Monomer Characterization and Studies of Self-Association of the Major β-Casein of Human Milk

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ABSTRACT

The casein form that has four organic phosphoryl groups, β-casein (CN)-4P, is the major constituent (~35%) of the β-CN fraction of human milk and should play an important role in micelle structure and formation. In 3.3 *M* urea, the monomer is present with a molecular mass of 24,500 Da and a sedimentation coefficient of 1.3 S (Svedberg units, 10^{-13}s). In 0.02 *M* NaCl and 0.01 *M* imidazole (low salt buffer) at pH 7, the sedimentation coefficient was 1.5 S, which increased to 14 S at 37°C. Laser light scattering in low salt buffer and 9 mg/ml of protein indicated monomers with a radius of about 4 nm at 4°C. The size of the radius increased as temperature increased, and, at 37°C, the radius was about 12 nm. The molecular mass suggested the presence of about 47 monomers per polymer. In 0.25 *M* NaCl and with 10 m*M* Ca^{2+} prior to precipitation, the polymer attained a maximum radius of about 15 nm, which perhaps is the size of the smallest human milk micelles. The low value for reduced viscosity of 8.2 ml/g for the calcium-induced polymer was independent of protein concentration, suggesting a spherical shape and fixed size. Calcium apparently binds strongly to the phosphates; the dissociation constant was 8.1×10^{-4} *M*. Other constituents of milk, such as inorganic orthophosphate, may contribute to differences in the manner by which β-CN, with various phosphorylation levels, participate in micelle formation.

(**Key words**: human milk, human β-casein, protein self-association, protein-ion interactions)

Abbreviation key: $D_{20,w}$ = diffusion coefficient corrected to 20°C and water solution, **LSB** = low salt buffer, M_r = relative molecular mass, η_{red} = reduced viscosity, $s_{20,w}$ = sedimentation coefficient corrected to 20°C and water solution.

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INTRODUCTION

In the bovine milk system, the phosphate groups of the α_{s1}-, α_{s2}-, and β-CN are apparently necessary for micelle formation, but the phosphate group of κ-CN is not (23). The level of phosphorylation determines the calcium-binding ability of these components (22). Similarly, in the human milk system, only a minor amount of protein corresponds to α_{s1}-CN (11); the phosphoryl groups of β-CN, varying from 0 to 5 (5, 7), are therefore important for the formation and function of human casein micelles. Self-association properties have been reported for most of the phosphorylation levels of human β-CN [i.e., the fully phosphorylated (β-CN-5P) (14), nonphosphorylated (β-CN-0P) (15), triply phosphorylated (β-CN-3P) (16), doubly phosphorylated (β-CN-2P) (17), and singly phosphorylated (β-CN-1P) (18)]. Although fluorescence spectroscopy and laser light scattering measurements of self-association have suggested a role for the phosphate esters on the molecule in the formation of micelles (13), a comparison of the primary sequences of β-CN from six species revealed residues that were common in all species examined and therefore pivotal in protein folding and conformation, intermolecular hydrophobic interactions, and ion bridge formation with calcium and inorganic phosphate (8).

The β-CN-4P form, which is the major component (~35%) of human β-CN, has four serine groups near the N-terminus, all of which are phosphorylated. This report presents results of studies that were designed to emphasize the role of the hydrophobic and electrostatic interactions of this major constituent of human β-CN in the process of micelle assembly.

MATERIALS AND METHODS

Methods for the collection of human milk samples, for the isolation and purification of human β-CN fractions with different levels of phosphorylation, and for the study of the physical properties of each fraction as well as its protein-protein and protein-ion interactions, are briefly presented. Details have been given in earlier papers (8, 13, 14, 15, 16, 17, 18). All

chemicals were reagent grade and were used as such, except urea, which was purified and deionized before use (14).

Protein Purification and Chemical Characterization

The modified procedure of Groves and Gordon (7), as described elsewhere (14), was used for the preparation of β-CN-4P. A further purification, which yielded a virtually pure sample of the protein, was carried out by HPLC on the anion exchanger (Mono Q; Pharmacia LKB, Piscataway, NJ), as described by Slattery et al. (13). After exhaustive dialysis with deionized water and lyophilization, the degree of phosphorylation was determined; the amount of phosphorous present in a given amount of protein was measured by the method of Long and Yardley (9). To determine the number of major Ca^{2+}-binding sites at 37°C, the rapid equilibrium method of Colowick and Womack (4) was used, followed by Scatchard analysis (12). The extinction coefficient and the partial specific volume were determined by standard methods, as described previously (14).

