Structure of Bovine $\kappa$-Casein: Spectrophotometric Titration and Molecular Size Changes in Alkaline Solution

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Received July 26, 1972

The ionization of tyrosyl groups in bovine $\kappa$-casein and S-carboxyamidomethyl-$\kappa$-casein (CAM-$\kappa$) was studied by spectrophotometric titration at 295 m$\mu$. In the denaturing solvent 8 m urea, the titration curves are reversible and the $pK_{app}$ values of eight tyrosyl groups both in $\kappa$-casein and in CMA-$\kappa$-casein are 10.7. In 0.2 M KCl solution, $\kappa$-casein has six tyrosyl groups with normal $pK_{app}$ value of 10.5 and two groups with higher $pK_{app}$ value of 11.4. CAM-$\kappa$-casein has eight tyrosyl groups with $pK_{app}$ value of 10.6 in 0.2 M KCl solution. These observations suggest that -S-S- bondings in $\kappa$-casein are concerned with the 'masking' of the tyrosyl groups. The evidence of the rupture of intermolecular -S-S- bondings of $\kappa$-casein in alkaline solution was shown by the study of gel chromatography on Sephadex G-150. One of the possible explanation is that the ionization of tyrosyl groups with higher $pK_{app}$ value is associated with the destruction of hydrophobic regions, and this destruction is due to the rupture of intermolecular -S-S- bondings under alkaline conditions.

Bovine $\kappa$-casein is known to be the substrate of rennin (EC 3.4.4.3.). As the result of the rupture of peptide linkage between -Phe-Met- by rennin, $\kappa$-casein is separated into para-$\kappa$-casein part and glycopeptide part which contains carbohydrates. The extent of hydrophobic amino acid residues in the para-$\kappa$-casein part is larger than that in the glycopeptide part, and the Tyr and Cys residues are contained only in the para-$\kappa$-casein part.1-3 This casein molecule is considered to be similar to the model of detergent.1-3 Conformational studies by Herskovits suggest that $\kappa$-casein has little organized structure in aqueous solutions.4 And it is reported that $\kappa$-casein has randomly formed intermolecular disulfide bonds but other major casein components have not. These intermolecular disulfide bonds could lead to the formation of a polydisperse material of which minimum molecular weight was about 56,000.5 Moreover, Payens postulated the possibility of hydrophobic interactions as a principal cause of aggregation of $\kappa$-casein.7

The purpose of the work reported here is to investigate the ionization of tyrosyl groups in bovine $\kappa$-casein and the effects of denaturing solvent and of disulfide bonds on the titration behavior. For the alkylation of reduced $\kappa$-casein, carboxyamidomethylation was performed, because there is no change of the number of ionizable groups of carboxyamidomethylated protein. And gel filtration on Sephadex G-150 was carried out to investigate the variation of molecular size of $\kappa$-casein in alkaline solutions.

MATERIALS AND METHODS

Bovine $\kappa$-casein was isolated from acid precipitated whole casein by the urea-sulfuric acid method of Zittle and Custer8 and purified further from ethanolic solution according to McKenzie and Wake.9 Polyacrylamide gel electrophoresis was used to check the purity of $\kappa$-casein. Electrophoretic patterns were obtained using 7.5% polyacrylamide gel in 4.5 M urea with Tris-glycine buffer (pH 9.4). 2-Mercaptoethanol was added to samples, which were not alkylated, before electrophoresis.10 Carboxyamidomethyl-$\kappa$-casein (CAM-$\kappa$) was prepared according to the method reported by Woychik et al.11 The elution profile of modified $\kappa$-casein obtained by gel filtration on Sephadex G-150 is shown in Fig. 1. As this pattern shows the polydispersity of molecular size, the samples used for spectrophotometric measurements were obtained by separation from fractions eluted at the void volume.
The ordinate indicates the transmittance at 280 nm. Eluent was 0.01 M imidazole buffer containing 4 M urea (pH 7.0) and flow rate was 10~15 ml/hr. The broken line shows the elution pattern of disulfide bond-intact κ-casein. The arrow mark means the void volume. The mark - - - shows the fraction range that was used for measurements.

CAM-κ-casein obtained with gel filtration showed the same electrophoretic pattern as unmodified κ-casein showed in the presence of 2-mercaptoethanol, though 2-mercaptoethanol was not added to the CAM-κ-casein solution.

