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Effect of self-association of α_{s1} -casein and its cleavage fractions
 α_{s1} -casein(136–196) and α_{s1} -casein(1–197),¹ on aromatic circular dichroic
spectra: comparison with predicted models

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and κ -casein, in the ratios of 4:1:4:1 [2]. A number of factors point toward the importance of α_{s1} -casein self-associations and α_{s1} - κ -casein interactions in stabilization of the bovine casein system. While β -casein is readily separable from the other fractions by mild procedures, synthetic micelles roughly resembling those of parent micelles can be formed by the α_{s1} - and κ -casein fractions alone [3].

The signature feature of α_{s1} -casein is its self-association. Both hydrophobic and electrostatic interactions are likely to play a sizable role in the pronounced self-association of the α_{s1} -casein monomer in aqueous solution [4–6]. At pH 6.6 and conditions of low ionic strength (0.01 M) this self-association is depressed and the protein is nearly monomeric [4]. As the ionic strength is increased to 0.05 M, the molecule dimerizes. The highly acidic segment of α_{s1} -casein from residues 43 through 84, which contains seven phosphoserines and twelve carboxylic acid groups, can readily account for lack of association at low ionic strength through charge repulsions. At moderate ionic strength dimerization occurs due to charge screening. However, at elevated ionic strengths (>0.2 M) the polymer size increases to octamer and above, and at ionic strengths greater than 0.5 M, α_{s1} -casein is salted out of solution at 37°C. Although the association of α_{s1} -casein is found to be nearly temperature independent between 8°C and 30°C, concentration-dependent association is somewhat reversible at low ($<8^\circ\text{C}$) temperatures [4]. A high degree of hydrophobicity is exhibited by the carboxyl terminal half of the molecule (residues 100 to 199). Hydrophobic interactions involving this portion of the molecule may govern the stoichiometry of the protein self-association at high ionic strengths and low temperatures.

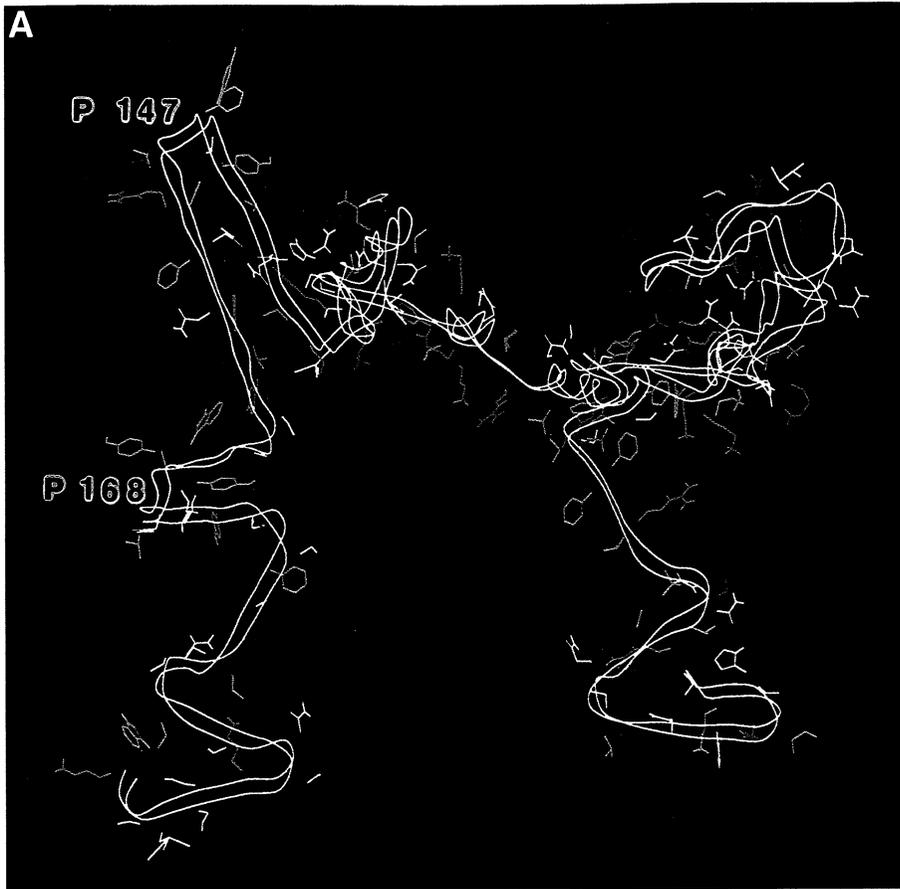
A major question is whether or not there are ‘specific’ sites in this aggregation which are related either to local secondary structure or to clustering of aromatic residues, particularly tyrosine and tryptophan. Since caseins have never been successfully crystal-

lized, detailed X-ray crystallographic structures will likely not be realized. Recently, three dimensional models refined via energy minimization techniques were constructed for α_{s1} -casein [7] and for its interactions in casein submicelles [8,9]. Predicted structures for the different forms of casein were built from sequence-based secondary structure prediction algorithms in conjunction with global secondary structure results obtained from vibrational spectroscopy experiments. A putative energy minimized structure generated for the prevalent B variant of α_{s1} -casein is given in Fig. 1A.

The α_{s1} -casein monomer contains 18 proline residues. Of particular interest are the prolines at positions 134, 147, 160, 168 and 177 of the primary sequence (Fig. 1B) which are conserved across species. Molecular modeling studies of the monomer suggest that these residues may be pivotal points for two stranded antiparallel sheet structures (see Fig. 1A), colloquially referred to as hydrophobic ‘arms’. These hydrophobic portions of α_{s1} -casein were predicted to be involved in sheet-sheet interactions to form α_{s1} -dimers (residues 136–159) or to act as κ - α_{s1} sites of interaction (residues 162–175) within the interior of the micelles [8]. Aromatic side chain groups, particularly those from Phe, Tyr and Trp which are principal components in the hydrophobic cores of globular proteins, also occur in this region of α_{s1} -casein and like key prolines are somewhat conserved across species (Fig. 1B). The chains of native caseins are considerably more extended and flexible, which likely precludes strong packing of aromatic clusters in casein monomers. Nevertheless, aromatic side chain interactions may influence casein self-association and help to stabilize certain secondary structures through *intra*-strand (β hairpin turns) and *inter*-strand (helices and β -sheets) contacts, and thus serve to report on these interactions.

The influence of the two Trp residues on the hydrophobic properties of α_{s1} -casein may be contrasted by comparison of the physical chemical behavior of

Fig. 1. Putative energy minimized structure for the monomer of α_{s1} -casein B [7]. Color plate (A) gives the structure with backbone as a double ribbon, anionic groups (carboxyl and serine phosphate) in red, cationic groups in purple and hydrophobic groups in green. (B) Sequence of bovine α_{s1} -casein C compared to those of ewe, goat and rat. The alignment follows [7]. α_{s1} -Casein B represents replacement of glycine (G) 192 with glutamic acid in the bovine protein.



B

| | | | | | | |
|------|-------|----------|----------|----------|-----------|------------------|
| | 1 | 10 | 20 | 30 | 40 | |
| Cow | RPK | ---HP | IKHQGLP | ---Q | EVLNENL | LRFFVAPFPEVFGKEK |
| Ewe | RPK | ---HPIKH | OGLD | ---P | EVLNENL | LRFFVAPFPEVFRKEN |
| Goat | RPK | ---HPI | NHQGLS | ---P | EVLNENL | LRFFVAPFPEVFRKEN |
| Rat | LPR | AHRRNAV | SQ | TQ | ENSSSEEQE | IVKQPKYLS |
| | | 10 | 20 | 30 | 40 | 50 |
| | | 60 | 70 | 80 | 90 | |
| Cow | EDQ | AMED | IKQMEAES | ISSSEE | ---IV | PNSVEQKH |
| Ewe | EDQ | AMED | AKQMKAGS | SSSSEE | ---IV | PNSAEQKY |
| Goat | EDQ | AMED | AKQMKAGS | SSSSEE | ---IV | PNSAEQKY |
| Rat | EEQ | ATASA | QEDSSSS | SSSSEESK | DA | IP |
| | 70 | 80 | 90 | 100 | 110 | 120 |
| | | | | | | |
| Cow | --- | --- | --- | --- | --- | --- |
| Ewe | --- | --- | --- | --- | --- | --- |
| Goat | --- | --- | --- | --- | --- | --- |
| Rat | AQQAS | LAQQAS | LAQQAL | LAQQAS | LAQQAS | LAQQAS |
| | 130 | 140 | 150 | 160 | 170 | 180 |
| | | | | | | |
| Cow | --- | --- | --- | --- | --- | --- |
| Ewe | --- | --- | --- | --- | --- | --- |
| Goat | --- | --- | --- | --- | --- | --- |
| Rat | AQQAS | LAQQAS | LAQQAL | LAQQAS | LAQQAS | LAQQAS |
| | 130 | 140 | 150 | 160 | 170 | 180 |
| | | | | | | |
| Cow | ERL | HS | MKEGI | HAQ | QKEP | MI |
| Ewe | EQL | HS | MKEG | PAHQ | KQPM | I |
| Goat | EQL | HS | MKEG | PAHQ | KQPM | I |
| Rat | EOP | YR | MNAYS | QMR | HPMS | V |
| | 200 | 210 | 220 | 230 | 240 | 250 |
| | | | | | | |
| Cow | SDI | PNPT | GSEN | SGKTT | MPLW | |
| Ewe | SDI | PNPI | GSEN | SGKIT | MPLW | |
| Goat | SDI | PNPI | GSEN | SGKTT | MPLW | |
| Rat | LNT | FKP | I | APK | D | A |
| | 260 | 270 | | | | |

the parent protein with that of the carboxypeptidase A cleavage peptide α_{s1} -casein(1–197), from which only the Leu198 and Trp199 amino acids have been excised. The other sites predicted to be essential to α_{s1} -casein polymerization and casein micellar stabilization are both contained in the cyanogen bromide cleavage peptide α_{s1} -casein(136–196). The hydrophobic portion of α_{s1} -casein can therefore be separated from the hydrophilic portion (residues 1 to 100) through the isolation of the α_{s1} -casein(136–196) peptide. The participation of aromatic side chain groups, Tyr and Trp, in the association of α_{s1} -casein and related peptides is the subject of this paper. The potential role of local secondary structure in directing these hydrophobic α_{s1} -casein associations will be evaluated in the following paper in this issue [10].

2. Materials and methods

2.1. Materials

All reagents used were of analytical grade or 'ACS certified' from Baker, Fisher, Sigma and Aldrich. Carboxypeptidase A, treated with diisopropyl fluorophosphate to inactivate chymotryptic activity, was purchased from Sigma Chemicals (St. Louis, MO). Cyanogen bromide was also purchased from Sigma Chemicals.

Two liters of fresh, uncooled milk were obtained from the entire milking of a single Holstein cow. The animal was in midlactation, in good health, and part of a commercial herd. Phenylmethylsulfonyl fluoride (0.1 g/l) was added immediately to retard proteolysis. The milk was transported to the laboratory and skimmed twice by centrifugation at $4000\times g$ for 10 min at room temperature. Skim milk (500 ml) was diluted with an equal volume of distilled water and warmed to 37°C . Casein was precipitated by careful addition of 1 N HCl to pH 4.6. The precipitate was homogenized with a Polytron ST-10 homogenizer at low speed and dissolved by addition of NaOH to yield a solution of pH of 7.0. The casein was reprecipitated, washed, then resuspended. Purified α_{s1} -casein was obtained by urea fractionation [11] followed by anion-exchange chromatography on a DEAE-cellulose column in urea [12]. The integrity of the samples was confirmed by sodium dodecyl sulfate-poly-

acrylamide gel electrophoresis (SDS-PAGE) [13]. Alkaline polyacrylamide gel electrophoresis in the presence of urea together with standard caseins of known genetic variance [11,12] showed that the sample consisted of α_{s1} -BB, β -AA and κ -AA caseins.

Purified α_{s1} -casein was reacted with carboxypeptidase A at a ratio of 100 parts of protein to 1 part of enzyme. The pH of the solution (10 mg/ml) was adjusted to 8.2 with NH_4HCO_3 , and digestion was for 2 h at 37°C . The samples were cooled and centrifuged at $10\,000\times g$ to remove any suspended enzyme. Samples were then warmed to 25°C and precipitated isoelectrically at pH 4.6. Centrifugation at $10\,000\times g$ removed the bulk of the precipitate. The supernatant was lyophilized, and the precipitate was dissolved in water at pH 7.0 and dialyzed exhaustively for 3 days at 4°C . The samples were adjusted to pH 7.0 and lyophilized. The freeze-dried supernatant was extracted with 0.1 N HCl, and filtered on a Waters Pico Tag free amino acid filtration device (Waters-Millipore, Milford, MA). Only two amino acids were present: leucine and tryptophan in a 1:1 ratio. The sample showed one band on electrophoresis on a 20% homogeneous Phast gel (Pharmacia Biotech, Uppsala, Sweden) in SDS.

Cyanogen bromide (CNBr) cleavage of α_{s1} -casein was accomplished essentially by the method of Phillips and Azari [14]. Purified α_{s1} -casein was dissolved in 70% formic acid at 30 mg/ml and nitrogen gas was bubbled gently through the solution for 10 min. The samples were in amber containers with teflon lined screw-cap lids. A 60-fold excess of CNBr in 70% formic was added (6 ml of protein solution to 1 ml of reagent). The samples were gently flushed with nitrogen, closed and allowed to react with stirring in the dark for 18 h. The container holding the sample vials was continuously flushed with nitrogen. The samples were next diluted 10-fold with water and lyophilized.

Purification of the CNBr peptide was accomplished in two steps. The lyophilized reaction mixture (100 mg) was dissolved in 2 to 3 ml of 0.5 M NH_4HCO_3 and applied to DEAE-Sephacel CL-6B in a K9 (0.9×12 cm) column (Pharmacia Biotech) which was equilibrated with the same buffer. The column was monitored at 280 nm with a Pharmacia UV-1 system. Two large peaks eluted immediately, and when the trace returned to baseline, a

gradient (30 ml, total) from 0.5 to 1.25 M NH_4HCO_3 was applied. After the gradient, the column was eluted isocratically with 1.25 M NH_4HCO_3 . The last peak to emerge contained the peptide 136–196. This fraction was lyophilized, dissolved in 0.1 M NH_4HCO_3 and applied to a G-50 Sephadex column (1.6 × 15 cm) equilibrated in the same buffer. A small degree of purification occurred, but the desalting which occurred here was necessary for later physical chemical studies.

