Comparison of Hydration Behavior of Bovine and Caprine Caseins As Determined by Oxygen-17 Nuclear Magnetic Resonance: Temperature Dependence of Colloidal Stability

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Oxygen-17 nuclear magnetic resonance (NMR) transverse relaxation rates for bovine and caprine casein micelles at various temperatures were analyzed by nonlinear regression analysis and a protein activity model. The dependence of the NMR transverse relaxation rates was markedly nonlinear due to interactions between protein molecules. Temperature dependences of the hydration parameters of the bovine and caprine casein micelles were in accordance with the hypothesis that hydrophobic interactions are the predominant forces responsible for the self-association of the caseins. Relaxation differences between reconstituted micelles of bovine and caprine caseins strongly suggest that important structural dissimilarities exist between these milk proteins that are due to differences in the ratios of α_{s1}- to β-casein. A higher degree of hydration, characteristic of a more open and looser structure, is observed for caprine casein micelles high in α_{s1}-casein at 21 and 37 °C. The observed hydration behavior of bovine casein micelles at all three temperatures is consistent with the hydration values determined previously by deuterium NMR studies of bovine casein micelles in D_2O containing 1,4-piperazinediethanesulfonic acid. The correspondence between the deuterium and oxygen-17 results suggests that both experiments detect exchangeable water "trapped" within the casein micelles. The dependence of the second virial coefficient B_0 on temperature was different for bovine and caprine casein micelles, suggesting the importance of "net" electrostatic charges of these milk proteins in their interactions with calcium and water.

Keywords: *NMR; ^{17}O; water binding; calcium binding; temperature effects; bovine casein; caprine casein; caprine α_{s1}-casein*

INTRODUCTION

The structure of the bovine casein micelles in milk has long been of interest to investigators in the field of milk proteins. Considerable information has been accumulated with regard to the structure of these micelles (Waugh, 1971; Byler et al., 1988; Eigel et al., 1984; Farrell, 1988). The composition of the micelles is a function of temperature and the natural variability of milk composition, and they have been estimated to contain 38% α_{s1}-casein, 10% α_{s2}-casein, 36% β-casein, and 13% κ-casein. In addition to proteins, they are known to contain colloidal calcium phosphate, with a calcium phosphate ratio of 1.5−1.8 (Eigel et al., 1984; Farrell, 1988).

To understand why the casein micelles appear to be stable in milk, one needs to examine the possible molecular interactions involved. The stability of casein micelles in an aqueous environment is determined not only by the primary structures and composition of the individual proteins but also by the effect of secondary bonding forces. In this context, hydrophobic forces are thought to play a great role in casein micelle organization (Waugh, 1971; Schmidt and Payens, 1976; Farrell, 1988). Ion pair formation between κ- and α_{s1}-caseins may also contribute to the stability of the milk micelles (Kumosinski et al., 1994a). The nature of protein−water and protein−protein interactions is also of critical importance in the stability of the milk micelles. Bovine micelles are porous and highly solvated (~1.9 g of water/g of protein; Thompson et al., 1969). In contrast, caprine micelles (Thompson et al., 1969) demonstrate a lower degree of solvation. Factors that affect protein−water interactions include the number and type of binding sites, the strengths of the interactions between water molecules and the binding sites, orientation of water molecules in the binding sites, mobility of bound water molecules, and protein conformation (Kumosinski et al., 1994b).

Recently, there has been great interest in the hydration of milk proteins (Kneifel et al., 1991). Deuterium NMR studies were carried out on bovine casein micelles (Kumosinski et al., 1987; Farrell et al., 1989); however, the hydration of caprine casein micelles has not yet been investigated by NMR relaxation techniques. Because the oxygen nucleus is the best probe for studying water binding, molecular motions of water, and water−protein interactions in solution (Halle et al., 1981; Denisov and Halle, 1995), we intend to investigate the hydration properties of bovine and caprine casein micelles by oxygen-17 NMR relaxation measurements. It was of interest to study the hydration of reconstituted micellar bovine and caprine casein at 4, 21, and 37 °C because the solubility of the caseins is strongly dependent on temperature (Farrell and Kumosinski, 1988; Farrell et al., 1988; Mora-Gutierrez et al., 1993a,b). The extent of protein−water and protein−protein interactions for bovine and caprine casein micelles was determined in deuterated piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) −KCl buffer (pD 6.98) containing 15 mM $CaCl_2$. The

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results of the present work allows us to determine the role of protein composition and structure in water−protein interactions.

