Role of Tyrosine Residues in Interactions between Casein Components

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Received November 26, 1973

It was indicated from ultraviolet difference spectra and ultracentrifugal experiments that associations occurred between two casein components (α_s - and κ -caseins, β - and κ -caseins and α_s - and β -caseins) at lower CaCl_2 concentrations (2~3 mM) and that aromatic amino acid residues participated in the associations. Chemical modification studies with 2-hydroxy-5-nitrobenzylbromide indicated that tryptophane residues of each casein component were not essential for these associations. It was also demonstrated by nitration of tyrosine residues with tetranitromethane that tyrosine residues of κ -casein were essential for α_s - κ -association and for β - κ -association and that tyrosine residues of α_s -casein were important to α_s - β -association.

Interactions between casein components were also studied at higher CaCl_2 concentration (10 mM) which is enough for micelle formation. It was found that tyrosine residues of κ -casein played an important role for the stabilization of α_s - and β -caseins. Properties of the nitrated- β -casein were almost the same as that of the native β -casein except the absorption spectrum. α_s - β -Interaction in the presence of 10 mM CaCl_2 was investigated by use of the nitrated- β -casein instead of the native β -casein. It was proved that α_s -casein was stabilized by the nitrated- β -casein and that precipitation of the nitrated- β -casein increased in the presence of α_s -casein.

The mechanism of interactions between casein components at higher CaCl_2 concentration (10 mM) are discussed in connection with the associations at lower CaCl_2 concentrations (2~3 mM).

In order to elucidate mechanism of the casein micelle formation, studies on interactions between casein components are important. It was found by Waugh *et al.*¹⁾ that α_s -casein was stabilized by κ -casein from precipitation in the presence of CaCl_2 . This interaction is understood to be the most fundamental step of the casein micelle formation. The association of α_s - and κ -caseins was also observed in the absence of CaCl_2 and have been studied by many workers.^{2~6)} α_s - β -Interaction^{7~9)} and β - κ -interaction⁸⁾ have been reported and these interactions are also considered to be important for the casein micelle formation. However, the knowledges on these interactions are limited.

Ultraviolet difference spectra were obtained in our laboratory by mixing α_s - and κ -caseins, β - and κ -caseins and α_s - and β -caseins. These facts suggest that the associations occur between

these pairs of casein components and that aromatic amino acid residues participate in the associations. The participation of tyrosine residues of κ -casein in α_s - κ -interaction was reported in previous paper.^{5,6)}

In this paper, the interactions between casein components (α_s - and κ -caseins, β - and κ -caseins and α_s - and β -caseins) were investigated at two different concentration levels of CaCl_2 with chemical modification of aromatic amino acid residues.

MATERIALS AND METHODS

Preparation of casein components. α_s - and β -Caseins were prepared from individual Holstein cow by the methods of Zittle *et al.*^{11,12)} and Hipp *et al.*¹³⁾ respectively. κ -Casein was prepared by the method of Zittle *et al.*^{11,12)} with minor modification. Acid casein (wet weight: 350 g) was dissolved in 1 liter of 6.6 M urea. After the addition of 2 liter of water and 200 ml

of 7 N H_2SO_4 the solution was allowed to stand for 2 hr at 25°C. The resulting precipitate was removed by centrifugation. The supernatant was subjected to ammonium sulfate fractionation and the fraction precipitated at 0.5~1.0 M was collected. Further purifications were performed according to the Zittle's procedure. Protein concentrations were determined from optical density. The $E_{1\text{cm}, 280\text{nm}}^{1\%}$ values used were 10.0 for α_s - and κ -caseins and 4.7 for β -casein.^{3,9)} Values of 27,000, 24,000 and 20,000 were used for monomer molecular weights of α_s - and β - and κ -caseins, respectively.^{14~16)}

Chemicals. 2-Hydroxy-5-nitrobenzylbromide was purchased from Seikagaku Co. Tetranitromethane (Extra-pure grade) and other reagents (Guaranteed reagent grade) were purchased from Nakarai Chemicals Co.

