

Secondary Structural Studies of Bovine Caseins: Structure and Temperature Dependence of β -Casein Phosphopeptide (1–25) as Analyzed by Circular Dichroism, FTIR Spectroscopy, and Analytical Ultracentrifugation¹

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The defining structural feature of all of the caseins is their common phosphorylation sequence. In milk, these phosphoserine residues combine with inorganic calcium and phosphate to form colloidal complexes. In addition, nutritional benefits have been ascribed to the phosphopeptides from casein. To obtain a molecular basis for the functional, chemical, and biochemical properties of these casein peptides, the secondary structure of the phosphopeptide of bovine β -casein (1–25) was reexamined using Fourier transform infrared (FTIR) and circular dichroism (CD) spectroscopies. Both methods predict secondary structures for the peptide which include polyproline II elements as well as β -extended sheet and turn-like elements. These structural elements were highly stable from 5° to 70°C. Reexamination of previously published ¹H NMR data using chemical shift indices suggests structures in accord with the CD and FTIR data. Dephosphorylation showed little or no secondary structural changes, as monitored by CD and FTIR, but the modified peptide demonstrated pronounced self-association. The polymers formed were not highly temperature sensitive, but were pressure sensitive as judged by analytical ultracentrifugation at selected rotor speeds. Molecular dynamics (MD) simulations demonstrated relatively large volume changes for the dephosphorylated peptide, in accord with the pressure dependent aggregation observed in the analytical ultracentrifuge data. In contrast the native peptide in MD remained relatively rigid. The physical properties of the peptide suggest how phosphorylation can alter its biochemical and physiological properties.

KEY WORDS: Casein structure; protein functionality; milk proteins.

1. INTRODUCTION

The caseins of milk form a unique calcium-phosphate transport complex in the mammary gland. These colloidal complexes sequester calcium in the Golgi apparatus and, as secreted, are the chief source of calcium for the mammalian neonate. At the heart of the interactions which lead to casein micelle formation, is the binding of Ca^{2+} to the phosphorylated caseins α_{s1} -, β -, and α_{s2} -caseins

(Farrell, 1999). β -Casein represents 36% of bovine casein (Farrell and Thompson, 1988) and has a variety of functional properties which in some cases are similar to but mostly different from those of α_{s1} - and κ -caseins (Horne, 1998). Moreover, the relative amount of β -casein differs significantly from species to species; e.g., in human milk in which small amounts of α_{s1} -casein occur, β - is the major casein component (Farrell and Thompson, 1988). The bovine β -casein molecule is a single chain of known sequence (Ribadeau-Dumas *et al.*, 1972) with five phos-

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Abbreviation key: CD = circular dichroism, FTIR = Fourier transform infrared, RMS = root mean square, NMR = nuclear magnetic resonance, MD = molecular dynamics, M_w = weight average molecular weight, Pse = phosphoserine, Σ = the phosphorylated form of S (serine) or T (threonine).

phoserine residues and a molecular weight of 23,980 Da. The charge frequency, hydrophobicity, and net charge for the various segments of β -casein are presented in Table I. Analysis of these data indicates that β -casein could be a linear amphiphile and thus much more "soap-like" than other caseins (Farrell, 1988). The N-terminal portion of the β -casein molecule (residues 1 to 25) contains four of the five phosphoserine residues which bind calcium, and carries essentially much of the protein's net charge. In addition to their role in the formation of casein micelles, phosphopeptides from caseins can form complexes with calcium and phosphate and are of interest because they have been implicated as nutritionally active ingredients (Holt *et al.*, 1996). Phosphopeptides from casein have been suggested to improve Ca^{2+} absorption in animals (Kitts and Yauns, 1992) and eggshell quality in hens (Ashida *et al.*, 1996). Addition of phosphopeptides also effected oocyte maturation in culture and improved sperm survival in vitro (Nagai *et al.*, 1996). Finally, calcium-phosphopeptides are thought to help protect against human dental carries (Reynolds, 1987; Rose, 2000). However, in some cases casein phosphopeptides have been found to be allergenic to humans (Bernard *et al.*, 2000).

The amino acid sequences for these primary Ca^{2+} binding sites are highly conserved (Fig. 1) not only in caseins but in other highly phosphorylated proteins (Farrell and Thompson, 1998). The conformation of the β -casein phosphopeptide has been studied by NMR (Tsuda *et al.*, 1991; Wahlgren *et al.*, 1993; Cross *et al.*, 2001) and by CD³ (Chaplin *et al.*, 1988). These four studies drew contradictory conclusions in terms of their structural analyses; we have examined the structure of the β -casein phosphopeptide with both CD and FTIR, and compared the results to the previously reported NMR data (Tsuda *et al.*, 1991; Wahlgren *et al.*, 1993; Cross *et al.*, 2001).

Table I. Profile of the β -Casein Molecule Derived from Its Primary Structure^a

Residues considered	Net charge ^b	Charge frequency ^b	Average hydrophobicity ^c
1–25	–12	0.55	842
26–45	–4	0.5	640
44–92	–1½	0.1	1370
93–135	+1	0.28	1173
136–177	+2	0.12	1467
178–209	0	0.13	1738

^a Derived from the data of Ribadeau-Dumas *et al.* (1972).

^b Serine phosphate = –2, histidine = +0.5; charges (+ or –) per residue.

^c Calculated as described by Bigelow (1967) and expressed in kcal per residue.

By these comparisons we hope to present a better understanding of the polypeptide conformation of the primary calcium-binding sites of the caseins.

2. MATERIALS AND METHODS

2.1. Materials

All reagents used were of analytical grade or 'ACS certified' from Sigma (St. Louis, MO). Isolation and purification of both β -casein and its native and dephosphorylated peptide have been presented in some detail elsewhere (Szymanski and Farrell, 1982). Poly-L-glutamic acid (750–1500 MW) was purchased from Sigma (St. Louis, MO). Characterization by HPLC and sequencing followed the methods given in Alaimo *et al.* (1999a).

2.2. Predictions of Secondary Structures

Prediction of conformational states for the individual amino acid residues was accomplished by comparing the results of sequence-based predictive techniques, primarily those of Chou and Fasman (1978), Garnier *et al.* (1978), and Cohen *et al.* (1978, 1983). Proline-based turn predictions were made using the data of Rose *et al.* (1985) and Ananthanarayanan *et al.* (1984). In addition, polyproline II structures were predicted by the method of Adzhubei and Sternberg (1989).

2.3. Molecular Modeling

The SYBYL program (Tripos, St. Louis, MO) version 6.7 was used for all molecular modeling of native β -casein (1–25) and its dephosphorylated form. The construction of the native model was accomplished by drawing the predicted behavior of the polypeptide chain from its amino acid sequence and reconciling these predictions with our spectroscopic data. The Kollman all-atom force field and Kollman charges were used throughout all minimizations and simulations (Weiner *et al.*, 1986). The conjugated gradient algorithm was used for all energy minimizations. The TIP3P model was used for water. A precomputed explicit solvent box was used. A truncation scheme of 8 Å was used for van der Waals and electrostatic nonbonded interactions. The integration time step was 1 fs, and the nonbonded list was updated every 25 steps. All MD simulations were performed with the application of periodic boundary conditions.

The simulations were performed and analyzed on a SGI Octane 2 workstation equipped with dual processors.