MATERIALS AND METHODS

Materials. All reagents used were of analytical grade or ACS certified from Fisher and Sigma. Deuterium oxide (99.8 atom % D) was obtained from Sigma (St. Louis, MO).

Bovine milk was obtained from an individual Jersey cow. Caprine milk was obtained from individual French-Alpine goats selected to represent high and low levels of α_{s1}-casein as determined by reversed-phase high-performance liquid chromatography (RP-HPLC; Mora-Gutierrez et al., 1991). From each animal, 2 L of fresh, uncooled milk was obtained. The animals were in midlactation, in good health, and were part of a commercial herd. Phenylmethanesulfonyl fluoride (0.1 g/L) was added to the milk immediately to retard proteolysis. The milk was transported to the laboratory and skimmed twice by centrifugation at 4000*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 resuspended with a homogenizer at low speed and dissolved by addition of NaOH to yield a solution of pH 7.0. The casein was reprecipitated, washed, and then resuspended. The sodium caseinate was subsequently cooled to 4 °C and centrifuged at 100000*g* for 30 min to remove residual fat. Finally, the casein suspensions were exhaustively dialyzed versus cold deionized water at 4 °C for 72 h, with three changes, adjusted to pH 7.0, and then lyophilized. The integrity of the samples was confirmed by RP-HPLC.

The lyophilized caseins were dissolved in D_2O containing 25 mM 1,4-piperazinediethanesulfonic acid (PIPES; pD, 6.98), 15 mM $CaCl_2$, and 85 mM KCl. Conversion of pH to pD values was made according to the relation pD = pH + 0.4 (Covington et al., 1968), where pH is the pH-meter reading for a solution in D_2O with the electrode calibrated in standard H_2O buffers.

NMR Measurements. Oxygen-17 NMR measurements were obtained on a Varian XL-200 spectrometer (Varian Associates, Palo Alto, CA) operating at 4.7 T. The oxygen-17 NMR spectra were recorded at 27.1 MHz. The number of scans required for a good signal-to-noise ratio (>100:1) in oxygen-17 NMR spectra of bovine and caprine casein micelles was ~1000. Fourier transforms were carried out with a Varian 4000 series data system computer with Pascal software (v. 6.3), and a 10-mm broadband series 3 probe. Other conditions were as follows: 90° pulse width of 19 μs, acquisition time of 0.50 s, and spectral width of 5 kHz.

The oxygen-17 spin−spin (T_2) relaxation parameter was determined by measuring the line widths at half-height for each spectrum at each concentration. To correct for any residual magnetic field inhomogeneity, the net line broadening ($\Delta\upsilon_B$) was calculated by subtracting the line width of liquid D_2O ($\Delta\upsilon_{free}$) from that of the sample ($\Delta\upsilon_{obs}$). The net or differential transverse relaxation rate (ΔR_2, s^{-1}), which is the inverse of ΔT_2, was calculated from the line widths by

$$\Delta R_2\ (s^{-1}) = \pi\Delta\upsilon_B\ (s^{-1}) \qquad (1)$$

Microcomputer Analysis of Experimental Data. The oxygen-17 NMR relaxation data were fitted with a Systat (SYSTAT, Inc., Evanston, IL) nonlinear regression program that utilizes a Quasi-Newton algorithm (Systat version 5.1) to obtain confidence intervals for the iterated parameters. The program was run on an Apple-Macintosh II microcomputer, with a 68020 CPU and a 68882 (16 MHz) mathematical coprocessor. This program minimizes the standard deviation (SD) points from the curve, also known as the root mean square (RMS), where the RMS is defined as

$$\mathrm{RMS} = \{[\sum(R_{2obs} - R_{2calc})^2]/ (\text{no. of data points} - \text{no. of fitting parameters})\}^{1/2} \qquad (2)$$

Here R_{2obs} and R_{2calc} are the observed and calculated transverse relaxation rates, respectively. RMS values were normalized to be within at least 5% error of the fit for all the data.