Instruments. Ultraviolet difference spectra of casein components were recorded with Hitachi double beam spectrophotometer model-124 equipped with a constant temperature cell holder. The output of the spectrophotometer (full range: 10 mV) was connected to the input of Hitachi automatic recorder model QPD 34 (full range: 1 mV). Thus ten fold expanded spectra were obtained.

Sedimentation experiments were performed with Beckman model E analytical ultracentrifuge equipped with RTIC control unit.

CaCl_2 Concentrations. Calcium ion is indispensable for the casein micelle formation. Micelle or precipitate is not formed at 2~3 mM CaCl_2 . Molecular interactions between casein components were studied under these conditions and these interactions are called associations in this paper. Casein micelle is formed at 10 mM CaCl_2 . Interactions between casein components were also studied under these conditions. These interactions are called the stabilization and the coprecipitation.

Chemical modification of casein components. Nitration of casein components was performed with tetranitromethane as described previously.^{5,17,18)} Numbers of modified tyrosine residues were calculated from the absorbance at 381 nm ($\epsilon=2200$).

Chemical modification of tryptophane residues of casein components by 2-hydroxy-5-nitrobenzylbromide was performed in 6 M urea at pH 3. 2-Hydroxy-5-nitrobenzylbromide dissolved in a minimum volume of acetone was added to the solution of each casein component (5 mg/ml) in a 50:1 molar ratio to the concentration of tryptophane residues. As the reaction proceeded, 1 N NaOH was added in order to keep pH 3. The reaction was stopped after 1 hr by passing the reaction mixture through Sephadex G-25 column equilibrated with 6 M urea containing 1 mM

HCl, and the protein fraction was neutralized. The protein solution was dialyzed against water and lyophilized. Numbers of modified tryptophane residues were calculated from the absorbance at 410 nm ($\epsilon=18,000$).¹⁹⁾

Precipitation of casein components. Precipitations of α_s -, β -caseins and their modified samples were tested as follows. The standard reaction mixture contained 2.5 mg of protein, 10 μ moles of imidazole buffer, pH 7.1 and 10 μ moles of CaCl_2 in a volume of 1.0 ml. After the precubation of protein solution for 20 min at 35°C, the precipitate was made by quick addition of 10 mM CaCl_2 . The suspension was incubated for 1 hr and the precipitate was removed by centrifugation at 3000 rpm, for 10 sec. The supernatant thus obtained was diluted with 0.05 M potassium citrate and the protein concentration was measured by the absorbance at 280 nm.

Stabilization of α_s - or β -casein by κ -casein. Stabilization of α_s - or β -casein by κ -casein was tested under the same conditions as those of precipitation experiments except that reaction mixture contained 0.25 mg κ -casein. The amount of α_s - or β -casein in the supernatant was calculated by subtracting the amount of κ -casein added from the total protein in the supernatant.

Interaction of α_s - and nitrated- β -caseins in the presence of 10 mM CaCl_2 . The interaction of α_s - and the nitrated- β -caseins was tested as follows. The reaction mixture contained 2.5 mg of α_s -casein, 2.5 mg of the nitrated- β -casein, 10 μ moles of imidazole buffer, pH 7.1 and 10 μ moles of CaCl_2 in a total volume of 1.0 ml. After the solution containing α_s - and the nitrated- β -caseins was preincubated for 20 min, CaCl_2 was added to the solution at temperatures indicated in Fig. 5. At the same temperature, the mixture was incubated for 1 hr and centrifuged at 3000 rpm, for 10 sec. The amount of the nitrated- β -casein in the supernatant was determined from the absorbance at 381 nm. From this result, the contribution of the nitrated- β -casein to the absorbance of the supernatant at 280 nm was calculated. Therefore, the amount of α_s -casein in the supernatant can be obtained from the absorbance at 280 nm.

RESULTS

Ultraviolet difference spectra

Changes of the ultraviolet absorption spectra caused by mixing two casein components were studied in the presence of 2 mM CaCl_2 at 20°C. As shown in Fig. 1, red shifts in the ultraviolet absorption spectra were observed in every

cases, although each difference spectrum had a different intensity. Difference of molar extinction coefficient at 286 nm were calculated to be 2000 per κ -casein monomer (curve I),

FIG. 1. Difference Spectra Produced by Mixing Two Casein Components.

