The Polymerization of Bovine $\alpha$-Casein B*

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SUMMARY

Osmotic pressure technique has been employed to investigate the polymerization of bovine $\alpha$,-casein B under different experimental conditions. Results at neutral pH indicate that the polymerization of $\alpha$,-casein B depends much more on ionic strength than on temperature. The number average molecular weight of $\alpha$,-casein B in 0.01 M KCl, pH 7, at 20° was found to be 29,400 which corresponds to the monomer, whereas the value at 0.1 M KCl corresponds to that of a trimer. Viscosity and diffusion data suggest that even though $\alpha$,-casein B is more flexible and possibly more solvated than a common globular protein, it is much more compact than a denatured protein. The results of the optical rotary dispersion study indicate that there is no gross conformational change as the protein is polymerized from monomer to trimer or tetramer.

Bovine $\alpha$,-casein, which makes up approximately 45% of the total casein components in milk (1), plays an important role in casein micelle formation. Thompson and Kiddy (2) have identified three genetic variants of $\alpha$,-casein which are known as A, B, and C according to their mobility in starch gel electrophoresis. Recently, many workers have been investigating casein interactions and micelle formation (3-10). According to their results, the set of interactions leading to casein micelle formation is intricate depending on pH, salt concentration, temperature, ratio of the casein components, and others. The purpose of this communication is to report some of our findings on the factors affecting the polymerization of bovine $\alpha$,-casein B.

EXPERIMENTAL PROCEDURE

Purified bovine $\alpha$,-casein B was prepared by the method of Waugh et al. (1) from fresh raw milk of cow homozygous for this variant.1 Lyophilized protein was first dissolved in 0.02 M potassium citrate and dialyzed against glass-distilled water. The protein solution was then deionized by means of mixed bed ion exchange resins (5). The pH of the protein solution was adjusted to approximately pH 7.6 by adding KOH. Finally, the protein solution was dialyzed against the appropriate concentration of KCl. Concentrations of protein were determined with a Zeiss PMQ II spectrophotometer by using $\epsilon_{280}$ = 10 (1).

Guanidine hydrochloride was obtained from J. T. Baker Company and was further purified by the method of Kawahara and Tanford (11). All of the other chemicals used were the best commercial grades available and were used without further purification.

The osmotic pressure was measured with a high speed membrane osmometer (model 503) manufactured by Mechronal, Inc. Optical rotatory dispersion measurements at 25° were made with a Jasco spectropolarimeter over the wave length range of 700 to 250 m$\mu$ at a protein concentration of 0.1%. Viscosities were measured with Cannon-Manning semimicroviscosimeters. The temperature variation of the water bath for the viscosity measurements was less than ±0.1°. Diffusion experiments were made in a Beckman/Spinco model H electrophoresis-diffusion instrument at 4°.

RESULTS AND DISCUSSION

Quite often, the osmotic pressure of a protein (up to moderate concentrations) can be represented by the modified van't Hoff equation. Included in this equation there is a term known as the osmotic second virial coefficient, B. Scatchard (12) related the second virial coefficient to variations in the excess chemical potentials of the components of the solution. In the limit as the protein concentration approaches zero, Scatchard's equation can be written, say for a protein, as subscript 2, in aqueous salt (KCl) as subscript 3

$$B = \frac{1000 \theta_i}{M_i} \left( \frac{Z_i^2}{4 m_i} + \frac{\beta_{23}}{2} - \frac{\beta_{2}^2 m_3}{4 + 2\beta_{2} m_3} \right)$$

