Oxygen-17 Nuclear Magnetic Resonance Studies of Bovine and Caprine Casein Hydration and Activity in Deuterated Sugar Solutions

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The hydration of bovine and genetically variable caprine caseins in D_2O solutions of sucrose and lactose was investigated by oxygen-17 NMR and fitted by nonlinear regression analysis. A charge−charge interaction model was employed to analyze the transverse relaxation ($1/T_2$) data. Lactose caused increased hydration of the bovine casein and the caprine casein naturally low in α_{s1}-casein, whereas sucrose led to increased hydration of the caprine casein naturally high in α_{s1}-casein. At pD 7.20 and 21 °C the effect of charge−charge repulsive interactions on the native caseins generally leads to decreased protein stability in bovine and caprine caseins. However, addition of sugars causes stronger (attractive) interactions yielding more stable casein complexes with increased hydration. The calculated preferential binding term $-(\partial g_s/\partial g_p)$ for casein mixtures suggests that sucrose and lactose are "preferentially" excluded from these milk proteins, yielding greater access to much of the aqueous compartment. This is consistent with the view that sugars lead to the stabilization of proteins in nonfrozen, aqueous systems.

Keywords: *NMR, ^{17}O; water binding; sucrose; lactose; bovine casein, caprine casein; α_{s1}-casein*

INTRODUCTION

Preferential hydration of proteins has been shown to occur in the presence of a diversity of chemically unrelated compounds including sugars, polyols, amino acids, methylamines, and inorganic salts (Aune and Timasheff, 1970; Timasheff et al., 1976; Pittz and Timasheff, 1978; Gekko and Morikawa, 1981; Gekko and Timasheff, 1981; Lee and Timasheff, 1981; Na and Timasheff, 1981; Timasheff, 1982; Arakawa and Timasheff, 1982a,b, 1983, 1984a−c, 1985a,b). Fortunately, through the efforts of Timasheff and his colleages, the mechanism by which these solutes exert their stabilizing influence on proteins in aqueous solutions has been determined. These researchers have observed experimentally that there is a deficiency of the stabilizing solute (relative to the bulk solution) in the immediate vicinity of the protein and that this leads to the preferential hydration of the protein. That is, these solutes are preferentially excluded from contact with the surface of the protein. Thus, as Timasheff and his colleagues explain, the presence of these solutes in a protein solution creates a thermodynamically unfavorable situation since the chemical potentials of both the protein and the additive are increased.

There have been many investigations that characterized the effectiveness of additives, usually sugars or polyols, as cryoprotectants for bovine milk proteins (Tumerman et al., 1954; Rose, 1956; Tessier et al., 1956; Minson et al., 1981; Lonergan et al., 1981). It is believed that soluble carbohydrates have a stabilizing effect on casein in frozen milk and that crystallization of lactose destroys its effectiveness as a cryoprotectant. Replacing the lactose with other sugars, such as raffinose, glucose, sucrose, and xylose, or hydrolysis of lactose with enzymes improved stability of casein during frozen storage (Guy, 1980; Minson et al., 1981; Lonergan et al., 1981). This was originally theorized by the latter authors as a direct interaction between a sugar and casein through hydrogen bonding. The mechanism established by Timasheff and co-workers for the stabilization of proteins in nonfrozen, aqueous systems is, however, thermodynamic in nature and indirect. It is of sufficient force to account for the stability afforded to proteins by a broad array of soluble carbohydrates and may explain why one carbohydrate is more effective as a cryoprotectant than another as a result of cosolute effects on surface tension (Arakawa and Timasheff, 1982a).

One approach toward evaluating the determinants of protein stability induced by the addition of sucrose and lactose to milk is to examine the effects of these molecules on the hydration properties of casein. In previous work (Mora-Gutierrez et al., 1995, 1996a,b), we have evaluated the effects of salt and pH/pD on the interactions of water with caseins by oxygen-17 NMR relaxation techniques. In this work, the response of oxygen-17 NMR relaxation rates of D_2O to the presence of lactose, sucrose, bovine casein, and two caprine caseins differing in their content of the α_{s1}-casein component at pD 7.20 and 21 °C will be evaluated. This will allow us to elucidate the effect of these carbohydrates on protein hydration and protein−protein interactions and to identify those interactions that play a unique role in protein stability.

THEORY

Interpretation of the NMR data is highly model-dependent (Finney et al., 1982), and the application of different models to the same data may lead to somewhat conflicting concepts. The isotropic two-state model for

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water−macromolecule interactions has been applied to several polymer systems (Child and Pryce, 1972; Cook and Wien, 1973; Oakes, 1976; Hansen, 1978; Derbyshire, 1982; Mora-Gutierrez and Baianu, 1990). This model predicts a linear relationship between the observed relaxation rate of water (R_{obs}) and changes in concentration of the macromolecule (Zimmerman and Brittin, 1957), assuming no additional contributions to relaxation are present. However, there are often nonlinear responses and the derivation of equations for a nonlinear three-component system using relaxation techniques has been given in detail by Kumosinski and Pessen (1982).