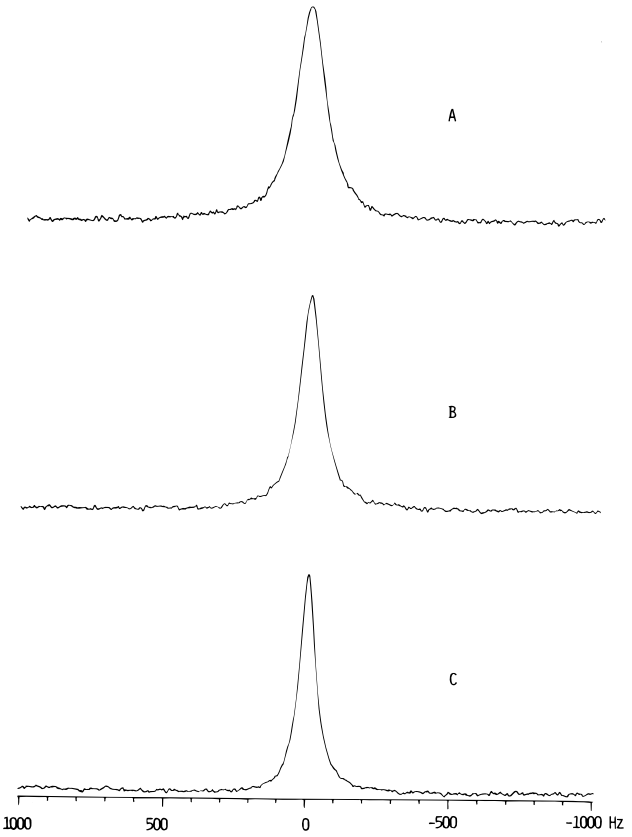

Figure 1. Oxygen-17 NMR spectra of caprine casein micelles high in the α_{s1}-component (13.20 mg/mL) in deuterated piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)−KCl buffer (pD 6.98) containing 15 mM $CaCl_2$ at (A) 4 °C, (B) 21 °C and (C) 37 °C.

RESULTS AND DISCUSSION

Oxygen-17 NMR Relaxation and the Fast-Exchange Model. The simplest model of protein hydration consistent with NMR relaxation data in very dilute solutions involves a fast exchange between bound and free (or bulk) water populations (Zimmerman and Brittin, 1957; Derbyshire, 1982). In this "two-state" model, the exchange rate of water molecules is fast compared with the nuclear spin relaxation rates of bound water in protein solutions. The observed relaxation rates are

$$R_{i,obs} = R_{i,B}P_B + (1 - P_B)R_{i,F} \qquad (3)$$

In eq 3, $i = 1, 2$, $R_i = 1/T_i$, the subscripts B and F stand, respectively, for bound and free water molecules, and P_B is the fraction of bound water molecules.

Such a model predicts single exponential relaxation for the water nuclei (or single Lorentzian water peaks in the frequency domain) in protein solutions. In the case of aqueous bovine and caprine casein micelle dispersions, the line shapes of the oxygen-17 NMR absorption peaks are approximately Lorentzian, which is in agreement with eq 3; an example is shown in Figure 1.