2.2. Protein analysis methods

Cleavage of α_{s1} -casein by carboxypeptidase A or cyanogen bromide was monitored by an HPLC system consisting of a Varian 5000 liquid chromatograph (Varian Associates, Palo Alto, CA), a 6-way Rheodyne (Cotati, CA) sample injector with a 20- μl sample loop, a 250 × 4.6-mm C4 HPLC column (Hi-Pore RP-304, Bio-Rad Laboratories, Richmond, CA) and a Varian Star data system. Casein samples were diluted with 0.1% trifluoroacetic acid (TFA) to a final protein concentration of 10 mg/ml; aliquots of 20 μl were injected onto the column and the chromatogram was developed with a linear gradient of acetonitrile (ACN), from 30% ACN, 0.1% TFA to 50% ACN, 0.1% TFA, over 31 min at a flow rate of 1.5 ml/min. Then the ACN addition rate was changed to bring ACN to 75% over 9 min and the column was regenerated by a reverse gradient to the starting solvent. The eluant absorbance was continuously monitored at 280 nm or 214 nm with a Varian 9050 variable wavelength detector.

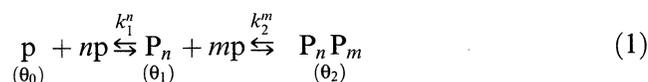
Bulk amino acid composition was determined using the Waters Pico Tag HPLC system. Protein samples were dissolved and appropriately diluted in 0.1 N HCl. Aliquots were placed in analysis tubes which had been pyrolyzed at 500°C, and dried in a Waters Pico Tag work station (Waters, Milford, MA). Gas phase hydrolysis was carried out at 110°C for 24 h with 6 N HCl containing 1.0% (v/v) phenol. Liberated amino acids were quantitated as their phenylthiocarbonyl derivatives.

The N-terminal amino acid sequences of the CNBr peptides were determined by automated Edman degradation on a pulse liquid sequencer with on-line phenylthiohydantoin amino acid analysis (Applied Biosystems 473A, Foster City, CA).

2.3. Circular dichroism (CD) measurements

Near-UV (250–320 nm) CD measurements were made for a peptide concentration series of α_{s1} -casein(136–196) at pH 6.75, 25°C under conditions of low ionic strength (10 mM dipotassium PIPES (piperazine-*N,N'*-bis(2-ethanesulfonic acid) buffer, $\mu=0.0175$ M) and high ionic strength (25 mM dipotassium PIPES/180 mM KCl buffer, $\mu=0.224$ M). Near-UV CD spectra were obtained for native α_{s1} -casein and α_{s1} -casein(1–197) at low and high ionic strength (same conditions as above), and also at medium ionic strength (25 mM dipotassium PIPES/40 mM KCl buffer, $\mu=0.0889$ M). Solvents for CD measurements were first filtered through a Millipore 0.22- μm pore filter. Dissolved peptide samples were filtered through centrifuge tubes containing a 0.45 μm pore regenerated cellulose filter. Circular dichroism spectra were recorded on an Aviv model 60DS spectropolarimeter (Aviv Associates, Lakewood, NJ) at 25°C, using appropriate path length cells (0.5 to 10 mm). Spectra were corrected for solvent contributions and are expressed in units of molar ellipticity $[\theta]$ vs. wavelength. Tyrosine and tryptophan absorbance maxima were estimated from second derivative absorbance spectra obtained on a Beckman DU-7 UV-Vis spectrophotometer (Palo Alto, CA). An extinction coefficient of 1.80 l g⁻¹ cm⁻¹ was calculated for the peptide from its sequence composition.

Characteristic changes in the degree of self-association of α_{s1} -casein occur as a function of both salt and protein contents [4]. In order to correlate changes in θ with these well-documented associations, we have made the following assumptions based in part on the concepts of Wyman's linked function theory [3]. For the biphasic behavior observed, we assumed two classes of association sites. 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 θ_0 , θ_1 and θ_2 are the ellipticities of the species indicated. For this study θ_1 and θ_2 will be relative to θ_0 . The mathematical relationship representing the above stoichiometry can be represented

according to the following:

$$\theta_{\text{app}} = \theta_0 f(p) + \theta_1 f(P_n) + \theta_2 f(P_n P_m) \quad (2)$$

where θ_{app} is the apparent observed ellipticity at a given protein concentration (P_T), $f(i)$ are the protein fractional components of species i and the θ values are previously defined. Incorporation of the apparent equilibrium constants (k_1 and k_2) as defined by Eq. 1 into Eq. 2 yields the following:

$$\theta_{\text{app}} = \frac{\theta_0 p}{p + k_1^n P^n} + \frac{\theta_1 k_1^n p P^n}{p + k_1^n P^n} + \frac{\theta_2 - \theta_1 k_2^m p P^m}{p + k_2^m P^m} \quad (3)$$

where p is the concentration in per cent of the unbound protein and P^n and P^m are the concentrations of bound protein of species P^n and $P^n P^m$, respectively. Cancellation of common terms yields:

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

It should be stressed here that the above expression is valid for sequential binding, i.e., $k_1 > k_2$ and n sites saturate prior to the binding of m sites on the protein; for simplicity, states n and m do not interact. Also, for n or m greater than 1, k_1 and k_2 represent an average value for each class of the n or m binding sites. In reality n or m moles of protein will associate with only an equilibrium constant (K_1), i.e., $K_1 = k_1^n$ and $K_2 = k_2^m$. The terms θ_i are equivalent to the maximum predicted molar ellipticity (θ_{max}).

Now, since only the total protein concentration is known and the free and associated protein concentrations at each state are required, solving directly for these constants is difficult. For these CD experiments when the total protein concentration is used in Eq. 4 instead of the free concentration, the values obtained are only approximations to be correlated in this study with the ultracentrifuge data and literature data [4]. The derivation of these equations and analysis using a Gauss-Newton nonlinear regression analysis program have been presented in detail elsewhere [15]. All profiles were analyzed by fixing the values of n and m and calculating the best least-squared fit for the optimum evaluated k_1 and k_2 values. The n and m values were then fixed to new integer values and the whole procedure repeated. 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 previ-

ously reported for ligand binding [3] and enzyme kinetics [16].

2.4. Analytical ultracentrifugation measurements

For analytical ultracentrifugation, the protein samples were dissolved at pH 6.75 at concentrations ranging from 1.0 to 3.0 mg/ml. Samples and solvents were filtered with a Waters (Milford, MA) HVLP 0.45- μm 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 26 000 to 36 000 rpm at 25°C. A 12-mm charcoal-epon 6 channel centerpiece was used with quartz windows in a wide aperture window holder. The solvent densities used in these experiments were 1.0016, 1.0060 and 1.0085 for the low, medium and high ionic strength experiments, respectively. These values were calculated from the data of Arakawa and Timasheff [17] using 0.564 cc/g as the partial specific volume of PIPES. The partial specific volume of α_{s1} -casein was taken as 0.728 [18] and that of the cyanogen bromide peptide was calculated to be 0.720 cc/g from amino acid sequence data. Data were collected at 280 nm or at 292 nm using the standard XL-A procedure. The absorption versus radius plots were analyzed directly for weight average molecular weight using the program IDEAL 1, 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 anticipated by the data of Schmidt [4,19] who clearly demonstrated self-association for the parent α_{s1} -casein. The following model was used by Schmidt and coworkers to analyze their data:



where α is the unbound protein and $i=1, 2, 3$ in consecutive steps. 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, 3 and 4 in Eq. 6 below. For the parent α_{s1} -casein the best fits were obtained by fixing the sequence molecular weight at 23 600 for the monomer and floating K values at the increasing integer values of n noted above. Analysis of the data was accomplished using the following equation:

$$\begin{aligned}
 A_r = & \text{EXP}[\ln(A_o) + H \times M \times (X^2 - X_o^2)] \\
 & + \text{EXP}[n_2 \times \ln(A_o) + \ln(K_{a2}) \\
 & + n_2 \times H \times M \times (X^2 - X_o^2)] + \text{EXP}[n_3 \times \ln(A_o) \\
 & + \ln(K_{a3}) + n_3 \times H \times M \times (X^2 - X_o^2)] \\
 & + \text{EXP}[n_4 \times \ln(A_o) + \ln(K_{a4}) \\
 & + n_4 \times H \times M \times (X^2 - X_o^2)] + E
 \end{aligned} \quad (6)$$

where A_r = total absorbance of all species at radius X , EXP = exponent, ln = natural log, A_o = absorbance of the monomer species at reference radius X_o , H = constant $[(1 - \nabla\rho)\omega^2]/2RT$, M = apparent monomer molecular weight (monomer for α_{s1} -casein, monomer or dimer for 136–196), X_o = reference radius, n_i = stoichiometry for species i (number of monomers), K_{ai} = association constant for the monomer– n mer equilibrium of species i , and E = baseline offset.

As determined above in Eq. 6, K_{ai} may be converted to molar units using:

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

where K_{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. In turn the molar constant, K_{conc} , can be converted to the weight constant k_n by:

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

here M_{monomer} is the monomer molecular weight and n has the same meaning as above. The k_n values reported here were determined in this fashion.

3. Results and discussion

3.1. Near-UV CD studies and self-association of α_{s1} -casein

The association of α_{s1} -casein depends strongly on pH and ionic strength. In-depth light scattering studies by Schmidt [4] have shown that at pH 6.6, α_{s1} -casein is present predominately in monomeric form at low ionic strength (0.01 M), with a monomer \leftrightarrow dimer equilibrium which increases to include oligomers at moderate ionic strength (0.05 M), and at high ionic strength (0.2 M) there exists an extended concentration dependent association to limiting polymer size of approximately $n = 8$ –10. Sedimentation equilibrium measurements made in this laboratory on purified α_{s1} -casein, at several ionic strengths, yield calculated association constants and apparent molecular weights, in PIPES KCl buffer. Table 1 compares association constants and apparent molecular weight data for α_{s1} -casein obtained in PIPES KCl with those obtained from light scattering [4,19] in phosphate.

The low salt sample ($\mu = 0.0175$) can best be described as a dimer in equilibrium with its component monomer; here only one association constant k_2 was needed to describe the system. The value of 0.88 l/g at 0.0175 ionic strength is in good agreement with the value obtained by Schmidt (0.44) in phosphate at pH 6.0 and $\mu = 0.01$. At medium ionic strength ($\mu = 0.0889$), fits to the data were described by adding two additional constants k_3 and k_4 . The values obtained in PIPES KCl for k_2 and k_3 are not in accord with those from light scattering; however the k_4 values are in moderate agreement. The algorithm used here is quite different from that of Schmidt [4,19] and the models are not identical, but the important fact is that at medium ionic strength the dominant associations of α_{s1} -casein are no longer that of monomer dimer, but of higher order oligomers. At high ionic strength our values of k_2 and k_3 obtained at 1.0 g/l, again differ significantly from light scattering values which were carried out over a much wider range of concentrations. However, k_4 values are in moderate agreement. The experiments here were designed to show that under conditions selected here (concentrations and solvents) there is at low ionic strength, a monomer–dimer equilibrium which responds to in-

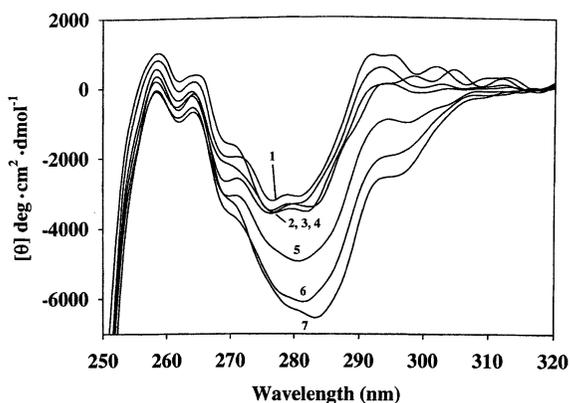


Fig. 2. Changes in near-UV CD spectra of α_{s1} -casein as a function of protein concentration at pH 6.75, 25°C and ionic strength = 0.0175. Protein concentrations were 0.05, 0.075, 0.15, 0.50, 1.0, 2.0 and 3.0 mg/ml, lines 1–7, respectively. Molar ellipticity $[\theta]$ is expressed in degrees·cm² per dmol.

CD bands for these side chains may occur at the same wavelengths as the corresponding absorption bands. Tyrosine and tryptophan side chains, along with cysteine disulfides, are the three major contributors to the near-UV CD of proteins [23] (note α_{s1} -casein contains neither cysteine nor cystine). It has been shown that an increasingly nonaqueous environment will give rise to both an increase in CD band intensity and a red shift of the band position for tyrosine [24]. These spectral changes most likely correspond to the burial of the side chain in a protein interior. Similar phenomena have been observed for tryptophan [25]. There is a gradual increase in molar ellipticity and a small red shift observed for the putative tyrosine band ($\lambda = 283$ nm) on going from 0.05 mg/ml to 3.0 mg/ml α_{s1} -casein (e.g. Fig. 2). There is also a change in sign and similar increase in ellipticity for the putative tryptophan band ($\lambda = 292$ nm) as a function of concentration. This indicates that both tyrosine and tryptophan side chains may play a role in the self-association of α_{s1} -casein at this ionic strength (0.0175 M). Analysis with Eq. 4 of the change in ellipticity in Fig. 2 at $\lambda = 283$ nm with change in concentration yields the apparent CD derived association constant for α_{s1} -casein at low ionic strength of 0.85 l/g; the fit is given in Fig. 3. Analysis by Eq. 4 also provides an assessment of the predicted maximum change in θ at these conditions. For the low ionic strength sample this is -6950 (Table 2). Under these conditions the protein

occurs as a monomer-dimer equilibrium system. Sedimentation equilibrium experiments allowed for the calculation of a k_2 of 0.88 l/g (Table 1), the apparent association constant of 0.85 as calculated from the change in θ at 283 nm with protein concentration (Fig. 3) is in good agreement with this value, indicating a correlation between self-association to dimer and restricted rotation of aromatic side chains.