These authors have shown that for the two-state fast exchange model, the change in R_{obs} (the observed relaxation rate) of water in the presence of various protein concentrations, C_p, is

$$R_{obs} - R_{free} = (R_b - R_f)\bar{\nu}_w a_p/W \quad (1)$$

where R_f is the appropiate relaxation rate of free water (R_1 or R_2), R_b is the corresponding relaxation rate of bound water, W is the total concentration of water, a_p is the activity of the protein, and $\bar{\nu}_w$ is the degree of hydration (i.e., basically, the average number of molecules of water bound per molecule of dry protein or, in units consistent with the concentration units employed, the number of grams of bound water per gram of dry protein). For ligands in general, $\bar{\nu}_w$ differs from n, the number of available binding sites for substrate molecule, the difference being a function of association constant and ligand concentration. In the case of water, however, which is a ligand present in such vast excess that the substrate is saturated with it, the distinction between $\bar{\nu}_w$ and n dissapears. In the following we will, for simplicity and convenience, use the expression "hydration" for short to indicate the quantity $\bar{\nu}_w$ in units of grams per gram. The term $R_{obs} - R_{free}$ has been termed the relaxation increment and will be used in this study to analyze all data.

In the case of polyelectrolytes such as proteins, departures from the linear behavior for the effects of protein on water relaxation are often observed as the protein concentration is increased. These departures indicate that new interactions are present which must be taken into account. Therefore, a model that includes such protein−protein and protein−solvent interactions (Kirkwood and Shumaker, 1952) present in nonideal solutions for the nonlinear concentration dependence of the NMR relaxation rates was developed (Pessen and Kumosinski, 1985). Such a model takes into account the chemical activity of the protein.

The activity of a protein (a_p) in solution is related to its concentration, C_p, by the activity coefficient, γ:

$$a_p = \gamma C_p \quad (2)$$

The activity coefficient can be obtained from the virial expansion of osmotic pressure as a function of concentration

$$\mathrm{d} \ln \gamma/\mathrm{d}C_p = 2B_0 + 3B_2C_p + ... \quad (3)$$

where the B parameters are the virial coefficients. The virial coefficients are a measure of the various molecular interactions (Tanford, 1963; Pessen and Kumosinski, 1985). The application of virial coefficients to the nonideality of macromolecules in solution is discussed by Richards (1980), Kumosinski and Pessen (1982), Bates (1982), Kumosinski et al. (1987), Myers-Betts and Baianu (1990), Kakalis et al. (1990a), and Mora-Gutierrez et al. (1995, 1996a,b).

Applying this relationship to casein NMR relaxation data (Mora-Gutierrez et al., 1995, 1996a,b) yields

$$R_{2obs} - R_{2F} = n_H(R_{2B} - R_{2F})C_p \exp[2B_0C_p + 2B_{0.5}C_p^{0.5} + 0.667B_{1.5}C_p^{1.5} + 1.5B_2C_p^2 + ...] \quad (4)$$

where R_{2obs} is the measured transverse relaxation rate corrected for inhomogeinity broadening, the subscripts "B" and "F" stand for "bound" and "free" water; n_H is the hydration number (i.e., the average number of water molecules "bound" per molecule of dry protein), and C_p is the varying protein concentration. The B_0 virial coefficient reflects the repulsive or attractive forces arising from the net protein charge Z, the protein excluded volume, and a preferential interaction term

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

where m_s is the sugar molarity, M_p is the average monomer molecular weight of casein, $\bar{\nu}_p$ is the average partial specific volume of the caseins, and $(\partial g_s/\partial g_p)^2$ is the preferential binding term (grams of preferentially bound sugar/gram of protein); this preferential interaction term must be included to take into account the preferential interaction of sugars and water at the protein interface (Arakawa and Timasheff, 1982a; Kumosinski et al., 1987). Unfortunately, the term in eq 5 is calculated from its square so that the sign of the term is lost. A negative term here represents preferential hydration, while a positive term represents preferential binding. However, in protein−sugar solutions the overwhelming body of evidence cited points to preferential hydration. Therefore, in this work it will be assigned a negative sign.

The B_2 virial coefficient represents attractive forces arising from fluctuating multipoles. Essentially, the exponential term of eq 4 represents the activity coefficient; therefore, determination of these virial coefficients will allow for quantitation of the protein−protein interactions or the protein activity.

