Thermodynamics of the Interaction between $\alpha_{s1}$- and $\kappa$-Caseins

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Pyrenebutyrate-conjugated $\alpha_{s1}$-casein was prepared and the complex formation between $\alpha_{s1}$- and $\kappa$-casein polymers was investigated by fluorescence polarization. The complex formation was also investigated by a microcalorimetric technique. The positive enthalpy and entropy changes and endothermic nature suggested the hydrophobic interaction between $\alpha_{s1}$- and $\kappa$-casein polymers.

The degree of polarization of $\kappa$-casein polymer decreased with the addition of 1-anilino-8-naphthalenesulfonate (ANS), while that of $\alpha_{s1}$-casein polymer and $\alpha_{s1}$-$\kappa$-casein complex was invariant. Moreover the reaction of $\kappa$-casein polymer and ANS was exothermic. These facts suggested that the intermolecular hydrophobic regions in $\kappa$-casein polymer were disrupted by the adsorption of ANS. The rotational relaxation time of pyrenebutyrate conjugated complex between cyanoethyl-$\kappa$-casein and $\alpha_{s1}$-casein polymer was smaller than that of cyanoethyl-$\kappa$-casein alone. From these results, it was postulated that the dissociation of $\kappa$-casein polymer by the complex formation with $\alpha_{s1}$-casein polymer might be caused by the disruption of the intermolecular hydrophobic bonds in $\kappa$-casein polymer.

In the preceding paper, it has been suggested that 4 moles of $\alpha_{s1}$-$\kappa$-casein complex are formed from 1 mole of $\kappa$-casein polymer. However, the mechanism of this interesting complex formation is obscure.

Nakai et al. have studied on the interaction between dimethylamino-naphthalenesulfonate(DNS)-conjugated $\alpha_{s1}$- and $\kappa$-caseins by fluorescence polarization technique. However, the application of this technique to very large protein (with a molecular weight greater than 150,000) is limited because of the short fluorescence lifetime of DNS-protein conjugate (10~20 nsec). On the contrary, the conjugates of proteins with pyrenebutyrate have a long fluorescence life time near 100 nsec.

In the present paper, conjugates of caseins with pyrenebutyrate were prepared and the mechanism of the complex formation between $\alpha_{s1}$- and $\kappa$-casein polymers was investigated by using the fluorescence polarization and microcalorimetric techniques.

MATERIALS AND METHODS

Caseins. Preparation of caseins was performed as described in the preceding paper. Buffer solution used in the experiments was 0.01 M imidazole-HCl-0.07 M KCl (pH 7.1). Cyanohethyl-$\kappa$-casein (CNE-$\kappa$-casein) was prepared by the method of Woychik. Fluorescence dye and labeling procedure to caseins. Pyrenebutyric acid was obtained from Eastman Kodak Company, Rochester, New York. 1-Anilino-8-naphthalenesulfonate (ANS) was obtained from Tokyo Kasei Company, Tokyo. The preparation of cyanohethyl anhydride (PB) was followed to Knopp and Weber. A method for the conjugation of dyes to caseins was described in the preceding paper. The number of dye molecules bound per mole of casein monomer was all less than 1.

Fluorescence polarization and intensity. Measurements of fluorescence polarization and intensity were made on a HITACHI Model MPF-3 fluorescence spectrophotometer as described in the preceding paper. Relative fluorescence intensity of the conjugates was determined with quinine bisulfate in 0.1 M H$_2$SO$_4$ as a standard.

Association constant. The formulation presented below for the quantitative interpretation of fluorescence polarization data is based on some assumptions. The first assumption is that the complex formation of $\alpha_{s1}$- and $\kappa$-casein polymer is written as follows according to the preceding paper.

$$\kappa + 8 \alpha_{s1} \rightleftharpoons 4 \text{complexes}$$

According to the preceding paper, if we write the dissociated $\kappa$-casein polymer as $\kappa''$, the reaction for
the formation of 1 mole of $\alpha_\text{S}1-\kappa$-casein complex is written as follows.

$$\kappa'' + 2 \alpha_{\text{S}1} \rightleftharpoons \text{complex}$$

(1)

The second assumption is that the molecular weights of $\alpha_{\text{S}1}$- and $\kappa$-casein polymers are $10 \times 10^4$ and $80 \times 10^4$, respectively. And the molecular weight of $\kappa''$ is assumed as $20 \times 10^4$.