Near-UV CD spectra were also obtained for α_{s1} -casein at medium (25 mM dipotassium PIPES/40 mM KCl buffer, $\mu = 0.0889$ M) and high ionic strength (25 mM dipotassium PIPES/180 mM KCl buffer, $\mu = 0.224$ M). All other conditions were kept the same as for the near-UV CD experiments on α_{s1} -casein at low ionic strength. Although the CD curves are not shown here, apparent association constants calculated from the CD data are given in Table 2. Experiments at higher ionic strengths reveal stronger casein associations in that the apparent k values increase with increased ionic strength (Table 2), and θ_{\max} increases proportionately. The near-UV CD spectra obtained for α_{s1} -casein at medium and high ionic strength look similar to the low ionic strength curves in Fig. 2, with proportionally larger band intensities at each protein concentration, but with somewhat increased 292 dichroism, as a function of increasing ionic strength. These results show that both tyrosine and tryptophan side chains are involved in progressive association steps of α_{s1} -casein. At medium ionic strength, the association constant, k_4 , calculated from sedimentation equilibrium (Table 2) shows excellent agreement with that calculated

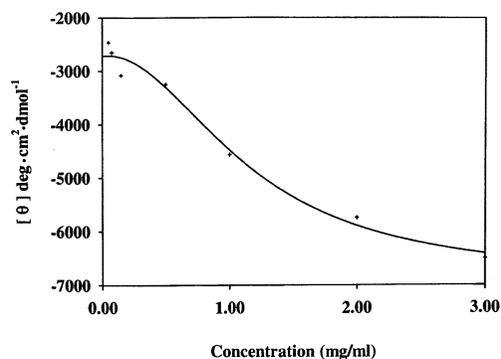


Fig. 3. Plot of change in $[\theta]$ at 283 nm as a function of protein concentration for α_{s1} -casein. The data were derived from Fig. 2 and fitted with Eq. 4; the calculated apparent constants are given in Table 2.

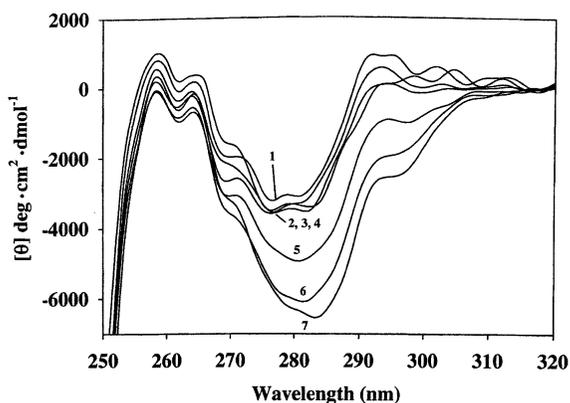


Fig. 2. Changes in near-UV CD spectra of α_{s1} -casein as a function of protein concentration at pH 6.75, 25°C and ionic strength = 0.0175. Protein concentrations were 0.05, 0.075, 0.15, 0.50, 1.0, 2.0 and 3.0 mg/ml, lines 1–7, respectively. Molar ellipticity $[\theta]$ is expressed in degrees·cm² per dmol.

CD bands for these side chains may occur at the same wavelengths as the corresponding absorption bands. Tyrosine and tryptophan side chains, along with cysteine disulfides, are the three major contributors to the near-UV CD of proteins [23] (note α_{s1} -casein contains neither cysteine nor cystine). It has been shown that an increasingly nonaqueous environment will give rise to both an increase in CD band intensity and a red shift of the band position for tyrosine [24]. These spectral changes most likely correspond to the burial of the side chain in a protein interior. Similar phenomena have been observed for tryptophan [25]. There is a gradual increase in molar ellipticity and a small red shift observed for the putative tyrosine band ($\lambda = 283$ nm) on going from 0.05 mg/ml to 3.0 mg/ml α_{s1} -casein (e.g. Fig. 2). There is also a change in sign and similar increase in ellipticity for the putative tryptophan band ($\lambda = 292$ nm) as a function of concentration. This indicates that both tyrosine and tryptophan side chains may play a role in the self-association of α_{s1} -casein at this ionic strength (0.0175 M). Analysis with Eq. 4 of the change in ellipticity in Fig. 2 at $\lambda = 283$ nm with change in concentration yields the apparent CD derived association constant for α_{s1} -casein at low ionic strength of 0.85 l/g; the fit is given in Fig. 3. Analysis by Eq. 4 also provides an assessment of the predicted maximum change in θ at these conditions. For the low ionic strength sample this is -6950 (Table 2). Under these conditions the protein

occurs as a monomer-dimer equilibrium system. Sedimentation equilibrium experiments allowed for the calculation of a k_2 of 0.88 l/g (Table 1), the apparent association constant of 0.85 as calculated from the change in θ at 283 nm with protein concentration (Fig. 3) is in good agreement with this value, indicating a correlation between self-association to dimer and restricted rotation of aromatic side chains.

Near-UV CD spectra were also obtained for α_{s1} -casein at medium (25 mM dipotassium PIPES/40 mM KCl buffer, $\mu = 0.0889$ M) and high ionic strength (25 mM dipotassium PIPES/180 mM KCl buffer, $\mu = 0.224$ M). All other conditions were kept the same as for the near-UV CD experiments on α_{s1} -casein at low ionic strength. Although the CD curves are not shown here, apparent association constants calculated from the CD data are given in Table 2. Experiments at higher ionic strengths reveal stronger casein associations in that the apparent k values increase with increased ionic strength (Table 2), and θ_{\max} increases proportionately. The near-UV CD spectra obtained for α_{s1} -casein at medium and high ionic strength look similar to the low ionic strength curves in Fig. 2, with proportionally larger band intensities at each protein concentration, but with somewhat increased 292 dichroism, as a function of increasing ionic strength. These results show that both tyrosine and tryptophan side chains are involved in progressive association steps of α_{s1} -casein. At medium ionic strength, the association constant, k_4 , calculated from sedimentation equilibrium (Table 2) shows excellent agreement with that calculated

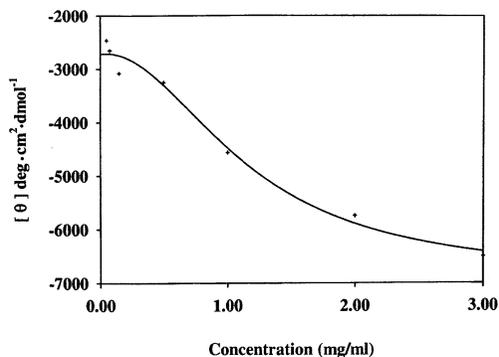


Fig. 3. Plot of change in $[\theta]$ at 283 nm as a function of protein concentration for α_{s1} -casein. The data were derived from Fig. 2 and fitted with Eq. 4; the calculated apparent constants are given in Table 2.

from CD data. At high ionic strength k_3 and k_4 from the sedimentation data again are in moderate agreement with the k_1 and k_2 from the CD data. Note that at low ionic strength the light scattering, ultracentrifuge and the CD-derived apparent association constants are all in reasonable agreement where the association (monomer-dimer) is relatively simple. The interpretation of the k values obtained from the non-linear regression programs used in the CD experiments is straight forward and the agreement with light scattering and ultracentrifuge data is quite good at lower concentrations. In contrast, the interpretation of the n values is cloudy, especially at high salt. They are nominally derived as stoichiometric values (Eq. 3), but even for simple enzyme substrate interactions or ligand binding [3,16] these values may represent some degree of cooperativity, a delay in the concentration-dependent onset of a transition, or be no more than fitting constants representing a range of possible values [16]. Examination of Table 2 bears this out in that the values for n and m which gave the best fit to the data differ from the ultracentrifuge data. However, taken together all of the data support increased changes in the CD of the aromatic regions of the protein, accompanying increased self-association of the protein.

3.2. Near-UV CD studies of carboxypeptidase A generated α_{s1} -casein(1-197)

Of the two tryptophan residues in α_{s1} -casein, Trp199 is the C-terminal residue and, from fluorescence studies, is not predicted to be directly involved protein self-association [21,26]. The other tryptophan in α_{s1} -casein, Trp164, is located in a hydrophobic region of the protein which may be a viable site for dimerization or the formation of even higher order aggregates. Changes in the near-UV CD spectrum for the carboxypeptidase A treated peptide α_{s1} -casein(1-197) as a function of concentration are shown in Fig. 4. All spectra in Fig. 4 were obtained at pH 6.75, 25°C, and low ionic strength (10 mM dipotassium PIPES buffer, $\mu = 0.0175$ M). The spectral features in Fig. 4 look much like those in Fig. 2. A general increase in molar ellipticity and concurrent red shift are observed for the putative tyrosine side chain band at $\lambda = 283$ nm. A change in sign and similar increase in ellipticity for tryptophan ($\lambda = 292$

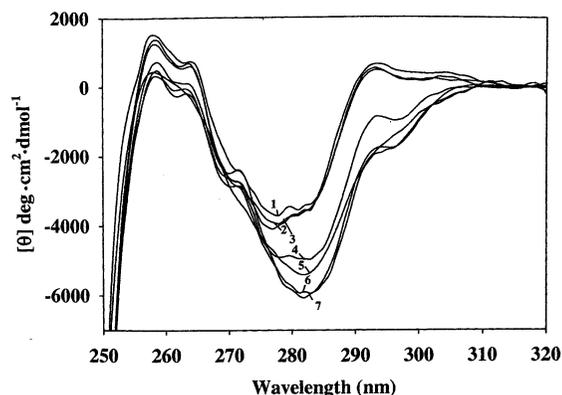


Fig. 4. Changes in the near-UV CD spectra of carboxypeptidase A-treated α_{s1} -casein(1-197) as a function of protein concentration at pH 6.75, 25°C and ionic strength = 0.0175. Protein concentrations were 0.075, 0.10, 0.15, 1.0, 1.5, 2.0 and 3.0 mg/ml, lines 1-7, respectively. Molar ellipticity $[\theta]$ is expressed in degrees \cdot cm² per dmol.

nm) as a function of concentration is likewise observed. Comparison of the data from Figs. 2 and 4 indicate that tyrosine and tryptophan residues clearly both contribute to self-association of α_{s1} -casein and α_{s1} -casein(1-197). While eight out of the ten tyrosine residues in α_{s1} -casein lie in the hydrophobic C-terminal half of the molecule, it is not clear from the data in Figs. 2 and 4 specifically which are involved in dimerization and/or higher degrees of association. However, Trp164 appears to be involved in the overall association of both α_{s1} -casein and α_{s1} -casein(1-197). Given that Trp164 lies in the middle of a series of eleven residues (Tyr159-Leu169) which constitute a strongly hydrophobic portion of the protein, this region is a likely site of hydrophobic aggregation. The absence of Trp 199 appears not to alter the CD spectra. After treatment with carboxypeptidase A, the degree of purity was checked by HPLC as described in Section 2. Under these conditions α_{s1} -casein has a retention time of 17.7 ± 0.1 min. The 1-197 polypeptide displays a reduced retention time (16.9 ± 0.1 min). This is marked enough that 1:1 mixtures are readily resolved. Thus, at acidic pHs (~ 2) Trp199 is surface accessible.

Near-UV CD of α_{s1} -casein(1-197) at high ionic strength (0.224 M), appeared similar to those of native α_{s1} -casein (data not shown). Band intensities were proportionally larger at each protein concentration as a function of increasing ionic strength as was

observed for the intact protein, and θ_{\max} values (Table 2) for the two are comparable. The CD band for tyrosine ($\lambda = 283$ nm) shows the largest change as a function of both ionic strength and polypeptide concentration, though the tryptophan spectrum shows similar changes indicating the influence of Trp164 in progressive association steps at higher ionic strengths. The apparent association constants and θ_{\max} values calculated from the CD data are quite comparable for the intact protein and (1–197) at both low and high ionic strengths (Table 2). The lack of influence of Trp199 in affecting associations of whole α_{s1} -casein (cf. Fig. 2 vs. Fig. 4) compares favorably with fluorescence studies [21] of casein and with casein micelle digestion with carboxypeptidase A by Ribadeau-Dumas and Garnier [27]. In the latter study, carboxypeptidase A was shown to quantitatively remove the C-terminal tryptophan of α_{s1} -casein in native and reconstituted micelles. The general availability of Trp199 to proteolytic digestion is in accord with results presented here, illustrating little participation of the two C-terminal residues in associations of α_{s1} -casein with itself or in casein colloids.

3.3. Self-association studies of cyanogen bromide generated α_{s1} -casein(136–196)

Although the self-association of α_{s1} -casein is governed to a large extent by hydrophobic attractions, electrostatic interactions cannot be neglected. The degree of ionization of the protein and the screening effect of the supporting electrolyte have been shown to influence associations [17]. Much of the hydrophilicity of native α_{s1} -casein arises from the N-terminal half of the molecule. The particular significance of tyrosine and tryptophan side chains in forming α_{s1} -casein aggregates may be better interpreted by investigation of the hydrophobic portion of the protein, namely the α_{s1} -casein(136–196) peptide. Isolation of this sizable portion of the C-terminal half of the protein is achieved by chemical cleavage with cyanogen bromide and purification on DEAE–Sepharose as described in Section 2. Reversed phase HPLC on a C4 column was used to monitor the separation of the CNBr fragments; four major peaks were observed. The peptide of interest (136–196) was identified by collection of the HPLC peak, amino acid analysis,

Table 2
Comparison of association constants (l/g) and apparent molecular weights for α_{s1} -casein and its cleavage fragments derived from ultracentrifuge and circular dichroism analysis

| | Analytical ultracentrifuge (l/g) | | | Circular dichroism (l/g) | | | |
|----------------------------|--------------------------------------|-------------------------------------|---------------------------------|------------------------------|----------------------|--------------------|--|
| | MW _w ^a (Da) | $k_2(n_2)^{b,d,e}$ | $k_3(n_3)^b$ | $k_4(n_4)^b$ | $k_1(n)^c$ | $k_2(m)^c$ | θ_{\max} (deg·cm ² per dmol) ^c |
| Low salt $\mu = 0.0175$ | | | | | | | |
| α_{s1} -Casein | 34 700 | 0.88 (2) ^b | | | 0.85 ± 0.13 (2) | | –6950 |
| α_{s1} -1 to 197 | – | – | | | 0.78 ± 0.11 (2) | | –5810 |
| α_{s1} -136 to 196 | 14 600 | 1.34 ± 0.18 (2) ^d | 2530 ± 830 (6) ^d | | 3.94 ± 0.53 (3) | | –4060 |
| Medium salt $\mu = 0.0889$ | | | | | | | |
| α_{s1} -Casein | 56 200 | 0.007 (2) ^b | 0.020 (3) | 6.82 (4) | 5.95 ± 0.42 (1) | | –7410 |
| High salt $\mu = 0.224$ | | | | | | | |
| α_{s1} -Casein | 68 000 | 0.021 (2) ^b | 0.32 (3) | 12.1 (4) | 0.63 ± 0.03 (16) | 8.1 ± 2.5 (1) | –10 100 |
| α_{s1} -1 to 197 | – | – | – | – | 0.61 ± 0.18 (16) | 5.2 ± 0.38 (4) | –11 100 |
| α_{s1} -136 to 196 | 37 900 | 0.0486 ± 0.151 (2) ^e | 2220 ± 720 (5) ^e | – | 8.80 ± 0.65 (10) | | –6000 |

^aWeight average molecular weight, three determinations $\pm 5\%$.