Casein components were dissolved in 10 mM imidazole buffer, pH 7.1, containing 2 mM CaCl_2 . In the case of curve I (α_s -casein + κ -casein), each compartment (light path: 4.5 mm) of reference tandem cell was filled with solutions of α_s -casein (2 mg/ml) and κ -casein (2 mg/ml), respectively, and both compartments of sample tandem cell were filled with a mixture of α_s -casein and κ -casein (concentration of each component: 1 mg/ml). Curve II (β -casein + κ -casein) and curve III (α_s -casein + β -casein) were obtained by the same procedure as described above.

1600 per κ -casein monomer (curve II) and 500 per α_s -casein monomer (curve III). These spectra suggest that casein components interact under these conditions and that there occurred some changes in the environments of aromatic amino acid residues of both or one of the two components. The main peak at 286 nm and the small peak or shoulder at 293 nm in the difference spectra suggest that the contribution of tryptophane residues to the difference spectra is relatively small compared with that of tyrosine residues in every cases.

Association between casein components

Associations between casein components

were studied by ultracentrifugal experiments. Although associations between casein components were hardly observed in 10 mM imidazole buffer, pH 7.1, associations were promoted by the addition of CaCl_2 . Partial associations were observed at 20°C in the same buffer containing 2 mM CaCl_2 . Almost complete associations were observed at 35°C in the presence of 3 mM CaCl_2 . Therefore, studies on associations were performed at 35°C in 10 mM imidazole buffer, pH 7.1, containing 3 mM CaCl_2 (Fig. 2).

i) $\alpha_s \cdot \kappa$ -Association. The mixture of α_s -casein (5.2 S) and κ -casein (19.4 S) gave a single peak with a sedimentation coefficient of 11.8 S. This means that there occurred an association between both components. $\alpha_s \cdot \kappa$ -Association seems to be accompanied by the dissociation of κ -casein into smaller unit.

ii) $\beta \cdot \kappa$ -Association. The mixture of β - and κ -caseins gave a single peak with a sedimentation coefficient, 13.1 S which is smaller than that of both components (15.7 S and 19.4 S). $\beta \cdot \kappa$ -Association might be accompanied by the dissociation of both components into smaller units.

iii) $\alpha_s \cdot \beta$ -Association. The mixture of α_s - and β -caseins gave a single peak with a similar sedimentation coefficient value (5.1 S) to that of α_s -casein (5.2 S). On the basis of the area of the 5.1 S peak and of the absence of the peak corresponding to β -casein, the 5.1 S peak is considered to be a complex of α_s - and β -caseins.

Sedimentation coefficient of modified casein components

Sedimentation patterns of modified casein components were investigated at 35°C in 10 mM imidazole buffer, pH 7.1, containing 3 mM CaCl_2 . Sedimentation coefficient of the nitrated casein components was different from that of the native components. The sedimentation coefficient of α_s -casein decreased from 5.2 S to 4.2 S, and that of κ -casein from 19.4 S to 11.5 S after the nitration of tyrosine residues.

FIG. 2. Sedimentation Patterns.

Each component (4 mg/ml) was dissolved in 10 mM imidazole buffer, pH 7.1 containing 3 mM CaCl_2 . Centrifugations were performed at 35°C, 60,000 rpm. Photographs were taken 18 ± 1 min after reaching maximum speed. Numbers of modified tyrosine residues per mole of each casein component were as follows; nitrated- α_s -casein: 8.5, nitrated- κ -casein: 7.1, nitrated- β -casein: 3.8.

These decreases may result from reduction of Ca^{2+} -dependent self-association of these casein components. In the case of β -casein, the sedimentation coefficient increased from 15.7 S to 18.1 S and the sedimentation pattern was poly-disperse after the nitration of tyrosine residues. The native β -casein was completely dissociated at 4°C.²⁰⁾ Dissociation of β -casein also occurred in the presence of 0.5% SDS.²¹⁾ The sedimentation pattern of the nitrated- β -casein was poly-disperse and its sedimentation coefficient was slightly larger than that of the native β -casein at 4°C or in the presence of 0.5% SDS. Therefore, these changes in sedimentation profiles induced by reaction with tetranitromethane may be derived from covalent cross-linking between β -casein molecules as observed in other proteins.^{22,23)}

Sedimentation coefficient of individual casein components modified with 2-hydroxy-5-nitrobenzylbromide (HNB-caseins) were almost the same as the native components.

