

# Thermal and Alkaline Denaturation of Bovine $\beta$ -Casein

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The secondary structure of bovine  $\beta$ -casein was characterized using circular dichroism (CD) and FTIR spectroscopies under physiologically relevant conditions. Analytical ultracentrifugation technique was used to follow the highly temperature, pH and concentration dependent self-association behavior. CD measurements provide convincing evidence for short segments of polyproline II-like structures in  $\beta$ -casein in addition to a wide range of secondary structure elements, such as 10–20%  $\alpha$ -helix, ~30% turns, 32–35% extended sheet. Results obtained at extreme pH (10.5) revealed structural destabilization in the monomeric form of the protein. At least four distinct structural transitions at 10, 33, 40 and 78°C were observed at pH 6.75 by CD analysis, compared to only two transitions, 26 and 40°C, at pH 10.5. Calculations from analytical ultracentrifugation suggest that the transitions at lower temperature ( $\leq 30^\circ\text{C}$ ) occur primarily in the monomer. It is hypothesized that the transition at 10°C and neutral pH may represent a general conformational change or cold denaturation. Those middle ranged transitions, i.e. 33 and 40°C are more likely the reflection of hydrophobic changes in the core of  $\beta$ -casein. As  $\beta$ -casein undergoes self-association and increases in size, the transition at higher temperature (78°C) is perhaps caused by the apparent conformational change within the micelle-like polymers. It has been shown that  $\beta$ -casein binds the hydrophobic fluorescent probe ANS with high affinity in much similar fashion to molten globular proteins. The effect of urea denaturation on the bound complex effectively supports this observation.

**KEY WORDS:**  $\beta$ -casein; denaturation; polyproline II; secondary structure; self-association; unfolding.

## 1. INTRODUCTION

The caseins are a group of phosphoproteins whose biological function include efficiently transporting inorganic calcium and phosphate to the neonate (Farrell, 1999). Inherent in this process are protein–protein interactions, protein–protein self-association and protein–salt interactions. The collective result of these interactions leads to the formation of stable colloidal complexes termed casein micelles (Swaisgood, 1992). In some species, notably human milk  $\beta$ -casein is the major calcium binding protein (Greenberg *et al.*, 1984).  $\beta$ -Casein has long been

the subject of research not only because of its importance in the formation and stabilization of food emulsions (Dickinson *et al.*, 2001), but also because of its implication in functionality studies such as milk allergens (Chatchatee *et al.*, 2001) heart disease (McLachlan, 2001) and tumor apoptosis (Grusby *et al.*, 1990). The molecular basis for the known and imputed functions of  $\beta$ -casein remains elusive, even

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<sup>3</sup> Abbreviations: CD, circular dichroism; FTIR, fourier transform infrared spectroscopy; ANS, 1-anilino-8-naphthalene sulfonic acid; ORD, optical rotatory dispersion; BSA, bovine serum albumin; ACS, american chemical society; ESI-MS, electron-spray ionization mass spectrometry, PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid); CAPS, 3-(cyclohexylamino) propane-sulfonic acid; PBS, phosphate buffered saline; MW, molecular weight; UV-vis, ultra violet-visible; SDS, sodium dodecyl sulfate; PPII, polyproline II; *r.m.s.*: root mean square deviation; MG, molten globule.

though its primary structure, molecular weight and degree of posttranslational modification are well documented (Eigel *et al.*, 1984). The cause of this uncertainty lies in the controversial nature of its secondary and tertiary structure.

It is long known (Andrews *et al.*, 1979; Schmidt and Payens, 1976; Tai and Kegeles, 1984) that  $\beta$ -casein undergoes an endothermic self-association that reaches a limiting size depending on the ionic strength. The critical micelle model (Takase *et al.*, 1980; Waugh *et al.*, 1970) has been applied to describe this highly concentration and temperature dependent process. It has been difficult, however, to obtain precise and detailed information on its three-dimensional structure because of, primarily, this aggressive self-association behavior. Under physiological condition (aqueous solution, neutral pH and low ionic strength),  $\beta$ -casein was originally predicted (Noelken and Reibstein, 1968) as a random coil with little or no regular secondary structure (10%  $\alpha$ -helix). Recently published CD (Caessens *et al.*, 1999) and Raman optical activity studies (Syme *et al.*, 2002) concurred with this assumption. Holt and Sawyer proposed (Holt and Sawyer, 1993) the "rheomorphic" hypothesis and suggested that all structures in caseins are completely flexible and form in response to flow. Previous analyses by optical rotatory dispersion (ORD) (Garnier, 1966; Herskovits, 1966), CD (Creamer *et al.*, 1981; Graham *et al.*, 1984) and Raman (Byler *et al.*, 1988), however, argued for possible existence of defined secondary structural elements in  $\beta$ -casein.

Recently, we reported (Farrell *et al.*, 2001) thermal-induced changes in the secondary structure of  $\beta$ -casein. In this work, we study the loss of structural integrity with concomitant loss of self-association of  $\beta$ -casein by applying three modes of denaturation: temperature, alkaline and urea. The results show that extreme pH (10.5) not only significantly decreases self-association of  $\beta$ -casein but also affects its secondary structural constituents.

## 2. MATERIALS AND METHODS

### 2.1. Materials

1-Anilino-8-naphthalene sulfonic acid (ANS) of high purity was purchased from Molecular Probes (Eugene, OR). Fatty acid free bovine serum albumin (BSA) crystalline was obtained from Calbiochem Co. (Darmstadt, Germany). All other

reagents used were of analytical grade or 'ACS certified' from Sigma (St. Louis, MO). Bovine  $\alpha$ -lactalbumin was prepared in house (Farrell *et al.*, 2002). Isolation and purification of  $\beta$ -casein have been presented in detail elsewhere (Thompson, 1966). The genetic variant used in this work was  $\beta$ -casein A<sup>1</sup> as determined by electrospray ionization mass spectrometry (ESI-MS) (Qi *et al.*, 2004).

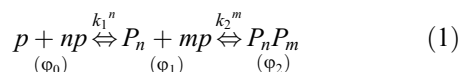
### 2.2. Circular Dichroism (CD) Spectroscopy

Far-UV CD experiments were carried out with  $0.17 \pm 0.02$  mg/ml ( $\sim 7.20$   $\mu$ M)  $\beta$ -casein in a 2 mM PIPES (piperazine-*N,N'*-bis(2-ethanesulfonic acid)), 4 mM KCl buffer at pH 6.75 or in 33 mM potassium phosphate buffer at pH 6.75 (50 mM ionic strength). CAPS (3-(cyclohexylamino) propanesulfonic acid) was titrated with NaOH and adjusted with KCl to yield buffer of pH 10.5 and 50 mM ionic strength. Homopolypeptides (poly-L-proline ( $n=4$ ), poly-glutamate (MW=750–1500) were used at concentrations of 0.50–0.70 mg/ml. Successive measurements in the far UV region, 190–250 nm, were completed with overlapping samples at 2–70°C. All solvents for CD measurements were first filtered through a Millipore 0.22  $\mu$ m pore filter. Dissolved protein and peptide samples were filtered through a 0.45  $\mu$ m pore regenerated cellulose filter. Concentrations of  $\beta$ -casein were calculated from absorbance spectra obtained on a Beckman DU-7 UV-Vis spectrophotometer (Palo Alto, CA), using an extinction coefficient of  $0.465$  l g<sup>-1</sup> cm<sup>-1</sup> (Eigel *et al.*, 1984). Concentrations of peptides were calculated from nitrogen values supplied by the manufacturer (Farrell *et al.*, 2002). CD spectra were recorded on an Aviv model 60DS spectrophotometer (Aviv Associates, Lakewood, NJ) using cells of appropriate path lengths and a scan time of 4.0 s/nm. The jacketed cells were attached to a circulating constant temperature bath. The time for equilibrating samples was calculated to be 30 min for a 30°C change in the bath temperature. All spectra are corrected for solvent contributions and are expressed in units of mean residue ellipticity (far-UV) (deg cm<sup>2</sup> dmole<sup>-1</sup>) versus wavelength. Analysis of peptide secondary structure from CD spectra was completed using the CONTIN procedure of Provencher and Glöckner (1981) using the Sreerama and Woody (1993) database.