	1									10								20							
Cow	R	E	L	E	E	L	N	V	P	G	E	I	V	E	Σ	L	Σ	Σ	Σ	E	E	S	I	T	R
Swine	R	A	K	E	E	L	N	Σ	--	G	E	Σ	V	E	Σ	L	Σ	Σ	Σ	E	E	S	I	T	H
Human	R	--	--	--	--	--	--	--	--	--	E	Σ	I	E	Σ	L	Σ	Σ	Σ	E	E	S	I	P	E
Rat	R	E	K	D	A	F	Σ	V	Σ	Σ	E	Σ	--	G	Σ	I	--	Σ	Σ	E	E	S	V	E	H

2.4. Circular Dichroism (CD) Measurements

Characteristic changes in the degree of self-association of β -casein (1–25) could occur as a function of temperature, salt and protein contents (Farrell *et al.*, 2001). In order to correlate changes in θ with these as-

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

2.5. Infrared (FTIR) Spectroscopic Measurements

For infrared (FTIR) measurements, β -casein peptides were dissolved as 2.5% w/w aqueous solutions at pH 6.75 in 25 mM dipotassium PIPES (piperazine-N, N'-bis (2-ethanesulfonic acid) buffer with 8 mM KCl (ionic strength, 1 = 0.05 M). Spectra were obtained at 5, 10, 15, 25, 37, 50 and 70°C using a Nexus 870 FTIR spectrometer (Thermo-Nicolet, Madison, WI) operated with Thermo-Nicolet's Omnic© software (version 5.2). Samples were introduced in a temperature controlled demountable cell (Specac Inc., Smyrna, GA). The cell had CaF₂ windows that were separated by a 6- μ m Mylar spacer. Resolution was 1 cm⁻¹, with 1024 double-sided interferograms collected for each spectra. Two levels of

zero fill were employed. The double-sided interferograms were co-added, phase corrected (Mertz), apodized (Happ-Genzel), and fast Fourier transformed.

Secondary structural features were calculated from the amide I envelope after a nonlinear regression fitting of Gaussian peaks to the 1950–1150 cm^{-1} region. Initial peak locations were made using Bayesian and maximum-likelihood statistical methodology utilizing the Razor Library (Spectrum Square Associates, Ithaca, NY) contained in the Cutting Edge software package (Cutting Edge Technologies, Orono, ME). The identified peak locations were then used as starting points for the nonlinear regression fitting. The selected peaks were refitted to the original spectra twice. First using the iterative curve-fitting procedure previously reported (Kumosinski and Unruh, 1996) by this laboratory. Second, using a maximum-likelihood nonlinear fitting in the Cutting Edge program. The Cutting Edge program uses the Razor Fit algorithm, an iterative peak-fitting algorithm incorporating maximum-likelihood statistics. A “fit” was considered acceptable when the results from the first and second methods agreed and agreement was achieved between the second derivatives of the fit and original spectra. The data are reported as the sum of assigned spectral elements gathered from a 20-protein database (Kumosinski and Unruh, 1996).

2.6. Analytical Ultracentrifugation Measurements

For analytical ultracentrifugation, the peptide samples were dissolved in 25 mM PIPES plus 8 mM KCl at pH 6.75 (ionic strength = 0.05 M) at concentrations of 0.46 ± 0.02 mg/ml. Samples and solvents were filtered with a Waters (Milford, MA) HVLP 0.45- μm membrane filter; 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 12,000 to 46,000 rpm at selected temperatures. 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 1.004 ± 0.003 depending on the temperature of the experiment. These values were calculated from the data using 0.564 cc/g as the partial specific volume of PIPES (Alaimo *et al.*, 1999a). The partial specific volume of β -casein phosphopeptide was calculated to be 0.687, the dephosphorylated peptide was calculated to be 0.725 (McMeekin *et al.*, 1948). Data were collected at 230 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 (MW) for the total cell contents were obtained. Where self-association was apparent, 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. 5. For the dephosphorylated β -casein (1–25) the best fits were obtained by first fixing the sequence molecular weight at 2803 for the monomer or 5606 for dimer and floating one K value at the increasing integer values of n noted above; in practice only n_1 was employed. Analysis of the data was accomplished using the following equation:

$$A_r = \text{EXP}[\ln(A_0) + H \times M \times (X^2 - X_0^2)] + \text{EXP}[n_2 \times \ln(A_0) + \ln(K_{a2}) + n_2 \times H \times M \times (X^2 - X_0^2)] + \text{EXP}[n_3 \times \ln(A_0) + \ln(K_{a3}) + n_3 \times H \times M \times (X^2 - X_0^2)] + \text{EXP}[n_4 \times \ln(A_0) + \ln(K_{a4}) + n_4 \times H \times M \times (X^2 - X_0^2)] + E. \quad (2)$$

where A_r is total absorbance of all species at radius x , EXP is exponent, $\ln$ is natural log, A_0 is absorbance of the monomer species at reference radius X_0 , H is constant $[(1 - \bar{v}\rho) \omega^2 / 2RT]$, M is apparent monomer molecular weight (monomer or protomer for the peptide), X_0 is reference radius, n_i is stoichiometry for species i (number of monomers), K_{ai} is association constant for the monomer-nmer equilibrium of species i , and E is baseline offset.

As determined above in Eq. (2), K_{ai} may be converted to molar units using:

$$K_{\text{conc}} = K_{\text{abs}} \left[\frac{e1}{n} \right]^{n-1} \quad (3)$$

where K_{abs} is K_{ai} of the software, e is the molar extinction coefficient, 1 is the pathlength 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:

$$k_n = \frac{K_{\text{conc}}}{\left[\frac{M_{\text{monomer}}}{n} \right]^{n-1}} \quad (4)$$

here M_{monomer} is the monomer or protomer 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. Estimation of Secondary Structure of β -Casein Phosphopeptide by FTIR and CD

The Amide I region of the FTIR spectrum of poly-L-glutamate at 25°C and in PIPES-KCl at pH 6.75 is shown in Fig. 2A along with that of the phosphopeptide. For both peptides, there is a dispersion of absorbances centering on 1650 cm^{-1} . The spectrum of the synthetic polypeptide is only slightly less as broadly dispersed as that of the β -casein (1–25). The overall similarity of the two spectra suggest that poly-L-glutamate may be a good model for a portion of the β -casein (1–25). When the polyglutamate spectrum was fitted with Gaussian peaks (as was done for the native and dephosphorylated β -casein peptides), three major areas were delineated as shown in Fig. 2B and quantitated in Table II. For poly-L-glutamate, the ratio of the area at 1560 to that at 1650 cm^{-1} is 2.4, which is expected for the ratio of the combined N-H and C-O stretch of the backbone and side chain carbonyls to the backbone carbonyls (theoretically 2.6 for an 8-residue peptide, Byler and Farrell, 1989). For the native and dephosphorylated casein peptides, the experimental ratio is 0.5 and 0.8, respectively; sequence analysis of amide N-H plus side chain carbonyl to peptide carbonyl yields 0.7. As seen quantitatively in Table II, dephosphorylation of the peptide yields a somewhat more compact Amide I envelope, centering on 1653 cm^{-1} , with some 20% excess absorption at this wavenumber.

Bands centering on 1650 ± 5 might theoretically be attributed to elements of α -helix, but combined CD and FTIR experiments on other polypeptides (Wilder *et al.*, 1992) have suggested that bands centered on 1656 ± 2 cm^{-1} might arise from large loop rather than from α -helical structures, while Kumosinski and Unruh (1996) showed similar bands for the “random” form of poly-L-aspartate. However, for β -casein and other nonglobular proteins there is a growing body of evidence to suggest that these peptides and proteins contain significant amounts of polyproline II (PPII) structures (Farrell *et al.*, 2001; Syme *et al.*, 2002). Historically, Tiffany and Krimm (1968) were first to suggest this structural element (PPII) for the non- α -, non- β -structures of polyamino acids at neutral pH. Table II suggests a 20% increase in PPII for the dephosphorylated peptide perhaps at the expense of turn (1681 cm^{-1}).