Hydrodynamic Methods

Viscosity measurements to monitor changes in size and shape were carried out as described by Sood et al. (14). An analytical ultracentrifuge (model E; Beckman Instruments, Fullerton, CA) was used to determine sedimentation coefficients, which were corrected to standard conditions of 20°C and water solution ($s_{20,w}$). Relative molecular mass (M_r) was determined by the sedimentation equilibrium method with protein at 0.3 to 0.4 mg/ml (14). The possibility that polymerization or depolymerization occurred because of pressure effects in the analytical ultracentrifuge was tested at 37°C in low salt buffer (**LSB**; 0.02 *M* NaCl and 0.01 *M* imidazole, pH 7) with protein at 3 mg/ml. Two procedures were used: 1) determination of $s_{20,w}$ at rotor speeds of 24,000, 36,000, and 48,000 rpm and 2) determination of $s_{20,w}$ at 48,000 rpm after the solution column in the cell (0.5 ml) was overlaid with two different thicknesses of mineral oil (0.1 and 0.2 ml). To eliminate any change in the properties of the aqueous solution caused by possible salt extraction by the oil, the mineral oil was shaken with twice its volume of LSB and allowed to equilibrate for 8 h before use.

Laser light scattering (Nicomp model 370 submicron particle sizing system and Nicomp analysis software; Pacific Scientific, Silver Spring, MD) was used to determine particle size distributions and diffusion coefficients, which were also corrected to standard conditions of 20°C and water solution ($D_{20,w}$). Measurements following a temperature change were taken only after stable readings were obtained that showed that aggregation was no longer occurring, usually within about 2 h.

RESULTS AND DISCUSSION

In the human β-CN-1P, the serine residue at either position 9 or at position 10 from the N-terminus is phosphorylated. For the 2P form, serine residues at both 9 and 10 are phosphorylated; serine residues at positions 8, 9, and 10 are phosphorylated for the 3P form; serine residues at positions 6, 8, 9, and 10 are phosphorylated for human β-CN-4P (6).

Monomer Physical Properties

The physical properties of human β-CN-4P are presented in Table 1. Comparison with the results for other phosphorylation levels (14, 15, 16, 17, 18) shows that phosphorylation level did not substantially affect monomeric physical properties, such as absorbance at 280 nm, partial specific volume, $s_{20,w}$, and the monomer molecular mass of the molecule that was determined from sedimentation equilibrium both in 3.3 *M* urea and in LSB. The laser light scattering measurements also revealed homogenous particle size, and the mean diameter (8 nm) for all phosphorylation levels was similar. With the assumption that the molecule is spherical, a combination of

TABLE 1. Physical properties of human β-CN-4P.

	$\bar{X}$	SE[1]
Absorbance, $E^{1\%}_{1\ cm,280\ nm}$[2]	6.2	0.3
Partial specific volume, $\bar{v}$, ml/g	0.75	0.01
Sedimentation coefficient in Svedberg units, S (10^{-13} s)		
In 3.3 *M* urea at 20°C	1.32	0.02
In 0.02 *M* NaCl		
At 4°C	1.50	0.01
At 20°C	1.50	0.01
At 37°C	14.2	0.3
Molecular mass by sedimentation equilibrium, Da		
In 3.3 *M* urea at 20°C	24,500	150
In 0.02 *M* NaCl		
At 4°C	24,600	200
At 20°C	25,100	250

[1]Standard error of the mean is calculated from at least three determinations.

[2]The value of $E^{1\%}_{1\ cm,280\ nm}$ represents the absorbance of a 1% (wt/vol) solution of protein measured through a path length of 1 cm at a wavelength of 280 nm.

the $s_{20,w}$ and $D_{20,w}$ gave a monomeric M_r of 25,900 Da for β-CN-4P, which was very close to that value obtained from sedimentation equilibrium and similar to that reported for other phosphorylation levels under identical conditions (14, 15, 16, 17, 18). The M_r from the amino acid composition is 23,938 Da (6).