### 2.3. Non-linear Regression Analysis

We have used a series of equations derived from the thermodynamic linkage theory (Wyman, 1964) in previous studies on protein–protein interactions (Alaimo *et al.*, 1999), ligand binding (Farrell *et al.*, 1988) and enzyme kinetics (Farrell *et al.*, 1990). These equations have been further extended to analyze many physical chemical reactions and their derivation has been presented in some detail elsewhere (Rusling and Kumosinski, 1996). By their nature, they are a series of saturation binding isotherms with exponential functions to allow cooperativity (Farrell *et al.*, 1990).

It has been found that characteristic changes in the degree of self-association of  $\beta$ -casein occur as a function of temperature, salt and protein concentration (Andrews *et al.*, 1979; Schmidt, 1979). To correlate changes in experimental observable, such as absorbance, ellipticity ( $\theta$ ) and fluorescence intensity ( $F$ ) with these well-documented associations, we initially made the following assumptions based in part on Wyman's linkage function theory as written for protein–protein interactions (Alaimo *et al.*, 1999). We assumed two classes of association sites for the observed biphasic behavior. For protein–protein interactions, we next assumed that the following equilibria occur:



where  $p$  is the unbound protein,  $n$  and  $m$  are the apparent number of moles aggregated to species  $P_n$  and  $P_n P_m$ , and  $\varphi_0$ ,  $\varphi_1$  and  $\varphi_2$  are the measurable physical properties of the species. For this study, both  $\varphi_1$  and  $\varphi_2$  are relative to  $\varphi_0$ . The above stoichiometry can be expressed according to the following:

$$\varphi_{\text{app}} = \varphi_0 f(p) + \varphi_1 (f(P_n) - f(P_n P_m)) + \varphi_2 f(P_n P_m) \quad (2)$$

where  $\varphi_{\text{app}}$  is the apparent observable at a given protein concentration ( $P_T$ ),  $f(i)$  are the protein fractional components of species  $i$ . Incorporation of the apparent equilibrium constants ( $k_1^n$  and  $k_2^m$ ) as defined by Eq. (1) into Eq. (2) yields the following where  $P^n$  and  $P^m$  are the concentrations of the associated species  $P_n$  and  $P_n P_m$  respectively:

$$\varphi_{\text{app}} = \frac{\varphi_0}{1 + k_1^n P^n} + \frac{\varphi_1 k_1^n P^n}{1 + k_1^n P^n} + \frac{(\varphi_2 - \varphi_1) k_2^m P^m}{1 + k_2^m P^m} \quad (3)$$

It should be noted that the above expression is valid for sequential binding only, *i.e.*, assuming  $k_1^n \gg k_2^m$  and  $k_1^n \gg 1$ ;  $n$  sites saturate prior to the binding of  $m$  sites on the protein; and for simplicity, states  $n$  and  $m$  do not interact. Moreover, for  $n$  or  $m$  greater than one,  $k_1^n$  and  $k_2^m$  represent an average value for each class of the  $n$  or  $m$  binding sites. The terms  $\varphi_i$  are equivalent to the maximum predicted observable ( $\varphi_{\text{max}}$ ).

Since the total protein concentration remains constant throughout all experiments and the free and associated protein concentrations at each state are required, solving directly for these constants is difficult. In addition, changes in  $\varphi$  may not necessarily relate to actual changes in the associated state but may reflect changes in the monomeric state. In the analysis of the CD data, it became clear that the most important changes in  $\theta$  were with temperature rather than concentration. The temperature-dependent transitions were fitted with these equations. Our primary interest here is to compare transition mid-points, we therefore semi-empirically substitute  $T$  for  $P$  in Eq. (3), then the following relationship holds true:

$$\theta_{\text{app}} = \frac{\theta_0}{1 + k_1^n T^n} + \frac{\theta_1 k_1^n T^n}{k_1^n T^n} + \frac{(\theta_2 - \theta_1) k_2^m T^m}{1 + k_2^m T^m} \quad (4)$$

This equation is closely related to the linkage between temperature and conformational changes as derived by Wyman (1964). The application of this equation is not *a priori* but occurs after a series of curve fitting attempts. The empirical meaning of the terms is as follows:  $\Theta_i = \Theta_{\text{max}}$  or  $\Theta_{\text{min}}$ , the maximum or minimum extrapolated  $\Theta$  at infinite  $T$ , and  $k_i$  is the half point of the transition. The term  $n$  may reflect a delay in onset, cooperativity, or could just be a fitting function (Farrell *et al.*, 1990). All fitting routines were accomplished by fixing the values of  $n$  and  $m$  and calculating the best least squared fit for the optimum evaluation of  $k_1$  and  $k_2$  values. The  $n$  and  $m$  values were then fixed to new integer values and the whole procedure reiterated. The  $n$  and  $m$  values, which yielded the minimum root-mean-square and lowest error values for  $k_1$  and  $k_2$  were then reported following the procedures previously

reported for ligand binding (Farrell *et al.*, 1988) and enzyme kinetics (Farrell *et al.*, 1990).

#### 2.4. Fourier Transform-Infrared (FT-IR) Spectroscopy

For FT-IR measurements, 3–5 mg of  $\beta$ -casein ( $3.1 \pm 0.3\%$ ) or polyproline peptides ( $3.5 \pm 0.1\%$ ) samples were dissolved in PIPES buffer for pH 6.75 or CAPS buffer for pH 10.5 at ionic strength of 50 mM. Spectra were recorded at 5, 10, 25, 35, 50 and 70°C using a Nicolet Nexus 670 FT-IR spectrometer (Madison, WI) equipped with Nicolet Omnic software. Data collection was carried out after a nitrogen purge of the sample chamber. Samples were introduced in a temperature controlled demountable cell with  $\text{CaF}_2$  windows separated by a 6  $\mu\text{m}$  Mylar spacer (Graseby Specac, Smyrnce, GA). Each spectrum consists of 1024 double-sided interferograms, co-added, phase-corrected, apodized with a Happ–Genzel function, and fast-Fourier transformed. Normal resolution was one data point every  $0.5\text{ cm}^{-1}$ . Secondary structural elements were derived from the amide I envelope by nonlinear regression fitting of Gaussian peaks to the original spectra. The iterative curve-fitting procedure has been described in detail by Kumosinski and Unruh (1996). Data were reported as the sum of assigned spectral elements gathered from a 14-protein database in  $\text{H}_2\text{O}$ .

#### 2.5. Fluorescence Spectroscopy

Fluorescence spectra were recorded with a Perkin–Elmer LS-50B bioluminescencemeter in a 1-cm path length quartz cell at room temperature (20–24°C). ANS binding curves were generated by titrating increased amount of ANS into a fixed concentration of protein (5.0  $\mu\text{M}$ ) in either PIPES buffer, pH 6.75 ( $I=50\text{ mM}$ ) or CAPS buffer, pH 10.5 ( $I=50\text{ mM}$ ). An excitation wavelength of 380 nm was used, and fluorescence emission was recorded from 400 to 600 nm. All the spectra were corrected for solvent and protein contributions to ANS fluorescence. The apparent association constants ( $K_a$ ) were calculated by plotting  $F$  (fluorescence intensity) vs. ANS concentration and fitted with Eq. (3). Although an uncertainty is highly likely, the apparent  $K_a$  values determined in this fashion serve as a diagnostic tool to distinguish folded protein from

those proteins may share similar properties as the molten globule state (Bailey *et al.*, 2001).