Oxygen-17 NMR Relaxation and the Protein Activity Model. Although the exchange is fast between the free and bound water populations, marked deviations from this linear model are found for reconstituted bovine and caprine casein micelles when one determines the relaxation rates of water as a function

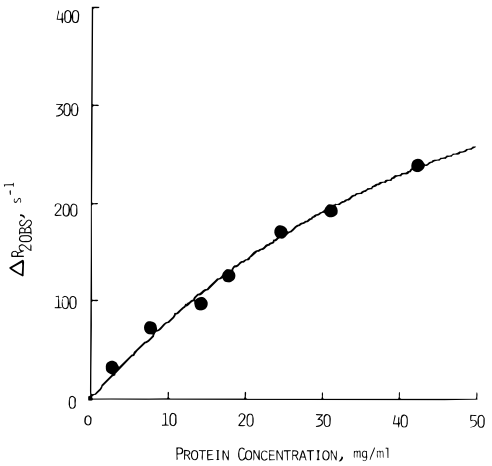

Figure 2. Concentration dependence of 27.1 MHz oxygen-17 NMR transverse relaxation rates for bovine casein micelles in deuterated piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)−KCl buffer (pD 6.98) containing 15 mM $CaCl_2$ at 4 °C. Data were fitted by eq 4. Results are in Table 1.

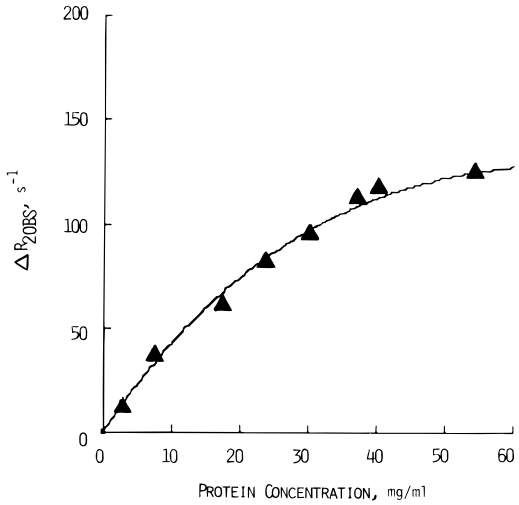

Figure 3. Concentration dependence of 27.1 MHz oxygen-17 NMR transverse relaxation rates for caprine casein micelles low in the α_{s1}-component at 21 °C. Conditions were the same as in Figure 2. Data were fitted by eq 4. Results are in Table 1.

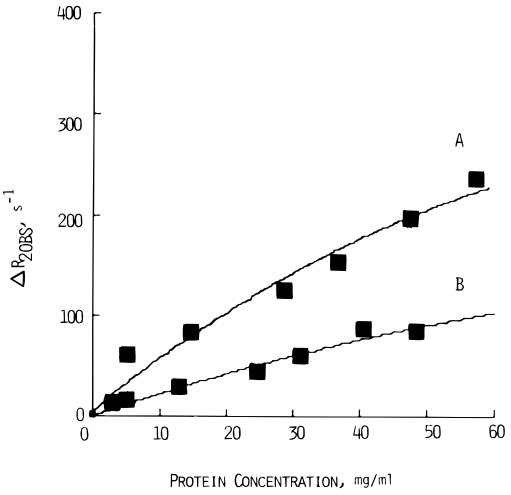

Figure 4. Concentration dependence of 27.1 MHz oxygen-17 NMR transverse relaxation rates for caprine casein micelles high in the α_{s1}-component at (A) 4 °C and (B) 37 °C. Conditions were the same as in Figure 2. Data were fitted by eq 4. Results are in Table 1.

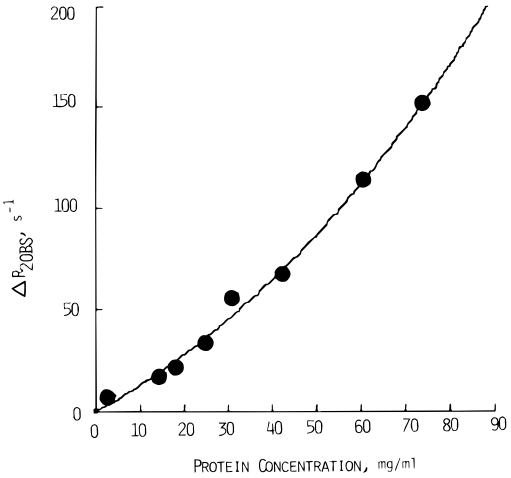

Figure 5. Concentration dependence of 27.1 MHz oxygen-17 NMR transverse relaxation rates of bovine casein micelles at 37 °C. Conditions were the same as in Figure 2. Data were fitted by eq 4. Results are in Table 1.

