Formation of Artificial Casein Micelles

Tomotada ONO, Tomomi FURUYAMA and Satoshi ODAGIRI

*Department of Agricultural Chemistry, Iwate University,
Ueda, Morioka, Iwate 020, Japan*

Received May 10, 1982

The reformation of casein micelles was investigated by dialysis of simulated milk ultrafiltrate or skim milk at a fixed temperature or by increasing the temperature from 5°C to 37°C. When submicelles were dialyzed at a fixed temperature the micelles were not reformed, but were when the submicelles were dialyzed as the temperature was increased from 5°C to 37°C. The salt content, casein composition and size distribution of the reformed micelles were approximately 70% of original micelles when the submicelles were dialyzed against skim milk. The submicelles obtained from large micelles reformed into large micelles while submicelles from small micelles reformed into small micelles.

The casein micelles of bovine milk are broken down into casein submicelles on removal of calcium.¹⁾ On the other hand, micelle-like-colloids are obtained from sodium caseinate and the halides of alkaline earth metals.²⁾ The formation of the colloids has been shown to be from α_s -casein, κ -casein and calcium ion.³⁾ Schmidt and Van der Spek⁴⁾ obtained artificial micelles from caseins by the addition of calcium. The size distribution of the micelles, however, differed from native micelles which may be explained by the presence and absence of colloidal calcium phosphate. In subsequent studies the introduction of colloidal calcium phosphate into the casein mixtures and the role of casein components in micelles were investigated.^{5~7)} In these studies artificial micelles differed from the native micelles in their stability towards ethanol, dialysis, pressure and heat. There may be two causes of this decreased stability, 1) the lack of minor caseins in the casein system and 2) the casein mixtures, without minor caseins, may differ from the native submicelles. Therefore, the objective of the present study was to investigate the formation of artificial casein micelles by the introduction of colloidal calcium phosphate to the submicelles. The submicelles were obtained by the removal of calcium phosphate from

small and large casein micelles.

MATERIALS AND METHODS

Submicelle solution and casein. Small micelle-submicelles (SMS) and large micelle-submicelles (LMS) were prepared from large and small micelles of skim milk, respectively. The large micelles were obtained by centrifugation at $15,000 \times g$ from skim milk, and the small micelles were obtained by centrifugation at $100,000 \times g$ from the supernatant of skim milk centrifuged at $60,000 \times g$ as described previously.⁸⁾ The micelle fractions were dispersed into 0.01 M imidazole buffer containing 0.07 M NaCl and 0.02% NaN_3 (pH 7.0) (Im-buffer). After dialysis against Im-buffer at 5°C for 70 hr, the suspensions were centrifuged at $100,000 \times g$ for 30 min.⁸⁾ The supernatants were used for the submicelle solutions. The supernatants were further dialyzed against distilled water for 48 hr. The submicelle caseins were obtained by lyophilization of the dialyzates. Heated submicelle caseins were obtained from submicelle solutions by dialysis and lyophilization after heating at 90°C for 10 min.

Simulated milk ultrafiltrate. Simulated milk ultrafiltrate (SMUF) was prepared at 5°C by the methods of Jenness and Koops.⁹⁾ Sodium azide (0.02%) was added to this solution to inhibit microbial growth.

Ultrafiltrate of skim milk. The ultrafiltrate was obtained from skim milk containing 0.02% NaN_3 with an ultrafiltration cell (Toyo Kagaku model UHP-76) with a membrane filter UK-200 (cut-off molecular weight, 200,000) at room temperature.

Formation of casein micelles from submicelle caseins.

Skimmilk and SMUF were stirred for 48 hr at a fixed temperature and then filtered through Toyo Roshi No. 5 papers. The filtrates were used for formation of casein micelles. Three methods were used for the formation of casein micelles.

1. Submicelle solutions containing 3% protein were dialyzed against SMUF for 72 hr at the temperature at which SMUF was equilibrated.

2. Submicelle caseins were dissolved in SMUF, and stirred for 72 hr at the temperature at which SMUF was equilibrated.

3. Submicelle solutions were dialyzed against SMUF or skimmilk at 5°C for 72 hr and subsequently dialyzed at 37°C for 72 hr.

Submicelle solutions treated as above were centrifuged at $100,000 \times g$ for 30 min, and the precipitates, which were the reformed micelles, were dispersed in water with a Potter type homogenizer. The quantity and components of protein as well as salt contents were analyzed.

Determination of protein. After the suspension of reformed micelles was dissolved in 10% NaOH, the protein content was determined by the method of Gornall *et al.*¹⁰⁾

Determination of calcium and inorganic phosphorus. After the protein was removed from the suspensions of reformed micelles by the addition of an equal volume of 20% trichloroacetic acid, calcium and inorganic phosphorus were determined by atomic absorption¹¹⁾ and Nakamura's

modified method,¹²⁾ respectively.