For urea titration experiments, proteins were prepared as follows.  $\beta$ -casein was used at 5.0  $\mu\text{M}$  and ANS at 40  $\mu\text{M}$  in PIPES buffer, pH 6.75. BSA was prepared in (phosphate buffered saline) PBS, pH 7.0. Molten globule form of  $\alpha$ -lactalbumin was prepared in 0.1 M NaCl, pH 2.0. Both BSA and  $\alpha$ -lactalbumin were used at 1  $\mu\text{M}$  and ANS at 50  $\mu\text{M}$ . Protein–ANS solutions were titrated with a urea stock solution made to 8.0 M. The ANS fluorescence was measured at an excitation wavelength of 380 nm and emission recorded at 480 nm. The data were presented as the ANS fluorescence intensity ( $F$ ) relative to that in the absence of urea ( $F_0$ ). Urea concentration was corrected for viscosity according to Kawahara and Tanford (1966).

#### 2.6. Analytical Ultracentrifugation

For analytical ultracentrifugation, the protein samples were dissolved at pH 6.75 and pH 10.5 at concentrations ranging from 1.0 to 3.0 mg/ml. Samples and solvents were filtered with a Waters (Milford, MA) HVLP 0.45  $\mu\text{m}$  membrane filter. Phast gel electrophoresis in SDS showed a nearly identical pattern of protein components before and after filtration; less than 1% of the material was retained on the filter as ascertained by UV spectroscopy. Sedimentation equilibrium experiments were performed using a Beckman Optima XL-A (Palo Alto, CA) analytical ultracentrifuge at speeds ranging from 6000 to 12,000 rpm at various temperatures. A 12 mm charcoal-epon 6-channel centerpiece was used with quartz windows in a wide aperture window holder. The solvent density used in these experiments was 1.0044. This value was calculated from the data as previously described (Alaimo *et al.*, 1999) using 0.564 cc/g as the partial specific volume of PIPES. The partial specific volume of  $\beta$ -casein was taken as 0.728 (Eigel *et al.*, 1984). Data were collected at 280 nm using the standard XL-A procedure. The plots of absorption vs. radius were analyzed directly for weight average molecular weight using the program IDEAL1; a part of the Optima XL-A data analysis software. As the absorbance offsets were not allowed to float in these analyses, weight average molecular weights were obtained. Increased molecular weight with increased ionic strength was supported by the data of Schmidt (1979) who clearly demonstrated self-association of

$\beta$ -casein. Schmidt and coworkers used the following model to analyze their data:

$$i\beta \xrightleftharpoons{k} \beta i \quad (5)$$

where  $i\beta$  refers to the unbound protein, and  $i \gg 1$ , typically ranges from 20 to 50. Other models such as the formation of a self-associated species through a critical micelle concentration have also been used (Tai and Kegeles, 1984). Analysis of the current data was accomplished using ASSOC4, which is for a system of up to four species. This model represents a parallel association scheme where monomer is simultaneously in equilibrium with dimer, trimer and tetramer when  $n_2$ ,  $n_3$  and  $n_4$  are the integers 2–4 in Eq. (6). In our experiments with  $\beta$ -casein the best fits were obtained by fixing the sequence molecular weight at 24,600 for the monomer and floating  $K$  values at the increasing integer values of  $n$  noted above; only one constant was required to fit the data. Analysis of all the data was accomplished using only the  $n_2$  term of the following equation:

$$\begin{aligned} A_t = & \exp[\ln(A_0) + H \times M \times (X^2 - X_0^2)] \\ & + \exp[n_2 \times \ln(A_0) + \ln(K_{a2}) \\ & + n_2 \times H \times M \times (X^2 - X_0^2)] \\ & + \exp[n_3 \times \ln(A_0) + \ln(K_{a3}) \\ & + n_3 \times H \times M \times (X^2 - X_0^2)] \\ & + \exp[n_4 \times \ln(A_0) + \ln(K_{a4}) \\ & + n_4 \times H \times M \times (X^2 - X_0^2)] + E \end{aligned} \quad (6)$$

where  $A_t$  is total absorbance of all species at radius  $X$ ;  $\exp$  is exponent;  $\ln$  is natural log;  $A_0$  is absorbance of the monomer species at reference radius  $X_0$ ;  $H$  is constant  $[(1-\nabla\rho)\omega^2]/2RT$ ;  $M$  is apparent monomer molecular weight;  $X_0$  is reference radius;  $n_i$  is stoichiometry for species  $i$  (number of monomers);  $K_{ai}$  is association constant for the monomer- $n$ mer equilibrium of species  $i$ ; and  $E$  is baseline offset.

As determined from Eq. (6),  $K_{ai}$  may be converted to molar units using:

$$K_{\text{conc}} = K_{\text{abs}} \left[ \frac{el}{n} \right]^{n-1} \quad (7)$$

where  $K_{\text{abs}}$  is  $K_{ai}$  of the software;  $e$  is the molar extinction coefficient;  $l$  is the path length in cm; and  $n$  is the fitted integer of the software.

Subsequently, the weight constant,  $k_n$ , can be converted from the molar constant,  $K_{\text{conc}}$ , by the following relationship:

$$k_n = \frac{K_{\text{conc}}}{[M_{\text{monomer}}/n]^{n-1}} \quad (8)$$

$M_{\text{monomer}}$  is the monomer molecular weight and  $n$  was defined as above.

### 3. RESULTS AND DISCUSSION

#### 3.1. pH Effect on the Self-association of $\beta$ -Casein

The critical micelle model has long been established (Tai and Kegeles, 1984; Takase *et al.*, 1980; Thurn *et al.*, 1987) to describe the dominant physical chemical temperature and concentration mediated self-association of  $\beta$ -casein. It has also been shown that the polymerization process is highly salt-dependent (Schmidt and Payens, 1976). The shape of the monomer, and the contributions of conformational change in the early stages of the polymerization, however, remained controversial. Thurn *et al.* (1987) found a molecular weight of 420,000 and an  $R_H$  of 13.2 nm for  $\beta$ -casein at 10°C; and the values increased to 1,400,000 and 16.3 nm at 25°C, respectively by static and dynamic light scattering. Monomers of  $\beta$ -casein have been reported to range from spherical particles of 10 nm diameter as viewed by electron microscopy (Buchheim and Schmidt, 1979) to long threads with an axial ratio of 12:1 as calculated from viscosity by Payens and van Markwijk (1963). The classical calculations of Waugh *et al.* (1970) clearly showed that on the average the  $\beta$ -casein monomer, within the polymer, must encompass a lower volume than most proposed monomer models. Thus, a conformational change to a more compact structure during the self-association process seems necessary. Perhaps one reason for the controversy over the nature of the  $\beta$ -casein monomer is that much of the physical-chemical data has been collected at protein concentrations (1–3% w/v) or ionic strengths (0.2–0.4 M) which generally favor the formation of self-associated polymers. In contrast, spectroscopic data are often collected on more dilute samples. Accordingly, we have reexamined the  $\beta$ -casein self-association process at reduced concentrations (1–2 mg/ml) and lower ionic strength (50 mM) at neutral (6.75) and extreme pH values (10.5).