of casein concentration at 4, 21, and 37 °C (Figures 2−5). Such observations indicate the need for a more complex model to explain the hydration behavior of the bovine and caprine casein micelles. Nonidealities of protein solutions are often related to the presence of significant protein−protein interactions or to "protein activity" (Derbyshire, 1982; Pessen and Kumosinski, 1985). By taking into account the protein activity, one can regain the linearity required by eq 3, if activities are employed instead of concentrations. In this case, protein activities are determined by nonlinear regression analysis of the NMR relaxation data. This analysis involves replacing eq 3 with eq 4:

$$R_{i,obs} - R_{i,F} = n_H(R_{i,B} - R_{i,F})C_p \exp(2B_0C_p) \quad (4)$$

In eq 4, n_H is the protein hydration, $\Delta R = R_{2B} - R_{2F}$, C_p is the macromolecular concentration, and B_0 is the second virial coefficient of the protein activity.

The sign of the second virial coefficient B_0 may serve as an indication of the type of protein−protein interactions: B_0 is positive for repulsive effects and negative for attractive ones. The B_0 (mL/mg) virial coefficient reflects interactions related to the "net" protein charge Z, the protein excluded volume $\bar{v}_p$ (mL/mg), and a preferential interaction term $\partial g_s/\partial g_p$ (mg of bound salt/mg of protein; Arakawa and Timasheff, 1982; Kumosinski et al., 1987):

$$2B_0 = Z^2/(4m_sM_p) + \bar{v}_p/1000 - (\partial g_s/\partial g_p)^2(1/m_s) \quad (5)$$

Oxygen-17 NMR transverse relaxation rates, ΔR_2 (s^{-1}), of bovine and caprine casein micelles were measured at concentrations ranging from 0 to 85 mg/mL. The caprine micelles were characterized by high and low content of the α_{s1}-component. The ratios of α_{s2}-, α_{s1}-, β-, and, κ-casein for the samples studied have been previously reported (Mora-Gutierrez et al., 1993b). β-Casein predominates in the caprine samples, and caprine micelles highest in α_{s1}-casein represent only one-fourth of the α_{s1}-content of bovine casein (Mora-Gutierrez et al., 1991). Clearly, ΔR_2 values vary in a nonlinear fashion with increasing concentration (Figures 2, 3, and 4). This nonlinear increase is the behavior one would expect if attractive protein−protein interactions of caseins existed in solution, leading to casein self-association.

Table 1. Calculated Hydration Products $n_H\Delta R$ and Virial Coefficients B_0 from Nonlinear Regression Analysis of Oxygen-17 NMR Transverse Relaxation Data for Bovine and Caprine Casein Micelles in Deuterated Piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)−KCl Buffer (pD 6.98) Containing 15 mM $CaCl_2$ Using Eq 4

micelle	temp, °C	$n_H\Delta R$,[a] mL mg^{-1} s^{-1}	B_0, mL mg^{-1}
bovine	4	8.77 ± 0.051	−0.0053 ± 0.0002
	21	5.36 ± 0.045	−0.0033 ± 0.0002
	37	1.22 ± 0.034	0.0035 ± 0.0008[b]
caprine high in α_{s1}-casein	4	6.65 ± 0.050	−0.0040 ± 0.0002
	21	6.01 ± 0.058	−0.0068 ± 0.0003
	37	2.36 ± 0.044	−0.0027 ± 0.0005
caprine low in α_{s1}-casein	4	9.56 ± 0.087	−0.0110 ± 0.0003
	21	4.94 ± 0.067	−0.0071 ± 0.0004
	37	1.87 ± 0.037	0.0040 ± 0.0006

[a] The protein concentration was in mg of protein/mL of solvent. [b] Deuterium NMR experiments at 30 °C yielded a value of 0.0032 (Kumosinski et al., 1987).