Effect of chemical modifications on the association between casein components

Associations between native and modified casein components were investigated in the presence of 3 mM CaCl_2 .

i) $\alpha_s \cdot \kappa$ -Association. The association of α_s - and the nitrated- κ -caseins was not observed. On the other hand, the association of κ - and the nitrated- α_s -caseins was observed, although the sedimentation coefficient of the complex was smaller than that of the native complex (Fig. 2). These results are similar to the facts observed in 10 mM imidazole buffer, pH 7.1, containing 0.07 M KCl.⁶⁾

ii) $\beta \cdot \kappa$ -Association. The association of β - and κ -caseins was also inhibited by the nitration of tyrosine residues of κ -casein. Tyrosine residues of κ -casein seem to be essential for the association. The association of κ - and the nitrated- β -caseins was almost intact and brought a formation of a complex

with the same sedimentation coefficient value as that of the native complex of β - and κ -caseins (Fig. 2). Thus tyrosine residues of β -casein seem to be not essential for β - κ -association.

iii) α_s - β -Association. The nitrated- β -casein associated with α_s -casein and gave a complex. The sedimentation coefficient of the complex was almost the same as that of the native complex. The sedimentation pattern of the mixture of β - and the nitrated- α_s -casein was complicated. In this case, the complex formation seems to be only partial. The 10.6 S peak may correspond to β -casein. The 3.8 S peak may be the complex and/or nitrated- α_s -casein. Discrepancy in the sedimentation coefficients of β -casein (15.7 S and 10.6 S) may be ascribed to the difference in the self-association at different concentrations of free β -casein (Fig. 2).

The associations of native and modified casein components of which tryptophane residues were almost completely modified with 2-hydroxy-5-nitrobenzylbromide were intact in each combinations (κ - and HNB- α_s -caseins, HNB- κ - and α_s -caseins, β - and HNB- κ -caseins, HNB- β - and κ -caseins, α_s - and HNB- β -caseins and HNB- α_s - and β -caseins).

It was concluded that for the individual associations (α_s - and κ -caseins, β - and κ -caseins and α_s - and β -caseins) tyrosine residues of κ -, κ - and α_s -caseins play important roles, respectively. It is supposed that the ultraviolet difference spectra (Fig. 1) are caused by environmental changes of these essential tyrosine residues.

Properties of modified casein components in the presence of 10 mM CaCl₂

The effects of chemical modifications on casein components were investigated in the presence of 10 mM CaCl₂ which is enough for casein micelle formation. The properties of the HNB-casein components were essentially the same as that of the native casein components. The properties of the nitrated casein components were as follows.

i) *Nitrated- κ -casein.* The stabilizing ability of κ -casein against α_s - or β -casein was tested

at a weight ratio of 1:10. As shown in Fig. 3, the nitration of κ -casein resulted in the loss of stabilizing ability against β -casein as well as α_s -casein. Both stabilization (Fig. 3) and association (Fig. 2) were inhibited by the nitration of tyrosine residues of κ -casein. These facts suggest that α_s - κ -association and β - κ -association are prerequisite to the stabilization of α_s - and β -caseins by κ -casein.

FIG. 3. Effect of Nitration on Stabilizing Ability of κ -Casein.

Stabilization was tested in 10 mM imidazole buffer, pH 7.1 containing 10 mM CaCl₂ at 35°C. Protein concentrations were as follows; α_s -casein; 2.5 mg/ml, β -casein; 2.5 mg/ml, κ -casein; 0.25 mg/ml.

