283 (1979).
- 7) F. Voort, C. Ma and S. Nakai, *Arch. Biochem. Biophys.*, **195**, 596 (1979).
- 8) D. F. Clarke and S. Nakai, *Biochemistry*, **10**, 3353 (1971).
- 9) S. Dosako, S. Kaminogawa and K. Yamauchi, *Agric. Biol. Chem.*, **39**, 2347 (1975).
- 10) S. Kaminogawa, T. Koide and K. Yamauchi, *ibid.*, **41**, 697 (1977).
- 11) C. A. Zittle, J. Cerbuis, L. Pepper and E. S. Della Monica, *J. Dairy Sci.*, **42**, 1897 (1959).
- 12) C. A. Zittle and J. H. Custer, *ibid.*, **46**, 1183 (1963).
- 13) S. Dosako, T. Kimura, S. Taneya, T. Sone, S. Kaminogawa and K. Yamauchi, *Agric. Biol. Chem.*, **44**, 2443 (1980).
- 14) D. G. Schmidt and W. Buchheim, *Neth. Milk Dairy J.*, **30**, 17 (1976).
- 15) R. M. Parry, Jr. and R. J. Carroll, *Biochim. Biophys. Acta*, **194**, 138 (1969).
- 16) W. Buchheim and U. Welsh, *Neth. Milk Dairy J.*, **27**, 163 (1973).
- 17) S. Dosako, S. Kaminogawa, S. Taneya and K. Yamauchi, *J. Dairy Res.*, **47**, 123 (1980).
- 18) T. G. Parker and D. G. Dalglish, *ibid.*, **44**, 70 (1977).
- 19) T. G. Parker and D. G. Dalglish, *Biopolymers*, **16**, 2533 (1977).
- 20) C. H. Yoon, S. Kaminogawa, K. Yamauchi, S. Dosako, T. Kimura and S. Taneya, Abstracts of Papers, 55th Annual Meeting of Agricultural Chemical Society of Japan, Fukuoka, April, 1980, p. 79.
- 21) S. Kaminogawa, K. Yamauchi and C. H. Yoon, *J. Dairy Sci.*, **63**, 223 (1980).