^b k_n and n_i as described in Table 1.

^c k_i , θ_{\max} , and n or m as described in Eqs. 1 and 4; the error is the error of the fit, and n and m values represent the integers at which the lowest RMS is obtained for the fit [3,16] of the model to the data.

^dThe sequence weight of α_{s1} -casein(136–196) is 6995, which was used in Eq. 6 to determine k_n ; average of six determinations, $n_3 = \pm 1$.

^eThe peptide ‘protomer’ molecular weight here was 14000 for Eq. 6; average of six determinations, $n_3 = \pm 1$.

and N-terminal sequencing. In the sequencing procedure, starting with 700 pmol of peptide the first 36 N-terminal residues were sequenced with statistical confidence. This peptide has an HPLC retention time of 16.3 ± 0.2 min, just slightly less than α_{s1} -casein(1–197). The retention time indicates the strong binding of this portion of α_{s1} -casein to the C4 reversed phase media. In fact, the late elution of the relatively low charged peptide from DEAE–Sephacose also points to the extreme hydrophobicity of the peptide. The calculated molecular weight from sequencing for this peptide is 6995.

The association properties of this peptide as a function of ionic strength may give insight into the nature of the properties of its parent, α_{s1} -casein. Fig. 5 shows a plot of peptide absorbance (at $\lambda = 283$ nm) vs. radial distance from a sedimentation equilibrium experiment for a 1 mg/ml (0.15 mM) solution of α_{s1} -casein(136–196) at 25°C, pH 6.75 at low ionic strength (0.0175 M) in 10 mM dipotassium PIPES buffer. The weight average molecular weight was 14 600, indicating predominantly dimer but with some higher associative species indicated by deviation of the last group of data points at large radius values (> 7.14 cm). An improved statistical fit is shown in Fig. 5, where the regression fit is to a three-component model, a monomer of 7000, a dimer of 14 000, and an oligomer, where n_3 for the oligomer is 6. The average effective molecular weights and association constants calculated from six such experiments are given in Table 2. It should be noted that there is some statistical uncertainty in the calculation of $n = 6$ for the oligomeric aggregation state, due to the statistical weighting of a relatively few data points at larger radial distance values; this leads to a variance of ± 1 for repeated experiments. Thus at low ionic strength for α_{s1} -casein(136–196), there is a low population of monomer, a notable population of dimer, and a small population of associated species which may include higher-order aggregates.

Sedimentation equilibrium measurements for α_{s1} -casein(136–196) at high ionic strength (0.224 M) showed a much higher degree of peptide association. Association constants and effective molecular weights are given in Table 2. The weight average molecular weight here is 37 900. The best nonlinear regression fit to the data (not shown) was by using a three component model, beginning with the 14 000

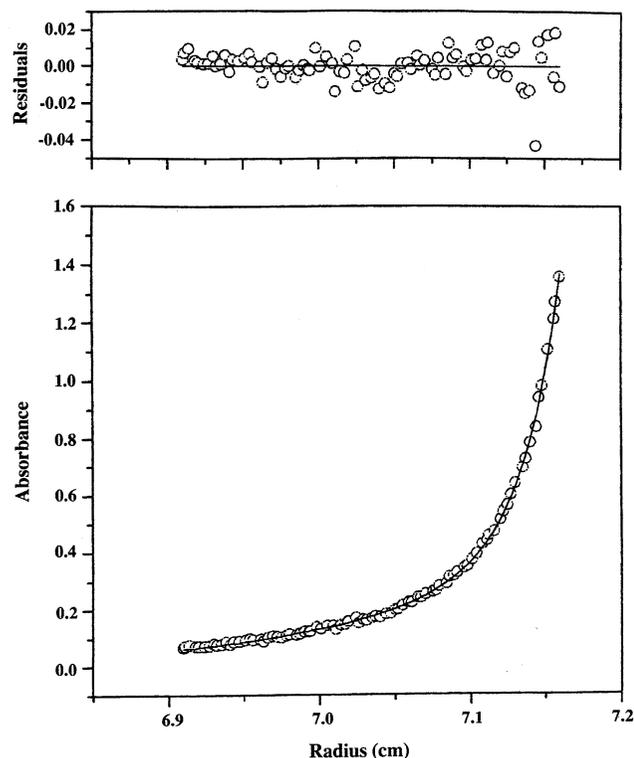


Fig. 5. Analytical ultracentrifugation analysis of α_{s1} -casein(136–196) at 25°C in PIPES buffer; $\mu = 0.0175$. The lower graph shows the fit to the absorbance vs. radius plot for Eq. 6 at 26 000 rpm; the upper plot shows the residuals for the fit showing no pattern. The chi-square value for this fit was 1×10^{-4} ; average parameters for k and n are given in Table 2. The apparent ‘monomer’ molecular weight near the meniscus was 7000 ± 1000 .

dimer (protomer) and extending to tetramer and higher oligomers. Here no fits could be made using 7000 as the starting point for fitting. Note that for k_3 , $n = 5 \pm 1$ as noted above. At 0.224 M ionic strength there appear to be no monomeric forms of α_{s1} -casein(136–196), indicating the predominance of a strong hydrophobically-driven self-association under these conditions.

3.4. Near-UV CD studies of cyanogen bromide generated α_{s1} -casein (136–196)

Fig. 6 shows the near-UV CD spectra for various concentrations of α_{s1} -casein(136–196) at low ionic strength ($\mu = 0.0175$ M). Spectra were obtained at pH 6.75, $T = 25^\circ\text{C}$ in a 10 mM dipotassium PIPES buffer. There is a strong increase in molar ellipticity with increasing peptide concentration for tyrosine

side chains monitored at $\lambda=283$ nm. This change in band intensity, along with an observed red shift, again, indicate that these residues are in a less-exposed environment. The magnitude of change in band intensity and red shift is not quite as large for tryptophan side chains ($\lambda=292$ nm). The results of a similar near-UV CD study of α_{s1} -casein(136–196) at high ionic strength ($\mu=0.224$ M) are shown in Fig. 7. The spectra were obtained in a 0.2 M KCl solution in dipotassium PIPES at pH 6.75 and $T=25^\circ\text{C}$. Perturbations in the tyrosine and tryptophan CD spectra are considerably more noticeable. The ellipticity change for tyrosine at $\lambda=283$ nm as a function of peptide concentration is quite large, with a noteworthy jump in band intensity and red shift between 0.059–0.13 mg/ml. The most remarkable feature of the near-UV CD behavior of α_{s1} -casein(136–196) at high ionic strength is the exceptional change in the tryptophan spectrum at $\lambda=292$ nm. Between 0.030 and 0.059 mg/ml peptide there is little change in the tryptophan spectrum. Above 0.059 mg/ml the sign of the tryptophan band changes, and the magnitude of the tryptophan band intensity increases substantially. Although the tryptophan band position does not appear to change to any great extent, the large jump in molar ellipticity between 0.059 and 0.13 mg/ml indicates a contribution of Trp164 region to the formation of peptide aggregates at high ionic strengths. This correlates with the ultracentrifuge

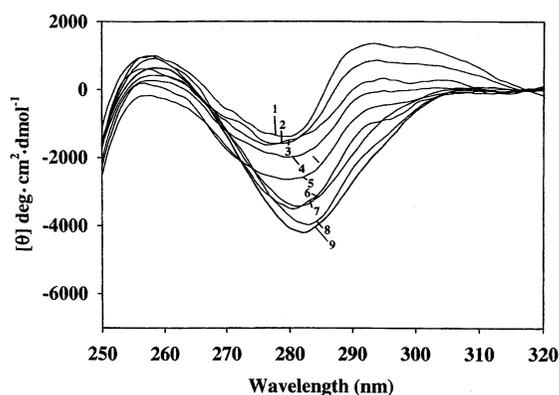


Fig. 6. Changes in the near-UV CD spectra of α_{s1} -casein(136–196) as a function of peptide concentration in PIPES at pH 6.75, 25°C and $\mu=0.0175$. Peptide concentrations were 0.03, 0.05, 0.13, 0.21, 0.26, 0.29, 0.44, 0.59 and 0.90 mg/ml, lines 1–9, respectively. Molar ellipticity $[\theta]$ is expressed in degrees-cm² per dmol. The concentrations of peptide were chosen to cover the molar concentration range used for intact protein.

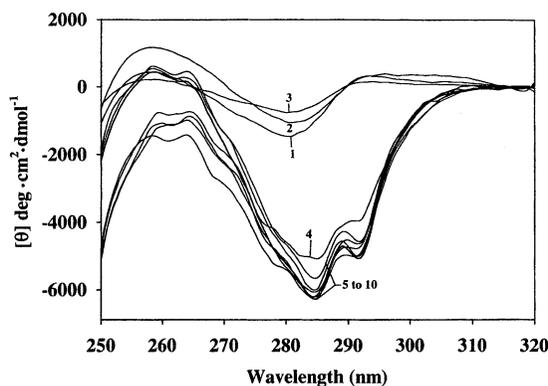


Fig. 7. Changes in the near-UV CD spectra of α_{s1} -casein(136–196) as a function of peptide concentration in PIPES at pH 6.75, 25°C and $\mu=0.224$. Molar ellipticity $[\theta]$ is expressed in degrees-cm² per dmol. Peptide concentrations were 0.030, 0.044, 0.059, 0.13, 0.21, 0.26, 0.30, 0.44, 0.59 and 0.87 mg/ml, lines 1–10, respectively. The concentrations of peptide were chosen to cover the molar concentration range used for the intact protein.

data which show the peptide to be dimer at low ionic strengths (weight average molecular weight = 14 600) and highly associated at high ionic strength (37 900 weight average molecular weight).

The results shown in Fig. 6 reveal that aromatic side chains are involved in hydrophobically-driven aggregation of α_{s1} -casein(136–196), with tyrosine appearing to be the predominant associating aromatic species at low ionic strength, where the peptide is predominantly dimer. In the previously published [7] putative molecular model for native α_{s1} -casein shown in Fig. 1, several proline-based turn structures were hypothesized to be effective sites of dimerization. There are three tyrosines (Tyr144, 146 and 153) in proximity to the Pro147-based turn, making this a probable site of dimerization. Additionally, there are three phenylalanine residues (Phe145, 150 and 152) in this region. These hydrophobic residues may also help to stabilize peptide-peptide interactions, although there is no direct spectral evidence from this study to support this latter speculation, because of the lower extinction of the Phe residues. There are three tyrosines (Tyr159, 165 and 166) and one tryptophan (Trp164) in proximity to the Pro168-based turn in the putative model. Based on the near-UV CD results in Fig. 6, this region may also be a site for the formation of dimers and small oligomers ($n=6$). However, in view of results from the near-UV CD study at high ionic strength (Fig. 7), it is

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Effect of self-association of α_{s1} -casein and its cleavage fractions
 α_{s1} -casein(136–196) and α_{s1} -casein(1–197),¹ on aromatic circular dichroic
spectra: comparison with predicted models

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and κ -casein, in the ratios of 4:1:4:1 [2]. A number of factors point toward the importance of α_{s1} -casein self-associations and α_{s1} - κ -casein interactions in stabilization of the bovine casein system. While β -casein is readily separable from the other fractions by mild procedures, synthetic micelles roughly resembling those of parent micelles can be formed by the α_{s1} - and κ -casein fractions alone [3].

The signature feature of α_{s1} -casein is its self-association. Both hydrophobic and electrostatic interactions are likely to play a sizable role in the pronounced self-association of the α_{s1} -casein monomer in aqueous solution [4–6]. At pH 6.6 and conditions of low ionic strength (0.01 M) this self-association is depressed and the protein is nearly monomeric [4]. As the ionic strength is increased to 0.05 M, the molecule dimerizes. The highly acidic segment of α_{s1} -casein from residues 43 through 84, which contains seven phosphoserines and twelve carboxylic acid groups, can readily account for lack of association at low ionic strength through charge repulsions. At moderate ionic strength dimerization occurs due to charge screening. However, at elevated ionic strengths (>0.2 M) the polymer size increases to octamer and above, and at ionic strengths greater than 0.5 M, α_{s1} -casein is salted out of solution at 37°C. Although the association of α_{s1} -casein is found to be nearly temperature independent between 8°C and 30°C, concentration-dependent association is somewhat reversible at low ($<8^\circ\text{C}$) temperatures [4]. A high degree of hydrophobicity is exhibited by the carboxyl terminal half of the molecule (residues 100 to 199). Hydrophobic interactions involving this portion of the molecule may govern the stoichiometry of the protein self-association at high ionic strengths and low temperatures.

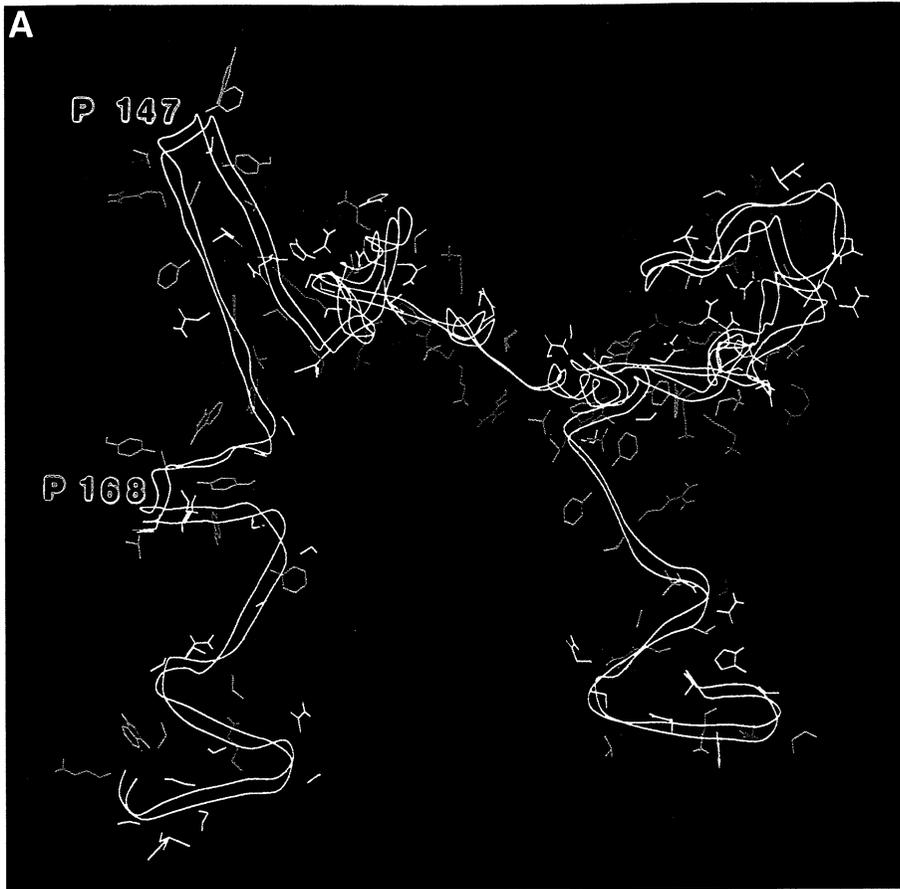
A major question is whether or not there are ‘specific’ sites in this aggregation which are related either to local secondary structure or to clustering of aromatic residues, particularly tyrosine and tryptophan. Since caseins have never been successfully crystal-

lized, detailed X-ray crystallographic structures will likely not be realized. Recently, three dimensional models refined via energy minimization techniques were constructed for α_{s1} -casein [7] and for its interactions in casein submicelles [8,9]. Predicted structures for the different forms of casein were built from sequence-based secondary structure prediction algorithms in conjunction with global secondary structure results obtained from vibrational spectroscopy experiments. A putative energy minimized structure generated for the prevalent B variant of α_{s1} -casein is given in Fig. 1A.