Sedimentation equilibrium analysis was carried out from 2 to 37°C at various rotor speeds, as described in the Materials and Methods section. At pH 6.75, even at 25°C and 50 mM ionic strength, a significant portion of polymer is clearly present as signified by the upward curvature of the data (Fig. 1A). At pH 10.5 and similar conditions, on the other hand,  $\beta$ -casein is predominantly monomeric. The association constants ( $K_{\text{conc}}$ ) and the degree of self-association ( $n$ ) derived from experimental data at pH 6.75 were published previously (Farrell *et al.*, 2001). To overcome the temperature limitation of the sedimentation experiments, a plot of  $\log K_a$  (absorbance data) relative to temperatures was made (Fig. 2A). It is apparent that the curve is non-linear, and the transformation to  $1/T$  does not overcome this feature. The plot of  $\log K_a$  versus  $T$  does follow the type of analysis used in Eq. (4). When  $\log K_a$  is substituted for  $\Theta$ , the data may be fitted with Eq. (4), and  $K_{a \text{ max}}$  and  $T_{1/2}$  calculated.  $K_a$  values at 50 and 70°C could thus be obtained from extrapolation. In a similar fashion,  $n$  versus  $T$

yielded values for the degree of polymerization at elevated temperatures (Fig. 2B). This results in  $n=29$  and  $n=30$  for 50 and 70°C, respectively, and agrees with earlier calculations of Thurn *et al.* (1987) from light scattering data and recent dynamic (DLS) and static (SLS) light scattering measurements (O'Connell *et al.*, 2003) at 40°C. Conversion to  $k_n$  and  $k_{\text{conc}}$  are therefore possible (Farrell *et al.*, 2001). From  $k_{\text{conc}}$ ,  $\Delta G^\circ$  can be calculated at each temperature. The values at 20°C are considerably lower than those obtained by Takase *et al.* (1980) at 0.4 M ionic strength. This suggests that charge screening in the formation of  $\beta$ -casein polymers of high  $n$  ( $>20$ ) might be important. However at 10°C where polymer formation is just beginning ( $n=10$ ), the value of  $\Delta G^\circ$  is in reasonable agreement with Takase's work ( $-58.6$  kcal/mol) and with the Payens and van Markwijk's (1963) value of  $-48$  kcal/mol at 8.5°C.

Andrews *et al.* (1979) studied the aggregation of  $\beta$ -casein by performing small-angle X-ray scattering experiments at 4 and 21°C at protein concentrations

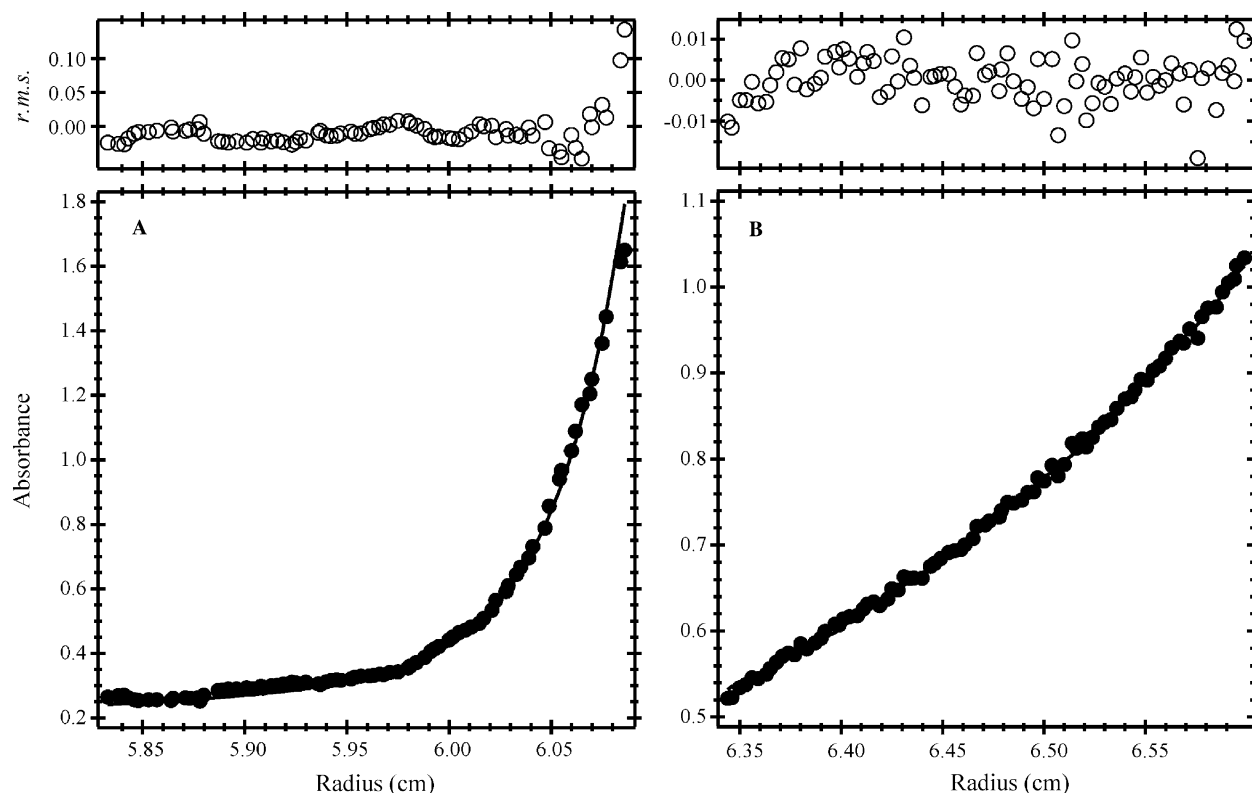


**Fig. 1.** Analytical ultracentrifugation analysis of native  $\beta$ -casein at 25°C and  $I=50$  mM (A) at pH 6.75 in PIPES buffer and (B) at pH 10.5 in CAPS buffer. The fits to the absorbance versus radius plot for Eq. (6), and the residual (r.m.s.) for the fits were also shown. The apparent “monomer” molecular weights near the meniscus were 24,600 Da.

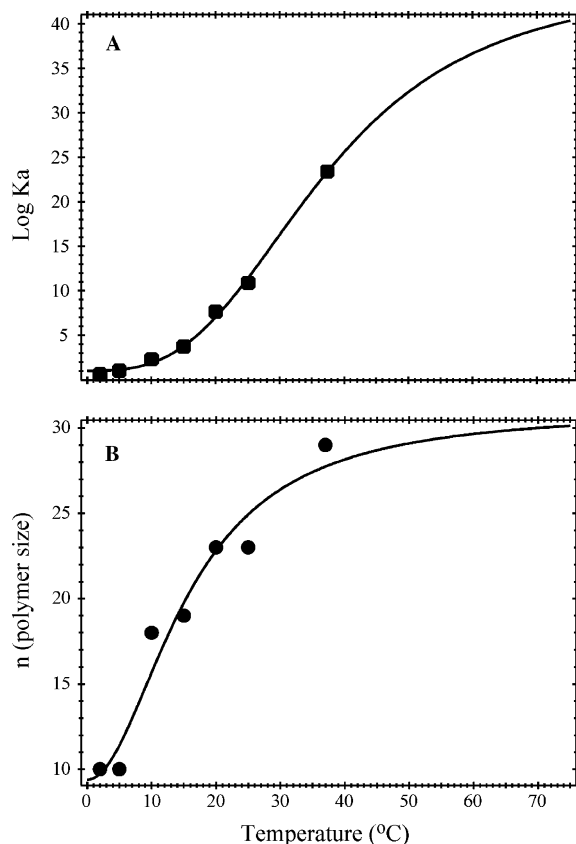


**Fig. 2.** Temperature dependence of  $\log K_a$  (A) and  $n$  (polymer size) (B) for  $\beta$ -casein at pH 6.75.  $K_a$  and  $n$  were obtained by fitting the analytical ultracentrifugation data to a multi-component equilibrium (Eq. (6)).  $\log K_a$  and  $n$  were then fitted with Eq. (4) as a function of temperature.

$\geq 10$  mg/ml. They calculated an  $R_g$  of  $4.6 \pm 0.2$  nm ( $R_H = 5.9$  nm) for a presumed monomer of  $\beta$ -casein at 4°C, which is somewhat larger than the effective Stokes radius of 3.7 nm derived from their gel chromatography data. These hydrodynamic studies are clearly in disagreement with the EM estimate of 10 nm in diameter for  $\beta$ -casein monomer at 4°C, obtained by Buchheim and Schmidt (1979). Several studies offered more conflicting views of the size and shape of  $\beta$ -casein monomer. Payens and van Markwijk (1963) calculated an axial ratio of 12 to 1 for  $\beta$ -casein at 4°C and Waugh *et al.* (1970) calculated a ratio of 9 to 1. By neutron reflection studies of  $\beta$ -casein adsorbed on self-assembled monolayers, Fragneto *et al.* (1995) reported that the  $\beta$ -casein monomer is an ellipsoid with half axes of 2 nm  $\times$  1 nm with a less dense 3.5 nm hydrophilic tail.