Far-UV (190–250 nm) CD measurements were made using a 43 μM aqueous solution of β -casein (1–25) at pH 6.75 in a low ionic strength buffer (2 mM potassium PIPES + 4 mM KCl). Spectra of the native and dephosphorylated peptides were recorded at 6°C and

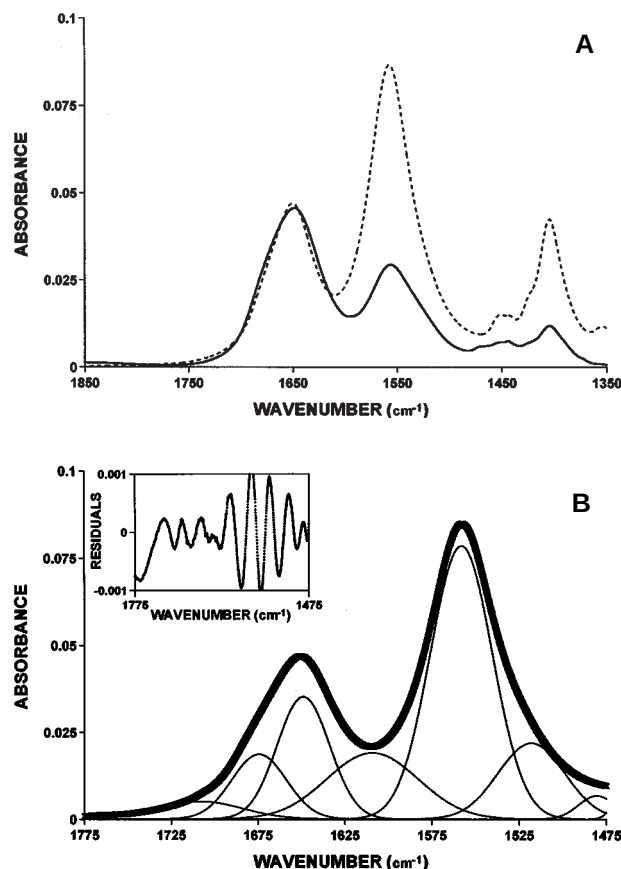


Fig. 2. A, Original FTIR spectra of poly-L-glutamate (dashed line) in 25 mM PIPES with 8 mM KCl at 25°C, and β -casein (1–25) (solid line) under the same conditions. B, Starting with the FTIR spectrum of poly-L-glutamate as in (A), amide I and II regions were fitted with Gaussian curves at selected peak positions as described in Methods and Materials. Following the initial assignments, nonlinear regression analysis was used to fit the data. Crosses represent experimental data; solid lines represent fitted individual Gaussian components and their sum. Upper inset is the corresponding residual plot obtained by comparison of the fitted with the original spectrum.

are shown in Fig. 3. The latter spectra are compared with poly-L-glutamate (750–1500 MW) under the same conditions. There are two significant differences in θ_r between the synthetic peptide and the β -casein peptides; these occur at 198 nm and in the depth of the trough at 224 nm. The spectra of the casein peptides, especially the trough at 224 nm, qualitatively suggest some sheet or turn in addition to PPII-like structure (Farrell *et al.*, 2001; Siligardi *et al.*, 1991; Makarov *et al.*, 1984). Analysis of the averaged spectra by CONTIN, indicates the presence of approximately 34% β -sheet, 29% turns, 32% remainder, and 5% helix for both casein peptides. Note that 5% helix in a 25-residue peptide amounts to less than two residues. CONTIN analysis of poly-L-glu-

Table II. Comparison of the Major Fitted Band Areas of the Amide I Region of Poly-L-Glutamic Acid with β -Casein (1–25) Native and Dephosphorylated

Wave number ($\text{cm}^{-1} \pm 2$)	Poly-L-glutamate ^a	β -Casein	
		Native ^a	Dephosphorylated ^a
		% Area	
1734	6.0 ± 1.0	4.1 ± 1.0	3.0 ± 1.0
1707	10.0 ± 2.1	13.4 ± 1.1	3.8 ± 1.2
1681	31.6 ± 2.8	26.5 ± 2.1	9.1 ± 2.0
1653	59.0 ± 4.3	63.5 ± 1.3	82.2 ± 0.8
Ratio: 1560:1650	2.5	0.7	0.5

^a Average seven determinations, two samples. All samples in PIPES-KCl at pH 6.75 and 25°C.

tamate, which is in the PPII state at pH 6.7 and at 6°C, gives 0% helix, 15% sheet, 30% turn, and 55% remainder; therefore the CD method may predict PPII structure for the β -casein peptides using poly-L-glutamate as a model, and taking the remainder term (32%) as a predictor of PPII structure (Sreerama and Woody, 1994).

Although the calculated percentages of structural elements from FTIR differ significantly from those obtained from far-UV CD, both methods may predict PPII structures. The lack of α -helix was clearly demonstrated in three NMR studies of this peptide (Tsuda *et al.*, 1991; Wahlgren *et al.*, 1993; Cross *et al.*, 2001) as well as the above CD results. Therefore the FTIR bands centering on 1650 (representing $\geq 60\%$ of the structure) may arise from segments of PPII. Comparison of the CD analysis of all three peptides suggest a similar lack of α -helix.

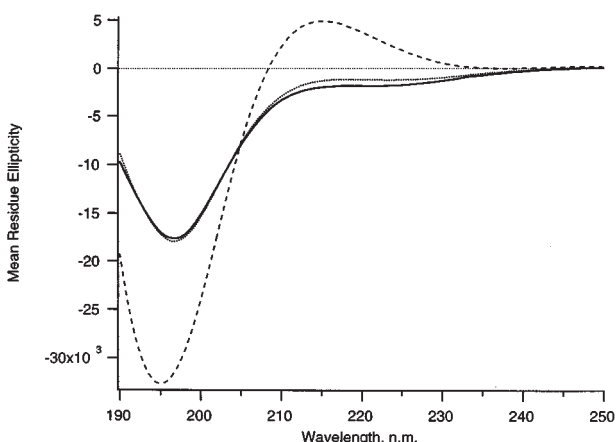


Fig. 3. Mean residue ellipticity $[\theta]$ as a function of wavelength at 6°C for the far-UV CD of native β -(1–25) 4P (dotted line), dephosphorylated β -(1–25) (solid line), and poly-L-glutamate (dashed line). Buffer conditions were 2 mM PIPES 4 mM KCl at pH 6.75. Concentrations of peptide were 0.260 ± 0.020 mg/ml. The ellipticities are expressed as mean residue weight-degree-cm²/dmol.

The N-terminal sequence of the phosphopeptide is rich in glutamic acid and could reflect the presence of a short run of a polyglutamate like structure. It was suggested by Tiffany and Krimm in 1968 that, at neutral pH, charged peptides such as poly(L-lysine) and poly(L-glutamate) are not truly random but contain elements of a left handed 3_1 -helix. Two spectral features of these charged polypeptides are IR bands between 1656 and 1652 cm^{-1} (Farrell *et al.*, 2001) and CD spectra similar to those shown for the β -casein peptides (Marakov *et al.*, 1984; Siligardi *et al.*, 1991). Additionally, Dukor and Keiderling (1991) reported similar assignments for poly-L-glutamate by vibrational CD. Finally, Adzhubei and Sternberg (1993) studied the frequency of occurrence of PPII in globular proteins and developed a database for the prediction of PPII. The predictive values range from 0 to 1 or greater and a run of values ≥ 1 are predictive of PPII in analogy with Chou-Fasman predictions. Table III shows that with the exception of Gly10 and Ile12 and 23 the majority of residues present in β -casein (1–25) are capable of attaining PPII structure.