●—●, α_s -casein; ○—○, β -casein.

ii) *Nitrated- α_s -casein.* The native α_s -casein precipitated completely in 10 mM imidazole buffer, pH 7.1, containing 10 mM CaCl₂. When 4 among 8 tyrosine residues of α_s -casein were nitrated, the nitrated- α_s -casein did not precipitate under the same conditions as reported previously.⁶⁾ Therefore, the stabilization of the nitrated- α_s -casein by κ - or β -casein could not be estimated.

iii) *Nitrated- β -casein.* The effect of nitration of tyrosine residues on the properties of β -casein was investigated. As shown in Fig. 4 A and B, nitration of 3.8 tyrosine residues among 4 residues resulted in little alteration in the calcium-dependent precipitation of β -casein. This suggests that β -casein has a differ-

FIG. 4. Effect of Nitration on Solubility of β -Casein.

Concentrations of β -casein and nitrated- β -casein were 2.5 mg/ml. Number of nitrated tyrosine residues was 3.8 per mole of β -casein. Experiment A was performed in 10 mM imidazole buffer, pH 7.1, containing various concentrations of CaCl_2 at 35°C. Experiment B was performed in the same buffer containing 10 mM CaCl_2 at various temperatures.

●—●, β -casein; ○—○, nitrated- β -casein.

ent mechanism of calcium-dependent precipitation from that of α_s -casein which does not precipitate after the nitration of tyrosine residues.

As shown in Table I, it was also found that the nitrated- β -casein as well as the native β -casein was stabilized by κ -casein. The nitrated- β -casein associated with α_s - or κ -casein (Fig. 2). Therefore, the nitrated- β -casein seems to retain the properties of the native β -casein except for having absorbance in the visible region.

TABLE I. STABILIZATION OF β -CASEIN BY κ -CASEIN

Stabilization was tested in 10 mM imidazole buffer, pH 7.1, containing 10 mM CaCl_2 at 35°C. Protein concentrations were as follows; β -casein: 2.5 mg/ml, nitrated- β -casein: 2.5 mg/ml, κ -casein: 0.25 mg/ml.

β - or Nitrated- β -casein in the supernatant	
β	12%
$\beta + \kappa$	86
Nitrated- β	19
Nitrated- $\beta + \kappa$	90

The amount of this colored derivative of β -casein could be easily determined in a mixture with other components.

Interaction of α_s - and nitrated- β -casein in the presence of 10 mM CaCl_2

The interaction of α_s - and the nitrated- β -caseins was investigated as a model of α_s - β -interaction (Fig. 5A and B). Most of α_s -casein precipitated in all temperature tested and the nitrated- β -casein was completely soluble below 20°C in the presence of 10 mM CaCl_2 . When equal amount of both components were mixed, solubility of α_s -casein was enhanced (stabilization) and that of the nitrated- β -casein decreased (coprecipitation). As shown in Fig. 5B, stabilization of α_s -casein by the nitrated- β -casein was maximum at 10°C, and coprecipitation of the nitrated- β -casein with α_s -casein was maximum at 25°C. The supernatant thus obtained was opalescent. Stabilization of α_s -casein by the nitrated- β -casein was tested in various weight ratio at 10°C. Stabilization of α_s -casein increased with increasing amounts of the nitrated- β -casein (Fig. 6). Maximum stabilization of α_s -casein appeared at a weight ratio near unity. The stabilizing ability of the nitrated- β -casein against α_s -casein was about 1/10 compared with that of κ -casein. Coprecipitation of the nitrated- β -casein by α_s -casein was tested in various weight ratio at 20°C. Coprecipitation of the nitrated- β -casein with α_s -casein exhibited its maximum value at a ratio of 1:2 (Fig. 7). It is interesting that this weight ratio is same to

modification was due to changes in conformation or in net charges induced by chemical modification of non-essential tyrosine residues. However, both the difference spectra and the inhibition of association by chemical modification were observed in every combinations. Therefore, the essential role of tyrosine residues for respective associations seems to be valid as described in the results.

The essential role of tyrosine residues of κ -casein for $\alpha_s \cdot \kappa$ -interaction was indicated by chemical modification and other experiments in the previous paper.⁶⁾ These results are not contradictory to the results of ultraviolet difference spectra in this paper.

It was also found that tyrosine residues of κ -casein were also essential for $\beta \cdot \kappa$ -association and for the stabilization of β -casein by κ -casein.