Two possible explanations may be proposed for the inconsistencies among the experimentally observed data and theoretical calculations for

$\beta$ -casein monomer. The first, attributable initially to Garnier (1966) by ORD analysis, is that a conformational change occurs that increases the degree of secondary structure in  $\beta$ -casein. This more structured molecule (perhaps similar to the model presented by Kumosinski *et al.* (1993) participates more readily in the aggregation reaction. The second is that  $\beta$ -casein monomer may remain unstructured at 2°C, suggested by Pearce from fluorescence studies (Pearce, 1975), but may change conformation because of polymer formation with increasing temperature. Javor *et al.* (1991) observed a slight increase in the fluorescence intensity of ANS bound with triply phosphorylated human  $\beta$ -casein between 5 and 25°C and a more significant increase in the region of 25–40°C, and suggested conformational changes in response to polymer formation.

The analytical ultracentrifugation experiments at extreme pH (10.5) and low ionic strength (50 mM) illustrated that there is no apparent polymer formation within the same temperature range (2–37°C), suggesting that  $\beta$ -casein largely exists as monomers independent of temperature under these conditions. The self-association process has been largely abolished presumably because of alkaline denaturation or neutralization of the positively charged side chains of  $\beta$ -casein at high pH (10.5). The net charges of the protein presumably changed from -12 per molecule at neutral pH (Swaisgood, 1992) to -22 at pH 10.5.

### 3.2. Alkaline Denaturation of $\beta$ -Casein by FT-IR

FT-IR spectra of  $\beta$ -casein in  $H_2O$  at pH 6.75 and 10.5 were recorded at various temperatures as described in the experimental section. The spectra (not shown) are reminiscent of those found for globular proteins containing a mixed distribution of secondary structural elements that represents a variety of C=O stretch frequencies with amide I peak centering on  $1650\text{ cm}^{-1}$  and shoulders at about  $1660$  and  $1640\text{ cm}^{-1}$ . The amide I envelope is neither strongly monotonal as is found for polyproline II (Farrell *et al.*, 2001) nor strongly helical as found in proteins such as myoglobin. Peak assignments were made tentatively based on the calculations of Krimm and Bandekar (1986), further refinement and estimation of the secondary structural elements that comprise this broad distribution were based upon experimental data collected for 14 globular proteins (Kumosinski and Unruh, 1996). For the amide I region, the following structures were

initially assigned: turns, 1700 to 1660  $\text{cm}^{-1}$ ;  $\alpha$ -helix,  $1652 \pm 2 \text{ cm}^{-1}$ ; irregular structures,  $1642 \pm 2 \text{ cm}^{-1}$ ; and  $\beta$ -sheet or non-stranded extended structure, 1640–1620  $\text{cm}^{-1}$ . Upon data reduction, the protein's spectra contained a band centering on 1658  $\text{cm}^{-1}$ ; such bands might be attributed theoretically to  $\alpha$ -helix or  $\beta$ -turns. Combined circular dichroism and FT-IR experiments on other proteins (Wilder *et al.*, 1992) have suggested that bands centered on  $1656 \pm 2 \text{ cm}^{-1}$  might arise from large loop or extended turn structures rather than from  $\alpha$ -helical structures and be sensed by FT-IR but not by CD.

Accordingly, the elements centering on 1658  $\text{cm}^{-1}$  are attributed to a separate category termed large loop (Curley *et al.*, 1998). The FT-IR results and peak assignments of  $\beta$ -casein at 25°C and pH 6.75 were published previously (Farrell *et al.*, 2001). Peaks measured at pH 10.5 were assigned accordingly. The estimated bulk secondary structure content of  $\beta$ -casein was calculated by summing the areas of the FT-IR bands and shows a considerable amount of  $\alpha$ -helix and large loop ( $\sim 25\%$ ).

In depth analysis of globular proteins with known crystal structures has shown that short runs of polyproline II-like structure (4–6 residues) do occur frequently (Adzhubei and Sternberg, 1993). It was hypothesized by Tiffany and Krim (1968) that unfolded proteins such as charged peptides, poly(l-lysine) and poly(l-glutamate), for example, are not truly random coils but contain short stretches of left-handed polyproline II (PPII) helix-like elements. The major FT-IR spectral feature of these charged polypeptides are the bands between 1656 and 1652  $\text{cm}^{-1}$ . Additionally, Dukor and Keiderling (1991) reported these structures by vibrational CD and suggested that short runs of poly-L-proline (3–6 residues) would yield FT-IR spectra with maxima at 1656 and 1620  $\text{cm}^{-1}$  in  $\text{D}_2\text{O}$ . For long runs of polyproline the 1620  $\text{cm}^{-1}$  peak dominates; this was indeed confirmed in our FT-IR experiments on polyproline peptide ( $n=4$ ) and  $\beta$ -casein in  $\text{H}_2\text{O}$  both at pH 6.75 and 10.5. *Ab initio* calculations for the charged polypeptides by Torii and Tasumi (1998) are in good agreement with this assignment. Furthermore, the polyproline II bearing 1620  $\text{cm}^{-1}$  peak for  $\beta$ -casein seems much more prominent at high pH, suggesting that there are apparent secondary structural changes in  $\beta$ -casein because of side-chain charge screening and neutralization in a similar fashion as a 7-residue lysine peptide studied by Rucker and Creamer (2002). More evidence to

support this observation is presented by CD analyses (see below).

The importance of these short polyproline II structures in protein-protein interactions has been disclosed recently. Studies of profilin, intracellular actin and other signal transduction proteins (Kay *et al.*, 2000) have shown that peptides containing polyproline II structures bind to these proteins and can alter their function significantly. The active binding site of SRC-protein, as elucidated by X-ray crystallography requires a 5-residue (left-handed  $3_1$ - $\alpha$ -helix) polyproline II structure. Peptides with similar structural motifs can be found in  $\beta$ -casein. The predictions for these sequences being in a polyproline II-like motif come from combination of Chou and Fasman (1978) and a published survey by Stapley and Creamer (1999) based on 274 nonhomologous proteins with known structures. The number of  $\beta$ -casein residues predicted likely to adopt short stretches of polyproline II is about 30 or 14% of total residues. Analysis of the amide I region of FT-IR spectrum of  $\beta$ -casein indicated that there is about 20% of the total polyproline II structures in  $\beta$ -casein at pH 6.75 and 25°C. Though this is slightly higher than predicted (14%) for  $n \geq 4$ , a significant amount of short runs ( $n \geq 4$ ) of polyproline II-like structure may be present in  $\beta$ -casein. This assessment is now supported by the recent ROA data published by Syme *et al.* (2002).

Quantitative comparison of FT-IR spectra of  $\beta$ -casein at pH 6.75 and pH 10.5 at 25°C based on overlapping areas between second-derivatives (Kendrick *et al.*, 1996) revealed high similarity correlation ( $\sim 80\%$ ). The secondary structural content analyses for both pH values are compared in Table 1. The only significant difference lies in the sum of  $\alpha$ -helix and loop, possibly attributable to the conformational changes caused by pH, as discussed below. Although there appears little or insignificant temperature effect on FT-IR spectra of  $\beta$ -casein and its overall secondary structure content at pH 6.75 in the range of 5–70°C, elevated temperature does seem to decrease the region that signifies  $\alpha$ -helical and loop, and increase the extended structure considerably at pH 10.5.