Casein composition. The casein composition of reformed micelles was analyzed by disc gradient acrylamide-gel (4~8%) electrophoresis (pH 8.3).¹³⁾ The relative ratio of casein components was calculated from the densitometric traces of the electrophoretic pattern. The densitometric traces were obtained with a Fujiriken FD-A IV densitometer.

Size distribution. Size distribution of the micelles was determined by transmission electron microscopy. The micelle specimens were prepared by the method of Carroll *et al.*¹⁴⁾ using glutaraldehyde fixation. Details of this method and of the statistical processing of the experimental data were described previously.¹⁵⁾

Clotting time measurement. The native and reformed micelles which were centrifuged at $100,000 \times g$ for 30 min were dispersed in ultrafiltrate of skimmilk to a protein concentration of 3%. The clotting time of these suspensions was determined as the time taken from the addition of chymosin to the formation of aggregates at 35°C.

RESULTS

Submicelles from large and small micelles

The large and small micelles were dissociated by dialysis at 5°C for 70 hr. Small

PLATE I. Electron Micrographs of Large and Small Micelles before and after Dialysis against 0.01 M Imidazole Buffer.

Large micelles: (A) before dialysis; (B) after dialysis. Small micelles: (C) before dialysis; (D) after dialysis.

└─ shows 200 nm in photographs.

homogeneous size particles (below 40 nm) were observed (Plate I). It is impossible to distinguish between particles obtained from large and small micelles. These particles were the submicelles and were used for the reformation of micelles.

Dialysis of the submicelles against SMUF at a fixed temperature

When the submicelles from large micelles (LMS) were dialyzed against SMUF equilibrated at various temperatures, a precipitate was formed above 20°C (Fig. 1). The precipitate, however, was not formed when the heated submicelle solution was dialyzed. Similar results were obtained on dialysis against Im-buffer without calcium and phosphate. The formation of precipitates seems to be caused by unstable peptides derived on decomposition of caseins by protease. Casein micelles were not reformed when submicelles were dialyzed at a constant temperature.

Formation of casein micelles from submicelles in SMUF when the temperature was increased from 5°C to 37°C

The amounts of calcium and inorganic

FIG. 1. The Reformation of Micelles on Dialysis of Submicelles against SMUF or Imidazole Buffer at Fixed Temperatures.

Submicelles from large micelles were dialyzed against SMUF (a) or 0.01 M imidazole buffer (b) for 72 hr at 5, 10, 15, 20, 25, 30 and 37°C. The precipitates were obtained from the dialyzates by centrifugation at $100,000 \times g$ for 30 min. Unheated (○) and heated (●) LMS were used for samples.

phosphorus in SMUF varied with temperature as shown in Fig. 2. The amounts decreased with increasing temperature. Precipitates of calcium phosphate were formed due to the decreased solubility of calcium phosphate. Therefore, the introduction of calcium phosphate into submicelles was added by an increase in temperature of SMUF. After the LMS was dissolved in SMUF and stirred at 5°C, the temperature of the solution was increased to 37°C. A micelle-like-colloid was formed with no evident precipitate. The formation efficiency of the micelle colloid was 50%, and it made no difference whether heated or unheated submicelles were used (Table I). The salt contents of the reformed micelles were 20 mg Ca/g casein and 8 mg Pi/g casein, corresponding to 70 and 40% of those found in

FIG. 2. The Quantities of Calcium and Inorganic Phosphorus in SMUF at Each Temperature.

□, calcium; ▨, inorganic phosphorus.

TABLE I. THE COMPOSITIONS OF REFORMED MICELLES FROM HEATED OR UNHEATED LMS IN SMUF ON INCREASING THE TEMPERATURE FROM 5°C TO 37°C

Proportion reformed (%)	Native micelles	Reformed micelles	
		Heated	Unheated
Micelles	100	52	48
Calcium*	100	72	68
	(28)	(20)	(19)
Inorganic phosphorus*	100	37	35
	(23)	(8.5)	(8.0)
α_s -Casein	54	61	60
β -Casein	28	28	23
κ -Casein fraction	18	11	17

* () is mg/g casein.