Calcium Binding

The Scatchard plot for equilibrium dialysis experiments at 37°C yielded a mean value of 4.0 ± 0.3 for the number of major calcium-binding sites on this protein. Phosphorous analysis gave a mean of 4.0 ± 0.2 for the number of phosphoryl groups on this molecule, confirming the previous observations that there is one major calcium-binding site for each phosphoryl group. The mean $D_{20,w}$ was 8.1×10^{-4} *M*, which was approximately 70% of that reported for the human β-CN-1P (18) or the 2P (17) forms and was almost double that reported for the 5P form (14). Thus, in all of the human β-CN, as the number of phosphoryl groups increased, the mean $D_{20,w}$ decreased.

Protein Solubility

In the absence of calcium ions, the protein was readily soluble up to very high concentrations (30 mg/ml) in 3.3 *M* urea and stayed as a clear solution up to 37°C. As indicated by comparisons with previous studies, in LSB at pH 7, the solubility increased as phosphate groups were added to the protein. For example, human β-CN-0P took more than 3 h to become completely solubilized at low temperature (4 or 5°C), was slightly turbid at 23°C, and was almost opaque at 37°C (14). However, the 3P form easily became solubilized at low temperatures, stayed clear at room temperature (24°C), but was slightly turbid at 37°C (16). In this study, the 4P form not only became solubilized readily, both at low temperature and at room temperature, but also produced a clear solution even at 37°C.

The solubility of protein at 3 and 9 mg/ml in LSB at pH 7 was also examined as a function of temperature at 5, 10, 100, and 300 m*M* Ca^{2+} concentration. All solutions were clear at low temperatures (<15°C), but cloudiness and precipitation occurred as the temperatures were increased. Precipitation began at a lower temperature as Ca^{2+} concentration increased and as the protein concentration increased. The point of initial precipitation was elaborated further with light scattering, as described in the next section. For the β-CN-4P protein, the amount of precipitation also increased as the Ca^{2+} concentration increased from 100 to 300 m*M*. For the 0P, 1P, and 2P forms, solubility reversed markedly or increased as Ca^{2+} increased from 100 to 300 m*M* (15, 17, 18) but not for the 3P form (16) or for the 4P form at both 3 and 9 mg/ml of protein concentration. The resolubilization was probably due to a charge reversal caused by calcium binding, which was perhaps prevented by crosslinking of the molecules that had higher phosphorylation levels through the formation of calcium bridges.

A study (10) comparing calcium-induced association of bovine and caprine whole caseins pointed out that the increase in protein salting-in (resolubilization) in the presence of higher $CaCl_2$ concentrations was directly related to the casein composition, particularly to the decrease in the relative amount of α_{s1}-CN in the system. Because the α_{s2}-CN content was higher in the low α_{s1}-CN samples, the mean phosphorous content decreased only slightly; this decrease was not an important factor. However, as is evident from these results and from our studies of the other phosphorylated forms of purified human β-CN with no α_{s1}-, α_{s2}-, or κ-CN present (15, 16, 17, 18), the resolubilization at higher concentrations of $CaCl_2$ depended on the number of organic phosphates, and a decrease in only one phosphoryl group made a large difference. Azuma et al. (2), while reporting the calcium-binding ability and calcium-dependent precipitability of the human β-CN, found that both properties increased as phosphorus content increased. The data here, along with similar information from other levels of phosphorylation, confirm that, as the number of phosphoryl groups on the molecule increased, the temperature at which a slight cloudiness or precipitate appeared also increased, although an increase in protein concentration (from 3 to 9 mg/ml) lowered this temperature in all cases. These results indicate that the initial precipitates were due to temperature-dependent hydrophobic interactions that were prevented by the negatively charged phosphoryl groups until the charge was neutralized by calcium binding.

Aoki et al. (1), while studying artificial casein micelles formed with human β-CN and bovine κ-CN, suggested that only three phosphate esters were necessary for crosslinking of the caseins by colloidal calcium phosphate. Thus, the human casein system appears to differ from the bovine or caprine system in the number of phosphoryl groups required for stability. For each of the different forms of human β-CN, the precipitate that formed in the presence of $CaCl_2$ at high temperatures dissolved on cooling to low temperatures, which suggested that hydrophobic bonding is a principle force for self-association of human β-CN

even in the presence of Ca^{2+}, as has been reported for bovine β-CN (20).