### 3.3. Secondary Structure Analysis of $\beta$ -Casein by far-UV CD

The far-UV CD spectrum of  $\beta$ -casein is characterized by a low ellipticity in the 222 nm region and

**Table 1.** Analysis of Secondary Structural Elements of  $\beta$ -casein at 25°C by Various Methods

|                               | $\alpha$ -Helix | $\beta$ -Structure | Turns | PPII | Unspecified |
|-------------------------------|-----------------|--------------------|-------|------|-------------|
| Creamer <i>et al.</i> (1981)  | 20              | 17                 | —     | —    | 63          |
| Graham <i>et al.</i> (1984)   | 7               | 33                 | 19    | —    | 42          |
| Caessens <i>et al.</i> (1999) | 13              | 15                 | —     | —    | 72          |
| Byler <i>et al.</i> (1988)    | 13              | 22                 | 35    | —    | 30          |
| This work CD, pH 6.75         | 20              | 32                 | 28    | —    | 21          |
| This work CD, pH 10.5         | 10              | 36                 | 27    | —    | 27          |
| This work FT-IR, pH 6.75      | 25 <sup>a</sup> | 12                 | 29    | 20   | 14          |
| This work FT-IR, pH 10.5      | 12 <sup>a</sup> | 17                 | 36    | 19   | 16          |

<sup>a</sup> It includes the region centering on  $1652 \pm 6 \text{ cm}^{-1}$  and the sum of loop and  $\alpha$ -helix.

a large negative peak in the 200 nm region, often observed in an unfolded polypeptide chain. A detailed analysis, however, shows that  $\beta$ -casein, being considerably distorted, is still far from to be completely unfolded and preserved some residual structure. The CONTIN procedure (Provencher and Glockner, 1981) has been applied to analyze the CD data and estimate secondary structure content of proteins and peptides. Though the database of 15 proteins of known structures plus a polyglutamate is less than complete and perfect for it fails to represent left handed polyproline II (PPII) structural elements, it serves as a comparative tool to correlate quantitatively CD spectrum to the secondary structure of proteins. Analysis of far-UV CD data of  $\beta$ -casein at room temperature (25°C) and pH 6.75 indicates the presence of approximately 20%  $\alpha$ -helix, 32%  $\beta$ -sheet, 28% turns, and 21% unordered (Table 1) at low ionic strength and pH 6.75, while each element becomes 10, 36, 27, and 27%, respectively, at pH 10.5. The calculated percentages of sheet and turns from FT-IR differ somewhat from those obtained from far-UV CD, but both techniques support the assumption that extended sheet and turns comprise a significant portion (~60%) of the protein structure. The presence of the large-loop designation in FT-IR is apparently absent in CD (Wilder *et al.*, 1992). Even though residues 1–6 of  $\beta$ -casein were strongly predicted to adopt  $\alpha$ -helix conformation in the secondary structure prediction, the lack of  $\alpha$ -helix in the N-terminal portion of  $\beta$ -casein was clearly demonstrated in the NMR studies of 1–25 peptide and 1–42 peptide (Wahlgren *et al.*, 1994). Our recent work (Farrell *et al.*, 2002) on native 1–25 peptide and its dephosphorylated form revealed possible existence of polyproline II and turns in the N-terminal region. Fig. 3 compares the CD spectra of  $\beta$ -casein, 1–25 peptide and polyproline peptides ( $n=4$ ) at pH 6.75

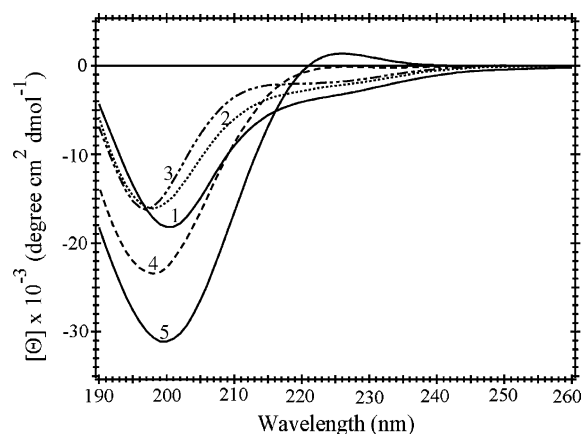


**Fig. 3.** CD spectra of polyproline peptides, and  $\beta$ -casein at 25°C and ionic strength  $I=50 \text{ mM}$  (1),  $\beta$ -casein at pH 6.75. (2),  $\beta$ -casein at pH 10.5. (3),  $\beta$ -casein 1–25 peptide. (4), polyproline ( $n=4$ ) and (5), polyproline ( $n=4$ ) in 60% MeOH.

and 25°C. Clearly, there is a strong similarity between  $\beta$ -casein, 1–25 peptide and polyproline ( $n=4$ ) in 60% MeOH. As suggested previously (Farrell *et al.*, 2001), the similarity between  $\beta$ -casein and  $\beta$ -lipotropic hormone (Makarov *et al.*, 1992), which has been thought to contain short segments (3–6) of polyproline II, is also apparent.

### 3.4. Thermal and Alkaline Denaturation of $\beta$ -Casein by far-UV CD

The analysis of CD spectra of  $\beta$ -casein at various temperatures showed that there is little change in the calculated amounts of unordered, turns, or sheet structure, indicating that there is little thermal unfolding of these local structures as the protein is heated at both pH 6.75 and 10.5. The amounts of  $\beta$ -turn and extended structure remain statistically invariant at about 28 and 31% respectively. A

similar conservation of extended turns at high temperature had been previously observed for  $\beta$ -casein (A<sup>1</sup> variant) by Graham *et al.* (1984). The only significant change is an apparent increase in  $\alpha$ -helical content (at 222 nm) ongoing from 2 to 70°C at both pH values. This behavior is totally different from that of a typical globular protein, which shows temperature-induced reduction of  $\alpha$ -helical content. This may be accounted for, not by  $\alpha$ -helix but by an increase in polyproline II structure and self-association beginning to take place with increased temperature as noted above. The movement of polyproline II model peptide from aqueous to a more apolar (hydrophobic) environment (Tiffany and Krimm, 1968) mirrors the changes seen for  $\beta$ -casein in Fig. 4. CONTIN analysis of the temperature dependence of CD spectra at pH 10.5 showed virtually no significant secondary structural changes occurred during heating. Recently emerged concept of “natively unfolded” proteins (Dunker and Obradovi, 2001; Uversky, 2002) suggested that such effect of heating might be ascribed to the heat-induced intensification of the hydrophobic interactions. We propose that this can arguably be attributed to a possible increase in polyproline II and a simultaneous decrease in  $\alpha$ -helix at pH 10.5. Analytical ultracentrifugation studies revealed that at higher pH self-association has only minimal effect on the overall CD spectra, even at high temperature ( $\geq 50^\circ\text{C}$ ). Similar results (data not shown for reasons of clarity) were also obtained in analyzing the temperature dependence of CD spectra of a polyglutamate (MW750–1500) peptide at neutral pH, which has been regarded as mainly containing polyproline II conformations (Park *et al.*, 1997; Tiffany and Krimm, 1968). Systemin that has been demonstrated (Toumadje and Johnson, 1995) to contain PPII elements also displayed a similar temperature dependent behavior.