Urea, 2-mercaptoethanol (purchased from Wako Pure Chemical Industries, Ltd.) and iodoacetamide (purchased from Nakarai Chemicals, Ltd.), which were guaranteed reagents, were used without further purification. All other chemicals were reagent grade. κ-Casein was dissolved in a solution of 0.01 M potassium citrate and was dialyzed against deionized water or desirable salt solution for at least 48 hr with several changes of outer solvent at 2~4°C. Dialyzed solution was then filtered with Sartorius-Membranfilter (pore size ~0.45 μ).

Absorbance measurements were made with a Hitachi recording spectrophotometer model EPS-3T with a thermostated cell holder at 25±1°C, using 1-cm quartz cells. The absorbance of the alkaline solution of κ-casein was read against a neutral solution of κ-casein as a reference. The correction of scattered light was performed by the method of Leach and Scheraga, as necessary.

The pH of these solutions was determined in a thermostated vessel (25°C) using a Hitachi-Horiba pH meter type F-5 standardized at pH 6.86 and 10.02 with standard buffer. Either HCl or KOH (1 N or 5 N) was added from a micrometer syringe of 0.500 ml capacity. The pH measurement was carried out in an atmosphere of nitrogen.

In the gel filtration procedure, 2~4% κ-casein solution was applied to the Sephadex G-150 column (1.5 × 45 cm) and then developed with 0.01 M imidazole-4 M urea (pH 7.0 or 7.5) at a constant flow rate using a Perista-minipump (Mitsumi SJ-1210 type) at room temperature. Urea was used in order to prevent the interactions, such as hydrogen bonds and hydrophobic bonds, from making aggregates. The eluted solution was monitored by the absorption at 280 mμ with Uvicord II (LKB Produkter, Stockholm) using a 3-mm flow cell. The preparative gel filtration was performed on the 3.2 × 60 cm column.

Protein concentration was estimated spectrophotometrically using the extinction coefficient of E_{1%1cm}^{280}= 12.2 at 280 mμ for κ-casein, or determined by the semimicro Kjeldahl method. A nitrogen factor of 15.3% was used for both κ-casein and CAM-κ-casein.

**RESULTS**

The equation describes the spectrophotometric titration curves of tyrosyl groups. a is the fraction of ionized form of tyrosyl groups and this is determined from the equation

\[ \text{pH} = \text{pK}_{\text{app}} + \log \left( \frac{a}{1-a} \right) \]  

where \( \frac{\text{J O.D.}}{\text{J O.D.}_{\text{max}}} \) is the difference absorption coefficient at any pH and \( \text{J O.D.}_{\text{max}} \) is the maximum difference absorption coefficient of the titration curve. The term of electrostatic free energy is not considered in the equation (1). Assuming that equation (1) is applicable to the titration of κ-casein, the adjustable parameters \( \frac{\text{J O.D.}}{\text{J O.D.}_{\text{max}}} \) and \( \text{pK}_{\text{app}} \) are computed in each case to provide the best fit with the experimental points by the least square method. All calculations were performed with a FACOM 230~60 FORTRAN computer in the Hokkaido University Computing Center. And the number of ionized form of tyrosyl groups \( r \) is calculated from \( r = n \times a \), where \( n \) is the total number of tyrosyl groups. The values of 19,000 and 8 were used for molecular weight and \( n \) of functional κ-casein monomer, respectively.

The results of spectrophotometric titration of κ-casein at 295 mμ are shown in Fig. 2. The ionization of tyrosyl groups of native κ-casein (in 0.2 M KCl) proceeded in two stages. The first ionization occurred between

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**Fig. 1.** Gel Filtration of CAM-κ-casein on the Sephadex G-150 Column (1.5 × 45 cm).
Tyr Ionization in κ-Casein and CAM-κ-casein

FIG. 2-a. Spectrophotometric Titration of κ-Casein in 0.2 M KCl at 295 m/s at 25°C.

The concentration of κ-casein; 0.046%. Curves A, B, C, and A+B are theoretical curves. Curve A+B is the sum of curves A and B. The lower points were obtained by subtracting the corresponding values of curve A from experimental points. Curve C is obtained, assuming to be the single stage of ionization.

FIG. 2-b. Spectrophotometric Titration of κ-Casein in 8 M Urea at 295 m/s at 25°C.