Light Scattering

Studies to determine gross light scattering as particle size increased were performed at 400 nm as a function of temperature and at a protein concentration of 7 mg/ml of LSB, both in the absence and presence of 2.5 to 10 m*M* calcium. The results are presented in Figure 1. In the absence of Ca^{2+} or with 2.5 m*M* Ca^{2+}, only a small increase above 26°C was noticed in absorbance value. With 5 m*M* Ca^{2+} a sudden increase in the absorbance value was observed at 22°C, indicating the formation of larger aggregates that led to precipitation at higher temperatures. With higher levels of Ca^{2+}, formation of larger aggregates began somewhere below 20°C. Formation was examined further by means of laser light scattering.

In LSB at pH 7 and at a protein concentration of 9 mg/ml for the β-CN-4P, laser light scattering gave a single homogenous distribution representing a radius of about 4 nm at 4°C, which was characteristic of a monomer. The size increased as temperature increased, and, at 37°C, a polymer with a radius of about 12 nm was indicated. Under these conditions, the combination of $D_{20,w}$ and $s_{20,w}$ gave a M_r for the polymer that was equivalent to about 47 monomers. At a protein concentration of 3 mg/ml and in the presence of 10 m*M* $CaCl_2$, aggregation at 15°C yielded particles that had a radius of about 12 nm (Table 2). The radius of the particles increased to 15 nm at 17°C, and precipitation occurred at 18°C and above. At a concentration of 9 mg/ml of protein and 4°C, a single homogenous distribution was obtained, representing a radius of about 4.5 nm, which is almost identical to the radius obtained in the absence of Ca^{2+}. At 15°C, the particle size increased over time, and a maximum stable radius of about 106 nm was obtained within 2 h. At 16°C, the particles had a radius of about 250 nm, which was approximately the size of the normal casein micelles in skimmed human milk, as determined by laser light scattering. At 17°C and above, precipitation occurred. At 3 mg/ml of protein concentration, precipitation started at 18°C. We concluded that aggregation in the presence of $CaCl_2$ depends on the protein concentration as well as the temperature.

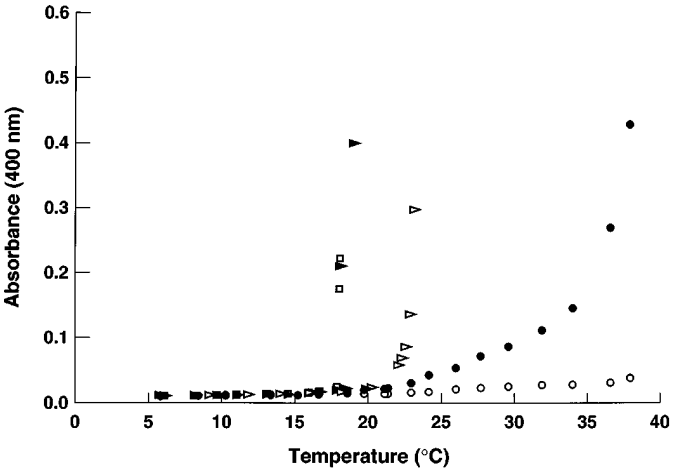

Figure 1. Absorbance at 400 nm of human β-CN-4P as a function of temperature. Protein was 7 mg/ml in low salt buffer. No $CaCl_2$ (o), 2.5 m*M* $CaCl_2$ (●), 5.0 m*M* $CaCl_2$ (open triangle), 7.5 m*M* $CaCl_2$ (closed triangle), and 10 m*M* $CaCl_2$ (□).

TABLE 2. Effect of temperature on the particle size of human β-CN-4P in low salt buffer, pH 7.0, and 10 m*M* $CaCl_2$.

Temperature	Radius at protein concentration	
	3 mg/ml	9 mg/ml
(°C)	(nm)	
4	4.5	4.5
12	...	14.9
13	...	15.6
15	12.0	106.0
16	14.5	258.0
17	15.0	Pr[1]
18	Pr	Pr

[1]Precipitation occurs.

Because Table 3 shows that the radius (4.5 nm) of particles in 0.25 *M* NaCl plus 0.01 *M* imidazole buffer at pH 7.0 and 4°C with a β-CN-4P protein concentration of 9 mg/ml was about the same as that obtained in LSB under similar conditions of protein concentration, both in the absence and presence of 10 m*M* $CaCl_2$ (Table 2), the predominant role of hydrophobic interactions in protein-protein association under all these conditions is indicated. Under these high salt conditions, laser light scattering measurements indicated a single homogenous particle size of about

TABLE 3. Effect of temperature of the particle size of human β-CN-4P at high ionic strength.[1]

Temperature	Radius
(°C)	(nm)
4	4.7
20	8.1
23	9.4
37	14.7

[1]The protein was in 0.01 *M* imidazole buffer (pH 7.0) and 0.25 *M* NaCl at a concentration of 9 mg/ml.