The α_{s1} -casein monomer contains 18 proline residues. Of particular interest are the prolines at positions 134, 147, 160, 168 and 177 of the primary sequence (Fig. 1B) which are conserved across species. Molecular modeling studies of the monomer suggest that these residues may be pivotal points for two stranded antiparallel sheet structures (see Fig. 1A), colloquially referred to as hydrophobic ‘arms’. These hydrophobic portions of α_{s1} -casein were predicted to be involved in sheet-sheet interactions to form α_{s1} -dimers (residues 136–159) or to act as κ - α_{s1} sites of interaction (residues 162–175) within the interior of the micelles [8]. Aromatic side chain groups, particularly those from Phe, Tyr and Trp which are principal components in the hydrophobic cores of globular proteins, also occur in this region of α_{s1} -casein and like key prolines are somewhat conserved across species (Fig. 1B). The chains of native caseins are considerably more extended and flexible, which likely precludes strong packing of aromatic clusters in casein monomers. Nevertheless, aromatic side chain interactions may influence casein self-association and help to stabilize certain secondary structures through *intra*-strand (β hairpin turns) and *inter*-strand (helices and β -sheets) contacts, and thus serve to report on these interactions.

The influence of the two Trp residues on the hydrophobic properties of α_{s1} -casein may be contrasted by comparison of the physical chemical behavior of

Fig. 1. Putative energy minimized structure for the monomer of α_{s1} -casein B [7]. Color plate (A) gives the structure with backbone as a double ribbon, anionic groups (carboxyl and serine phosphate) in red, cationic groups in purple and hydrophobic groups in green. (B) Sequence of bovine α_{s1} -casein C compared to those of ewe, goat and rat. The alignment follows [7]. α_{s1} -Casein B represents replacement of glycine (G) 192 with glutamic acid in the bovine protein.



B

| | | | | | | |
|------|-------|----------|----------|--------|-----------|------------------|
| | 1 | 10 | 20 | 30 | 40 | |
| Cow | RPK | ---HP | IKHQGLP | ---Q | EVLNENL | LRFFVAPFPEVFGKEK |
| Ewe | RPK | ---HPIKH | OGLD | ---P | EVLNENL | LRFFVAPFPEVFRKEN |
| Goat | RPK | ---HPI | NHQGLS | ---P | EVLNENL | LRFFVAPFPEVFRKEN |
| Rat | LPR | AHRRNAV | SQ | TQ | ENSSSEEQE | IVKQPKYLS |
| | | 10 | 20 | 30 | 40 | 50 |
| | | 60 | 70 | 80 | 90 | |
| Cow | EDQ | AMED | IKQMEAES | IS | SSEE | --- |
| Ewe | EDQ | AMED | AKQMKAGS | SS | SSEE | --- |
| Goat | EDQ | AMED | AKQMKAGS | SS | SSEE | --- |
| Rat | EEQ | ATASA | QEDSSSS | SS | SSEESKDA | IP |
| | 70 | 80 | 90 | 100 | 110 | 120 |
| | | | | | | |
| Cow | --- | --- | --- | --- | --- | --- |
| Ewe | --- | --- | --- | --- | --- | --- |
| Goat | --- | --- | --- | --- | --- | --- |
| Rat | AQQAS | LAQQAS | LAQQAL | LAQQAS | LAQQAS | LAQQAS |
| | 130 | 140 | 150 | 160 | 170 | 180 |
| | | | | | | |
| Cow | --- | --- | --- | --- | --- | --- |
| Ewe | --- | --- | --- | --- | --- | --- |
| Goat | --- | --- | --- | --- | --- | --- |
| Rat | AQQAS | LAQQAS | LAQQAL | LAQQAS | LAQQAS | LAQQAS |
| | 130 | 140 | 150 | 160 | 170 | 180 |
| | | | | | | |
| Cow | ERL | HS | MKEGI | HA | QKKE | PMI |
| Ewe | EQL | HS | MKEG | PA | HQ | KQPMI |
| Goat | EQL | HS | MKEG | PA | HQ | KQPMI |
| Rat | EOP | YR | MNAYS | QMR | HP | MSV |
| | 200 | 210 | 220 | 230 | 240 | 250 |
| | | | | | | |
| Cow | --- | --- | --- | --- | --- | --- |
| Ewe | --- | --- | --- | --- | --- | --- |
| Goat | --- | --- | --- | --- | --- | --- |
| Rat | LNT | FKP | I | APK | D | AENTNV |
| | 260 | 270 | | | | |
| | | | | | | |
| Cow | SDI | PNPT | GSEN | SGKTT | MPLW | |
| Ewe | SDI | PNPI | GSEN | SGKIT | MPLW | |
| Goat | SDI | PNPI | GSEN | SGKTT | MPLW | |
| Rat | LNT | FKP | I | APK | D | AENTNV |
| | 260 | 270 | | | | |

the parent protein with that of the carboxypeptidase A cleavage peptide α_{s1} -casein(1–197), from which only the Leu198 and Trp199 amino acids have been excised. The other sites predicted to be essential to α_{s1} -casein polymerization and casein micellar stabilization are both contained in the cyanogen bromide cleavage peptide α_{s1} -casein(136–196). The hydrophobic portion of α_{s1} -casein can therefore be separated from the hydrophilic portion (residues 1 to 100) through the isolation of the α_{s1} -casein(136–196) peptide. The participation of aromatic side chain groups, Tyr and Trp, in the association of α_{s1} -casein and related peptides is the subject of this paper. The potential role of local secondary structure in directing these hydrophobic α_{s1} -casein associations will be evaluated in the following paper in this issue [10].

2. Materials and methods

2.1. Materials

All reagents used were of analytical grade or 'ACS certified' from Baker, Fisher, Sigma and Aldrich. Carboxypeptidase A, treated with diisopropyl fluorophosphate to inactivate chymotryptic activity, was purchased from Sigma Chemicals (St. Louis, MO). Cyanogen bromide was also purchased from Sigma Chemicals.

Two liters of fresh, uncooled milk were obtained from the entire milking of a single Holstein cow. The animal was in midlactation, in good health, and part of a commercial herd. Phenylmethylsulfonyl fluoride (0.1 g/l) was added immediately to retard proteolysis. The milk was transported to the laboratory and skimmed twice by centrifugation at $4000\times g$ for 10 min at room temperature. Skim milk (500 ml) was diluted with an equal volume of distilled water and warmed to 37°C . Casein was precipitated by careful addition of 1 N HCl to pH 4.6. The precipitate was homogenized with a Polytron ST-10 homogenizer at low speed and dissolved by addition of NaOH to yield a solution of pH of 7.0. The casein was reprecipitated, washed, then resuspended. Purified α_{s1} -casein was obtained by urea fractionation [11] followed by anion-exchange chromatography on a DEAE-cellulose column in urea [12]. The integrity of the samples was confirmed by sodium dodecyl sulfate-poly-

acrylamide gel electrophoresis (SDS-PAGE) [13]. Alkaline polyacrylamide gel electrophoresis in the presence of urea together with standard caseins of known genetic variance [11,12] showed that the sample consisted of α_{s1} -BB, β -AA and κ -AA caseins.

Purified α_{s1} -casein was reacted with carboxypeptidase A at a ratio of 100 parts of protein to 1 part of enzyme. The pH of the solution (10 mg/ml) was adjusted to 8.2 with NH_4HCO_3 , and digestion was for 2 h at 37°C . The samples were cooled and centrifuged at $10\,000\times g$ to remove any suspended enzyme. Samples were then warmed to 25°C and precipitated isoelectrically at pH 4.6. Centrifugation at $10\,000\times g$ removed the bulk of the precipitate. The supernatant was lyophilized, and the precipitate was dissolved in water at pH 7.0 and dialyzed exhaustively for 3 days at 4°C . The samples were adjusted to pH 7.0 and lyophilized. The freeze-dried supernatant was extracted with 0.1 N HCl, and filtered on a Waters Pico Tag free amino acid filtration device (Waters-Millipore, Milford, MA). Only two amino acids were present: leucine and tryptophan in a 1:1 ratio. The sample showed one band on electrophoresis on a 20% homogeneous Phast gel (Pharmacia Biotech, Uppsala, Sweden) in SDS.

Cyanogen bromide (CNBr) cleavage of α_{s1} -casein was accomplished essentially by the method of Phillips and Azari [14]. Purified α_{s1} -casein was dissolved in 70% formic acid at 30 mg/ml and nitrogen gas was bubbled gently through the solution for 10 min. The samples were in amber containers with teflon lined screw-cap lids. A 60-fold excess of CNBr in 70% formic was added (6 ml of protein solution to 1 ml of reagent). The samples were gently flushed with nitrogen, closed and allowed to react with stirring in the dark for 18 h. The container holding the sample vials was continuously flushed with nitrogen. The samples were next diluted 10-fold with water and lyophilized.

Purification of the CNBr peptide was accomplished in two steps. The lyophilized reaction mixture (100 mg) was dissolved in 2 to 3 ml of 0.5 M NH_4HCO_3 and applied to DEAE-Sephacel CL-6B in a K9 (0.9×12 cm) column (Pharmacia Biotech) which was equilibrated with the same buffer. The column was monitored at 280 nm with a Pharmacia UV-1 system. Two large peaks eluted immediately, and when the trace returned to baseline, a

gradient (30 ml, total) from 0.5 to 1.25 M NH_4HCO_3 was applied. After the gradient, the column was eluted isocratically with 1.25 M NH_4HCO_3 . The last peak to emerge contained the peptide 136–196. This fraction was lyophilized, dissolved in 0.1 M NH_4HCO_3 and applied to a G-50 Sephadex column (1.6 × 15 cm) equilibrated in the same buffer. A small degree of purification occurred, but the desalting which occurred here was necessary for later physical chemical studies.

2.2. Protein analysis methods

Cleavage of α_{s1} -casein by carboxypeptidase A or cyanogen bromide was monitored by an HPLC system consisting of a Varian 5000 liquid chromatograph (Varian Associates, Palo Alto, CA), a 6-way Rheodyne (Cotati, CA) sample injector with a 20- μl sample loop, a 250 × 4.6-mm C4 HPLC column (Hi-Pore RP-304, Bio-Rad Laboratories, Richmond, CA) and a Varian Star data system. Casein samples were diluted with 0.1% trifluoroacetic acid (TFA) to a final protein concentration of 10 mg/ml; aliquots of 20 μl were injected onto the column and the chromatogram was developed with a linear gradient of acetonitrile (ACN), from 30% ACN, 0.1% TFA to 50% ACN, 0.1% TFA, over 31 min at a flow rate of 1.5 ml/min. Then the ACN addition rate was changed to bring ACN to 75% over 9 min and the column was regenerated by a reverse gradient to the starting solvent. The eluant absorbance was continuously monitored at 280 nm or 214 nm with a Varian 9050 variable wavelength detector.

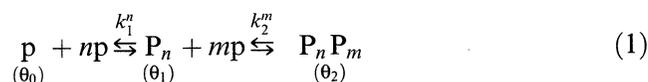
Bulk amino acid composition was determined using the Waters Pico Tag HPLC system. Protein samples were dissolved and appropriately diluted in 0.1 N HCl. Aliquots were placed in analysis tubes which had been pyrolyzed at 500°C, and dried in a Waters Pico Tag work station (Waters, Milford, MA). Gas phase hydrolysis was carried out at 110°C for 24 h with 6 N HCl containing 1.0% (v/v) phenol. Liberated amino acids were quantitated as their phenylthiocarbonyl derivatives.

The N-terminal amino acid sequences of the CNBr peptides were determined by automated Edman degradation on a pulse liquid sequencer with on-line phenylthiohydantoin amino acid analysis (Applied Biosystems 473A, Foster City, CA).

2.3. Circular dichroism (CD) measurements

Near-UV (250–320 nm) CD measurements were made for a peptide concentration series of α_{s1} -casein(136–196) at pH 6.75, 25°C under conditions of low ionic strength (10 mM dipotassium PIPES (piperazine-*N,N'*-bis(2-ethanesulfonic acid) buffer, $\mu=0.0175$ M) and high ionic strength (25 mM dipotassium PIPES/180 mM KCl buffer, $\mu=0.224$ M). Near-UV CD spectra were obtained for native α_{s1} -casein and α_{s1} -casein(1–197) at low and high ionic strength (same conditions as above), and also at medium ionic strength (25 mM dipotassium PIPES/40 mM KCl buffer, $\mu=0.0889$ M). Solvents for CD measurements were first filtered through a Millipore 0.22- μm pore filter. Dissolved peptide samples were filtered through centrifuge tubes containing a 0.45 μm pore regenerated cellulose filter. Circular dichroism spectra were recorded on an Aviv model 60DS spectropolarimeter (Aviv Associates, Lakewood, NJ) at 25°C, using appropriate path length cells (0.5 to 10 mm). Spectra were corrected for solvent contributions and are expressed in units of molar ellipticity $[\theta]$ vs. wavelength. Tyrosine and tryptophan absorbance maxima were estimated from second derivative absorbance spectra obtained on a Beckman DU-7 UV-Vis spectrophotometer (Palo Alto, CA). An extinction coefficient of 1.80 l g⁻¹ cm⁻¹ was calculated for the peptide from its sequence composition.