If PB is conjugated to $\alpha_{\text{S}1}$-casein polymer, the association constant, $K$, in Eqn. (1) is given by

$$K = \frac{1/2(1-x_{\alpha_{\text{S}1}})M_{\alpha_{\text{S}1}}}{(M_{\kappa''} - 1/2)(1-x_{\alpha_{\text{S}1}})M_{\alpha_{\text{S}1}}}{(x_{\alpha_{\text{S}1}}M_{\alpha_{\text{S}1}})^2}$$

(2)

where $x_{\alpha_{\text{S}1}}$ is the mole fraction of free PB-$\alpha_{\text{S}1}$-casein polymer, and $M_{\alpha_{\text{S}1}}$ and $M_{\kappa''}$ are total molar concentrations of PB-$\alpha_{\text{S}1}$-casein polymer and $\kappa''$, respectively. According to Weber, the observed polarization, $P$, is given by

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{\sum_{i} f_i}{1 + 3\tau_i/\rho_i}\right)^{-1} = R$$

(3)

where $\rho_i$ is the rotational relaxation time of component $i$, $\tau$ is the life time and $f_i$ is the contribution of the $i$th component to the total fluorescence intensity, and given by

$$f_i = F_i / \sum_i F_i$$

(4)

where $F_i$ is the fluorescence intensity of $i$th component. In this case, the fluorescent components are free PB-$\alpha_{\text{S}1}$-casein polymer and complex species, and Eqn. (3) becomes as

$$\frac{1}{R} = \frac{f_{\alpha_{\text{S}1}}}{1 + 3\tau_i/\rho_{\alpha_{\text{S}1}}} + \sum_{i=2}^{a} \frac{f_i}{1 + 3\tau_i/\rho_i}$$

(5)

where $f_{\alpha_{\text{S}1}}$ and $\rho_{\alpha_{\text{S}1}}$ are the contribution and the rotational relaxation time of free PB-$\alpha_{\text{S}1}$-casein polymer. According to Steiner's theory, Eqn. (5) becomes as

$$R_{\alpha_{\text{S}1}}/R = f_{\alpha_{\text{S}1}} + R_{\alpha_{\text{S}1}} (1-f_{\alpha_{\text{S}1}}) / R_0$$

(6)

where $R_{\alpha_{\text{S}1}}$ is the value of $R$ for free PB-$\alpha_{\text{S}1}$-casein polymer and $R_0$ is the mean value of $R$ for the complex species. $R_{\alpha_{\text{S}1}}$ and $R_0$ are given by

$$R_{\alpha_{\text{S}1}} = 1 + 3\tau_i/\rho_{\alpha_{\text{S}1}}$$

(7)

$$R_0 = 1 - f_{\alpha_{\text{S}1}} \sum_{i=2}^{a} f_i / \sum_{i=2}^{a} f_i$$

(8)

From Eqn. (6), $f_{\alpha_{\text{S}1}}$ is given by

$$f_{\alpha_{\text{S}1}} = R_{\alpha_{\text{S}1}} (R - R_0) / (R_{\alpha_{\text{S}1}} - R_0)$$

(9)

On the other hand, the fluorescence intensity of free PB-casein polymer, $F_{\alpha_{\text{S}1}}$, is given by

$$F_{\alpha_{\text{S}1}} = x_{\alpha_{\text{S}1}} Q_{\alpha_{\text{S}1}}$$

(10)

where $Q_{\alpha_{\text{S}1}}$ is the molar fluorescence intensity of free PB-casein polymer. Since the total concentration of fluorescent components is $x_{\alpha_{\text{S}1}} + 1/2 (1-x_{\alpha_{\text{S}1}})$, $M_{\alpha_{\text{S}1}}$, the total fluorescence intensity, $\sum_i F_i$, is given by

$$\sum_i F_i = x_{\alpha_{\text{S}1}} Q_{\alpha_{\text{S}1}} + 1/2 (1-x_{\alpha_{\text{S}1}}) Q_{\alpha_{\text{S}1}} M_{\alpha_{\text{S}1}}$$

(11)

where $Q_{\alpha_{\text{S}1}}$ is the mean molar fluorescence intensity of the complex species. Substituting Eqn. (10) and (11) into Eqn. (4), we obtain

$$x_{\alpha_{\text{S}1}} = \frac{f_{\alpha_{\text{S}1}}}{Q_{\alpha_{\text{S}1}}/Q_e + \left(1 - Q_{\alpha_{\text{S}1}}/Q_e\right) f_{\alpha_{\text{S}1}}}$$

(12)

Therefore, from Eqn. (9), (12) and (2), we can calculate the association constant for the complex formation between $\alpha_{\text{S}1}$- and $\kappa$-casein polymers.