3.2. Reanalysis of NMR Results for β -Casein Phosphopeptide (1–25) and Comparison with CD and FTIR Analyses

Studies on β -casein (1–25) by ^1H NMR, conducted by Wahlgren *et al.* (1993), showed little classical secondary structure, particularly from NOE connectivity. They suggested however that because trifluoroethanol can induce helix, that the phosphopeptide area could represent a nascent α -helix. A similar study was conducted by Tsuda *et al.* (1991), who concluded that no classical hydrogen bonded structures were present, so the peptide lacked any defined structure. In the latest NMR study Cross *et al.* (2001) suggested some defined structural elements for the phosphopeptide.

Table III. Structural Predictions for Native β -Casein (1–25): Polyproline II Predictions (PPII)^a as Well as Predictions from Chemical Shift Index (CSI)^b and ¹H-N NMR Relative to Random Coil Peptides: $\Delta = (\text{ppm}_{\text{obs}} - \text{ppm}_{\text{rc}})^c$

No.	PP II ^a	CSI ^b	$\Delta\text{N-H}^c$
E-2	0.93	+1	0.60
L3	1.02	+1	0.70
E4	0.93	0	0.20
E5	0.93	0	0.20
L6	1.02	+1	0.30
N7	0.96	0	0.20
V8	0.86	+1	0.15
P9	5.09	0	. . .
G10	0.37	+1	0.20
E11	0.93	+1	−0.10
I12	0.63	+1	0.25
V13	0.86	+1	0.33
E14	0.93	+1	0.20
$\Sigma 15^d$	1.00	+1	0.50
L16	1.00	+1	0.35
$\Sigma 17^d$	1.00	+1	0.50
$\Sigma 18^d$	1.00	+1	0.50
$\Sigma 19^d$	1.00	+1	0.55
E20	0.93	+1	0.02
E21	0.93	0	0.10
S22	1.00	0	0.15
I23	0.63	+1	0.15
T24	0.80	−1	0.20
R25	1.24	0	−0.10

^a Polyproline II Chou Fasman predictions from Adzhubei and Sternberg (1993), values ~ 1 or greater predict PPII.

^b Chemical Shift Index (CSI) is positive for β -structures and negative for α -helical structures as given in by Wishart and Sykes (1994); it was applied to the average chemical shifts of Tasude *et al.* (1991), Wahlgren *et al.* (1993), and Cross *et al.* (2001).

^c ppm_{rc} ; ppm_{obs} (Wishart and Sykes, 1994); a positive value >0.29 predicts β -structure while a negative value below -0.19 predicts α -helix.

^d Σ = Phosphoserine.

The chemical shift index (CSI), which was developed by Wishart and Sykes (1994), can be used for the qualitative determination of protein secondary structure based on amino acid C_{α} proton chemical shift values of proteins. CSI analysis was applied to the data of Tsuda *et al.* (1991) and Wahlgren *et al.* (1993) by Cross *et al.* (2001). The average CSI values for all three studies are given in Table III. A chemical shift value less than the range (−1) may indicate that the residue lies in a region of α -helix, a zero value implies random coil, while a value greater than the range (+1) may indicate β -strand or extended structure. The CSI for β -casein (1–25) as given in Table III, suggests that the sequence from Glu 11 through Pse 19 contains β -sheet. Overall, all of the residues show some propensity for an extended conformation in that there is only one helical predictor (−1 value) near the C terminal.

Information can also be gained from the amide proton data. The temperature dependence of amide proton chemical shifts can be strongly affected by hydrogen bonding. Low temperature coefficients (<4.0 ppb/ $^{\circ}\text{K}$) can result from *interresidue* hydrogen bonds in α -helices and reverse turns and *interstrand* hydrogen bonding in β -pleated sheets (Dyson *et al.*, 1988). All of the amide temperature coefficients for β -casein (1–25) casein, reported by Tsuda *et al.* (1991), were in the 6–9 ppb/ $^{\circ}\text{K}$ range, indicating that these residues are fully solvent exposed, and not involved in conventional α -helix or pleated β -sheet hydrogen bonds. By comparison completely random structures, however, have temperature coefficients ≥ 12 ppb/ $^{\circ}\text{K}$. This most likely means that on the NMR time scale the peptide can access only a relatively narrow range of conformational states with quite similar free energies. Moreover, the peptide has moderate temperature coefficients, but contains little backbone hydrogen bonds. It is interesting to note that PPII structures do not contain backbone hydrogen bonds (Adzhubei and Sternberg, 1993).

In general, amide N-H proton chemical shifts ($\Delta\text{N-H}$) for β -sheet structures average $+0.29$ ppm from random coil, while those of α -helices are -0.19 ppm from random (Wishart and Sykes, 1994). The $\Delta\text{N-H}$ values for the averaged data of Wahlgren *et al.* (1993) and Cross *et al.* (2001) are calculated in Table III. Residues 12–19 are strongly indicative of extended or β -sheet (note the $C_{\alpha\text{H}}$ indices are as well); thus this area may represent extended sheet with no apparent hydrogen bonding. The NMR evidence from medium-range NOEs and temperature coefficients further supports the conclusion that the peptide in solution does not adopt conventional helical or pleated sheet structures. However, as noted by Cross *et al.* (2001), these values are not indicative of a totally random chain, since both the CSI and amide proton shifts do argue for an extended structure for the phosphorylated region. A similar motif appears to be present in the N-terminal peptide (1–23) of α_{s1} -casein (Malin *et al.*, 2001).

The calculation of Tori and Tasumi (1998) suggests that poly-L-glutamate contains left-handed 3_1 -helical elements and would resemble PPII structures. They would have $\phi \psi$ angles of -100° and 170° ; that is, the upper left quadrant of a Ramachandran plot, near β -sheet. It had previously been shown from Ramachandran calculations that an individual amino acid residue in a “random coil” peptide (e.g., polyglutamate) does spend up to 95% of its time in the broad β -sheet region (Feeney *et al.*, 1972). These PPII elements may be represented on the NMR timescale by structures with N-H shifts such as shown in Table III for the N-terminal region of the phosphopeptide. Thus these elements would have positive N-H

chemical shifts close to β -sheet and FTIR bands around $1653 \pm 3 \text{ cm}^{-1}$ as shown in Fig. 2, and quantitated in Table II. The CD spectra for polypeptides with mixtures of PPII and extended structures are quite characteristic (Makarov *et al.*, 1984; Siligardi *et al.*, 1991), and resemble those shown for the β -casein peptides (Fig. 4). This would suggest a significant amount of PPII like structure for the β -phosphopeptide. Additionally, PPII structures are open to solvent and not hydrogen bonded (Adzhubei and Sternberg, 1993; Creamer, 1998), which is in agree-

ment with all of the NMR studies. Finally, a recent Raman optical activity study has suggested that PPII motifs may occur in caseins (Syme *et al.*, 2001).

No classic four residue β -turn occurs in Pro 9 through Glu 11 or in the C-terminal region of the β -casein (1–25), as judged by NOE values (Tsuda *et al.*, 1991; Whalgren *et al.*, 1993). However, all secondary structural algorithms predict some type of turn for these areas especially the C-terminal region, and so turn elements seen in CD could be represented in part by open nonhydrogen-bonded turns.