Table 2. Hydration Estimates of Bovine and Caprine Casein Micelles[a]

micelle	temp, °C	hydration,[b] g of water/g of protein
bovine	4	0.02479 (2 °C, 0.0282)[c]
	21	0.01012 (15 °C, 0.0225)[c]
	37	0.00159 (30 °C, 0.0165)[c]
caprine high in α_{s1}-casein	4	0.01944
	21	0.01250 (0.00572)[d]
	37	0.00331
caprine low in α_{s1}-casein	4	0.03264
	21	0.00923 (0.00257)[d]
	37	0.00231

[a] From oxygen-17 NMR data at 4, 21, and 37 °C and at pD 6.98, according to a two-state, isotropic model (Mora-Gutierrez et al., 1995). [b] Assuming τ_c = 45.1 ns (4 °C), 51.1 ns (21 °C), and 63.6 ns (37 °C) for bovine casein micelles (Kumosinski et al., 1987). [c] Numbers in parentheses for deuterium NMR (Kumosinski et al., 1987). [d] Numbers in parentheses for oxygen-17 NMR with NaCl at 21 °C (Mora-Gutierrez et al., 1995).

Temperature-Dependent Effects on Hydration. The NMR data of bovine and caprine casein micelles at 4, 21, and 37 °C (Figures 2−5) were analyzed according to eq 4. The results of this analysis, presented in Table 1, indicate there is an overall decrease in the hydration product $n_H\Delta R$ with increasing temperature for bovine and the two caprine casein micelles. This temperature-dependent change in the hydration product correlates well with the rheological properties of whole skim milk (Whitnah and Rutz, 1959; Dewan et al., 1972; Farrell et al., 1989). The relative viscosity of skim milk decreases with increasing temperature. The specific nature of this change in viscosity has been ascribed to a lowered hydration as a result of a decrease in the volume occupied by the micelles (Whitnah and Rutz, 1959; Dewan et al., 1972; Farrell et al., 1989). The ΔR_2 values of casein micelle solutions at 37 °C are indeed significantly lower than those at 4 °C (Figure 4), presumably because of the decreasing viscosity at higher temperature (37 °C). The decreased viscosity, in turn, would decrease the correlation time of bound water, tumbling together with the caseins.

The caseins are also highly hydrophobic and quite capable of association through hydrophobic interactions not only involving like caseins but also different casein species (Farrell, 1988). Several investigators have noted that β-casein and, to a lesser extent, κ- and α_{s1}-casein diffuse out of the micelle at low temperature (1 °C). This behavior is consistent with the hydrophobic character of these proteins because the oustanding feature of β-casein is its temperature-dependent association (Farrell, 1988). An increase in association with an increase in temperature is, therefore, to be expected. High temperatures would favor casein self-association, presumably through hydrophobic bonding, and this self-association would substantially decrease overall micelle hydration, as we observed in the $n_H\Delta R$ values (Table 1). Our observations are consistent with the current view that the solubility, and therefore, degree of solvation of the caseins mainly depends on temperature (Farrell and Kumosinski, 1988; Farrell et al., 1988; Mora-Gutierrez et al., 1993a,b). At 4 °C, where hydrophobic interactions are minimized, caprine casein micelles high in α_{s1}-casein are less hydrated than bovine and caprine casein micelles low in α_{s1}-casein, as exemplified in the $n_H\Delta R$ values in Table 1. The lower hydration level of caprine casein micelles high in the α_{s1}-component at 4 °C and pD 6.98 is in agreement with the lower solubility at comparable ionic strengths found by Mora-Gutierrez et al. (1993b).