Figure 4A shows temperature dependence of ellipticity at 222 nm ( $\Theta_{222}$ ) at both pH values. When fitted with Eq. (4), three parameters can be found, the predicted maximum change at temperatures greater than 70°C, the minimum  $\Theta$  at 0°C and the mid-point for the transition ( $T_{\Theta_{222}}$ ). The maximum change in  $\Theta_{222}$  is  $-3740$  and  $-3260^\circ\text{cm}^2/\text{dmole}$  with mid-point transition of 33 and 39°C for pH 6.75 and pH 10.5, respectively. Finally, the value of  $n=2$  fitted from Eq. (4) points to the apparent sigmoidal nature of the curve and suggests some degree of cooperativity in the transition. Because the mid-point for this transition occurs at 33°C and  $\beta$ -casein

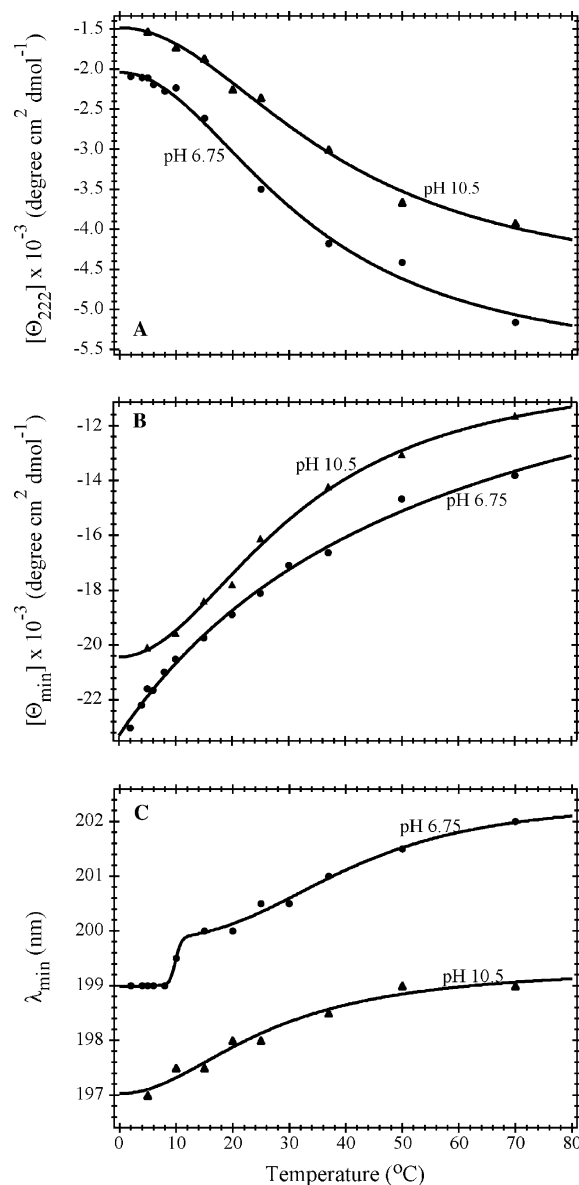


**Fig. 4.** Temperature dependence of the CD spectra of  $\beta$ -casein at pH 6.75 and pH 10.5. (A) ellipticity at 222 nm ( $\Theta_{222}$ ), (B) maximum ellipticity ( $\Theta_{\min}$ ), and (C) ellipticity maxima ( $\lambda_{\min}$ ) in the far-UV region. Data were fitted with Eq. (4).

is known to associate under these conditions, the initial explanation for this transition might be cooperative self-association leading to more hydrophobic environment of the polymer: the rheomorphic hypothesis (Holt and Sawyer, 1993). However, the CD experiments were conducted at  $0.17 \pm 0.02 \text{ mg/ml}$ . Calculations of the monomer content from the analytical ultracentrifugation data at neutral pH (Fig. 2B) demonstrated that  $\beta$ -casein remains

>98% monomer at the concentrations used in CD experiments even at 37°C. Extrapolation of the data to 50 and 70°C (Fig. 2A and B) showed 71% monomer and 46% monomer at these respective temperatures for the same range of concentrations. As noted above, the  $\beta$ -casein is predominantly monomeric at pH 10.5 independent of temperature. The transitions observed in Fig. 4A at both pH values are therefore likely attributed to changes in the structure of the  $\beta$ -casein monomer, and not structural accommodation to the self-associated state. If the changes in the CD spectra indeed reflect movement to a more hydrophobic environment, then the monomer may be becoming more compact with increasing temperature. This model reconciles with the apparent thread-like monomeric structures at 2–4°C which may become more compact in the presence of polymer at 8.5°C (Payens and van Markwijk, 1963). The apparent midpoint transition at high pH (10.5) is only slightly lower than that at pH 6.75, suggesting  $\beta$ -casein monomer is perhaps less stable and compact at alkaline pH. Moreover, it is important to point out that the transitions observed in Fig. 4 are highly reversible and showed little or no hysteresis.

Figure 4B shows a sharp decrease in the maximum ellipticity ( $\Theta_{\min}$ ) as a function of temperature in the far-UV region at both pH values. This decrease is accompanied by a shift to longer wavelength (Fig. 4C). When the change at its maximum is plotted versus temperature, it has the shape of a smooth monotonic transition ( $n=1$ ) at pH 6.75 but remains sigmoidal ( $n=2$ ) at pH 10.5 in Eq. (4). Unlike Fig. 4A the predicted maximum change in  $\Theta_{\min}$  is nearly two fold greater at neutral pH, *i.e.*  $-20,500$  and  $-10,500^\circ \text{cm}^2/\text{dmole}$  for pH 6.75 and 10.5 respectively (Fig. 4B). The mid-point for this transition ( $T_{\Theta_{\min}}$ ) is 78°C at pH 6.75 and 32°C at pH 10.5. These differences in both  $\Theta_{\min}$  and  $T_{\Theta_{\min}}$  between the two pH values are likely caused by the pH-dependent self-association behavior of  $\beta$ -casein. At neutral pH and elevated temperature,  $\beta$ -casein starts to associate even at CD concentrations evidenced by a large decrease in  $\Theta_{\min}$  and  $T_{\Theta_{\min}} = 78^\circ\text{C}$  can perhaps be attributed by changes occurred in the associated state. At pH 10.5,  $T_{\Theta_{\min}} = 32^\circ\text{C}$  may be accounted for by an actual conformational change in  $\beta$ -casein monomer that is somewhat even more unfolded than at neutral pH.

Graham *et al.* (1984) have calculated that the far-UV CD spectrum of  $\beta$ -casein at  $\sim 200$  nm arises from larger Cotton effects in its ORD curves in the

near vacuum UV area. If this hypothesis holds true, the red shift in  $\lambda_{\min}$  observed here (Fig. 4C) could suggest meaningful structural changes in the backbone. A close examination of  $\lambda_{\min}$  found that two transitions could be fitted with Eq. (4) for pH 6.75,  $T_{\lambda 1} = 10^\circ\text{C}$  and  $T_{\lambda 2} = 41^\circ\text{C}$  while only one,  $T_{\lambda} = 26^\circ\text{C}$ , was derived for pH 10.5, as shown in Fig. 4C. Using Eq. (4), a detailed analysis of Garnier's ORD data (1966) showed a similar change in  $A_{436}$  with temperature from 5 to 10°C. The ORD data would represent nearly 40% monomer using our association constants. The changes observed in the CD at 10 and 33°C at neutral pH, as noted above occur at >98% monomer. Changes at higher temperatures, however, are most likely the result of the self-association of  $\beta$ -casein. In contrast, at pH 10.5, all transitions are perhaps a more realistic reflection of conformational changes in the  $\beta$ -casein monomer. Clearly, pH not only has a significant effect on the self-association process of  $\beta$ -casein but also appears to alter the stability of its monomeric form profoundly. The temperature dependence behavior of  $\lambda_{\min}$  at pH 10.5 in Fig. 4C grossly parallels to a "typical" protein denaturation curve with a hint of cooperativity. CONTIN analysis of the differences in CD spectra between the two pH values in the temperature range when  $\beta$ -casein exists predominately as a monomer, suggests a slight but visible loss of  $\alpha$ -helix, PPII and  $\beta$ -sheet content (see Table 1); and perhaps a lesser increase in unordered structures. By using surface quasielastic light scattering techniques, Cicuta and Hopkinson (2001) too found an effective swelling of  $\beta$ -casein at the air-buffer interface at high pH ( $\sim 10$ ) and low salt concentration. Nonetheless, the existence of PPII structures in  $\beta$ -casein is evident even at pH 10.5. Rucker and Creamer (2002) recently hypothesized that even unfolded proteins possess considerable PPII helical conformation based on their CD studies of a 7-residue polylysine peptide.