On the basis of above theory, the experiments to determine the association constants were performed as follows. $\kappa$-Casein (2 mg/ml) and PB-$\alpha_{\text{S}1}$-casein (2 mg/ml) solution were incubated at desired temperature for over 1 hr, separately. Subsequently, equal volume of both solutions were mixed, and after the reaction mixture reached to equilibrium, the degree of fluorescence polarization was measured. $R_{\alpha_{\text{S}1}}$ and $Q_{\alpha_{\text{S}1}}$ are easily calculated from a measurement of the $\alpha_{\text{S}1}$-casein conjugate alone. However, because of the small polarization of the $\alpha_{\text{S}1}$-casein polymer conjugated with PB, it is difficult to obtain the accurate $\rho_{\alpha_{\text{S}1}}$. Therefore, the rotational relaxation time of DNS-conjugated $\alpha_{\text{S}1}$-casein polymer is used as substitute for that of PB-$\alpha_{\text{S}1}$-casein polymer. The rotational relaxation times of both DNS and PB conjugated $\alpha_{\text{S}1}$-casein polymer should be identical in principle. $R_0$ and $Q_e$ are determined from measurements on a state in which the complete complex species at various weight ratios are formed. Since complete complex can not be realized physically, a hypothetical complex, incubated for 1 hr at 37°C, is used for the calculations of $R_0$ and $Q_e$. Values for $R_{\alpha_{\text{S}1}}$ and $R_0$ were calculated from Table III, IV and Fig. 6 in the preceding paper. The experiments were performed at 15, 20, 25, 30 and 35°C.

**Calorimetric measurements.** The calorimeter used in the experiments was an Oyodenki Lab. Co. Model MCF-1F microcalorimeter. In principle, it is a heat conduction type calorimeter with a twin compartment giving the possibility of compensating for the blank reaction in a reference vessel, so that the output recorded is already corrected for the heats of mixing and dilution of buffer. The partition type glass vessels were used. The sample vessel was charged with $\alpha_{\text{S}1}$- and $\kappa$-casein solutions in the 0.01 M imidazole-HCl-0.07 M KCl buffer (pH 7.1) containing 0.02% of sodium azide, separately. In the reference vessel, each compartment was charged with equal volume of buffer solution to the sample vessel. In separate experiments, the determination of heat of dilution of the two protein solutions were performed. Each casein solution was charged in the sample vessel and diluted with an equal volume of buffer solution.

**RESULTS**

The association constants calculated with
Eqn. (9), (12) and (2) are given in Table I.

Thermodynamic parameters, $\Delta G^\circ$, $\Delta H^\circ$ and $\Delta S^\circ$ were calculated from the following equations,

\[
\Delta G^\circ = -R'T \ln K \quad (13)
\]
\[
\log K = -(\Delta H^\circ/2.303R'T) + C \quad (14)
\]
\[
\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T \quad (15)
\]

where $R'$ is the gas constant, $T$ is the absolute temperature, $K$ is the association constant and $C$ is the integration constant. Thermodynamic parameters are given in Table II.

Table I. Association Constants for the Interaction between PB-$\alpha_{s1}$-Casein and $\kappa$-Casein