The concentration of κ-casein; 0.043%. The curved line is a theoretical curve.

pH 8 and 12, and the second ionization occurred above pH 11. Time-dependent changes of the ionization were not occurring at any pH. Reversibility of the titration was changed at near pH 11. When the final pH values of solution were below 11, the backward titration points were found to lie on the forward titration curve. But when the final pH values were above 11, the titration curve did not coincide with the forward curve. The best fit values of pK_app and r to the data obtained at pH range 7 to 11, were 10.5 and 6, respectively. Curve A in Fig. 2-a was drawn according to equation (1) with pK_app = 10.5 and r = 6. Curve C is in the case of K_p = 10.5 and r = 8 and this curve is not consistent with experimental data. The values of pK_app and r for the second stage of ionization were calculated to be 11.4 and 2 respectively, from the lower points of Fig. 2-a, that is the points subtracted the corresponding values of curve A from experimental points. Curve B was obtained from equation (1) by assuming pK_app = 11.4 and r = 2. Curve A+B is the sum of

FIG. 3-a. Spectrophotometric Titration of CAM-κ-casein in 0.2 M KCl at 295 m/s at 25°C.

The concentration of CAM-κ-casein; 0.049%. The curved line is a theoretical curve.

FIG. 3-b. Spectrophotometric Titration of CAM-κ-casein in 8 M Urea at 295 m/s at 25°C.

The concentration of CAM-κ-casein; 0.040%. The curved line is a theoretical curve.
curve A and curve B, and the experimental points are found to lie on this synthesized curve. Figure 2-b is the spectrophotometric titration curve of $\kappa$-casein in 8 M urea at 295 m$\mu$. The titration showed complete reversibility and no time-dependent changes. The ionization of tyrosyl groups proceeded into a single stage and the $pK_{app}$ and $r$ were 10.7 and 8, respectively. The curved line was obtained from equation (1) by assuming the ionization of eight tyrosyl groups with $pK_{app}=10.7$. The experimental points were found to lie on the theoretical curve. The results of spectrophotometric titration of CAM-$\kappa$-casein were shown in Fig. 3. Both in 0.2 M KCl and in 8 M urea, the ionization of tyrosyl groups of CAM-$\kappa$-casein proceeded into a single stage. The best fit values of $pK_{app}$ of CAM-$\kappa$-casein in 0.2 M KCl and 8 M urea were 10.6 and 10.7, respectively. Time-dependent changes of the ionization were not observed and the titration was reversible. The curved lines are the theoretical curves obtained from equation (1) by assuming the ionization of eight tyrosyl groups with $pK_{app}=10.6$ and 10.7, respectively. The experimental points were found to lie on the theoretical curves.

The chromatographic pattern of $\kappa$-casein is shown in Fig. 1 (a broken line). $\kappa$-Casein was eluted with 0.01 M imidazole-4 M urea (pH 7.0) at the void volume (arrow mark in the figure) on the Sephadex G-150 column. On the basis of this data, it seemed reasonable to assume that $\kappa$-casein was present as a covalently coupled polymeric form of which molecular weight was more than 400,000 and that such covalent bond was disulfide linkage. In an effort to determine the effect of alkali addition, the variation of molecular size was studied at gel-chromatographic patterns. $\kappa$-Casein was dissolved into 1 M, 0.1 M, and 0.01 M KOH aqueous solutions and incubated for about 100 min at room temperature. And then 0.5 ml of the solution was eluted on the Sephadex G-150 column (Fig. 4). The experiments described above leave no doubt that the dispersion of the molecular size occurred with increased concentrations of KOH.

**FIG. 4. Gel Filtration of Alkali Treated $\kappa$-Casein on the Sephadex G-150 Column (1.5 x 45 cm).**

The ordinate indicates the transmittance at 280 m$\mu$ in arbitrary units. $\kappa$-Casein (5 mg) in KOH aq. solution (0.5 ml) was incubated for 100 min at room temperature and then chromatographed. The concentration of KOH: (a) 0.01 M, (b) 0.1 M, (c) 1.0 M. Eluent was 0.01 M imidazole buffer containing 4 M urea (pH 7.5) and flow rate was 5 ml/hr. The arrow mark means the void volume.

**DISCUSSION**

The spectrophotometric titration of $\kappa$-casein in 0.2 M KCl has revealed the occurrence of two kinds of tyrosyl groups with different $pK_{app}$ values. One of them ionized normally with a $pK_{app}$ value of 10.5, while the other has higher $pK_{app}$ value of 11.4. On the other hand, CAM-$\kappa$-casein had one kind of tyrosyl group with a single $pK_{app}$ value of 10.6 in 0.2 M KCl and both $\kappa$-casein and CAM-$\kappa$-casein had tyrosyl groups with a single $pK_{app}$ value of 10.7 in 8 M urea.