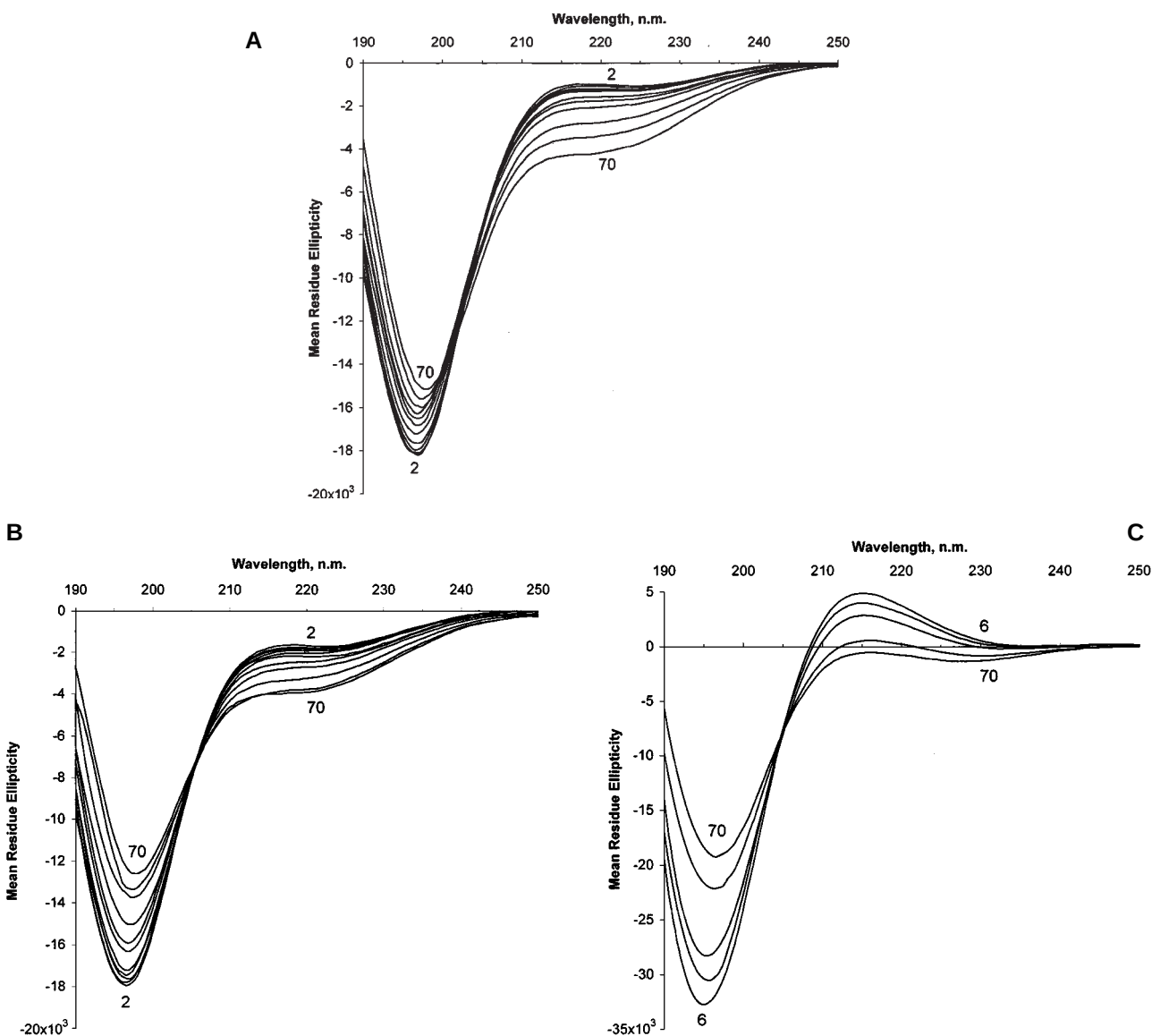


Fig. 4. Mean residue ellipticity $[\theta_r]$ as a function of wavelength for the far-UV CD of (A) β -casein (1–25) at 2°, 4°, 6°, 8°, 10°, 15°, 25°, 37°, 50°, and 70°C (solid lines labeled with high and low temperatures), buffer conditions were 2 mM PIPES 4 mM KCl at pH 6.75. B, Dephosphorylated (1–25) casein. C, Poly-L-glutamate at 6°, 15°, 25°, 50° and 70°C. Concentrations of peptides were $0.260 \pm 0.020 \text{ mg/ml}$. The ellipticities are expressed in degree $\cdot \text{cm}^2/\text{dmol}$: mean residue ellipticity.

3.3. Effects of Temperature on FTIR Analysis of Spectra of Native and Dephosphorylated β -Casein (1–25)

Secondary structural features were calculated for each FTIR spectrum taken at seven temperatures: 5°, 10°, 15°, 25°, 37°, 50°, and 70°C. As the peptide is heated, there are no statistical changes over the amounts of structure shown in Table II. This indicates that there is little thermal unfolding of local structures. Dephosphorylation of the peptide yielded similar results when the spectra were fitted and analyzed over the same temperature range. It appears, however, as though dephosphorylation has a slight effect on structural elements as quantitated from the Amide I region of the spectra. The dephosphorylated peptide has overall a slightly narrower Amide I envelope as >80% of the absorbance is centered on 1653 cm^{-1} compared with 60% for the native peptide regardless of temperature. There is some loss of area centering on 1681 cm^{-1} for the dephosphorylated peptide (Table II); this could be conversion from turn or β -extended to PPII. Overall, the relative insensitivity to temperature, as shown by FTIR, suggests that the native peptide is stabilized by its net negative charge and its predicted and persistent PPII structure.

3.4. Effects of Temperature on CD Spectra of Native and Dephosphorylated β -Casein (1–25)

Figure 4A shows the change in mean residue ellipticity as a function of wavelength in the far-UV CD for

β -casein (1–25) at 11 different temperatures. The calculated secondary structural features for each dichroic spectrum in Fig. 4A are given in Table IV. In agreement with the FTIR analysis, as the peptide is heated, there is little change in the calculated percentages of unordered, sheet or turn structure, indicating that there is little thermal unfolding of local structures. When dephosphorylated, Fig. 4B, the spectra of the peptide demonstrate qualitatively larger and more progressive temperature-dependent changes. There is a slight red shift, a decrease in the negative value of $[\theta_r]$ at λ_{max} , and a progressive increase in the negative ellipticity of the trough centering on 224 nm. However, CONTIN analysis did not yield significantly different results from those of Table IV for the native peptide. Figure 4C shows the effect of temperature on a short chain poly-L-glutamate under the same conditions; the changes mirror those found for the peptides of β -casein, but the trough at 224 nm deepens less. Again, the CONTIN analysis revealed no calculated temperature changes for poly-L-glutamate (Table IV). Thus, while visual inspection of all the spectra (Fig. 4) suggests temperature dependent change, the database analysis is negative.