<table>
<thead>
<tr>
<th>Temp. (°C)</th>
<th>$P$</th>
<th>$R$</th>
<th>$R_0$</th>
<th>$R_{\alpha_{s1}}$</th>
<th>$f_{\alpha_{s1}}$</th>
<th>$Q_{\alpha_{s1}}/Q_e$</th>
<th>$x_{\alpha_{s1}}$</th>
<th>log $K$</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0.0819</td>
<td>1.457</td>
<td>1.372</td>
<td>2.737</td>
<td>0.117</td>
<td>0.413</td>
<td>0.138</td>
<td>6.806</td>
</tr>
<tr>
<td>20</td>
<td>0.0824</td>
<td>1.448</td>
<td>1.395</td>
<td>3.085</td>
<td>0.067</td>
<td>0.400</td>
<td>0.082</td>
<td>7.266</td>
</tr>
<tr>
<td>25</td>
<td>0.0765</td>
<td>1.563</td>
<td>1.492</td>
<td>3.342</td>
<td>0.082</td>
<td>0.407</td>
<td>0.099</td>
<td>7.018</td>
</tr>
<tr>
<td>30</td>
<td>0.0734</td>
<td>1.631</td>
<td>1.556</td>
<td>3.643</td>
<td>0.080</td>
<td>0.431</td>
<td>0.092</td>
<td>7.112</td>
</tr>
<tr>
<td>35</td>
<td>0.0702</td>
<td>1.707</td>
<td>1.627</td>
<td>4.155</td>
<td>0.077</td>
<td>0.451</td>
<td>0.085</td>
<td>7.223</td>
</tr>
</tbody>
</table>

Figure 1 shows the Perrin plots (1/$P$ vs. $T/\eta$) of PB conjugated CNE-$\kappa$-casein and complex between CNE-$\kappa$-casein and $\alpha_{s1}$-casein polymer. The rotational relaxation time, $\rho_h$, was calculated by the Perrin equation:\[ (1/P-1/3)=(1/P_c-1/3)(1+3\tau/\rho_h) \quad (16) \]

where $P_c$ is the limiting polarization at $T/\eta=0$, $\tau$ is the mean life time and $\eta$ is the viscosity of the solvent. The rotational relaxation times of PB conjugated CNE-$\kappa$-casein and CNE-$\kappa$-$\alpha_{s1}$-casein complex were 340 and 180 nsec, respectively.

Changes of the fluorescence polarization as a function of ANS concentration are shown in Fig. 2. Because of the short fluorescence

FIG. 1. Perrin Plots of PB-CNE-$\kappa$-Casein (●) and PB-CNE-$\kappa$-$\alpha_{s1}$-Casein Complex (▲) in 0.01 M Imidazole-HCl-0.07 M KCl Buffer (pH 7.1). Samples were excited at 346 nm.

FIG. 2. Changes of Polarization as a Function of ANS Concentration in 0.01 M Imidazole-HCl-0.07 M KCl Buffer (pH 7.1) at 37°C.

The solid lines represent ANS-labeled caseins and the dotted lines represent PB labeled caseins. ●, $\alpha_{s1}$-casein polymer; ○, $\kappa$-casein polymer; ▲, $\alpha_{s1}$-$\kappa$-casein complex.
Lifetime of ANS-protein (10–20 nsec), accurate molecular sizes of \( \kappa \)-casein polymer and \( \alpha_{\text{s1}}-\kappa \)-casein complex are not necessarily reflected the observed polarization. Then, the changes of the polarization of PB conjugated \( \kappa \)-casein polymer and \( \alpha_{\text{s1}}-\kappa \)-casein complex are also inserted in Fig. 2. Figure 3 shows the increase of the relative fluorescence intensities of caseins with increasing the concentration of ANS. These results show that although the polarization of \( \alpha_{\text{s1}} \)-casein polymer and \( \alpha_{\text{s1}}-\kappa \)-casein complex is almost invariant to ANS concentration, that of \( \kappa \)-casein polymer is decreased by the adsorption of ANS to the intermolecular hydrophobic region in \( \kappa \)-casein polymer.

In the calorimetric measurements, the observed heat, \( Q_{\text{obs}} \), and the corrected heat, \( Q_{\text{cor}} \), which is corrected for the heats of dilution of the protein solutions, are given in Table III. The reaction heat of \( \kappa \)-casein polymer and ANS is also given in Table III. From Table III, it was shown that the complex formation between \( \alpha_{\text{s1}} \) and \( \kappa \)-casein polymers was endothermic, while the reaction of \( \kappa \)-casein polymer and ANS was exothermic.

### Discussion

Nemethy and Scheraga have postulated that the endothermic nature of interaction and a positive enthalpy and entropy could be due to hydrophobic interaction in the proteins. The enthalpy and entropy changes, \( \Delta H^0 \) and \( \Delta S^0 \), for the complex formation of \( \alpha_{\text{s1}} \) and \( \kappa \)-casein polymers are both positive (Table II). Moreover, calorimetric measurements show endothermic nature for the complex formation (Table III). These suggest hydrophobic nature of the interaction between \( \alpha_{\text{s1}} \) and \( \kappa \)-casein polymers. Our suggestion is in good agreement with Payens, Clarke and Nakai.