Characteristic changes in the degree of self-association of α_{s1} -casein occur as a function of both salt and protein contents [4]. In order to correlate changes in θ with these well-documented associations, we have made the following assumptions based in part on the concepts of Wyman's linked function theory [3]. For the biphasic behavior observed, we assumed two classes of association sites. 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 θ_0 , θ_1 and θ_2 are the ellipticities of the species indicated. For this study θ_1 and θ_2 will be relative to θ_0 . The mathematical relationship representing the above stoichiometry can be represented

according to the following:

$$\theta_{\text{app}} = \theta_0 f(p) + \theta_1 f(P_n) + \theta_2 f(P_n P_m) \quad (2)$$

where θ_{app} is the apparent observed ellipticity at a given protein concentration (P_T), $f(i)$ are the protein fractional components of species i and the θ values are previously defined. Incorporation of the apparent equilibrium constants (k_1 and k_2) as defined by Eq. 1 into Eq. 2 yields the following:

$$\theta_{\text{app}} = \frac{\theta_0 p}{p + k_1^n P^n} + \frac{\theta_1 k_1^n p P^n}{p + k_1^n P^n} + \frac{\theta_2 - \theta_1 k_2^m p P^m}{p + k_2^m P^m} \quad (3)$$

where p is the concentration in per cent of the unbound protein and P^n and P^m are the concentrations of bound protein of species P^n and $P^n P^m$, respectively. Cancellation of common terms yields:

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

It should be stressed here that the above expression is valid for sequential binding, i.e., $k_1 > k_2$ and n sites saturate prior to the binding of m sites on the protein; for simplicity, states n and m do not interact. Also, for n or m greater than 1, k_1 and k_2 represent an average value for each class of the n or m binding sites. In reality n or m moles of protein will associate with only an equilibrium constant (K_1), i.e., $K_1 = k_1^n$ and $K_2 = k_2^m$. The terms θ_i are equivalent to the maximum predicted molar ellipticity (θ_{max}).

Now, since only the total protein concentration is known and the free and associated protein concentrations at each state are required, solving directly for these constants is difficult. For these CD experiments when the total protein concentration is used in Eq. 4 instead of the free concentration, the values obtained are only approximations to be correlated in this study with the ultracentrifuge data and literature data [4]. The derivation of these equations and analysis using a Gauss-Newton nonlinear regression analysis program have been presented in detail elsewhere [15]. All profiles were analyzed by fixing the values of n and m and calculating the best least-squared fit for the optimum evaluated k_1 and k_2 values. The n and m values were then fixed to new integer values and the whole procedure repeated. 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 previ-

ously reported for ligand binding [3] and enzyme kinetics [16].

2.4. Analytical ultracentrifugation measurements

For analytical ultracentrifugation, the protein samples were dissolved at pH 6.75 at concentrations ranging from 1.0 to 3.0 mg/ml. Samples and solvents were filtered with a Waters (Milford, MA) HVLP 0.45- μm 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 26 000 to 36 000 rpm at 25°C. A 12-mm charcoal-epon 6 channel centerpiece was used with quartz windows in a wide aperture window holder. The solvent densities used in these experiments were 1.0016, 1.0060 and 1.0085 for the low, medium and high ionic strength experiments, respectively. These values were calculated from the data of Arakawa and Timasheff [17] using 0.564 cc/g as the partial specific volume of PIPES. The partial specific volume of α_{s1} -casein was taken as 0.728 [18] and that of the cyanogen bromide peptide was calculated to be 0.720 cc/g from amino acid sequence data. Data were collected at 280 nm or at 292 nm using the standard XL-A procedure. The absorption versus radius plots were analyzed directly for weight average molecular weight using the program IDEAL 1, 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 anticipated by the data of Schmidt [4,19] who clearly demonstrated self-association for the parent α_{s1} -casein. The following model was used by Schmidt and coworkers to analyze their data:



where α is the unbound protein and $i=1, 2, 3$ in consecutive steps. 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, 3 and 4 in Eq. 6 below. For the parent α_{s1} -casein the best fits were obtained by fixing the sequence molecular weight at 23 600 for the monomer and floating K values at the increasing integer values of n noted above. Analysis of the data was accomplished using the following equation:

$$\begin{aligned}
A_r = & \text{EXP}[\ln(A_o) + H \times M \times (X^2 - X_o^2)] \\
& + \text{EXP}[n_2 \times \ln(A_o) + \ln(K_{a2}) \\
& + n_2 \times H \times M \times (X^2 - X_o^2)] + \text{EXP}[n_3 \times \ln(A_o) \\
& + \ln(K_{a3}) + n_3 \times H \times M \times (X^2 - X_o^2)] \\
& + \text{EXP}[n_4 \times \ln(A_o) + \ln(K_{a4}) \\
& + n_4 \times H \times M \times (X^2 - X_o^2)] + E
\end{aligned} \quad (6)$$

where A_r = total absorbance of all species at radius X , EXP = exponent, ln = natural log, A_o = absorbance of the monomer species at reference radius X_o , H = constant $[(1 - \nabla\rho)\omega^2]/2RT$, M = apparent monomer molecular weight (monomer for α_{s1} -casein, monomer or dimer for 136–196), X_o = reference radius, n_i = stoichiometry for species i (number of monomers), K_{ai} = association constant for the monomer– n mer equilibrium of species i , and E = baseline offset.

As determined above in 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_{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. In turn the molar constant, K_{conc} , can be converted to the weight constant k_n by:

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

here M_{monomer} is the monomer molecular weight and n has the same meaning as above. The k_n values reported here were determined in this fashion.

3. Results and discussion

3.1. Near-UV CD studies and self-association of α_{s1} -casein

The association of α_{s1} -casein depends strongly on pH and ionic strength. In-depth light scattering studies by Schmidt [4] have shown that at pH 6.6, α_{s1} -casein is present predominately in monomeric form at low ionic strength (0.01 M), with a monomer \leftrightarrow dimer equilibrium which increases to include oligomers at moderate ionic strength (0.05 M), and at high ionic strength (0.2 M) there exists an extended concentration dependent association to limiting polymer size of approximately $n = 8$ –10. Sedimentation equilibrium measurements made in this laboratory on purified α_{s1} -casein, at several ionic strengths, yield calculated association constants and apparent molecular weights, in PIPES KCl buffer. Table 1 compares association constants and apparent molecular weight data for α_{s1} -casein obtained in PIPES KCl with those obtained from light scattering [4,19] in phosphate.

The low salt sample ($\mu = 0.0175$) can best be described as a dimer in equilibrium with its component monomer; here only one association constant k_2 was needed to describe the system. The value of 0.88 l/g at 0.0175 ionic strength is in good agreement with the value obtained by Schmidt (0.44) in phosphate at pH 6.0 and $\mu = 0.01$. At medium ionic strength ($\mu = 0.0889$), fits to the data were described by adding two additional constants k_3 and k_4 . The values obtained in PIPES KCl for k_2 and k_3 are not in accord with those from light scattering; however the k_4 values are in moderate agreement. The algorithm used here is quite different from that of Schmidt [4,19] and the models are not identical, but the important fact is that at medium ionic strength the dominant associations of α_{s1} -casein are no longer that of monomer dimer, but of higher order oligomers. At high ionic strength our values of k_2 and k_3 obtained at 1.0 g/l, again differ significantly from light scattering values which were carried out over a much wider range of concentrations. However, k_4 values are in moderate agreement. The experiments here were designed to show that under conditions selected here (concentrations and solvents) there is at low ionic strength, a monomer–dimer equilibrium which responds to in-

creases in salt with increased degrees of self-association. The rigorous data of Schmidt are in essence the definitive association constants for the system. The interpretation of the k_n values obtained from the nonlinear regression programs used in all of the current experiments supports the overall conclusion of a concentration-dependent, salt-dependent self-association mechanism for α_{s1} -casein. Examination of Table 1 bears this out in that the values for k_2 which are model independent gave the best agreement for the monomer dimer equilibrium at low salt; the weight average molecular weight supports the monomer-dimer argument. Similarly the k_4 values, representing perhaps the dominant terms in Eq. 7, are also in reasonable accord with the k_4 data of Schmidt and coworkers. The ultracentrifuge data here must be viewed as supportive of the literature values for the self-association of α_{s1} -casein [4,19].

Previous investigations of α_{s1} -casein associations have not focused on the influence of specific amino acid side chains in protein and peptide self-association. However, Ono et al. [20] noted that the change in extinction coefficient of α_{s1} -casein with aggregation could be commensurate with the 'burying' of tyrosine and tryptophan residues during the aggregation process, and Dagleish noted that calcium aggregation caused increased tryptophan fluorescence [21]. Near-UV CD spectropolarimetry can be a useful technique in determining the role of aromatic side chain groups in polypeptide aggregation. In particular, the near-UV CD spectra of tyrosine and tryptophan residues are dependent upon side chain conformation and environment, and can be used to monitor changes in structure during the self-association

of caseins. The α_{s1} -casein monomer contains two tryptophan residues at positions 164 and 199, respectively. Trp164 is located in a primarily hydrophobic portion of the molecule, near Pro168, which is located in a predicted turn structure in the putative molecular model (see Fig. 1), and may be an important site in the formation of hydrophobically-driven α_{s1} -casein aggregates. Enzymatic cleavage by carboxypeptidase A allows for removal of two carboxy terminal residues, including Trp199. Removal of the C-terminal tryptophan and leucine residues by carboxypeptidase A from each of the three genetic variants of α_{s1} -casein was demonstrated in the past as a method of end group analysis [22]. Studies of the carboxypeptidase A treated peptide α_{s1} -casein(1-197) can help determine the possible role of Trp164 in peptide aggregation.

Changes in the near-UV CD spectrum for native α_{s1} -casein as a function of concentration are shown in Fig. 2. The spectra in Fig. 2 were all obtained at pH 6.75, 25°C and low ionic strength (10 mM dipotassium PIPES buffer, $\mu=0.0175$ M). Prior to CD analysis, the identity and position of side chain absorption bands were determined from second-derivative UV-Vis spectra. A strong absorption band at $\lambda=283$ nm was tentatively ascribed to tyrosine. Tryptophan was tentatively assigned to an absorption band at $\lambda=292$ nm. However, tryptophan side chains may also contribute somewhat to the strong band at $\lambda=283$ nm. The second derivative band at 292 was diminished by half, relative to the parent protein, in the carboxypeptidase treated sample where 1 of 2 tryptophan residues is deleted, but the 283 band was little changed in value. The vibronic

Table 1
Comparison of association constants (l/g) and apparent molecular weights for α_{s1} -casein by ultracentrifuge and light scattering analysis

| | Sedimentation equilibrium (l/g) | | | Light scattering ^a (l/g) | | | |
|--|-----------------------------------|------------------------------------|-----------------------|-------------------------------------|-------|-------|-------|
| | MW _w ^b (Da) | $k_2(n_2)^c$ | $k_3(n_3)^c$ | $k_4(n_4)^c$ | k_2 | k_3 | k_4 |
| Low salt $\mu=0.0175$ α_{s1} -casein | 34 700 | 0.88 ± 0.08 (2) ^d | | | 0.44 | | |
| Medium salt $\mu=0.0889$ α_{s1} -casein | 56 200 | 0.007 ± 0.002 (2) ^d | 0.020 ± 0.007 (3) | 6.82 ± 0.28 (4) | 2.83 | 3.13 | 4.25 |
| High salt $\mu=0.224$ α_{s1} -casein | 68 000 | 0.021 ± 0.004 (2) ^d | 0.32 ± 0.08 (3) | 12.1 ± 0.3 (4) | 15.9 | 20.8 | 14.7 |

^aRefs. [4,19] following notation of Eq. 5.

^bWeight average molecular weight; three determinations $\pm 5\%$.

^c K_{ai} and n_i as described in Eq. 6, and converted to l/g by Eqs. 7 and 8. Sequence molecular weight of α_{s1} -casein is 23 600; the value represents the average of three determinations, each experiment containing three samples, one at each ionic strength.

^dThe monomer here was 23 600, the sequence weight; the values of n_i represent integer values of Schmidt and coworkers [4,19].

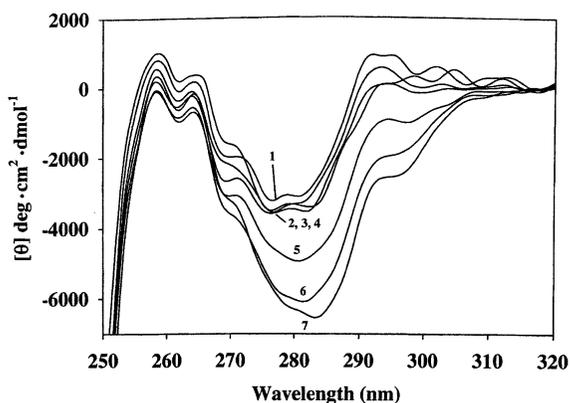


Fig. 2. Changes in near-UV CD spectra of α_{s1} -casein as a function of protein concentration at pH 6.75, 25°C and ionic strength = 0.0175. Protein concentrations were 0.05, 0.075, 0.15, 0.50, 1.0, 2.0 and 3.0 mg/ml, lines 1–7, respectively. Molar ellipticity $[\theta]$ is expressed in degrees·cm² per dmol.

CD bands for these side chains may occur at the same wavelengths as the corresponding absorption bands. Tyrosine and tryptophan side chains, along with cysteine disulfides, are the three major contributors to the near-UV CD of proteins [23] (note α_{s1} -casein contains neither cysteine nor cystine). It has been shown that an increasingly nonaqueous environment will give rise to both an increase in CD band intensity and a red shift of the band position for tyrosine [24]. These spectral changes most likely correspond to the burial of the side chain in a protein interior. Similar phenomena have been observed for tryptophan [25]. There is a gradual increase in molar ellipticity and a small red shift observed for the putative tyrosine band ($\lambda = 283$ nm) on going from 0.05 mg/ml to 3.0 mg/ml α_{s1} -casein (e.g. Fig. 2). There is also a change in sign and similar increase in ellipticity for the putative tryptophan band ($\lambda = 292$ nm) as a function of concentration. This indicates that both tyrosine and tryptophan side chains may play a role in the self-association of α_{s1} -casein at this ionic strength (0.0175 M). Analysis with Eq. 4 of the change in ellipticity in Fig. 2 at $\lambda = 283$ nm with change in concentration yields the apparent CD derived association constant for α_{s1} -casein at low ionic strength of 0.85 l/g; the fit is given in Fig. 3. Analysis by Eq. 4 also provides an assessment of the predicted maximum change in θ at these conditions. For the low ionic strength sample this is -6950 (Table 2). Under these conditions the protein

occurs as a monomer-dimer equilibrium system. Sedimentation equilibrium experiments allowed for the calculation of a k_2 of 0.88 l/g (Table 1), the apparent association constant of 0.85 as calculated from the change in θ at 283 nm with protein concentration (Fig. 3) is in good agreement with this value, indicating a correlation between self-association to dimer and restricted rotation of aromatic side chains.