Figure 5 shows the change in intrinsic viscosity of the solvent with temperature, and the accompanying changes in $[\theta_r]$ for native and dephosphorylated peptides and poly-L-glutamate. While the viscosity changes rapidly over the lower temperature range, the relative change in θ_r remains much lower for the native peptide but greater for the dephosphorylated peptide and greatest for the poly-L-glutamate. One interpretation of the data

Table IV. Effects of Temperature on CD Secondary Structural Estimates for the Native and Dephosphorylated β -Casein Phosphopeptide (1–25) and Poly-L-Glutamate

Method ^a	Temperature	% β -Sheet	% Turns	% Unspecified	% α -Helix
Native β -(1–25)	2°C	34	30	32	5
	4°C	33	30	32	5
	6°C	34	29	32	5
	8°C	33	30	32	5
	10°C	34	29	29	5
	15°C	33	29	30	6
	20°C	34	30	31	5
	25°C	34	28	29	5
	37°C	34	28	29	7
	50°C	34	27	29	6
	70°C	34	26	29	7
	Mean ^b	33.8 $\pm$ 0.5	28.7 $\pm$ 1.3	30.3 $\pm$ 1.4	5.5 $\pm$ 0.8
Dephosphorylated (1–25)	Mean ^b	33.1 $\pm$ 0.7	29.2 $\pm$ 0.9	30.6 $\pm$ 1.3	7.1 $\pm$ 0.9
Poly-L-glutamate	Mean ^b	30.0 $\pm$ 2.3	30.2 $\pm$ 1.3	37.4 $\pm$ 2.8	2.1 $\pm$ 1.7

^a For CD average fits (6 accumulations at each temperature), three overlapping experiments, triplicate runs at 10°, 37°, and 70°C.

^b Mean and standard deviation for all temperatures.

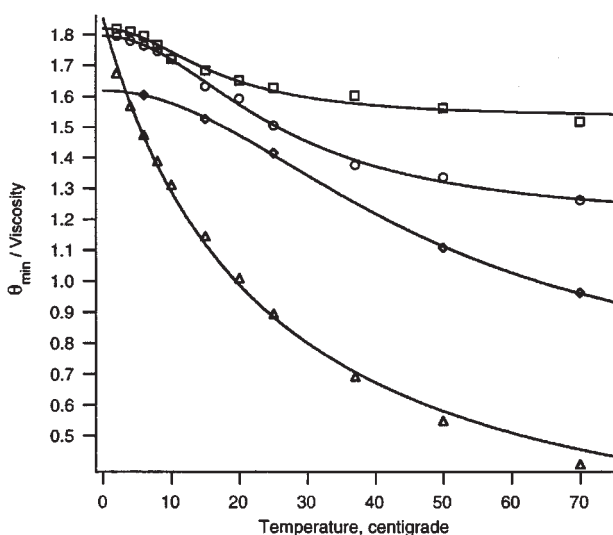


Fig. 5. Change in viscosity for 2 mM PIPES 4 mM KCl buffer pH 6.75 with temperature (Δ). The change in $[\theta_r]$ at $\theta_{\min}$ for the native ($\square$) and dephosphorylated ($\circ$) peptide β -casein (1–25) and poly-L-glutamate ($\diamond$) are also shown and scaled at times 10^{-4} for comparison.

could be that the PPII elements of the poly-L-glutamate respond to increased temperature because they are more flexible, while the native casein peptide is in contrast the most rigid. The loss of high net negative charge, following dephosphorylation of the peptide could lead to increased flexibility with increased temperature. Comparison of Fig. 4 (A, B, and C) suggests that the spectra of the dephosphorylated peptide (Fig. 4B) appear to have an isodichroic point at 205 nm, with a shift of $\theta_{\max}$ from lower to higher wavelengths; this also appears to be true for poly-L-glutamate but these changes are less obvious for the native peptide.

To quantitatively evaluate the observed changes, for the dephosphorylated peptide, the change in $[\theta_r]$ at $\lambda_{\max}$, as a function of temperature (Fig. 6), was fitted with Eq. (1). From this analysis, three parameters can be found, the predicted maximum change at temperatures $>70^\circ\text{C}$, the minimum θ at 0°C and the $1/2$ point for the transition (T_0). For the dephosphorylated peptide, the maximum absolute change in $[\theta_r]$ is $(-18,000$ to $-12,000)$ or 6000 degrees $\cdot \text{cm}^2/\text{dmol}$ representing 33% of the original value (for comparison the change in viscosity is 80% while the native peptide changes 16% and poly-L-glutamate 60%). The midpoint for the dephosphopeptide transition is 26°C , compared to 16°C and 47°C for similar analysis of the native peptide and polyglutamate respectively. Changes in $[\theta_r]$ could be the result of increased peptide flexibility at the decreased viscosity. Alternatively, they may arise from stronger hydrophobic attrac-

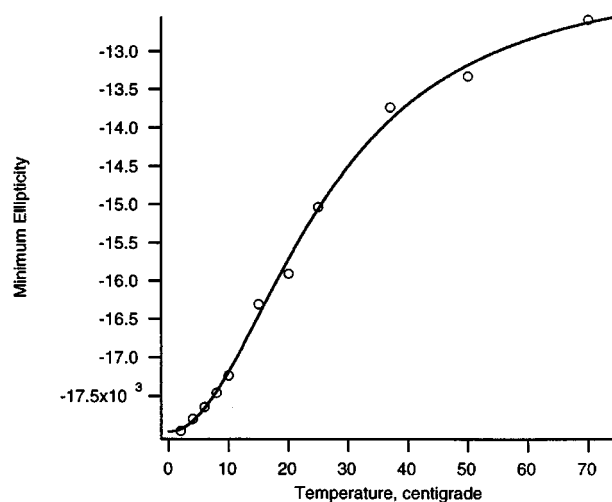


Fig. 6. Change in mean residue ellipticity of dephosphorylated (1–25) for β -casein $[\theta_r]$ at $\theta_{\min}$ as a function of temperature. The data were taken from Fig. 5 replotted and fitted with Eq. 1.

tions as the temperature is increased, which could suggest self-association as a function of temperature for the β -casein peptides. Finally the values of $n = 2$ in Eq. (1) for all three peptides point to the apparent sigmoidal nature of the curves and suggest either cooperativity or a lag in onset of the transitions (Fig. 5); conversely $n = 1$ for temperature changes in viscosity.

3.5. Analysis of the β -Casein (1–25) Peptides by Analytical Ultracentrifugation

Sedimentation equilibrium analysis of native β -casein (1–25) was conducted at three rotor speeds and three temperatures (Table V). Overall, within error, there was little change in weight average molecular weight with rotor speed. In addition there was no temperature dependence in weight average molecular weight, regardless of rotor speed. The average, molecular weight (weight average) for all runs was 3065 ± 80 , in good agreement with the sequence molecular weight of 3127.

In contrast, the dephosphorylated peptide showed a strong upward curvature at the base of the cell (Fig. 7); this is indicative of a self-association reaction. In addition, there was a speed dependence in its weight average molecular weight ranging from 2957 to 11021 (Table V). Here the degree of polymerization does not seem significantly temperature dependent suggesting that the mechanism of self-association may not be totally hydrophobic in nature. Increasing the ionic strength to 0.122 M had

Table V. Analytical Ultracentrifugation of Native and Dephosphorylated β -Casein (1–25); Effects of Temperature and Rotor Speed on Weight Average Molecular Weight