It was found that the complex formation between \( \alpha_{\text{s1}} \) and \( \kappa \)-casein polymers led to dissociation of the \( \kappa \)-casein polymer. And it has been known that \( \kappa \)-casein is polymerized through disulfide bonds and hydrophobic bonds. If we assumed that the disulfide bonds in \( \kappa \)-casein polymer are disrupted by the complex formation, the molecular size of CNE-\( \kappa \)-\( \alpha_{\text{s1}} \)-casein complex is smaller than that of CNE-\( \kappa \)-casein alone. However, Fig. 1 shows that the rotational relaxation time of PB conjugated CNE-\( \kappa \)-\( \alpha_{\text{s1}} \)-casein complex is smaller than that of CNE-\( \kappa \)-casein. Our result is in agreement with that of Slattery and Evard, who found that the molecular weight of the complex between reduced \( \kappa \)-casein and \( \alpha_{\text{s1}} \)-casein was smaller than that of reduced \( \kappa \)-casein alone. These results suggest that the
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In the interaction between \( \alpha_s \)- and \( \kappa \)-casein polymers, hydrophobic regions in \( \kappa \)-casein polymer are dissociated by the complex formation. On the other hand, since the adsorption of ANS to proteins is known to be hydrophobic, the decrease of polarization of \( \kappa \)-casein polymer with increasing ANS concentration (Fig. 2) indicates the disruption of the intermolecular hydrophobic regions in \( \kappa \)-casein polymer due to the adsorption of ANS to these regions. However, Sugimoto et al. reported that little effect of ANS on the sedimentation coefficient of \( \kappa \)-casein was observed. This contradiction may result from the low concentration of caseins used in their experiment.

The disruption of \( \kappa \)-casein polymer may be supported by means of calorimetric measurement. The reaction heat of \( \kappa \)-casein polymer and ANS, \( Q_{\kappa-\text{ANS}} \), is considered to be represented by sum of the dissociation heat of \( \kappa \)-casein polymer, \( Q_{\text{diss}} \), and the adsorption heat of ANS, \( Q_{\text{ANS}} \).

\[
Q_{\kappa-\text{ANS}} = Q_{\text{diss}} + Q_{\text{ANS}}
\]  

(17)

Although the value of \( Q_{\text{ANS}} \) is not clear, \( Q_{\text{ANS}} \) should be negative because of the hydrophobic nature in the adsorption of ANS to proteins. Since \( Q_{\kappa-\text{ANS}} \) is positive (Table III), \( Q_{\text{diss}} \) should be positive, which indicates that the dissociation of \( \kappa \)-casein polymer occurs in the hydrophobic regions. On the other hand, the main interaction between \( \alpha_s \)- and \( \kappa \)-casein polymers is considered to be hydrophobic as described above. Nevertheless, the intramolecular hydrophobic bonds between \( \alpha_s \)- and \( \kappa \)-casein polymers is stable against ANS (Fig. 2). In order to satisfy these experimental facts, it is convenient to consider that the dissociation of \( \kappa \)-casein polymer with complex formation may be caused by the disruption of the intermolecular hydrophobic regions in \( \kappa \)-casein polymer and that this disruption may be due to the adsorption of \( \alpha_s \)-casein polymer to these regions.

If we take the dissociation of \( \kappa \)-casein polymer into consideration, the heat of complex formation, \( Q_{\alpha_s-k} \), shown in Table III is given by

\[
Q_{\alpha_s-k} = Q_{\text{diss}} + Q_{\text{ass}}
\]  

(18)

where \( Q_{\text{ass}} \) is the association heat of \( \alpha_s \)- and \( \kappa \)-casein polymers. Since \( Q_{\text{diss}} \) is considered to be positive as described above and \( Q_{\alpha_s-k} \) is \(-0.9\) mcal/g (Table III), \( Q_{\text{ass}} \) should be smaller than \(-0.9\) mcal/g. Therefore, the enthalpy change for the association between \( \alpha_s \)- and \( \kappa \)-casein polymers should be greater than \(+2.24\) Kcal/mol, assuming that the molecular weight of \( \alpha_s \)-\( \kappa \)-casein complex is \( 40 \times 10^4 \).

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