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The Association of Bovine β-Casein. The Importance of the C-Terminal Region†

Gillian P. Berry and Lawrence K. Creamer*

ABSTRACT: Bovine β-casein exists in the monomer form in solution (pH 6.5, 0.1 *M* NaCl, 0.5% w/v) at low temperatures, but associates to form polymers at higher temperatures. Gel filtration chromatography at 36° showed that the polymer is large with a hydrodynamic size greater than that of a globular protein with a mol wt of 1.34×10^6. Removal of two C-terminal amino acids per molecule decreased the proportion of polymer in the solution, although the chromatographic behavior of the modified β-casein monomers and polymers was retained. Removal of a 20 amino acid peptide from the C terminus of the β-casein completely destroyed its ability to form polymers and removed the 8-anilino-1-naphthalenesulfonate binding site. However, deletion of segments of the protein from the N terminus did not decrease the ability of the modified β-casein to associate, nor did it affect the 8-anilino-1-naphthalenesulfonate binding site greatly. It seems likely that all, or some, of the 20 amino acids at the C terminus are responsible for the associative behavior of β-casein, possibly by the direct participation of their side chains in hydrophobic bond formation. However, removal of the C-terminal peptides may have disrupted the spatial structure of the native protein so that it could no longer associate normally.

The association of proteins has been a subject of interest for a considerable length of time. Studies on chymotrypsin, insulin, hemoglobin, and other crystalline proteins, for example, have shown which amino acids are at the site of association. The association of β-casein of bovine milk is reversible (Sullivan et al., 1955), being dependent on temperature and to a lesser extent on pH, ionic strength, and concentration. A further characteristic of this association is that apparently only the monomer (mol wt 24,000) and a polymer (mol wt ~600,000) co-exist (Waugh et al., 1970; Schmidt and Payens, 1972).

The monomer β-casein consists of 209 amino acids in a single chain with no cystine cross-links (Ribadeau-Dumas et al., 1972). It is a hydrophobic protein whose net charge of −13 at pH 7.0 resides in the N-terminal 50 amino acids. The remainder of the protein contains few hydrophilic residues and there is a high proportion of proline residues in the sequence. The spatial structure of the monomer protein is not known with certainty although optical rotatory disper-

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sion measurements at low temperature were explained in terms of the presence of poly(L-proline) II structure (Garnier, 1966; Herskovits, 1966). Viscosity (Noelken and Reibstein, 1968), nuclear magnetic resonance (Leslie et al., 1969; Evans et al., 1971), and titration studies (Creamer, 1971) suggest that most of the hydrophobic and all of the ionizable residues of both the monomer and the polymer are accessible to the solvent. A number of investigators have concluded that a protein conformational change takes place concomitant with aggregation (e.g. Garnier, 1966). However, there is no clear evidence of any conformational change which precedes or is a prerequisite for association.

The association of β-casein at constant temperatures has been studied using light-scattering and ultracentrifugation (Waugh et al., 1970; Payens and van Markwijk, 1963; Schmidt and Payens, 1972). A brief report indicated that removal of the three hydrophobic C-terminal amino acids of β-casein diminished the ability of β-casein to associate as shown by ultracentrifugation (Thompson et al., 1967). In this investigation β-casein peptides have been isolated and their associative properties measured by column chromatography and by the use of a fluorescent probe (Edelman and McClure, 1968; Stryer, 1968). It has thereby been possible to determine the importance of different segments of the protein sequence in the association of β-casein with itself and with α_{s1}-casein.

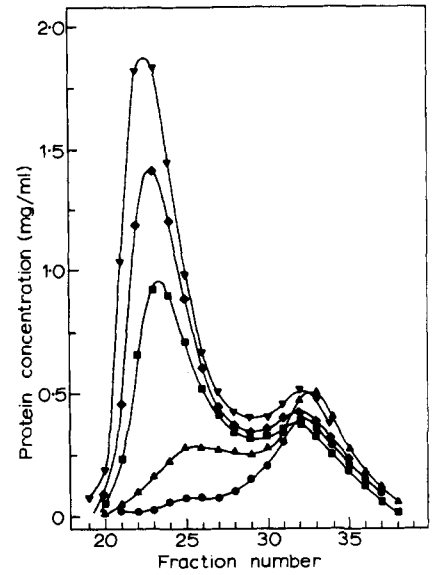

FIGURE 1: The effect of sample concentration on gel filtration pattern. The samples contained 6.5 mg (●), 13 mg (▲), 25 mg (■), 35 mg (♦), and 45 mg (▼) of β-casein. The samples were chromatographed on a 15 × 2 cm column of Sephadex G-10 followed by a 25 × 2 cm column of Sepharose 4B. The buffer was pH 6.65, 0.1 *M* NaCl-0.003 *M* NaN_3-0.025 *M* imidazole, and the elution rate was 35 ml/hr. Protein concentration was estimated from the 280-nm absorbance of the eluted fractions.