### 3.5. Temperature Effect on the Secondary Structure of $\beta$ -Casein in SDS Micelles

$\beta$ -Casein appears to display somewhat different CD spectra at micellar concentration of SDS (18 mM) (Fig. 5). The principle negative band at 202 nm remains in the same vicinity with nearly half the intensity ( $-10,000^\circ \text{cm}^2 \text{dmol}^{-1}$ ) compared to that in PIPS buffer ( $-18,000^\circ \text{cm}^2 \text{dmol}^{-1}$ , Fig. 5), while the 220–230 band becomes much more promi-

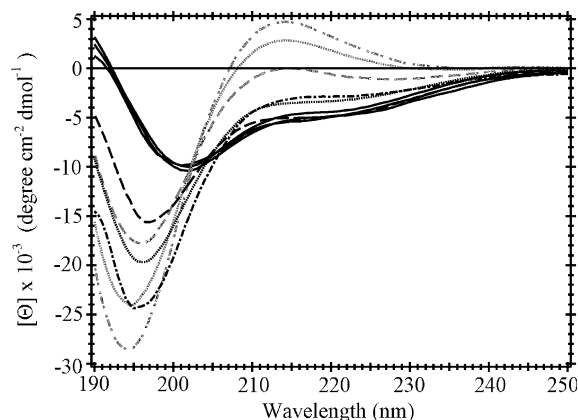


**Fig. 5.** CD spectra of  $\beta$ -casein in SDS (18 mM) micelles at pH 6.75 and 5, 25 and 70°C (dark solid lines). CD spectra of  $\beta$ -casein 1–25 peptide (dark dotted-dashed, dotted and dashed lines) and polyglutamate (MW750–1500) (grey dotted-dashed, dotted and dashed lines) in SDS (18 mM) micelles at pH 6.75, at respective temperatures were also plotted as a comparison.

ment. Interestingly, CONTIN analysis yielded a slight decrease in  $\alpha$ -helical content at 25°C. Low-level non-micellar concentrations of SDS were not used in this work due to  $\beta$ -casein's tendency to polymerize (Creamer, 1980). Furthermore, the CD spectra and secondary structure content of  $\beta$ -casein SDS showed temperature insensitivity from 10 to 50°C and only slight changes were observed below 10°C and above 50°C as attested in Fig. 5. This apparently is in contrast to the thermal denaturation behavior of  $\beta$ -casein at either pH 6.75 or 10.5, where the protein exhibits relatively larger changes in its CD spectra (Fig. 4). In comparison with the temperature sensitive behaviors of both polyglutamate (MW750–1500) and  $\beta$ -casein 1–25 peptide shown in Fig. 5,  $\beta$ -casein appears to be a much more rigid molecule. Because explanations on the temperature effect of SDS on PPII and  $\alpha$ -helix are poorly documented, we propose, tentatively, that this lack of temperature dependence of  $\beta$ -casein in SDS micelles is consistent with its corresponding conformational changes as a function of temperature as discussed above. The secondary structure of  $\beta$ -casein remains thermal stable in the SDS micelles.

### 3.6. ANS Binding to $\beta$ -Casein

ANS is known to bind compact denatured states such as molten globules (Ptitsyn, 1995). We have carried out studies on ANS binding to  $\beta$ -casein

and urea denaturation of the bound complex. The results were compared to globular protein–ANS and to molten globular protein–ANS complexes. At pH 6.75 and room temperature, it may be calculated that  $\beta$ -casein remains essentially monomeric (Fig. 2) at the concentration (5  $\mu$ M) used for fluorescence measurements. When ANS fluorescence intensity ( $F$ ) is plotted as a function of [ANS] then fitted to Eq. (3), an apparent dissociation constant  $K_d \sim 19.0 \pm 2.7 \mu$ M is determined. It is considerably greater than the  $K_d$  for BSA ( $K_d \sim 7.6 \pm 0.5 \mu$ M) but significantly smaller than that of molten globular  $\alpha$ -lactalbumin ( $K_d \sim 183 \pm 12 \mu$ M) obtained in a similar fashion by Bailly *et al.* (2001). This indicates the formation of a high affinity complex between  $\beta$ -casein and ANS. Although nonpreferential binding cannot be completely ruled out, there is a great deal of cooperativity in this binding process. Increasing the pH to 10.5 has resulted largely in the abolishment of the binding between  $\beta$ -casein and ANS. This observation further supports our previous assessment of the pH effect on the secondary structure of  $\beta$ -casein.

Protein–ANS complexes were titrated with increasing urea concentration, and the change in ANS fluorescence intensity ( $F/F_0$ ) was compared in Fig. 6. The results showed that natively folded globular protein, represented by BSA, comprising a sigmoidal  $F/F_0$  profile, a diagnosis of cooperative binding (Schellman, 1990). The model molten glob-

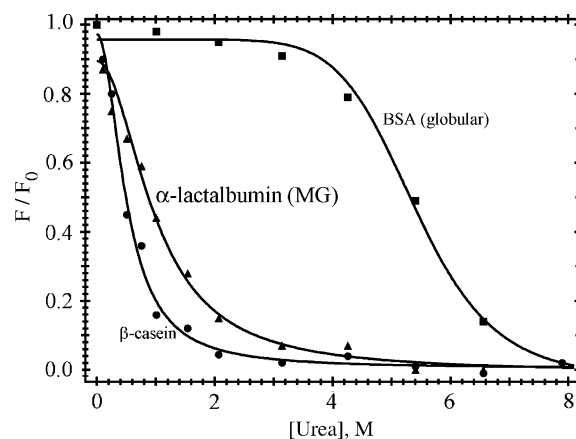


**Fig. 6.** Urea titration curves of protein–ANS complexes. Globular protein (BSA), the typical molten globule protein ( $\alpha$ -lactalbumin) and molten globule like protein,  $\beta$ -casein were incubated with ANS at concentrations indicated in the text and titrated with urea. The change in fluorescence ( $F/F_0$ ) of ANS ( $\lambda_{em} = 480$  nm) versus urea concentration is indicative of differences in proteins of rigid structured binding sites versus molten globular binding sites for the protein–ANS complexes.

ule (MG) protein,  $\alpha$ -lactalbumin at pH 2.0, displayed only a slight sigmoidal behavior. This is in close agreement with reported previously. The unfolding of  $\beta$ -casein-ANS complex as a function of urea, perhaps less stable, nevertheless bears a substantial resemblance to that of the molten globular protein (Fig. 6) with some cooperativity hinted, *i.e.*, when fitted with Eq. (3),  $n > 1$ . The urea concentrations at which  $F/F_0$  reached mid-point were  $\sim 5.4$  M for BSA,  $\sim 1$  M for molten globular  $\alpha$ -lactalbumin, and only  $\sim 0.5$  M was found for  $\beta$ -casein. These data indicated that ANS binding sites in  $\beta$ -casein exhibit less stability as those "typical" molten globular proteins, and are significantly less stable than those globular proteins.

Consistent with the transitions found in CD and the analysis of FT-IR data, ANS fluorescence measurements provide further support to the notion that  $\beta$ -casein contains limited yet defined and persistent secondary structures similar, to some degree, to those found in some molten globule states. This is in apparent contrast to the common belief that  $\beta$ -casein is a completely random and structureless protein.

#### 4. CONCLUSIONS

The changes observed in limited yet defined secondary structure of  $\beta$ -casein monomer, concomitant with loss of self-association (caused by alkaline condition) would argue for the role of preferred secondary structural elements in the process. The combination of results obtained from thermal dependence and urea denaturation of the ANS- $\beta$ -casein complex has led to the hypothesis that  $\beta$ -casein might share some properties similar to the molten globule state. The possibility of the caseins as "native pre-molten globules" places them in line with current "new views" on protein folding, *i.e.* unfolded state is less chaotic and "random" than widely believed (Dyson and Wright, 2002; Plaxco and Gross, 2001). This concept is also in accord with two other terminologies, which have been suggested for the caseins and/or similar proteins recently, namely, "natively unfolded" (Uversky, 2002) and "intrinsically unstructured" (Tompa, 2002). Furthermore, this work suggested that short run polyproline II helix is indeed a significant structural component of the growing family of "natively unfolded" proteins.

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