where $\theta_i$ is the partial specific volume of the solvent, $Z_i$ the average net charge per protein monomer, $m_3$ the molality of KCl solution, and the $\beta$'s are derivatives of the activity coefficient, i.e. $\beta_{ij} = (\partial \ln \gamma_{ij})/\partial m_i$. The excess chemical potential of the protein component is equal to $RT \ln \gamma_2 - RT \beta_2 m_3$, where $\gamma_2$ is the activity coefficient of the protein, $\beta_2$ is obtained from the limiting slope in the osmotic pressure plot (Fig. 1), $R$ is the gas constant, and $T$ is the absolute temperature. Included in $\beta_2$ would be the excluded volume effect and the interaction between charges on different protein molecules (12). If we follow Scatchard and adopt his definition of protein component, we can interpret the osmotic interaction parameter in light of the Donnan term, $Z_i^2/4 m_i$, and $\beta_2$ because $\beta_2$ can be assumed to be zero (12, 13). The protein component, under our experimental conditions, is $\underline{2}^{14} [K_2P(OH)_7]^{14-}$ Cl$_{14}$, (see below and Reference 5) and the charge, $Z_i$, must then include the bound ions.

Fig. 1 is the osmotic pressure plot of $\alpha$,-casein B at neutral pH in 0.01 M and 0.1 M KCl at 4° and 20°. The number average molecular weight is obtained by extrapolating the straight line in the plot of $\pi/\theta_i$ with respect to $c_2$ to zero protein concentrations, and the second virial coefficient is obtained from the slope of the line in this plot. Table 1 is a summary of our experimental parameters derived from the osmotic pressure data of $\alpha$,-casein B. According to the results of Ho and Waugh (5), $\alpha$,-casein binds 28 hydroxide ions and 5 potassium ions per monomer with a molecular weight of 27,800 under our experimental conditions. Conse-

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...the average net charge per monomer of \( \alpha_s \)-casein, under the present conditions, is \(-23\) protonic charges. The Donnan term at each concentration of KCl can now be calculated. The number average molecular weight of \( \alpha_s \)-casein B in 0.01 M KCl is about 30,000 which is the molecular weight of the monomer of this protein as determined by a number of physicochemical techniques (1, 10, 14). In the presence of 0.1 M KCl, the molecular weight of \( \alpha_s \)-casein corresponds to the "trimer" and "tetramer." The degree of polymerization appears to depend much more on the ionic strength than on temperature. Secondly, \( \beta_{20} \), which probably measures the tendency to polymerize, increases with salt concentration. The excess free energies, \( \beta_{20} \), are given in Table I. These values vary much more with salt concentration than with temperature. In addition, Ho and Waugh (5) reported that bovine \( \alpha_s \)-casein can be deionized by means of mixed bed ion exchange resins and that the deionized \( \alpha_s \)-casein is a colloid. The deionized \( \alpha_s \)-casein colloidal suspensions can be converted to small polymers or even to monomer by adding alkali or acid and this process is completely reversible.

These results suggest that the electrostatic interactions play an important role in the polymerization of \( \alpha_s \)-casein.²

Our next concern is the shape of \( \alpha_s \)-casein B in the monomeric and polymeric states. Since the intrinsic viscosity is a measure of the specific volume (in units of milliliters per g) of the domain of a macromolecule in solution, its measurement can give information about the gross conformation of the macromolecule in solution (15). Fig. 2 is a summary of our viscosity data. In the presence of 0.01 M KCl at pH 7.1, the intrinsic viscosities of the monomer of \( \alpha_s \)-casein vary from 11.8 ml per g to 10.2 ml per g as the temperature is increased from 4.9° to 37°. In the presence of 0.1 M KCl at pH 7, the intrinsic viscosities of the trimer or tetramer vary from 9.3 ml per g to 7.7 ml per g from 4° to 20°. It is well known that the common globular proteins are neither highly solvated nor very asymmetric and that their intrinsic viscosities vary from 3.3 to 4.0 ml per g (15). We have also carried out a diffusion study of \( \alpha_s \)-casein B in 0.1 M KCl at pH 7.08. The diffusion constant, \( D_{20,w} \), is \( 3.7 \times 10^{-9} \) cm² per sec, which is a factor of two less than that of the common globular proteins of similar molecular weight (15). Both the diffusion and the viscosity results suggest that \( \alpha_s \)-casein B is not a spherical molecule and could be much more solvated than a globular protein.