Materials and Methods

β_A[1]-, TS-A-, R-, and γ_A-caseins (designated as β-casein, β-casein 106–209, β-casein 108–209, and β-casein 29–209) were isolated and purified as described by Creamer (1975).

β-I (designated as β-casein 1–189) was separated from the products of the action of rennin (EC 3.4.4.3) on β-casein A[1] in solution at 10°, pH 6.5, by DEAE column chromatography (Creamer et al., 1971).

The C-terminal amino acids were removed from β-casein with carboxypeptidase A (Sigma Chemical Co.) using the procedure of Ambler (1972). After reaction, the solution was heated to 80° for 2 min and the protein was precipitated with acetic acid (30% v/v). The supernatant was assayed for free amino acids using a Locarte Mark IV amino acid analyzer to estimate the extent of reaction. The precipitated protein (designated β-casein 1–207) was washed, dissolved in buffer (0.1 *M* NaCl-0.025 *M* imidazole-0.002 *M* NaN_3 adjusted to pH 6.65 at 30°), and used for analysis by gel filtration and by fluorescence. The purity of the protein preparations was checked by gel electrophoresis. This was carried out using the disc system of Ornstein (1964) and Davis (1964) with 6 *M* urea in the gels and the samples, but not in the electrode chamber buffers.

Gel Filtration. The sample (in 3 ml of buffer solution unless otherwise stated) was initially passed down a 15 × 2.0 cm column of Sephadex G-10 at 36° so that the protein was in equilibrium with the buffer components. It was then passed down a 25 × 2.0 cm column of Sepharose 4B at 36°. The column effluent fractions were collected and their absorbance at 280 nm determined. When a mixture of proteins was chromatographed, the distribution of the individual proteins was determined by gel electrophoresis of each column fraction followed by densitometry of the gel patterns. The column was calibrated using a bacterial suspension of *Streptococcus cremoris* R_1 (for the void volume), thyroglobulin, γ-globulin, chymotrypsinogen, bovine serum albumin, myoglobin, and α-lactalbumin as described by Andrews (1970).

Fluorescence Measurements. The temperature dependence of the fluorescent intensity of Ans[1] in the presence of β-casein or one of its derivatives was measured as described previously (Creamer and Wheelock, 1975) using a Perkin-Elmer MPF 2A spectrophotofluorimeter. The sample solutions contained 8×10^{-5} *M* Ans and 0.95×10^{-5} *M* protein (ca. 0.25 mg/ml).

Results

β-Casein. The association properties of β-casein were examined at 36°, pH 6.65, in 0.1 *M* NaCl using columns of Sephadex G-10 and Sepharose 4B. The resultant elution patterns of several different sample concentrations are shown in Figure 1. When small quantities (<5 mg) of β-casein were chromatographed only a single peak was observed. As the quantity of β-casein was increased a second peak, which eluted before the previous peak, became apparent. Further increases in the quantity of β-casein in the sample increased the size of the front peak without altering its position greatly, while the rear peak did not significantly alter its height or position. Chromatography of a sample of the front peak gave rise to two similar peaks.

When a series of proteins of known molecular weight was passed down the columns, thyroglobulin (dimeric mol wt 1,340,000) eluted slightly behind the position of the front β-casein peak, and bovine serum albumin (mol wt 67,000) eluted at the same position as the rear β-casein peak; γ-globulin (mol wt 205,000; Andrews, 1970) eluted at an intermediate position. These results show that the front peak is a β-casein polymer, and under the conditions of these experiments it is hydrodynamically similar to a globular protein with a mol wt greater than 1.34×10^6. Ultracentrifuge studies by Waugh et al. (1970) showed that polymers had a mol wt of 650,000 or 820,000, depending on solution condi-

[1] Abbreviation used is: Ans, ammonium 8-anilino-1-naphthalenesulfonate.