TABLE II. THE COMPOSITIONS OF REFORMED MICELLES FROM HEATED LMS AND SMS ON DIALYSIS AGAINST SMUF OR SKIMMILK WHEN THE TEMPERATURE WAS INCREASED FROM 5°C TO 37°C

Proportion reformed %	LMS			SMS		
	Native	Reformed		Native	Reformed	
		SMUF	Skimmilk		SMUF	Skimmilk
Micelles	100	58	70	100	20	62
Calcium*	100	71	64	100	86	77
	(28)	(20)	(18)	(22)	(19)	(17)
Inorganic phosphorus*	100	43	48	100	71	71
	(23)	(10)	(11)	(14)	(10)	(10)
α_s -Casein	54	63	54	50	58	46
β -Casein	28	23	22	22	20	23
κ -Casein fraction	18	14	24	28	22	31

* () is mg/g casein.

native micelles, respectively. The reformed micelles from unheated submicelles contained more κ -casein fraction (containing γ -caseins) and less β -casein than heated submicelles. This suggests that β -casein was broken by protease and migrated to the κ -casein fraction on electrophoresis.

The formation of casein micelles on dialysis

In order to increase the formation efficiency of the micelle-like-colloid, the reformation of casein micelles by dialysis against SMUF or skimmilk was examined by increasing the temperature from 5°C to 37°C. Heated LMS and SMS were used for this experiment. The proportions of reformed micelles from LMS and SMS on dialysis against skimmilk were 70 and 60%, respectively (Table II), and were higher than those on dissolution into SMUF. The composition of the reformed micelles on dialysis against SMUF, however, was similar to that on dissolution into SMUF. The salt contents of the reformed micelles from SMS were 71 to 86% of the contents of native micelles. The inorganic phosphorus content of the reformed micelles from LMS was lower than that of native micelles. The casein composition of the reformed micelles was similar to that of native micelles, though the κ -casein content of the reformed micelles on dialysis against skim-

FIG. 3. The Size Distribution of Large Micelles and Reformed Micelles from LMS on Dialysis against SMUF or Skimmilk when the Temperature was Increased from 5 to 37°C.

a, large micelles; b, reformed micelles from LMS on dialysis against SMUF; c, reformed micelles from LMS on dialysis against skimmilk.

milk was larger than that of native micelles. The size distribution of reformed micelles is shown in Figs. 3 and 4. The reformed micelles from LMS were larger than those from SMS. The size of reformed micelles on dialysis against skimmilk was larger than that obtained on dialysis against SMUF, and similar to that of native micelles.

FIG. 4. The Size Distribution of Small Micelles and Reformed Micelles from SMS on Dialysis against SMUF or Skimmilk When the Temperature was Increased from 5 to 37°C.

a, small micelles; b, reformed micelles from SMS on dialysis against SMUF; c, reformed micelles from SMS on dialysis against skimmilk.

TABLE III. THE CLOTTING TIME OF NATIVE AND REFORMED MICELLES

	Native	Reformed
Large micelles	9 ± 1	14 ± 1 min
Small micelles	4 ± 1	7 ± 1 min

Measurements of clotting time

The clotting time of native and reformed micelles was measured and is shown in Table III. The reformed micelles have a longer clotting time than the native micelles. The small micelles clotted in half the time when compared to large micelles.

DISCUSSION

When the submicelles were equilibrated in SMUF at a fixed temperature the casein micelles were not formed, however, it has been reported that colloidal phosphate-free (CPF) casein is formed.¹⁶ CPF casein contains 25% of calcium found in native micelles but little of the inorganic phosphate, and the CPF casein is not a micelle.¹⁶ Particles formed during dialysis were below 40 nm (Plate), so they did not precipitate when centrifuged at $100,000 \times g$ for 30 min and could not be distinguished from submicelles.

The micelles formed on the addition of calcium to submicelles do not contain in-

organic phosphate, and the salt composition differs from that of native ones. Schmidt and Van der Spek⁴) reported that the micelles formed on the addition of calcium differed from native ones in the size distribution. On the other hand, it was reported that phosphate was critical in the formation of larger and more stable micelles.^{5,17,18} Schmidt *et al.*⁵⁻⁷) investigated the formation of micelles by the addition of calcium phosphate to casein and the role of the main casein components in micelles. The relation between micelles and submicelles, however, was not studied and their synthetic micelles did not contain the minor casein. The synthesized micelles differed from the native micelles in their stability towards ethanol, dialysis, pressure and heat. We attempted reformation of casein micelles not from isolated caseins or casein mixtures but from submicelles which were prepared by removal of calcium phosphate from large or small micelles.

The reformation of micelles was performed by a moderate method (dialysis and a temperature change from 5°C to 37°C). The yield of the reformed micelles was high (60 to 70%), and the calcium phosphate introduced into micelles was 70% that of native micelles (small micelles) (Table I). The yield of the reformed micelles on dialysis against skimmilk was higher than that on dialysis against SMUF. The transport of calcium phosphate into micellar casein is gradual when dialyzed against skimmilk, since the casein micelles in skimmilk have a buffering capacity according to the deposition and dissolution of calcium phosphate. The size distribution of the moderately reformed micelles was similar to that of native large or small micelles. Thus, the reformed micelles would seem to closely resemble the native micelles, with the exception that the clotting time of the reformed micelles was longer than that of the native micelles. The fluctuation of the time might be caused by slight differences in the salt content, casein composition and size populations between the reformed and native micelles.