15-nm radius at 37°C, which is the same as that for the 5P protein (14). The molecule may be considered to be more or less spherical, as suggested by the low value of the reduced viscosity (η_{red}) shown later. A corrected $D_{20,w}$ of 1.49×10^{-7} cm^2/s was obtained, which, when combined with $s_{20,w}$ gave a value of 2.1×10^6 Da for the M_r. This M_r suggests that, at this stage, the polymer contained about 87 monomers, which is similar to the number of monomers per polymer reported for other forms of human β-CN under similar conditions (14, 15, 16, 17, 18).

Analytical Ultracentrifugation

At a protein concentration for β-CN-4P of 3 mg/ml, studies of sedimentation velocity were carried out in LSB at pH 7 and at 4 to 37°C. The $s_{20,w}$ are presented in Figure 2 as a function of temperature. Significant polymerization was apparent after 26°C, and, at 28°C, there were two peaks, which had $s_{20,w}$ of about 5 and 1.5 S, respectively. The peak at 1.5 S was much smaller than the faster peak. The faster peak was quite skewed, indicating that the system was heterogeneous at this stage. A further increase in temperature resulted in a decrease in the area of the slower peak. At 37°C, a single polymer peak remained with $s_{20,w}$ of 14 S, which was lower than the values obtained for the other phosphorylated forms under similar conditions. No precipitation of the aggregated protein occurred. For all of the β-CN forms, as the number of phosphoryl groups increased, the $s_{20,w}$ and, hence, the size of the polymer at 37°C decreased. Other important information may be obtained from the concentration-dependent behavior of $s_{20,w}$ for these different forms. For the 0P form, the $s_{20,w}$ at 37°C decreased as the protein concentration increased (15). With additional phosphate groups, this behavior gradually disappeared, and, for the 4P form, the value was virtually independent of protein concentration. This result indicates that the highly phosphorylated forms aggregate to give roughly spherical polymers of fixed size, but those forms with less phosphorylation probably do not (3).

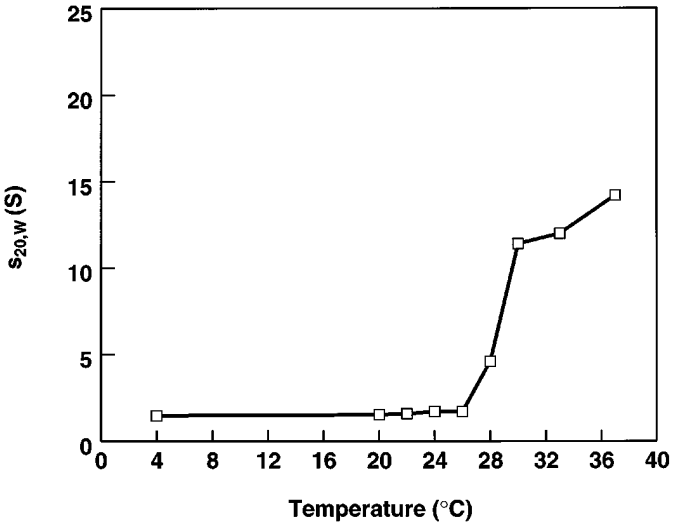

Figure 2. Sedimentation coefficient corrected to 20°C and water solution ($s_{20,w}$) of human β-CN-4P as a function of temperature. The protein was in low salt buffer and at a concentration of 3 mg/ml. S = Svedberg units (10^{-13} s).