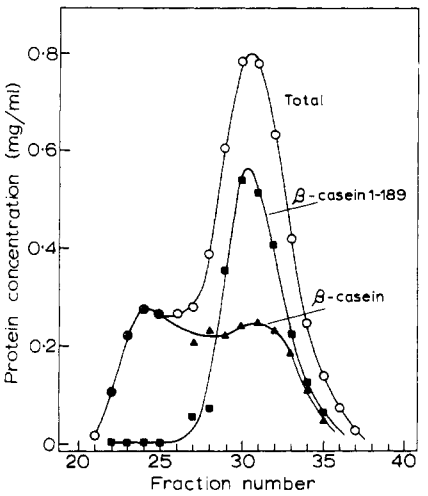

FIGURE 2: Gel filtration of β-casein and β-casein 1-189 in admixture. The sample contained 10 mg of each protein. Total protein (○) was estimated from 280-nm absorbance, and the elution pattern for each protein (▲ and ■) was determined from densitometry of the gel electrophoresis patterns of each fraction.

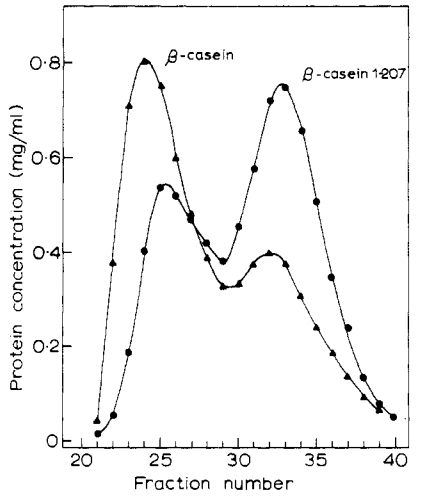

FIGURE 3: Gel filtration of β-casein 1-207. A sample (●) of 25 mg was used. A chromatogram of β-casein (▲) run under the same conditions is also shown.

tions. The second peak has elution properties similar to those of the casein monomer at low temperature (Downey and Murphy, 1970). These present results support the suggestion of Schmidt and Payens (1972) that β-casein association is reversible and of the detergent micelle type where the only species are the monomer and a single species of large polymer.

β-Casein 1-189. When β-casein 1-189 was chromatographed only a single peak was obtained. It had the same elution volume as monomeric β-casein. When β-casein was mixed with β-casein 1-189 and the mixture chromatographed, gel electrophoresis of the eluted fractions showed that β-casein and β-casein 1-189 behaved independently, i.e. β-casein eluted as two peaks while the β-casein 1-189 still eluted as a single peak with the same elution volume as before (Figure 2). Similar experiments were carried out using α_{s1}-casein and β-casein 1-189. The α_{s1}-casein eluted as a single fast-moving peak (Creamer and Berry, 1975), while the β-casein 1-189 eluted as before. These results suggested that the C-terminal 20 amino acids are possibly important to the monomer protein conformation and that

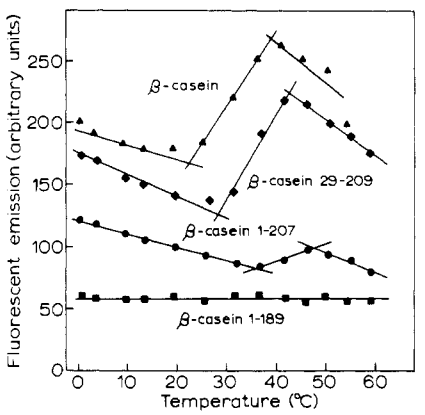

FIGURE 4: Effect of temperature on Ans fluorescence in the presence of modified β-caseins. Protein concentrations were 0.95×10^{-5} *M* (ca. 0.25 mg/ml) and the buffer was that used for column chromatography. Ans concentration was 8×10^{-5} *M*: (▲) β-casein; (●) β-casein 1-207; (■) β-casein 1-189; and (♦) β-casein 29-209.

their removal disrupts the native protein conformation and thus destroys the ability of the protein to associate. A second, more likely suggestion was that some or all of the 20 amino acids, which are mostly hydrophobic, are themselves involved in the associations that β-casein undergoes with other proteins.

β-Casein 1-207. Treatment of β-casein with carboxypeptidase released valine and isoleucine in the molar ratio of 1 to 1.1. The C-terminal sequence of β-casein is Pro-Ile-Ile-Val-COOH and it is likely that only the last two C-terminal amino acids were released. Gel electrophoresis in the presence of 6 *M* urea of the treated β-casein showed only a single band which had the same electrophoretic mobility as β-casein.