Near-UV CD spectra were also obtained for α_{s1} -casein at medium (25 mM dipotassium PIPES/40 mM KCl buffer, $\mu = 0.0889$ M) and high ionic strength (25 mM dipotassium PIPES/180 mM KCl buffer, $\mu = 0.224$ M). All other conditions were kept the same as for the near-UV CD experiments on α_{s1} -casein at low ionic strength. Although the CD curves are not shown here, apparent association constants calculated from the CD data are given in Table 2. Experiments at higher ionic strengths reveal stronger casein associations in that the apparent k values increase with increased ionic strength (Table 2), and θ_{\max} increases proportionately. The near-UV CD spectra obtained for α_{s1} -casein at medium and high ionic strength look similar to the low ionic strength curves in Fig. 2, with proportionally larger band intensities at each protein concentration, but with somewhat increased 292 dichroism, as a function of increasing ionic strength. These results show that both tyrosine and tryptophan side chains are involved in progressive association steps of α_{s1} -casein. At medium ionic strength, the association constant, k_4 , calculated from sedimentation equilibrium (Table 2) shows excellent agreement with that calculated

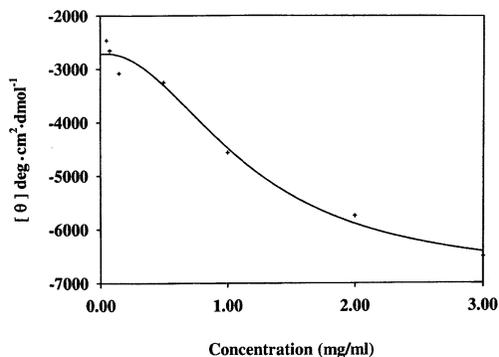


Fig. 3. Plot of change in $[\theta]$ at 283 nm as a function of protein concentration for α_{s1} -casein. The data were derived from Fig. 2 and fitted with Eq. 4; the calculated apparent constants are given in Table 2.

from CD data. At high ionic strength k_3 and k_4 from the sedimentation data again are in moderate agreement with the k_1 and k_2 from the CD data. Note that at low ionic strength the light scattering, ultracentrifuge and the CD-derived apparent association constants are all in reasonable agreement where the association (monomer-dimer) is relatively simple. The interpretation of the k values obtained from the non-linear regression programs used in the CD experiments is straight forward and the agreement with light scattering and ultracentrifuge data is quite good at lower concentrations. In contrast, the interpretation of the n values is cloudy, especially at high salt. They are nominally derived as stoichiometric values (Eq. 3), but even for simple enzyme substrate interactions or ligand binding [3,16] these values may represent some degree of cooperativity, a delay in the concentration-dependent onset of a transition, or be no more than fitting constants representing a range of possible values [16]. Examination of Table 2 bears this out in that the values for n and m which gave the best fit to the data differ from the ultracentrifuge data. However, taken together all of the data support increased changes in the CD of the aromatic regions of the protein, accompanying increased self-association of the protein.

3.2. Near-UV CD studies of carboxypeptidase A generated α_{s1} -casein(1-197)

Of the two tryptophan residues in α_{s1} -casein, Trp199 is the C-terminal residue and, from fluorescence studies, is not predicted to be directly involved protein self-association [21,26]. The other tryptophan in α_{s1} -casein, Trp164, is located in a hydrophobic region of the protein which may be a viable site for dimerization or the formation of even higher order aggregates. Changes in the near-UV CD spectrum for the carboxypeptidase A treated peptide α_{s1} -casein(1-197) as a function of concentration are shown in Fig. 4. All spectra in Fig. 4 were obtained at pH 6.75, 25°C, and low ionic strength (10 mM dipotassium PIPES buffer, $\mu = 0.0175$ M). The spectral features in Fig. 4 look much like those in Fig. 2. A general increase in molar ellipticity and concurrent red shift are observed for the putative tyrosine side chain band at $\lambda = 283$ nm. A change in sign and similar increase in ellipticity for tryptophan ($\lambda = 292$

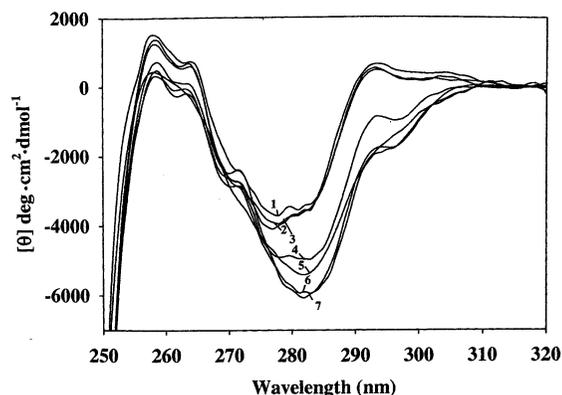


Fig. 4. Changes in the near-UV CD spectra of carboxypeptidase A-treated α_{s1} -casein(1-197) as a function of protein concentration at pH 6.75, 25°C and ionic strength = 0.0175. Protein concentrations were 0.075, 0.10, 0.15, 1.0, 1.5, 2.0 and 3.0 mg/ml, lines 1-7, respectively. Molar ellipticity $[\theta]$ is expressed in degrees \cdot cm² per dmol.

nm) as a function of concentration is likewise observed. Comparison of the data from Figs. 2 and 4 indicate that tyrosine and tryptophan residues clearly both contribute to self-association of α_{s1} -casein and α_{s1} -casein(1-197). While eight out of the ten tyrosine residues in α_{s1} -casein lie in the hydrophobic C-terminal half of the molecule, it is not clear from the data in Figs. 2 and 4 specifically which are involved in dimerization and/or higher degrees of association. However, Trp164 appears to be involved in the overall association of both α_{s1} -casein and α_{s1} -casein(1-197). Given that Trp164 lies in the middle of a series of eleven residues (Tyr159-Leu169) which constitute a strongly hydrophobic portion of the protein, this region is a likely site of hydrophobic aggregation. The absence of Trp 199 appears not to alter the CD spectra. After treatment with carboxypeptidase A, the degree of purity was checked by HPLC as described in Section 2. Under these conditions α_{s1} -casein has a retention time of 17.7 ± 0.1 min. The 1-197 polypeptide displays a reduced retention time (16.9 ± 0.1 min). This is marked enough that 1:1 mixtures are readily resolved. Thus, at acidic pHs (~ 2) Trp199 is surface accessible.

Near-UV CD of α_{s1} -casein(1-197) at high ionic strength (0.224 M), appeared similar to those of native α_{s1} -casein (data not shown). Band intensities were proportionally larger at each protein concentration as a function of increasing ionic strength as was

observed for the intact protein, and θ_{\max} values (Table 2) for the two are comparable. The CD band for tyrosine ($\lambda = 283$ nm) shows the largest change as a function of both ionic strength and polypeptide concentration, though the tryptophan spectrum shows similar changes indicating the influence of Trp164 in progressive association steps at higher ionic strengths. The apparent association constants and θ_{\max} values calculated from the CD data are quite comparable for the intact protein and (1–197) at both low and high ionic strengths (Table 2). The lack of influence of Trp199 in affecting associations of whole α_{s1} -casein (cf. Fig. 2 vs. Fig. 4) compares favorably with fluorescence studies [21] of casein and with casein micelle digestion with carboxypeptidase A by Ribadeau-Dumas and Garnier [27]. In the latter study, carboxypeptidase A was shown to quantitatively remove the C-terminal tryptophan of α_{s1} -casein in native and reconstituted micelles. The general availability of Trp199 to proteolytic digestion is in accord with results presented here, illustrating little participation of the two C-terminal residues in associations of α_{s1} -casein with itself or in casein colloids.

3.3. Self-association studies of cyanogen bromide generated α_{s1} -casein(136–196)

Although the self-association of α_{s1} -casein is governed to a large extent by hydrophobic attractions, electrostatic interactions cannot be neglected. The degree of ionization of the protein and the screening effect of the supporting electrolyte have been shown to influence associations [17]. Much of the hydrophilicity of native α_{s1} -casein arises from the N-terminal half of the molecule. The particular significance of tyrosine and tryptophan side chains in forming α_{s1} -casein aggregates may be better interpreted by investigation of the hydrophobic portion of the protein, namely the α_{s1} -casein(136–196) peptide. Isolation of this sizable portion of the C-terminal half of the protein is achieved by chemical cleavage with cyanogen bromide and purification on DEAE–Sepharose as described in Section 2. Reversed phase HPLC on a C4 column was used to monitor the separation of the CNBr fragments; four major peaks were observed. The peptide of interest (136–196) was identified by collection of the HPLC peak, amino acid analysis,

Table 2
Comparison of association constants (l/g) and apparent molecular weights for α_{s1} -casein and its cleavage fragments derived from ultracentrifuge and circular dichroism analysis

| | Analytical ultracentrifuge (l/g) | | | Circular dichroism (l/g) | | | |
|----------------------------|--------------------------------------|-------------------------------------|---------------------------------|------------------------------|----------------------|--------------------|--|
| | MW _w ^a (Da) | $k_2(n_2)^{b,d,e}$ | $k_3(n_3)^b$ | $k_4(n_4)^b$ | $k_1(n)^c$ | $k_2(m)^c$ | θ_{\max} (deg·cm ² per dmol) ^c |
| Low salt $\mu = 0.0175$ | | | | | | | |
| α_{s1} -Casein | 34 700 | 0.88 (2) ^b | | | 0.85 ± 0.13 (2) | | –6950 |
| α_{s1} -1 to 197 | – | – | | | 0.78 ± 0.11 (2) | | –5810 |
| α_{s1} -136 to 196 | 14 600 | 1.34 ± 0.18 (2) ^d | 2530 ± 830 (6) ^d | | 3.94 ± 0.53 (3) | | –4060 |
| Medium salt $\mu = 0.0889$ | | | | | | | |
| α_{s1} -Casein | 56 200 | 0.007 (2) ^b | 0.020 (3) | 6.82 (4) | 5.95 ± 0.42 (1) | | –7410 |
| High salt $\mu = 0.224$ | | | | | | | |
| α_{s1} -Casein | 68 000 | 0.021 (2) ^b | 0.32 (3) | 12.1 (4) | 0.63 ± 0.03 (16) | 8.1 ± 2.5 (1) | –10 100 |
| α_{s1} -1 to 197 | – | – | – | – | 0.61 ± 0.18 (16) | 5.2 ± 0.38 (4) | –11 100 |
| α_{s1} -136 to 196 | 37 900 | 0.0486 ± 0.151 (2) ^e | 2220 ± 720 (5) ^e | – | 8.80 ± 0.65 (10) | | –6000 |

^aWeight average molecular weight, three determinations $\pm 5\%$.

^b k_n and n_i as described in Table 1.

^c k_i , θ_{\max} , and n or m as described in Eqs. 1 and 4; the error is the error of the fit, and n and m values represent the integers at which the lowest RMS is obtained for the fit [3,16] of the model to the data.

^dThe sequence weight of α_{s1} -casein(136–196) is 6995, which was used in Eq. 6 to determine k_n ; average of six determinations, $n_3 = \pm 1$.

^eThe peptide ‘protomer’ molecular weight here was 14000 for Eq. 6; average of six determinations, $n_3 = \pm 1$.

and N-terminal sequencing. In the sequencing procedure, starting with 700 pmol of peptide the first 36 N-terminal residues were sequenced with statistical confidence. This peptide has an HPLC retention time of 16.3 ± 0.2 min, just slightly less than α_{s1} -casein(1–197). The retention time indicates the strong binding of this portion of α_{s1} -casein to the C4 reversed phase media. In fact, the late elution of the relatively low charged peptide from DEAE–Sephacose also points to the extreme hydrophobicity of the peptide. The calculated molecular weight from sequencing for this peptide is 6995.

The association properties of this peptide as a function of ionic strength may give insight into the nature of the properties of its parent, α_{s1} -casein. Fig. 5 shows a plot of peptide absorbance (at $\lambda = 283$ nm) vs. radial distance from a sedimentation equilibrium experiment for a 1 mg/ml (0.15 mM) solution of α_{s1} -casein(136–196) at 25°C, pH 6.75 at low ionic strength (0.0175 M) in 10 mM dipotassium PIPES buffer. The weight average molecular weight was 14 600, indicating predominantly dimer but with some higher associative species indicated by deviation of the last group of data points at large radius values (> 7.14 cm). An improved statistical fit is shown in Fig. 5, where the regression fit is to a three-component model, a monomer of 7000, a dimer of 14 000, and an oligomer, where n_3 for the oligomer is 6. The average effective molecular weights and association constants calculated from six such experiments are given in Table 2. It should be noted that there is some statistical uncertainty in the calculation of $n = 6$ for the oligomeric aggregation state, due to the statistical weighting of a relatively few data points at larger radial distance values; this leads to a variance of ± 1 for repeated experiments. Thus at low ionic strength for α_{s1} -casein(136–196), there is a low population of monomer, a notable population of dimer, and a small population of associated species which may include higher-order aggregates.

Sedimentation equilibrium measurements for α_{s1} -casein(136–196) at high ionic strength (0.224 M) showed a much higher degree of peptide association. Association constants and effective molecular weights are given in Table 2. The weight average molecular weight here is 37 900. The best nonlinear regression fit to the data (not shown) was by using a three component model, beginning with the 14 000

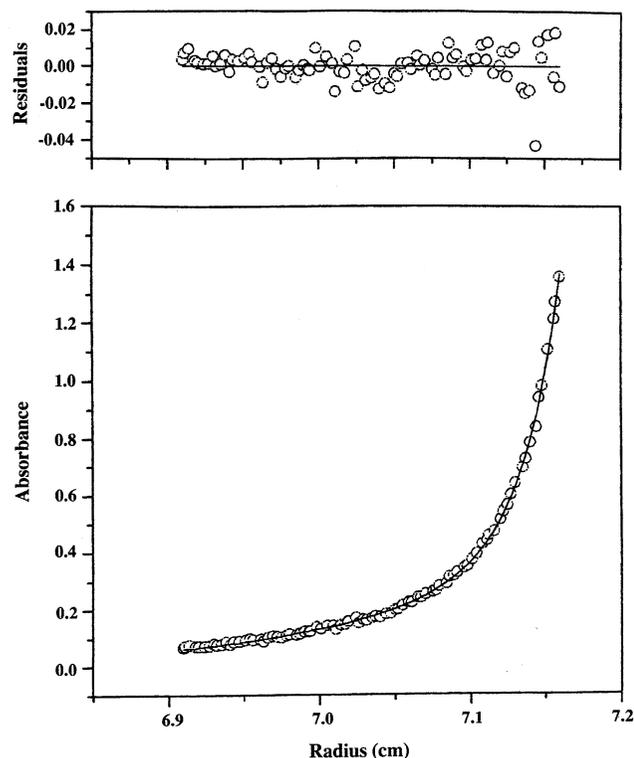


Fig. 5. Analytical ultracentrifugation analysis of α_{s1} -casein(136–196) at 25°C in PIPES buffer; $\mu = 0.0175$. The lower graph shows the fit to the absorbance vs. radius plot for Eq. 6 at 26 000 rpm; the upper plot shows the residuals for the fit showing no pattern. The chi-square value for this fit was 1×10^{-4} ; average parameters for k and n are given in Table 2. The apparent ‘monomer’ molecular weight near the meniscus was 7000 ± 1000 .

dimer (protomer) and extending to tetramer and higher oligomers. Here no fits could be made using 7000 as the starting point for fitting. Note that for k_3 , $n = 5 \pm 1$ as noted above. At 0.224 M ionic strength there appear to be no monomeric forms of α_{s1} -casein(136–196), indicating the predominance of a strong hydrophobically-driven self-association under these conditions.