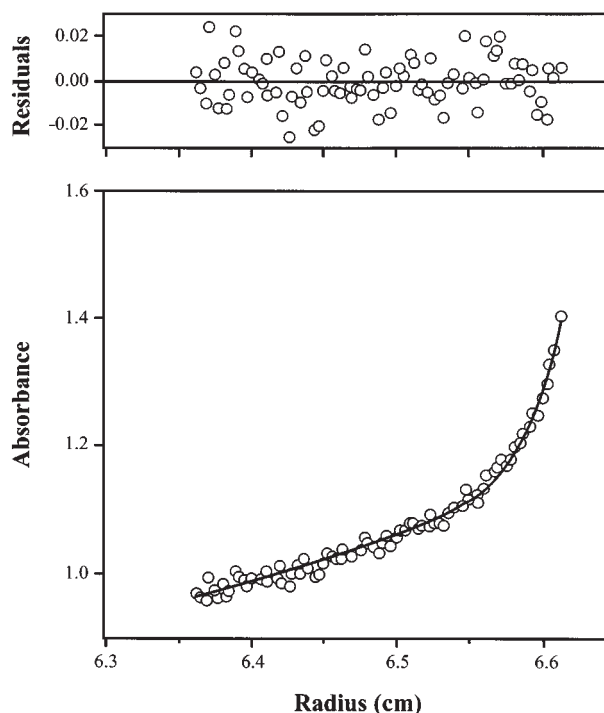
Rotor speed (1000 rpm)	Temperature (°C)	β -Casein (1–25)	
		Dephosphorylated M_w (Da)	Native M_w (Da)
46	10	3332 ^a	3075 ^a
	25	2976 ^a	3177 ^a
	37	2565 ^a	2815 ^a
25		2957 $\pm$ 313	3022 $\pm$ 152
	10	6389 ^a	3433 ^a
	25	5950 ^a	3137 ^a
	37	5683 ^a	2901 ^a
12		6007 $\pm$ 291	3157 $\pm$ 217
	10	12,106 ^a	3154 ^a
	25	11,688 ^a	3093 ^a
	37	9270 ^a	2801 ^a
		11,021 $\pm$ 1250 ^b	3016 $\pm$ 154

^a Weight average molecular weight three determinations $\pm$ 3% CV; sequence weights are 2803 and 3127, respectively.

^b Increasing the ionic strength to 0.122 M did not appreciably change this value.

little or no effect on the weight average molecular weight at 12,000 rpm. The highest amount of polymer was present at 12,000 rpm, where the pressure change across the channel is 1.9 atmospheres. In contrast the average pressure change is 27.9, and 8.3 atm at 46,000 and 25,000 rpm (Schachman, 1959). Thus at the highest pressure, the molecular weight of the dephosphorylated peptide approaches monomer while the aggregate size is greatest near atmospheric pressure (Table V). Pressure induced changes in size are most often the result of negative changes in volume (Josephs and Harrington, 1968) or $\bar{v}$ (Schachman, 1959). It may thus be hypothesized that the volume of the polymers of the dephosphorylated peptide are greater than the sum of the component monomers and aggregation is accompanied by a $+\Delta V$.

When the data were analyzed for self-association it was noted that the best fits with Eq. (2) occurred for the monomer $\rightarrow$ polymer model as used for β -casein (Farrell *et al.*, 2001; Schmidt, 1982). Under these conditions however, at 12,000 rpm the “protomer” in the self-association reaction was calculated to be dimer. The fitted value for this species was 5595 $\pm$ 136 as compared to the sequence weight of the dimer, 5606 (Table VI). As the rotor speed (pressure) was increased to 25,000 rpm, the protomer in the self-association reaction became monomeric. Thus the kinetically active species in the aggregation reaction is pressure dependent.

**Fig. 7.** Analytical ultracentrifugation analysis of dephosphorylated β -casein (1–25) at 25°C in PIPES buffer; $\mu = 0.050$. The lower graph shows the fit to the absorbance vs. radius plot for Eq. 2 at 12,000 rpm; the upper graph 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 VI. The apparent “monomer” molecular weight near the meniscus was 5,640 $\pm$ 600.

The results of the analysis of the data for k_2 and n_2 are given in Table VI. The best fits to the data were obtained with high values of n , which contain a significant

Table VI. Analytical Ultracentrifugation of Dephosphorylated β -Casein (1–25); Effect of Temperature and Rotor Speed on Apparent Association Constants and Degree of Polymerization

Rotor speed 1000 rpm	Temperature (°C)	k_2^a 1/g	n_2^a integer	Protomer (Da)
25	10	2.5×10^4	26	2965 ^b
	25	3.3×10^2	32	2897 ^b
	37	4.7×10^5	29	3082 ^b
12	10	3.2×10^{-7}	30	5700 ^c
	25	1.3×10^{-8}	48	5640 ^c
	37	1.5×10^{-7}	42	5447 ^c

^a Data were fitted with Eq. 2; three samples in each determination, RMS errors = 10^{-4} for each fit.

^b The protomer here was calculated to be near the monomer molecular weight (2803).

^c The protomer here was calculated from Eq. 2 and approximates a dimer.

degree of uncertainty (Alaimo *et al.*, 1999a). Nevertheless back calculation to the primary data showed self-consistency with the weight average numbers, and the patterns are commensurate with each other. At 12,000 rpm (1.9 atm) the peptide is essentially dimer with a small degree of high molecular weight polymer (2%), while at 25,000 rpm the peptide represents a monomer with a somewhat larger amount of polymer (6%). The polymeric species is also pressure dependent as increasing the speed to 46,000 rpm produced ~99% monomer. It may thus be concluded that the dephosphorylated peptide perhaps occupies a larger volume (is more flexible) than the native peptide as inferred above from CD analysis, and that this change in volume leads to pressure-dependent aggregation.

3.6. Molecular Modeling of β -Casein (1–25)

Various methodologies for sequence-based secondary structural predictions are currently available (Chou and Fasman, 1978; Garnier *et al.*, 1978; Cohen *et al.*, 1986), and these methods have been applied to β -caseins (Graham *et al.*, 1984; Kumosinski, *et al.*, 1993). In our initial approaches, a sequence-based prediction was generated from each of three basis sets for β -casein (1–25). The results were ambiguous; for some portions of the sequences, structures were consistently predicted, whereas in others they were not. PPII predictions were high for residues 2–9 and 11–22 (Table III), and all algorithms predicted a β -turn for residues 9–11 and 22–25, although as noted above no hydrogen bonding was observed by NMR.

The secondary structural predictions were further reconciled with our interpretations of the NMR, CD, and FTIR spectroscopic data as presented above. The C-terminal residues (1–11) were assigned the PPII angles. Then β -sheet was assigned to residues 12–21, because preliminary minimization studies showed that neither α -helix nor PPII could be maintained in the presence of the highly charged run of phosphoserines (Pse) and this assignment was suggested by CSI indexes (Table III).

A neutron reflection study of β -casein monolayers adsorbed on a hydrophobic surface (Fragneto *et al.*, 1995) suggests that the phosphopeptide may project outward into solution. Hence a flexible extended turn-like motif between residues 20–25 could act as a hydrated hinge to connect the hydrophilic peptide to the more hydrophobic section of the molecule. The FTIR absorbances in the turn region (1680 cm^{-1}) could be attributable to side bands of polyglutamate, as its overlay with the phosphopeptide (Fig. 3) shows the two molecules to be similar. A better argument for turn-like structures can be made from the

CD data (Table IV). Thus the C-terminal section of the molecule may contain a “nascent” turn-like structure.