Some differences in casein and salt com-

positions were noted between small and large micelles. Choate *et al.*¹⁹⁾ reported that the caseins from large and small micelles reformed into large and small micelles, respectively. However, their results are suspect since sedimentation coefficients were obtained with a polydispersed system. In our study it was clear that the LMS formed the large micelles and the SMS formed the small micelles. The reformed large and small micelles had compositions (Table II) and size distributions similar to those of the native large and small micelles (Figs. 3 and 4). The difference between SMS and LMS lies in the κ -casein content. SMS containing large quantities of κ -casein formed small micelles. Schmidt *et al.*⁵⁾ obtained synthetic small micelles from a casein mixture containing large quantities of κ -casein. It is postulated that κ -casein inhibits the formation of large micelles.²⁰⁾ On the other hand, we²¹⁾ reported the existence of some types of submicelles. One is the α_{s1} - κ -casein complex which is abundant in SMS, and the others are complexes and polymers of α_{s1} - and β -caseins which are found in LMS in large quantities. Micelles were formed from these submicelles by the deposition of colloidal calcium phosphate. Waugh and Noble³⁾ suggested that polymers of α_s - and β -casein are located in the core regions of casein micelles on addition of calcium, and that κ -casein is located in the surface region of casein micelles. We hypothesize that the core of casein micelles is composed of submicelles containing little κ -casein which are linked by calcium phosphate and that the periphery of each micelle is made from submicelles containing an abundance of κ -casein. It is probable that the LMS which contains many submicelles for the micelle core forms the large micelles and the SMS containing many submicelles for the mi-

celle coat forms the small micelles.

REFERENCES

- 1) W. K. Downey and R. F. Murphy, *J. Dairy Res.*, **37**, 47 (1970).
- 2) S. Adachi, *J. Dairy Sci.*, **46**, 743 (1963).
- 3) D. F. Waugh and R. W. Noble, Jr., *J. Am. Chem. Soc.*, **87**, 2246 (1965).
- 4) D. G. Schmidt and C. V. Van der Spek, *Milchwissenschaft*, **29**, 455 (1974).
- 5) D. G. Schmidt, J. Koops and D. Westerbeek, *Neth. Milk Dairy J.*, **31**, 328 (1977).
- 6) D. G. Schmidt and J. Koops, *Neth. Milk Dairy J.*, **31**, 342 (1977).
- 7) D. G. Schmidt, P. Both and J. Koops, *Neth. Milk Dairy J.*, **33**, 40 (1979).
- 8) T. Ono, T. Furuyama and S. Odagiri, *Agric. Biol. Chem.*, **45**, 511 (1981).
- 9) R. Jenness and J. Koops, *Neth. Milk Dairy J.*, **16**, 153 (1962).
- 10) A. G. Gornall, C. S. Bardwill and M. M. David, *J. Biol. Chem.*, **177**, 751 (1949).
- 11) T. Ono and S. Odagiri, *Jpn. J. Dairy Sci.*, **24**, A-133 (1975).
- 12) M. Nakamura, *Nippon Nôgeikagaku Kaishi*, **24**, 1 (1950).
- 13) A. H. Gordon, "Laboratory Techniques in Biochemistry and Molecular Biology," Vol. 1, ed. by T. S. Work and E. Work, North-Holland Publ. Co., Amsterdam, 1969, p. 50.
- 14) R. J. Carroll, M. P. Thompson and G. C. Mutting, *J. Dairy Sci.*, **51**, 1903 (1969).
- 15) T. Ono, T. D. Ho and S. Odagiri, *Agric. Biol. Chem.*, **42**, 1063 (1978).
- 16) C. V. Morr, R. V. Josephson, R. Jenness and P. B. Manning, *J. Dairy Sci.*, **54**, 1555 (1971).
- 17) L. K. Creamer and S. Yamashita, *New Zealand J. Dairy Sci. Technol.*, **11**, 257 (1976).
- 18) C. W. Slattery, *J. Dairy Res.*, **46**, 253 (1979).
- 19) W. L. Choate, F. A. Heckman and T. F. Ford, *J. Dairy Sci.*, **42**, 761 (1959).
- 20) C. W. Slattery and R. Evard, *Biochim. Biophys. Acta*, **317**, 529 (1973).
- 21) T. Ono, S. Odagiri and T. Takagi, Abstracts of Papers, 56th Meeting of Agricultural Chemical Society of Japan, April, 1981, p. 348.