When β-casein 1-207 was chromatographed on columns of Sephadex G-10 and Sepharose 4B, two peaks were obtained (Figure 3) as with β-casein. However, the relative sizes of the front and rear peaks were quite different with the rear peak for β-casein 1-207 higher than that for β-casein, indicating that the extent of association was less. The front peak had a similar elution position as the front peak of the β-casein chromatogram, indicating that the polymer species of β-casein 1-207 is about the same size as that of β-casein.

β-Casein 29-209. This protein, often referred to as γ-casein, was more difficult to study as it is not very soluble at pH 6.65, and it is difficult to obtain uncontaminated with β-casein 106-209 and β-casein 108-209. However, when β-casein 29-209 was chromatographed in admixture with β-casein it behaved interchangeably with β-casein, i.e. it was eluted as two peaks and the ratio of β-casein to β-casein 29-209 was constant across the whole chromatogram. When β-casein 29-209 was mixed with α_{s1}-casein and chromatographed, the two proteins were also eluted concurrently. Similar results were obtained with β-casein 106-209 and 108-209.

Fluorescence Measurements. When a solution of β-casein was mixed with Ans at pH 6.6 and 0.1 *M* NaCl, and the fluorescent emission at 465 nm was measured as a function of temperature, it was found that from 2 to 20° the emitted fluorescence decreased, from 25 to 40° it increased, and from 40 to 55° it again decreased. As with some other proteins (Brand and Gohlke, 1972) it has been suggested (Creamer and Wheelock, 1975) that the polymer form of β-casein causes a greater enhancement of Ans fluorescence

emission than the monomer, and that this is superimposed onto the normal steady decrease in fluorescence emission with increasing temperature, probably caused by the greater thermal agitation and decreased lifetime of the fluorescent species (Gally and Edelman, 1964). Thus, at low temperature (<20°) and at high temperature (>40°), the increase in quenching with increasing temperature was the predominant effect, while between 25 and 40° the increase in the proportion of β-casein in the polymer form with increasing temperature was the dominant effect.

Removal of C-terminal amino acids decreased the ability of the protein to enhance Ans fluorescence at low temperature (Figure 4). The removal of two amino acids (i.e. β-casein 1–207) had less effect than the removal of 20 (i.e. β-casein 1–189). The increase in fluorescence with increasing temperature is retained to a small degree by β-casein 1–207 but is lost by β-casein 1–189. It is likely that with the loss of the two amino acids the monomer and the polymer binding sites are disrupted and that with the removal of 20 amino acids the monomer binding site is lost.

Removal of the N-terminal 28 amino acids from β-casein did not greatly affect its ability to cause Ans to fluoresce or to alter its fluorescence with temperature.

Discussion

The interpretation of the present results depends partly on the assumptions made about the three-dimensional structure of β-casein. Waugh et al. (1970), in their micelle model building, assumed that a proportion of the β-casein molecule was in a well-defined structure under the conditions prevailing in milk (37 to 40°, $a_{Ca^{2+}} \sim 0.001$ *M*, pH 6.7, etc.). Other studies, however, suggest that β-casein has a much less ordered structure (Noelken and Reibstein, 1968; Leslie et al., 1969; Evans et al., 1971). If it is considered that perhaps 30% of the β-casein molecule is in a well-ordered structure, then the loss of a peptide of up to 20 amino acids from the C terminal of the protein could disrupt this structure. An alternative explanation would be that a few amino acids at the C terminus are responsible for both the enhanced Ans fluorescence and for the associative behavior of β-casein at 36°.

The present results do not allow a differentiation between these possibilities. Both of these patterns of associative behavior have been described before; Teller (1973) outlines a number of instances where C- or N-terminal sequences are important, and the studies on chymotrypsin (Neet et al., 1974) show the importance of the active-site amino acids in association. Tanford (1973) has estimated that each CH_2 residue transferred from a nonpolar to a polar environment involved a free energy change of 8–900 cal/mol at 25° and thus the transfer of all the CH_2 and CH_3 groups of the two C-terminal amino acids might involve a change of 5 to 6 kcal/mol. This is probably sufficient to explain the difference between the behavior of β-casein and β-casein 1–207 (Figures 3 and 4). The marked increase in Ans fluorescence in the presence of β-casein polymers (Figure 4) suggests that polymerization is accompanied by an increase in the hydrophobic region accessible to the Ans. The decrease in Ans fluorescence on removal of the two C-terminal amino acids suggests that the Ans binding site is removed concurrently with these amino acids.

Regardless of the detailed explanation of the present results, it is clear that a peptide sequence containing between 2 and 20 amino acids at the C terminus of β-casein is critical to the stability of the polymers that β-casein forms with both α_{s1}- and β-casein.

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