In 8 M urea, $\kappa$-casein and CAM-$\kappa$-casein are considered to be in the denatured state, so that all the tyrosyl groups are solvent-accessible. The tyrosyl groups of CAM-$\kappa$-casein in 0.2 M KCl are as solvent-accessible as
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those in 8 M urea, but all the tyrosyl groups of \( \kappa \)-casein in 0.2 M KCl were not equally solvent-accessible. Two of eight tyrosyl groups are masked or solvent-inaccessible in the pH region below 11.

Abnormal behavior of ionization of the tyrosyl groups has been reported in the cases of many proteins; e.g., lysozyme,\(^{15}\) cytochrome c,\(^{16}\) ribonuclease,\(^{17}\) and so on.\(^{18}\) In general, it is considered that the tyrosyl groups, which are not titrated in native state, are buried in the hydrophobic regions of the molecule. In view of the above facts, the most reasonable conclusion to be drawn from available data is that some tyrosyl groups in native \( \kappa \)-casein are buried in the hydrophobic regions and such hydrophobic regions are stabilized by intermolecular -S-S- bondings of \( \kappa \)-casein.

Ho and Waugh,\(^{19}\) and Creamer\(^{20}\) reported no evidence of abnormal ionization behavior of the tyrosyl groups in their titration studies of \( a_\kappa \)-casein and \( \beta \)-casein. Woychik et al. reported the titration of the tyrosyl groups in \( a_\kappa \)- and \( \kappa \)-casein by tetranitromethane.\(^{21}\) They concluded that a complete tyrosyl titration was achieved at low molar ratios of tetranitromethane so that these proteins were devoid of major secondary or tertiary structures.

Leslie et al. reported the NMR studies of \( a_\kappa \)-, \( \beta \)-, and \( \kappa \)-caseins.\(^{22}\) They suggested from the NMR spectra of \( \kappa \)-casein that the signals from aromatic and terminal methyl protons are fully developed only after heating or treatment with urea and the spectra are consistent with a structure for the \( \kappa \)-casein aggregate in which the aromatic and most of the non-polar residues are restricted in motion, but the hydrophilic groups have considerable molecular motion. They attempted to obtain an evidence on the possible role of the -S-S- bondings, and compared the NMR spectra of \( \kappa \)-casein and S-carboxymethyl-\( \kappa \)-casein (SCM-\( \kappa \)). They obtained the results that SCM-\( \kappa \)-casein in native state gave a better resolved spectrum than \( \kappa \)-casein itself but the general appearances of \( \kappa \)-casein and SCM-\( \kappa \)-casein spectra were similar.

Clarke and Nakai reported many interesting results from the fluorescent study of conjugate between \( \kappa \)-casein and 8-anilinonaphthalene-1-sulfonate.\(^{23}\) The presence of hydrophobic regions was indicated in native state of \( \kappa \)-casein and they also suggested the highly significant structural change above pH 11 on the bases of the large red shift in the wavelength of maximum emission.

Herskovits studied the conformation of caseins in solution from optical rotatory properties.\(^{4}\) He reported that the three major casein components, \( a_\lambda \)-, \( \beta \)-, and \( \kappa \)-caseins, have very low helical content and a low degree of structural organization. For example, the Moffitt \( b_0 \) parameter of \( \kappa \)-casein in neutral aqueous solution and in 8 M urea are \(-10\) and \(+20\), and the helical contents are \(2\%\) and \(0\%\), respectively.

From these results, it may be concluded that the exposure of two tyrosyl groups of \( \kappa \)-casein was not explained in terms of the transition from ordered to disordered structure but in terms of changes concerned with the state of \( \kappa \)-casein aggregates. The results of gel filtration supported this inference.

From the gel filtration, it is reasonable to assume that the variation of the molecular size observed was due to the rupture of intermolecular -S-S- bondings under alkaline conditions, because urea cannot disrupt the covalent bonds. This result coincided with those reported by Mackinlay and Wake\(^{5}\) who investigated the destruction of -S-S- bondings in \( \kappa \)-casein under alkaline conditions at various temperatures.

Donovan reported that the spectrophotometric observations of the alkaline hydrolysis of -S-S- bonds in ovomucoid were consistent with the reaction scheme described below.\(^{24}\)

\[
RS-SR+OH^- = RS^- + RSOH \quad (3)
\]
\[
2 RSOH = RSH + RSO_2H \quad (4)
\]
\[
2RS-SR+4OH^- = 3RS^- + RSO_2 + 2H_2O \quad (5)
\]
It is reasonable to assume that this reaction scheme is one of the possible mechanisms for the -S-S- bondings split by hydroxide ion in the case of \( \kappa \)-casein.
The spectrophotometric study of Cheese-
man and Knight on detergent binding to
casein gives a powerful support to interprete
the abnormal tyrosyl ionization. Their results
are as follows; a decrease in absorption when
\( \kappa \)-casein was dissociated indicated that the
tyrosyl and tryptophyl groups were not in
the region of the molecule to which the deter-
genent was bound and that in the \( \kappa \)-casein ag-
gregate these residues were in a more hydro-
phobic environment.