The initial structures were built with the following idealized secondary structure elements: residues 1–11 are polypyrrolone II lefthand helix, 12–21 are extended β -sheet, and 22–25 are type I turns. All starting structures were energy minimized in the presence of explicit water molecules (3000–5000) before being subjected to molecular dynamics simulations at 300 K. All hydrogen atoms were included during simulations. Molecular dynamics (MD) simulations on the native and dephosphorylated peptides, starting from the same idealized conformations, were conducted for up to 200 picoseconds. During the MD simulations, the conformations of both peptides changed from their starting structures, as shown by the trajectories of the radius of gyration and the *rms* of center of mass. While the trajectories of the simulations reach a relative constant value for the native peptide at about 100 ps, they continue to change for the dephosphorylated peptide during the course of MD simulations, suggesting a much more flexible and dynamic behavior for this peptide. This observation is further supported by the time dependence of the atomic distance between N-terminal Arg and C-terminal Arg residues. Figure 8 shows the change in distance between α C atoms of Arg-1 and Arg-25 residues. The distance for the native peptide undergoes fairly small variations in the range of 30–50 Å in comparison to its dephosphorylated form, which ranges from 10 to 50 Å. A similar result was also found in the distance change between ϵ N atoms of these two residues (data not shown). A close examination of the dihedral angles (ϕ , ψ) of each residue during

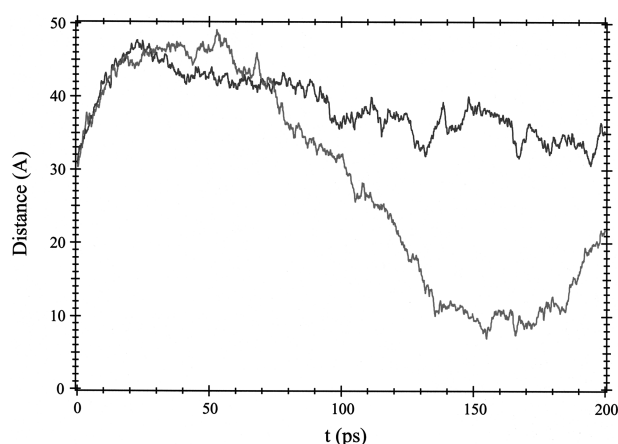
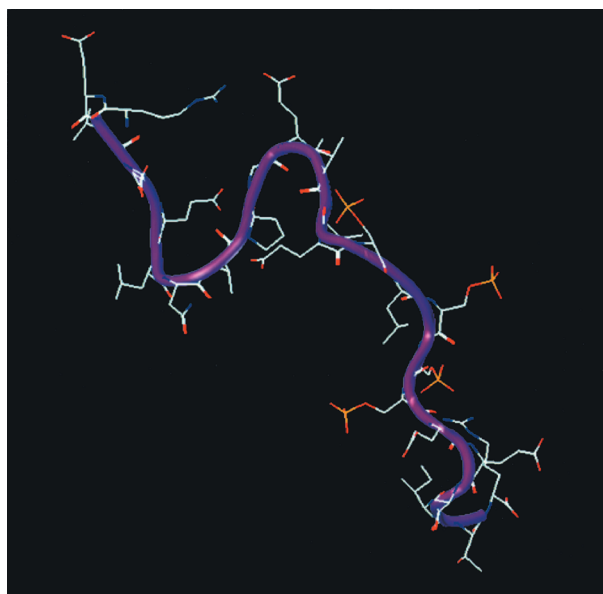
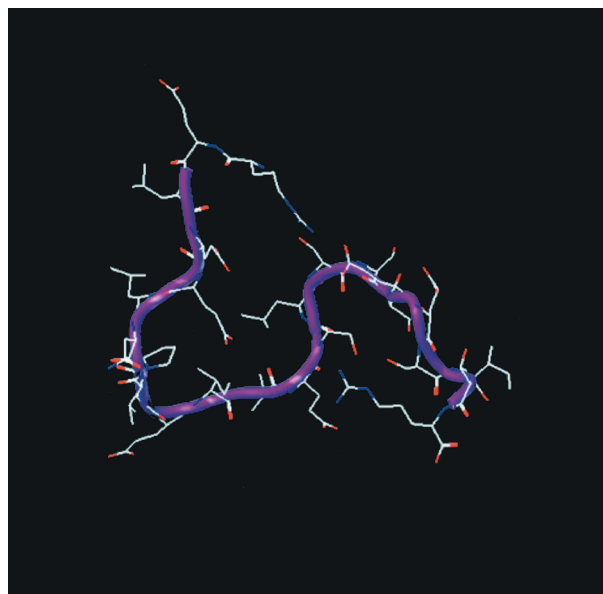


Fig. 8. Distance (Å) dependence of α C-atom of Arg-1 and Arg-25 during MD simulations with explicit solvent at 300 K. The native peptide is on top, and the dephosphorylated form is on the bottom.

A



B



flexible and dynamic structure (Fig. 8). Figure 10 compares the final peptide structures in water following MD and minimization. In this respect the models following MD simulations, as shown in Fig. 10, support the ultracentrifuge data. Docking two of the flexible semi-circular structures of the dephosphorylated peptide could

lead to a polymer with a $+\Delta V$, and self-association for this form of the peptide. In addition, the CD data predicted a more flexible response to temperature for the dephosphorylated peptide as well.

4. CONCLUSIONS

The β -casein peptide studied in this work represents the bulk of the hydrophilic residues of β -casein (Table I) and occurs in primarily extended sheet, extended turn, and polyglutamate-like motifs. Temperature studies for the native peptide in the far-UV CD indicate little thermal unfolding of local structures even at 70°C. Similarly, there is no loss in β -casein (1–25) in the bulk quantity of structure based on FTIR analysis by increasing temperature up to 70°C. The conformational stability of the backbone chain of the peptide along with NMR-derived thermal factors suggest a heat stable, core structure for the native peptide. It may be speculated these thermo stable structures in the peptide also behave in the same fashion in the intact protein. Similar structural stability with increased temperature was observed by CD for intact β -casein (Graham *et al.*, 1984; Farrell *et al.*, 2001), and heat stable secondary structures have also been observed for the peptide 136–196 of α_{s1} -casein (Alaimo *et al.*, 1999b) and for α_{s1} -casein (1–23) by Malin *et al.* (2001).

In studies of β -casein at oil–water interfaces or on hydrophobic surfaces, it has been suggested that the protein forms a hydrophobic core and that the phosphopeptide forms an extended structure which protrudes into the more hydrophilic environment (Horne, 1998). In a similar fashion, neutron reflectivity of β -casein at the hydrophobic–solid–water interface suggests a charged layer of ~ 3.5 nm that protrudes into solution (Fragneto *et al.*, 1995). From the solution studies presented above, it would appear as though the phosphopeptide may be quite rigid, while from MD simulations, the overall length for the native peptide (at apparent equilibrium) was found to be 3.5 nm (Fig. 8) in agreement with the neutron reflectivity experimental data for the length of the hydrophilic layer. These two motifs, a hydrophobic core and an extended hydrated arm, may account for functionality of the β -casein molecule in surface/interfacial interactions (Horne, 1998). By comparison (Fig. 1), the swine and bovine proteins may have quite similar extensions, while the rodent peptide is potentially more phosphorylated and the human form truncated.

It has been suggested that β -casein (1–25) can be a strong food allergen (Bernard *et al.*, 2000) and that dephosphorylation of this casein may reduce its food aller-

gic responses in some patients. As there is little structural change on dephosphorylation, it may be speculated that self-association may mask sensitive areas on the casein polypeptide chain, rendering them less stable and therefore less allergenic or less available for contact with the immune system.

Caseins have been thought to be random coil or completely unfolded proteins; however, more recent studies have suggested persistent regular structures for segments of the molecules (Alaimo *et al.*, 1999a, b; Malin *et al.*, 2001; Farrell *et al.*, 2001; Syme *et al.*, 2001; Cross *et al.*, 2001). Overall the data presented above suggest that the N-terminal segment of β -casein contains thermostable structures which may account for its functional properties in food systems and its consistent physical properties in calcium accretion during milk synthesis and in digestion.

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