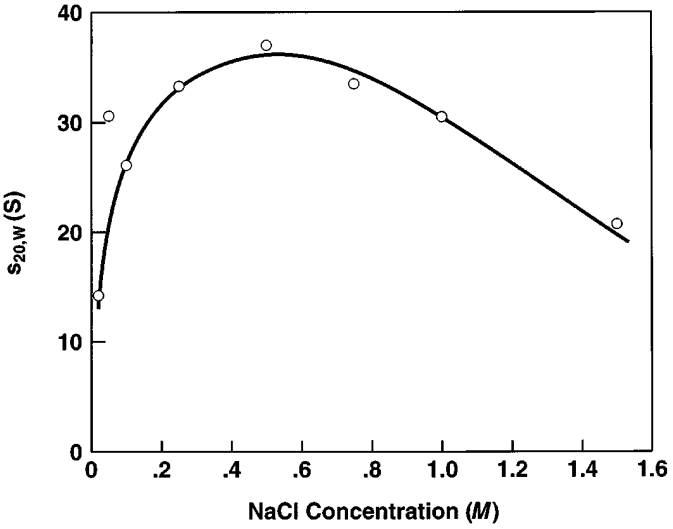

Figure 3. Sedimentation coefficient corrected to 20°C and water solution ($s_{20,w}$) of human β-CN-4P at 37°C as a function of NaCl concentration. The protein concentration was 3 mg/ml in 0.01 *M* imidazole at pH 7.0. S = Svedberg units (10^{-13} s).

Self-association properties of bovine β-CN were studied by Waugh et al. (21) as a function of ionic strength, contributing to the discovery and characterization of polymers that may function as submicelles. As NaCl was added to human β-CN-4P at 37°C (Figure 3), the $s_{20,w}$ of the polymer also reached a maximum value at an ionic strength near 0.2. Of all the forms of human β-CN, only the 0P form reached a maximum at a lower ionic strength of 0.1 *M* NaCl (15). The $s_{20,w}$ of 33 S for the 4P form was approximately equal to that for each of the other forms. In the experiments that tested for polymerization or depolymerization of the 4P protein because of pressure effects in the analytical ultracentrifuge, no significant difference in the $s_{20,w}$ was observed during any of the determinations, either with three different speeds or with two different thicknesses of overlaid

mineral oil, as described in the Materials and Methods. These results suggest that the monomer building blocks are similar in size, shape, and interaction modes.

Viscosity

Viscosity measurements provided information about the shape and hydration of the β-CN-4P polymers and, therefore, about possible mechanisms by which the proteins aggregate. Both in 3.3 *M* urea and in LSB at pH 7, the protein showed almost the same high value of η_{red} at 4°C (Figure 4). Because the protein was essentially monomeric in both solutions (Table 1), the degree of asymmetry (the molecular shape) was probably also similar and relatively high. In urea, the η_{red} gradually decreased as temperature increased. Because the mean M_r apparently did not change, at least up to 20°C, this decrease in η_{red} could have been due to an increase in hydration of the hydrodynamic particles, small conformational changes within the molecules, or both because both can give rise to slightly less asymmetry. The η_{red} decrease could also have been due to a change in the ability of the asymmetric molecules to orient themselves in the fluid flow in the viscometer as the temperature changed.

In LSB, the η_{red} began a similar gradual decrease as temperature increased but decreased suddenly between 24 and 30°C, after which the rate of decrease was constant again. This pattern of change suggests a rather large decrease in asymmetry for the protein particles over the temperature range, which apparently accompanied polymerization because the $s_{20,w}$ increased rapidly above 26°C and continued to increase (Figure 2). These data also imply that, as the temperature was increased above about 28°C, the polymer particles again maintained a relatively uniform shape, changing only slightly as they increased in size.

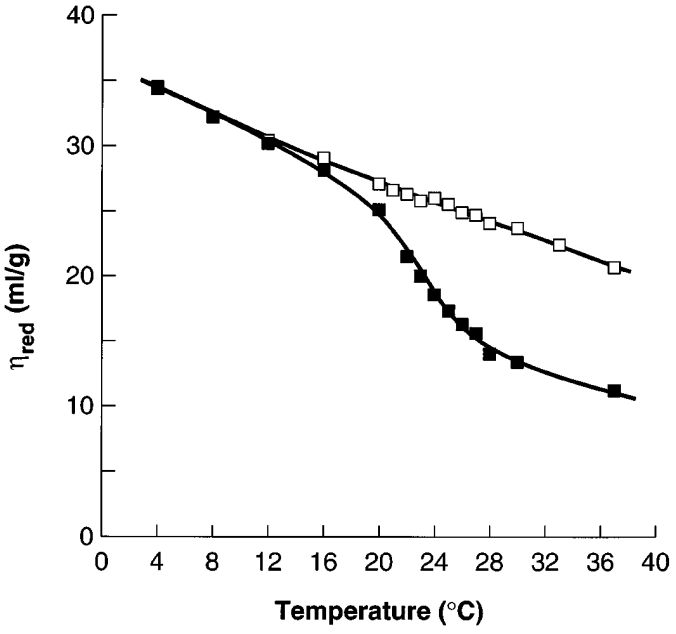

Figure 4. Reduced viscosity (η_{red}) of human β-CN-4P as a function of temperature. Protein concentration was 9 mg/ml (□) in 3.3 *M* urea and (■) in low salt buffer.