At temperatures well above 4 °C, however, both types of caprine casein micelles are more hydrated than the bovine casein micelles (Table 1). This increase in hydration might suggest that the caprine casein micelles have a tendency either to (partially) unfold as the temperature is raised, thus exposing active sites for water binding or to form a looser network, thus "trapping" more water. Although some minor conformational changes during casein self-association are predicted to occur by Raman spectroscopy (Byler et al., 1988), it is unlikely that the higher hydration levels ($n_H\Delta R$) of the caprine casein micelles (Table 1) are due to these predicted minor conformational changes. This process may more likely be related to differences in composition of the casein monomers. For bovine casein, the ratios of various caseins interacting in mixed associations will produce different results from the caprine caseins in which the β-casein component predominates in the ratio. In fact, Schmidt et al. (1977) studied synthetic bovine micelles made from different ratios of α_{s1}-, β-, and κ-caseins by electron microscopy. By alterating the ratios of the various components, micelle size could be altered. Micelles rich in β-casein were significantly smaller than those rich in α_s-casein. In this work, changes in the ratios of α_{s1}- to β-casein in the caprine micelles argue for a micellar effect. The low α_{s1}-casein micelles have a lower $n_H\Delta R$ term at 37 °C, perhaps resulting from a smaller, more compact structure. Curiously, the bovine casein micelles have the lowest hydration product. Another explanation is that the caprine casein micelles high in the α_{s1}-component might be "preferentially" hydrated at higher temperatures (i.e., 21 and 37 °C). Preferential interaction parameters were derived from oxygen-17 NMR measurements at 21 °C for bovine and caprine whole caseins in the presence of NaCl (Mora-Gutierrez et al., 1995). These results indicated that there is a greater "preferential" binding of salt (NaCl) for the caprine whole casein high in the α_{s1}-component. The observed results in the presence of calcium at 21 °C in this study on the hydration product $n_H\Delta R$ (Table 1) are in accordance with the order of preferential binding data for bovine and caprine whole caseins at 21 °C of Mora-Gutierrez et al. (1995).

Calculation of Apparent Hydration Number. Hydration estimates for bovine and caprine casein micelles are presented in Table 2. At all three temper-

atures, the estimated values of hydration (n_H) for the bovine casein micelles are quite close to the hydration values derived by deuterium NMR (Kumosinski et al., 1987). The hydration numbers were calculated assuming the correlation times found for bovine micelles at the appropiate temperatures. The magnitude of these numbers and their correspondence with the deuterium NMR values indicate that the oxygen-17 experiments sense the same "window" on the casein micelle that other NMR techniques reveal (i.e., the water observed is internal or trapped water associated with submicellar particles; Kumosinski et al., 1987; Farrell et al., 1989). This conclusion is borne out by the correspondence of the hydration number estimates found by Mora-Gutierrez et al. (1995) in the absence of $CaCl_2$ (Table 2). Although hydration does increase two- to threefold upon incorporation of the caseins into the micelle, a good deal of the bound water is primarily associated with the casein submicelles (casein aggregated to its maximum extent in the absence of $CaCl_2$). Therefore, these experiments report changes undergone by this "trapped" water and not water transiently bound to surface charges. Kumosinski et al. (1994b) proposed an apparent molecular mechanism to account for the nature of this "trapped" water by comparison of casein 3D models and small-angle X-ray scattering data. Regardless of the exact positioning of this "trapped" water, the oxygen-17 NMR data describe changes with environmental effects. Recent experiments by Denisov and Halle (1995) confirm this interpretation. The latter workers were able to mathematically separate the contributions to relaxation of oxygen-17 by various classes of exchangeable water through field dependency studies. For bovine pancreatic trypsin inhibitor (BPTI), the classes of water molecules included four internal (trapped) water molecules, 250 surface water molecules, and 2500 protein-influenced bulk water molecules. However, the four internal water molecules contribute disproportionally to the relaxation. Although we observe a weighted average for the relaxation and are studying a more complex protein system in our work, internal or "trapped" water undoubtedly dominates the experiment. This dominance is true for the submicelles alone, which provide many internal cavities and surface pockets for water, and most certainly for these particles when assembled into casein micelles.