All of tyrosine residues of κ -casein are located exclusively in para- κ region.^{24, 25)} These facts suggest that para- κ region of κ -casein molecules participates in $\beta \cdot \kappa$ -interactions (association and stabilization) as well as in $\alpha_s \cdot \kappa$ -interactions.^{6, 26)}

The stabilizing ability of κ -casein for β -casein was more sensitive to the nitration than for α_s -casein (Fig. 3). Peak in the difference spectra caused by $\beta \cdot \kappa$ -association was smaller than that caused by $\alpha_s \cdot \kappa$ -association (Fig. 1, peak I and II). These facts suggest that smaller number of tyrosine residues of κ -casein contribute to $\beta \cdot \kappa$ -interaction compared with the case of $\alpha_s \cdot \kappa$ -interaction.

Properties of the nitrated- β -casein are as follows; 1) Manners of precipitation of the nitrated- β -casein under the various conditions are almost the same as those of the native β -casein (Fig. 3). 2) The nitrated- β -casein has a little larger sedimentation coefficient than that of the native β -casein (Fig. 1). 3) The nitrated- β -casein associates with α_s - or κ -casein in similar manners to the native β -casein. These complexes have the same sedimentation coefficient as those of the native components (Fig. 2). 4) The nitrated- β -casein, as well as the native β -casein, is stabilized by κ -casein in the presence of 10 mM CaCl_2 .

Thus the nitrated- β -casein seems to retain

essentially identical properties to the native β -casein except for having a little larger sedimentation coefficient and absorbance in the visible region. In order to study quantitatively the $\alpha_s \cdot \beta$ -interaction at higher CaCl_2 concentration, determination of the ratio α_s - and β -caseins in the supernatant or the precipitate is necessary. The determination of the ratio α_s - and β -caseins is difficult because of the similar chemical nature of the both casein components. The nitrated- β -casein has almost the same properties as the native β -casein as described above. Therefore, the interaction between α_s - and the nitrated- β -caseins at higher CaCl_2 concentration was studied as a model of $\alpha_s \cdot \beta$ -interaction. As shown in Fig. 5 A, solubilities of α_s - and the nitrated- β -caseins were changed by the interaction of the both components. The amounts of stabilized α_s -casein and coprecipitated nitrated- β -casein changed markedly according to the temperature and showed their maxima at different temperatures. It is supposed that the contribution of hydrophobic binding forces at higher temperature alter the manner of the interaction between α_s - and the nitrated- β -caseins. The coprecipitation of the nitrated- β -casein by α_s -casein was maximum at 25°C. However, this would not mean that the interaction between α_s - and the nitrated- β -caseins was weakened above this temperature. Almost all the nitrated- β -casein precipitated at elevated temperatures even in the absence of α_s -casein. The nitrated- β -casein to be coprecipitated by α_s -casein was left in a little amount in the supernatant. This fact may be the cause for observed decrease of the coprecipitation of the nitrated- β -casein by α_s -casein above 25°C. This idea was supported by ultracentrifugal experiments. Dissociation of the complex between α_s - and the nitrated- β -caseins was not observed above 25°C (Fig. 2).

Interaction between α_s - and β -caseins was also studied by Waugh *et al.* They concluded that the comingled precipitate of α_s - and β -caseins was a core of casein micelle.⁹⁾ Coprecipitation of nitrated- β -casein by α_s -casein may be correlated to the comingled precipitate described by Waugh *et al.* Stabilization of α_s -

casein by the nitrated- β -casein at lower temperature may correspond to the metastable colloid described qualitatively by Waugh.²⁷⁾

The possibility that the stabilization of α_s -casein by the nitrated- β -casein may result from contaminating κ -casein was excluded by the fact that the stabilizing ability of κ -casein against α_s -casein is lost by the nitration of tyrosine residues of κ -casein.

Data indicating that the interaction between α_s - and the native β -caseins are essentially the same as the interaction between α_s - and the nitrated- β -caseins have been obtained in this laboratory (unpublished data). It is an interesting problem why such a temperature-dependent interaction occur between α_s - and β -caseins. Studies on the mechanism of α_s - β -interaction is in progress.

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