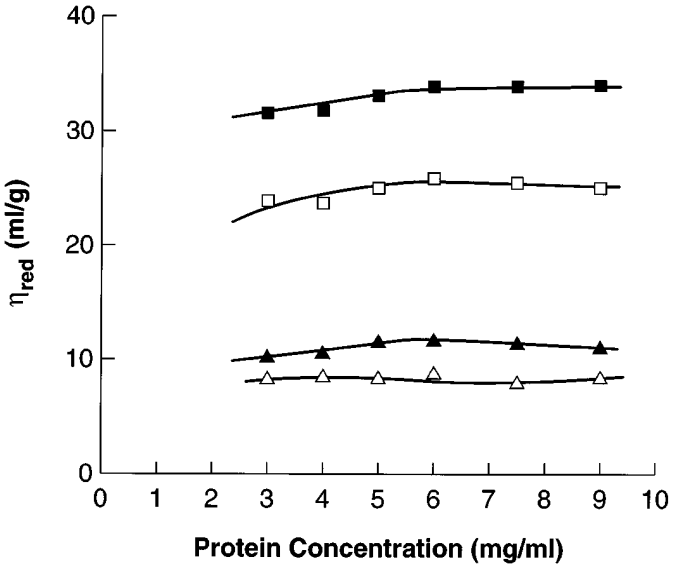

Figure 5. Reduced viscosity (η_{red}) of human β-CN-4P as a function of protein concentration at 4°C (■), at 20°C (□), at 37°C (▲), and in 0.25 *M* NaCl buffer at pH 7.0 at 37°C (△).

In Figure 5, the values for η_{red} of β-CN-4P that were determined at 4, 20, and 37°C in LSB were plotted as a function of protein concentration. Extrapolation from the most accurate data in Figure 5, which is at the higher concentrations, yields intrinsic viscosities of 33, 24, and 11 ml/g from low to high temperatures. These values were only slightly higher than those reported for the 2P form under similar conditions (16). The decrease in η_{red} suggests that an increase in temperature caused the polymer of each form to become more spherical in shape.

Figure 5 shows η_{red} values in 0.25 *M* NaCl plus 0.01 *M* imidazole at 37°C in the protein concentration range of 3 to 9 mg/ml. These values were relatively low and almost independent of the protein concentration, which may mean that the polymer was more or less spherical at this stage and of a fixed size. Extrapolation to 0 concentration yielded a value of 8.2 ml/g for intrinsic viscosity, which was slightly higher

than that reported for the 2P protein [6.7 ml/g; (17)]. Using Tanford's relation (19), a value of 2.7 g H_2O/g of protein was obtained for solvation. This value was very close to that suggested for the 5P form of the protein (3 g H_2O/g protein) under similar conditions (14).

CONCLUSIONS

The characteristics of the human β-CN-4P monomer and the mode of self-association that were observed in these hydrodynamic studies show an amphipathic molecule with charged, hydrophilic phosphoryl groups sequestered in one part of the molecule and separated from the exposed hydrophobic groups that are available for intermolecular interactions. Unless neutralized, the charges on the phosphoryl groups would tend to cause repulsion of the molecules. The hydrophobic interactions are weak at low temperatures and become stronger as the temperature is increased. High ionic strength from added NaCl would reduce the charge-charge repulsion and allow for the formation of polymers of limited maximum size. Calcium ions may provide bridges between phosphoryl groups on adjacent molecules within and between polymers and can lead to the formation of larger aggregates and eventual precipitation.

The behavior of the various forms of human β-CN in the presence and absence of calcium ions revealed more similarities than differences among the different forms. The monomers had approximately the same size, shape, and solvation, and the aggregated products at high ionic strength were also not very different. They behaved somewhat differently in the presence of calcium ions because of the difference in the net charge on these molecules. However, any differences in how the various phosphorylation levels participate in micelle formation seem to have been caused by the presence of other constituents of milk, such as inorganic orthophosphate.