Significance of the Second Virial Coefficient B_0 for Caseins. The values required for the second virial coefficient B_0 to produce a good fit of the oxygen-17 NMR data for bovine and caprine casein micelles (Table 1) suggest the presence of attractive forces between casein molecules. The overall increase of B_0 values upon increasing temperature of the casein micelle solutions (Table 1) could be caused by a decrease in the protein excluded volume ($\bar{v}_p$) due to the self-association process. If the concentration of salt is high enough, the value of B_0 is almost exclusively due to the excluded volume.

In the case of bovine and caprine casein micelles low in the α_{s1}-component at 37 °C, the presence of repulsive charge-charge interactions (positive B_0 values; Table 1) at first seems contradictory. The presence of such repulsive electrostatic interactions within the micelles could be due to any of the following reasons. (1) A change in quaternary structure. In this work, the type and degree of association (and thus hydration) would be influenced by the ratios of the different casein monomers participating in the interactions. (2) A change in secondary structure upon raising the temperature, which might be accompanied by either direct interactions with these charges or rearrangement of "trapped" water inside the casein complexes. The proteins may have a net negative or positive charge and, hence, repulsive interactions will exist within colloidal micellar structure. (3) Salts can affect both polar and apolar moieties in proteins and those salts might have positive or negative effects on the solubility and, therefore, on the degree of solvation. Mora-Gutierrez et al. (1993b) studied the effects of temperature and salt on calcium-induced solubilities of bovine and caprine caseins. Lower apparent binding affinities (greater solubility) were found for bovine and caprine caseins low in α_{s1}-casein; both of these caseins display positive B_0 values at 37 °C. In contrast, the caprine casein high in α_{s1}-casein was most sensitive to calcium-induced insolubility; note that this protein still displays a negative B_0 at 37 °C. This greater calcium sensitivity seems to be a determining factor in the more salted-out behavior of caprine casein with high content of the α_{s1}-component (Mora-Gutierrez et al., 1993a,b). The negative sign of the B_0 values derived from the NMR data (Table 1) suggests that attractive interactions dominate the hydration properties of caprine casein micelles high in the α_{s1}-component at all three temperatures and this protein system has the highest hydration product.

One explanation for the differences found in this study can be found by examination of the raw data. For bovine caseins, the data indicate a negative virial coefficient (B_0) at 4 °C (Figure 2). At this temperature, we expect that self-association is at a minimum and the complexes are loosely aggregated, resulting in higher hydration. As self-association increases with increasing temperature, the virial coefficient (B_0) for bovine casein approaches zero and finally becomes positive at 37 °C, where maximum self-association is achieved (Figure 5). The data for caprine casein low in α_{s1}-casein mirror these changes. For the caprine casein high in α_{s1}-casein, it could be that maximum self-association does not occur; the reconstituted micelles retain an open, highly hydrated structure. This system does not achieve full micellization (maximum protein−protein interaction), so it is the most readily destabilized (salted-out) system (Mora-Gutierrez et al., 1993b). Noting again that the water reported in this case is probably internal "trapped" water, the differences observed in these experiments are perhaps due to pronounced differences in self-association. Such differences can be inferred from the sign of their B_0 values at 37 °C (Table 1).

Conclusions. We have illustrated the usefulness of oxygen-17 NMR spectroscopy for the study of the hydration of bovine and caprine milk casein micelles. A complex protein system such as the casein micelle consists of subunits or submicelles containing all the casein components held together predominately by hydrophobic bonds. These subunits, in turn, are held in the micellar structure by colloidal calcium phosphate. The present results do not support reliable data interpretation at the molecular level; hence, work on purified individual casein components is required for that purpose. However, the changes in the hydration parameters as a function of temperature of bovine and caprine milk micelles presented here can be advantageous for applications in the manufacture of milk-based products and clearly demonstrate that alteration in casein composition can dramatically alter functional properties.

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