The ionization curve A in Fig. 2-a was
considerably flatter than the ionization curve
obtained in 8 M urea (Fig. 2-b). It may
be considered that this flatter curve was due
to the overlap of some stages of ionization
which we were unable to distinguish by the
procedure used in the present experiment.
Curve B shows a sharp curvature. This
suggests that the ionization above pH 11 was
accompanied by a structural change which
was concerned with the proton release. The
higher \( pK_{a} \) value in 8 M urea than that in
0.2 M KCl may be substantiated by the results
reported by Donovan et al. They indicated
that model compounds such as acetic acid,
phenol, imidazole, and \( n \)-butylamine have
higher \( pK \) values in 8 M urea than those in
0.2 M KCl.

To summarize the facts described above,
the scheme of the tyrosyl ionization was
postulated as follows. The tyrosyl groups
with lower \( pK_{a} \) values are located in the
solvent-accessible regions, and the other groups
with higher \( pK_{a} \) value are located in the
hydrophobic regions present in the interior
or at the intermolecular boundary of -S-S-
coupled polymers. The former groups can
ionize, but the latter groups cannot ionize
until the hydrophobic regions are destroyed.
Intermolecular -S-S- bondings stabilize the
formation of hydrophobic regions from de-
stabilizing factors, such as electrostatic re-
pulsions. When the pH of the solution goes
above 11, -S-S- bridges are ruptured and the
aromatic groups masked in the hydrophobic
regions are exposed to the solvent because of
the destruction of the hydrophobic regions.*
And the change from polymeric form to mono-
ermic form of \( \kappa \)-casein occurs simultaneously.

Acknowledgments. The authors wish to thank
Dr. Katsutoshi Nitta, Department of Polymer Science,
Faculty of Science, Hokkaido University, for many
helpful discussions and to Mr. Kiyoshi Hoshi, Depart-
ment of Civil Engineering, Faculty of Engineering,
Hokkaido University, for assistance in the formula-
tion of the computer programs.

REFERENCES

1) R. J. Hill and R. G. Wake, Nature, 221, 635
2) J. Jolliés, P. Jolles and C. Alais, ibid., 222, 668
(1969)
3) A.G. Mackinlay an R.G. Wake, “Milk Proteins:
Chemistry and Molecular Biology,” Vol. II, ed.
by H. A. McKenzie, Academic Press Inc., New
York, N.Y., 1971, p. 175.
5) A. G. Mackinlay and R. G. Wake, Biochim.
6) H. E. Swaygood, J. R. Brunner and H. A.
Lillewik, Biochemistry, 3, 1616 (1964)
8) C. A. Zittle and J. H. Custer, ibid., 46, 1183
(1963).
9) H. A. McKenzie and R. G. Wake, Biochim.
10) J. H. Woychik, Arch. Biochem. Biophys., 109,
542 (1965).
11) J. H. Woychik, E. B. Kalan and M. E. Noelken,
Biochemistry, 5, 2276 (1966).
12) S. J. Leach and H. A. Scheraga, J. Am. Chem.
Soc., 82, 4790 (1960).
13) H. A. McKenzie, Advan. Protein Chem., 22, 55
(1967).
14) J. Hermans, Jr., Biochemistry, 1, 193 (1962).
15) T. Tojo, K. Hamaguchi, M. Imanishi, and T.
17) C. Tanford, “Physical Chemistry of Macro-
18) M. J. Kronman and F. M. Robbins, “Biological
Macromolecules series 4: Fine Structure of
Proteins and Nucleic Acids,” ed. by G. D. Fasman
* The authors consider that one of the possible
changes may be drawn by equation (5).


Note added in proof.

Recently, Brignon *et al.* (1972) and Jolles *et al.* (1972) reported the complete primary structure of \( \alpha \)-casein B and A, respectively, and the number of tyrosyl groups in \( \kappa \)-casein was also determined to be nine. The result corrected on the basis of their data is that the number abnormal tyrosyl groups of \( \kappa \)-casein in 0.2 M KCl is 2.25 and that of normal groups is 6.75.

The genetic type of \( \kappa \)-casein used in this experiment was type A and there is no difference between the number of tyrosyl groups in \( \kappa \)-A and \( \kappa \)-B caseins.