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REFERENCES

1 Aoki, T., T. Umeda, and Y. Kako. 1992. The least number of phosphate groups for crosslinking of casein by colloidal calcium phosphate. J. Dairy Sci. 75:971.
2 Azuma, N., M. Aziawa, and K. Yamauchi. 1989. Effect of bound phosphate on the calcium-binding ability and calcium dependent precipitability of human β-casein. Agric. Biol. Chem. 53: 1037.
3 Bloomfield, V. A. 1968. Hydrodynamic studies of structure of biological macromolecules. Experimental and theoretical advances yield structural details of proteins, DNA, viruses, and polysomes. Science 161:1212.
4 Colowick, S. P., and F. C. Womack. 1969. Binding of diffusible molecules by macromolecules: rapid measurement by rate of dialysis. J. Biol. Chem. 244:774.
5 Greenberg, R., and M. L. Groves. 1979. Human β-casein. J. Dairy Res. 46:235.
6 Greenberg, R., M. L. Groves, and H. J. Dower. 1984. Human β-casein: amino acid sequence and identification of phosphorylation sites. J. Biol. Chem. 259:5132.
7 Groves, M. L., and W. G. Gordon. 1970. The major component of human casein: a protein phosphorylated at different levels. Arch. Biochem. Biophys. 140:47.
8 Javor, G. T., S. M. Sood, P. Chang, and C. W. Slattery. 1991. Interaction of triply phosphorylated human β-casein: fluorescence spectroscopy and light-scattering studies of conformation and self association. Arch. Biochem. Biophys. 289:39.
9 Long, V.J.W., and H. J. Yardley. 1970. Phospholipid in cultured guinea pig skin. J. Invest. Dermatol. 54:174.
10 Mora-Gutierrez, A., H. M. Farrell, Jr., and F. Kumosinski. 1993. Comparison of calcium induced association of bovine and caprine caseins and the relationship of α_{s1}-casein content to colloidal stabilization: thermodynamic linkage analysis. J. Dairy Sci. 76:3690.
11 Rasmussen, L. K., H. A. Due, and T. E. Petersen. 1995. Human α_{s1}-casein: purification and characterization. Comp. Biochem. Physiol. 111B:75.
12 Scatchard, G. 1949. The attraction of proteins for small molecules and ions. Ann. New York Acad. Sci. 51:660.
13 Slattery, C. W., S. M. Sood, and P. Chang. 1989. Hydrophobic interactions in human casein micelle formation: β-casein aggregation. J. Dairy Res. 56:427.
14 Sood, S. M., P. Chang, and C. W. Slattery. 1985. Interactions in human casein systems: self-association of fully phosphorylated human β-casein. Arch. Biochem. Biophys. 242:355.
15 Sood, S. M., P. Chang, and C. W. Slattery. 1988. Interactions in human casein systems: self-association of nonphosphorylated human β-casein. Arch. Biochem. Biophys. 264:574.
16 Sood, S. M., P. Chang, and C. W. Slattery. 1990. Interactions of triply phosphorylated human β-casein: monomer characterization and hydrodynamic studies of self-association. Arch. Biochem. Biophys. 277:415.
17 Sood, S. M., P. Chang, and C. W. Slattery. 1992. Interaction properties of doubly phosphorylated β-casein: a major component of the human milk caseins. J. Dairy Sci. 75:2937.
18 Sood, S. M., and C. W. Slattery. 1993. Interaction properties of singly phosphorylated human β-casein: similarities with other phosphorylation levels. J. Dairy Sci. 77:405.
19 Tanford, C. 1961. Page 390 *in* Physical Chemistry of Macromolecules. John Wiley & Sons, New York, NY.
20 Thompson, M. P., W. G. Gordon, R. T. Boswell, and H. M. Farrell, Jr. 1969. Solubility, solvation, and stabilization of α_{s1}- and β-caseins. J. Dairy Sci. 52:1166.
21 Waugh, D. F., L. K. Creamer, C. W. Slattery, and G. W. Dresdner. 1970. Core polymers of casein micelles. Biochemistry 9:786.
22 Yoshikawa, M., R. Sasaki, and H. Chiba. 1981. Effect of chemical phosphorylation of bovine casein components on the properties related to casein micelle formation. Agric. Biol. Chem. 45: 909.
23 Yoshikawa, M., M. Yamaki, E. Sugimoto, and H. Chiba. 1974. Effect of dephosphorylation on self-association and the precipitation of β-casein. Agric. Biol. Chem. 38:2051.