Tanford, Kawahara, and Lapanje (16) have shown that proteins, in the presence of 6 M guanidine hydrochloride, are true random coils, retaining no elements of their original native conformation. We have carried out a viscosity study of \( \alpha_s \)-casein B in 6 M guanidine hydrochloride. The intrinsic viscosity of this study at pH 7.1 and 20° is 19.2 ml per g (see Fig. 2), which is about twice the value in the absence of guanidine hydrochloride.

Payens and Schmidt (9) concluded from their results that hydrophobic interactions are mainly responsible for the association of \( \alpha_s \)-casein C. They used the Archibald ultracentrifuge method to determine the molecular weight. From the concentration dependence of the weight average molecular weight over the temperature range of 2-14°, they evaluated the thermodynamic parameters from the consecutive association constants which give positive enthalpy and entropy of association indicating the formation of hydrophobic bonds between the monomers. They assumed that the deviations from ideality depend only upon association. In addition, they used a different genetic variant of \( \alpha_s \)-casein, namely \( \alpha_s \)-casein C, and their experimental conditions were different from ours.

### Table I

<table>
<thead>
<tr>
<th>KCl</th>
<th>pH</th>
<th>Temperature</th>
<th>M₁</th>
<th>B</th>
<th>( \bar{Z}_v/\bar{m}^2 )</th>
<th>( \beta_{20}^b )</th>
<th>( \nu^b )</th>
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<td>20°</td>
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<td>-20,450</td>
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<td>-0.53</td>
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<tr>
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<td>20°</td>
<td>91,700</td>
<td>0</td>
<td>1,322</td>
<td>-2,644</td>
<td>-0.56</td>
</tr>
</tbody>
</table>

*The values were calculated on the basis of the monomer molecular weight of \( \alpha_s \)-casein as 27,300 (1, 14) and the net protonic charges as \(-23\) (see text and references).

**The values were calculated on the basis of a protein concentration of 1% with a molecular weight of 27,300.
ide. Consequently, we may conclude that α-casein B does not exist as a random coil characterizing the structure of a denatured protein and that the molecule of α-casein is relatively compact as compared to a denatured protein.

The next question that we would like to answer is whether there is a conformational change when α-casein is polymerized from monomer to trimer or tetramer. Since the optical rotation is highly sensitive to solvent and other environmental perturbations (17), the measurement of optical rotation offers a means to detect the conformational change in the polymerization of α-casein. It is a common practice to treat the optical rotatory dispersion data of proteins by means of the Moffitt-Yang equation (18). In the application of this equation to dispersion data, a critical problem is the selection of an optimal value for λ0. Our experimental results were calculated with an IBM 7090 computer, with the use of a Fortran program based on the statistical evaluation methods of Sogami, Leonard, and Foster (19). We used 119 as the mean residue molecular weight in our calculation. The best λ0 for α-casein B in 0.01 M KCl at pH 7.1 is 230 μm and the corresponding values of α0 and β0 are -306.7 ± 6.6 and +17.3 ± 2.8, respectively. In 0.1 M KCl at pH 7.1, the best λ0 is 231 μm and the corresponding values of α0 and β0 are -393.6 ± 1.9 and +25.9 ± 1.4, respectively. If we choose λ0 to be 212 μm, the best values for α0 and β0 are -404.8 ± 10.0 and -74.4 ± 16.1, respectively, for α-casein B in 0.01 M KCl at pH 7.1, and the corresponding values in 0.1 M KCl are -474.6 ± 3.0 for α0 and -39.3 ± 4.9 for β0. The values for β0 are quite small which suggest that there is no or very little α-helix in bovine α-casein B (17).4 There is about 16% difference in α0 values (in the case of λ0 = 212 μm) as α-casein is polymerized from monomer to trimer. This difference in α0 values could reflect the fact that there is a difference in solvent-protein interactions between the monomer and the polymer of α-casein B.

We are continuing our investigation on the polymerization of α-casein so as to cover a broader range of temperature, ionic strength, and pH. A detailed report will be given at a later date.

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