3.4. Near-UV CD studies of cyanogen bromide generated α_{s1} -casein (136–196)

Fig. 6 shows the near-UV CD spectra for various concentrations of α_{s1} -casein(136–196) at low ionic strength ($\mu = 0.0175$ M). Spectra were obtained at pH 6.75, $T = 25^\circ\text{C}$ in a 10 mM dipotassium PIPES buffer. There is a strong increase in molar ellipticity with increasing peptide concentration for tyrosine

side chains monitored at $\lambda=283$ nm. This change in band intensity, along with an observed red shift, again, indicate that these residues are in a less-exposed environment. The magnitude of change in band intensity and red shift is not quite as large for tryptophan side chains ($\lambda=292$ nm). The results of a similar near-UV CD study of α_{s1} -casein(136–196) at high ionic strength ($\mu=0.224$ M) are shown in Fig. 7. The spectra were obtained in a 0.2 M KCl solution in dipotassium PIPES at pH 6.75 and $T=25^\circ\text{C}$. Perturbations in the tyrosine and tryptophan CD spectra are considerably more noticeable. The ellipticity change for tyrosine at $\lambda=283$ nm as a function of peptide concentration is quite large, with a noteworthy jump in band intensity and red shift between 0.059–0.13 mg/ml. The most remarkable feature of the near-UV CD behavior of α_{s1} -casein(136–196) at high ionic strength is the exceptional change in the tryptophan spectrum at $\lambda=292$ nm. Between 0.030 and 0.059 mg/ml peptide there is little change in the tryptophan spectrum. Above 0.059 mg/ml the sign of the tryptophan band changes, and the magnitude of the tryptophan band intensity increases substantially. Although the tryptophan band position does not appear to change to any great extent, the large jump in molar ellipticity between 0.059 and 0.13 mg/ml indicates a contribution of Trp164 region to the formation of peptide aggregates at high ionic strengths. This correlates with the ultracentrifuge

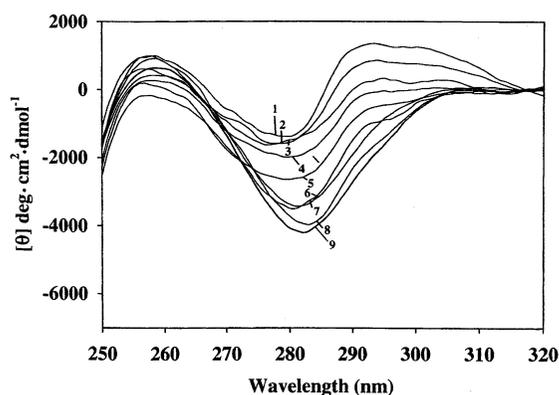


Fig. 6. Changes in the near-UV CD spectra of α_{s1} -casein(136–196) as a function of peptide concentration in PIPES at pH 6.75, 25°C and $\mu=0.0175$. Peptide concentrations were 0.03, 0.05, 0.13, 0.21, 0.26, 0.29, 0.44, 0.59 and 0.90 mg/ml, lines 1–9, respectively. Molar ellipticity $[\theta]$ is expressed in degrees-cm² per dmol. The concentrations of peptide were chosen to cover the molar concentration range used for intact protein.

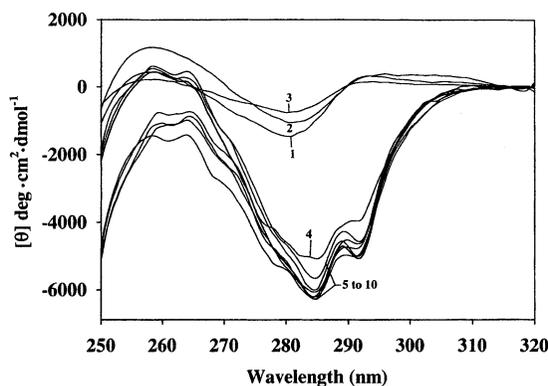


Fig. 7. Changes in the near-UV CD spectra of α_{s1} -casein(136–196) as a function of peptide concentration in PIPES at pH 6.75, 25°C and $\mu=0.224$. Molar ellipticity $[\theta]$ is expressed in degrees-cm² per dmol. Peptide concentrations were 0.030, 0.044, 0.059, 0.13, 0.21, 0.26, 0.30, 0.44, 0.59 and 0.87 mg/ml, lines 1–10, respectively. The concentrations of peptide were chosen to cover the molar concentration range used for the intact protein.

data which show the peptide to be dimer at low ionic strengths (weight average molecular weight = 14 600) and highly associated at high ionic strength (37 900 weight average molecular weight).

The results shown in Fig. 6 reveal that aromatic side chains are involved in hydrophobically-driven aggregation of α_{s1} -casein(136–196), with tyrosine appearing to be the predominant associating aromatic species at low ionic strength, where the peptide is predominantly dimer. In the previously published [7] putative molecular model for native α_{s1} -casein shown in Fig. 1, several proline-based turn structures were hypothesized to be effective sites of dimerization. There are three tyrosines (Tyr144, 146 and 153) in proximity to the Pro147-based turn, making this a probable site of dimerization. Additionally, there are three phenylalanine residues (Phe145, 150 and 152) in this region. These hydrophobic residues may also help to stabilize peptide-peptide interactions, although there is no direct spectral evidence from this study to support this latter speculation, because of the lower extinction of the Phe residues. There are three tyrosines (Tyr159, 165 and 166) and one tryptophan (Trp164) in proximity to the Pro168-based turn in the putative model. Based on the near-UV CD results in Fig. 6, this region may also be a site for the formation of dimers and small oligomers ($n=6$). However, in view of results from the near-UV CD study at high ionic strength (Fig. 7), it is

clear that the region near Trp164 plays a role in the formation of high-order aggregates at high ionic strength and higher (> 0.06 mg/ml) peptide concentrations. Based on comparison with association-reaction information obtained from the sedimentation experiment, we propose that the turn region about Pro147 is the initial site of hydrophobic aggregation (dimerization), with the turn region about Pro168 more involved in the formation of larger oligomers. As the tryptophan CD spectrum is perturbed to a much greater extent at high ionic strength for α_{s1} -casein(136–196) than it is for native α_{s1} -casein and α_{s1} -casein(1–197) under similar conditions, it is concluded that the region centering on Pro168, as reported upon by the CD of Trp164, is important in the stabilization of higher order hydrophobic α_{s1} -aggregates. The nature and importance of peptide secondary structure in the self-association of α_{s1} -casein(136–196) is the subject of the following paper in this issue [10].

The stability of the polypeptide chain of α_{s1} -casein around Pro147 is of additional interest for its potential role in cow's milk allergy (CMA), which has a prevalence of about 0.3–7.5% in infants [28]. The major allergens in bovine milk are α_{s1} -casein and the whey protein β -lactoglobulin. Both of these proteins are essentially absent from human milk [29]. A recent study identified three B-cell epitopes of bovine α_{s1} -casein [30]. Tested sera from 15 patients with acute clinical reactions to cow's milk showed reactions with each of three regions in α_{s1} -casein, corresponding to residues 19–30, 93–98 and 141–150. Whether the antigenic regions of α_{s1} -casein contain sequential epitopes or conformational epitopes remains somewhat uncertain, but conformationally stable peptides are often favored as antigens. The antigenic site at residues 141–150 is found in the cyanogen bromide cleavage peptide α_{s1} -casein(136–196) and may be the site of antibody interaction with the proline-directed hydrophobic 'arm' (residues 136–159).

4. Conclusion

It has been long recognized that the stability of casein submicelles is chiefly due to protein-protein interactions [19,29]. Aromatic side chains can pro-

mote stabilization of nonpolar regions of associated proteins. Near-UV CD results indicate that both tyrosine and tryptophan side chains are involved in self-association of α_{s1} -casein and α_{s1} -casein(1–197). CD and sedimentation data for the cyanogen bromide cleavage peptide, α_{s1} -casein (136–196) indicate that hydrophobic attractive forces predominate in peptide self-association. The tyrosine-rich area near Pro147 is presumed to be the site of dimerization at low ionic strength, while the hydrophobic region around Pro168, containing residue Trp164, is suggested to be a significant site for the formation of tetramers and higher-order aggregates. Hydrophobic amino acids play an important role in casein-casein interactions, and therefore determine to some extent a variety of milk product functional properties, such as foaming, gelation and emulsification.

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Effect of self-association of α_{s1} -casein and its cleavage fractions α_{s1} -casein(136–196) and α_{s1} -casein(1–197),¹ on aromatic circular dichroic spectra: comparison with predicted models

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Abstract

The self-association of native α_{s1} -casein is driven by a sum of interactions which are both electrostatic and hydrophobic in nature. The dichroism of aromatic side chains was used to derive regio-specific evidence in relation to potential sites of α_{s1} -casein polymerization. Near-ultraviolet circular dichroism (CD) revealed that both tyrosine and tryptophan side chains play a role in α_{s1} -casein associations. Spectral evidence shows these side chains to be in an increasingly nonaqueous environment as both ionic strength and protein concentration lead to increases in the degree of self-association of the protein from dimer to higher oligomers. Near-UV CD investigation of the carboxypeptidase A treated peptide, α_{s1} -casein(1–197), indicated that the C-terminal residue (Trp199) may be superficial to these interactions, and that the region surrounding Trp164 is more directly involved in an aggregation site. Similar results for the cyanogen bromide cleavage peptide α_{s1} -casein(136–196) indicated the presence of strongly hydrophobic interactions. Association constants for the peptides of interest were determined by analytical ultracentrifugation, and also were approximated from changes in the near-UV CD curves with protein concentration. Sedimentation equilibrium experiments suggest the peptide to be dimeric at low ionic strength; like the parent protein, the peptide further polymerizes at elevated (0.224 M) ionic strength. The initial site of dimerization is suggested to be the tyrosine-rich area near Pro147, while the hydrophobic region around Pro168, containing Trp164, may be more significant in the formation of higher-order aggregates. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Casein; Association; Cyanogen bromide cleavage; Circular dichroism; Analytical ultracentrifugation; Peptide interaction

1. Introduction

The chief source of calcium for neonatal mammals

Abbreviations: CD, circular dichroism; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid)

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¹ Mention of brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

is the colloidal calcium–phosphate transport complex of milk: the casein micelle. The major protein component of the bovine casein micelle is α_{s1} -casein. This protein is believed to form the framework for the colloidal casein micelle, in part because it does not exhibit highly temperature-dependent interactions [1]. Removal of calcium from bovine milk is thought to result in the dissociation of colloidal casein micelles into noncolloidal protein complexes called submicelles [1]. These submicelles consist of α_{s1} -, α_{s2} -, β -

clear that the region near Trp164 plays a role in the formation of high-order aggregates at high ionic strength and higher (> 0.06 mg/ml) peptide concentrations. Based on comparison with association-reaction information obtained from the sedimentation experiment, we propose that the turn region about Pro147 is the initial site of hydrophobic aggregation (dimerization), with the turn region about Pro168 more involved in the formation of larger oligomers. As the tryptophan CD spectrum is perturbed to a much greater extent at high ionic strength for α_{s1} -casein(136–196) than it is for native α_{s1} -casein and α_{s1} -casein(1–197) under similar conditions, it is concluded that the region centering on Pro168, as reported upon by the CD of Trp164, is important in the stabilization of higher order hydrophobic α_{s1} -aggregates. The nature and importance of peptide secondary structure in the self-association of α_{s1} -casein(136–196) is the subject of the following paper in this issue [10].

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Abstract

The self-association of native α_{s1} -casein is driven by a sum of interactions which are both electrostatic and hydrophobic in nature. The dichroism of aromatic side chains was used to derive regio-specific evidence in relation to potential sites of α_{s1} -casein polymerization. Near-ultraviolet circular dichroism (CD) revealed that both tyrosine and tryptophan side chains play a role in α_{s1} -casein associations. Spectral evidence shows these side chains to be in an increasingly nonaqueous environment as both ionic strength and protein concentration lead to increases in the degree of self-association of the protein from dimer to higher oligomers. Near-UV CD investigation of the carboxypeptidase A treated peptide, α_{s1} -casein(1–197), indicated that the C-terminal residue (Trp199) may be superficial to these interactions, and that the region surrounding Trp164 is more directly involved in an aggregation site. Similar results for the cyanogen bromide cleavage peptide α_{s1} -casein(136–196) indicated the presence of strongly hydrophobic interactions. Association constants for the peptides of interest were determined by analytical ultracentrifugation, and also were approximated from changes in the near-UV CD curves with protein concentration. Sedimentation equilibrium experiments suggest the peptide to be dimeric at low ionic strength; like the parent protein, the peptide further polymerizes at elevated (0.224 M) ionic strength. The initial site of dimerization is suggested to be the tyrosine-rich area near Pro147, while the hydrophobic region around Pro168, containing Trp164, may be more significant in the formation of higher-order aggregates. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Casein; Association; Cyanogen bromide cleavage; Circular dichroism; Analytical ultracentrifugation; Peptide interaction

1. Introduction

The chief source of calcium for neonatal mammals

Abbreviations: CD, circular dichroism; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid)

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is the colloidal calcium–phosphate transport complex of milk: the casein micelle. The major protein component of the bovine casein micelle is α_{s1} -casein. This protein is believed to form the framework for the colloidal casein micelle, in part because it does not exhibit highly temperature-dependent interactions [1]. Removal of calcium from bovine milk is thought to result in the dissociation of colloidal casein micelles into noncolloidal protein complexes called submicelles [1]. These submicelles consist of α_{s1} -, α_{s2} -, β -