MATERIALS AND METHODS

Materials. The following reagents were used: (1) sucrose (MW = 342.30), reagent grade (Sigma Chemical Co.); (2) α-lactose monohydrate (MW = 360.30) (Sigma), the content of the β-isomer being ~2%; and (3) deuterium oxide (99.8 atom % D) (Sigma). Other reagents were of reagent grade.

Sample Preparation. Bovine casein was obtained from the milk of a Jersey cow. The caprine caseins characterized by high and low content of the α_{s1}-casein component (Mora-Gutierrez et al., 1991) were obtained from the milk of an Anglo-Nubian and a French-Alpine goat, respectively. Caseins were isolated from 2 L of fresh, uncooled milk to which phenylmethanesulfonyl fluoride (0.1 g/L) was added immediately to retard proteolysis. The milk was centrifuged at 4000*g* for 10 min at room temperature to remove the cream fraction. Skimmed milk (500 mL) was diluted with an equal volume of distilled water and warmed to 37 °C. Casein was precipitated by careful addition of 1 N HCl to pH 4.6. The precipitate was homogenized with a Biospec 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 dialyzed exhaustively versus cold deionized water at 4 °C for 72 h and then lyophilized. The integrity of the samples was confirmed by

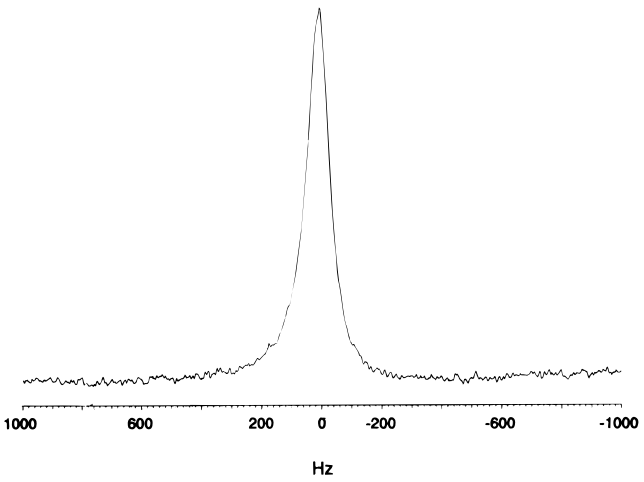

Figure 1. 27.1-MHz oxygen-17 NMR spectrum of D_2O in a 2.13% (w/v) of bovine casein solution with 100 mM lactose at pD 7.20 and 21 ± 1 °C.

Table 1. Comparison of the Percentage of Casein Distribution of Bovine and Caprine Caseins by Densitometry

		caprine casein	
casein type	bovine casein	high in α_{s1}-casein	low in α_{s1}-casein
α_{s2}-casein	12.1	9.2	29.2
α_{s1}-casein	39.5	25.1	5.9
β-casein	37.2	51.6	50.5
κ-casein	11.2	13.8	14.4

polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS), and densitometry was used to assess the relative concentrations of casein components (Basch et al., 1989). The compositions of the bovine and caprine caseins used in this study are given in Table 1.

The pD of the protein−sugar solutions was 7.20. Conversion to pD values was made according to the relation pD = pH + 0.4 (Covington et al., 1968). The protein solutions were prepared by stirring the appropiate amount of 99.8% deuterium oxide (D_2O) with casein and sugars. The samples were well mixed over a period of 3 h at 4 °C to reach maximum solubility (Mora-Gutierrez et al., 1993a). Samples were allowed to reach room temperature prior to the NMR measurements.

NMR Measurements. Natural abundance oxygen-17 (27.1 MHz) single-pulse NMR measurements were carried out with a Varian XL-200 spectrometer (Varian Associates, Palo Alto, CA) equipped with a high-resolution, narrow-bore (54 mm) 4.7-T superconducting magnet, and a Varian 4000 series data system computer with Pascal software (v. 6.3), and a 10-mm broadband series 3 probe.

The oxygen-17 NMR Fourier transform spectrum of 2.13% bovine casein−D_2O system is shown in Figure 1. The transverse relaxation rate, R_2 (s^{-1}) was calculated for the oxygen-17 nucleus by using the equation (Dwek, 1973)

$$R_2\ (s^{-1}) = \pi\Delta\nu_{obs} = 1/T_2 \qquad (6)$$

where $\Delta\nu_{obs}$ is the line width at half-height for each spectrum in the absence and in the presence of sugar and at each concentration of protein. For analysis of protein−water interactions, the relaxation rate increment (ΔR_2, s^{-1}) as defined above (eq 1) was calculated by subtracting the line width of liquid D_2O ($\Delta\nu_{free}$) from the line width of the sample ($\Delta\nu_{obs}$) before multiplying by π:

$$R_2\ (s^{-1}) = \pi(\Delta\nu_{obs} - \Delta\nu_{free}) \qquad (7)$$

About 4 mL of well-dispersed and thoroughly mixed protein in D_2O solutions (pD = 7.20) was run in 10-mm high-resolution NMR tubes (Wilmad, Buena, NJ). In all experiments two independent series of NMR measurements were conducted at 21 ± 1 °C. The oxygen-17 NMR 90° pulse width for D_2O was 19 μs, and 1024 scans were sufficient for a signal-to-noise ratio of 200:1 (with 5-Hz exponential line broadening applied). The spectral width was 5 kHz, the acquisition time was 0.50 s, and a 8K point−time−domain array was used for storing the data with adequate resolution.

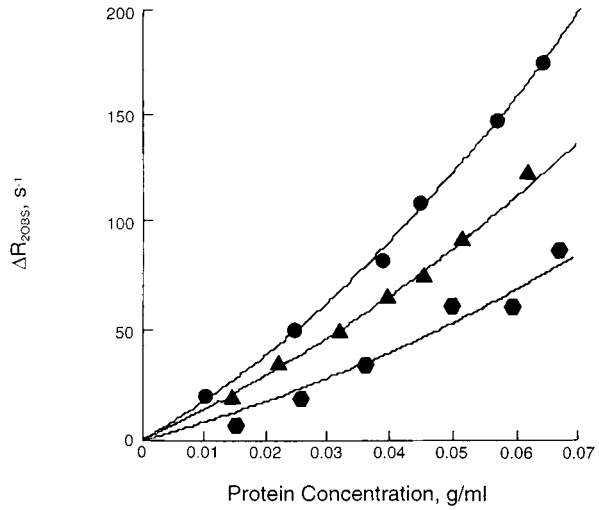

Figure 2. Dependence of the oxygen-17 NMR transverse relaxation rates, ΔR_2 (s^{-1}), on protein concentration (g/mL) for bovine casein (●), caprine casein low in α_{s1}-casein (▲), and caprine casein high in α_{s1}-casein (⬣) in the absence of sugar at pD 7.20 and 21 ± 1 °C. Data were fitted by eq 4. Results are in Tables 2 and 4.

Microcomputer Analysis of Experimental Data. Our treatment of the NMR relaxation data employed a nonlinear regression program that utilizes a Quasi-Newton algorithm. The program was run on a Macintosh II microcomputer (Apple Computer Inc., Cupertino, CA). Iteration of the concentration dependence for the NMR data yielded the virial coefficients of the protein activity according to eq 4 with a minimization of the standard deviation (SD). The SD of the experimental points from the curve, also known as the root mean square (RMS), is defined as

$$\mathrm{RMS} = \{[(R_{2calc} - R_{2obs})^2]/(\text{no. of data points} - \text{no. of fitting parameters})\}^{1/2} \qquad (8)$$

Here R_{2obs} and R_{2calc} are the observed and calculated transverse relaxation rates, respectively. The curves that represent the nonlinear regression best fit for the lowest RMS values are all within a relative standard deviation of 5% of the experimental data. Furthermore, the use of the Quasi-Newton algorithm allowed us to obtain the standard error of each parameter.

RESULTS AND DISCUSSION

^{17}O NMR and Derived B_0 Virial Coefficients. The change in transverse relaxation rates (ΔR_2) of water was measured by oxygen-17 NMR for bovine and caprine caseins as a function of protein concentration (Figure 2). Data for each casein alone show a nonlinear dependence on concentration. Such nonidealities of protein solutions are often related to the presence of significant protein−protein interactions in solution or to "protein activity" (Pessen and Kumosinski, 1985). Thus, the change in the gradient of the ΔR_2 relaxation rate as a function of protein concentration (Figure 2) could be attributed to the attraction or repulsion of the protein particles by charges on the molecular components of the protein. The upward curvature in the data is a preliminary indication of protein self-association (Mora-Gutierrez et al., 1995). As the caseins self-

Table 2. Calculated Virial Coefficients B_0 (Milliliters per Gram)[a] from Nonlinear Regression Analysis of Oxygen-17 NMR Transverse Relaxation Data for Bovine and Caprine Caseins in Deuterated Water Solutions of Sucrose and Lactose at pD 7.20 and 21 ± 1 °C Using Equation 4

casein	sucrose (mM)	B_0	lactose (mM)	B_0
bovine	0	3.9 ± 0.3	0	3.9 ± 0.3
	100	3.7 ± 0.2	100	0.7 ± 0.2
	200	1.2 ± 0.2	200	0.5 ± 0.2
	300	0.4 ± 0.1	300	1.1 ± 0.1
caprine				
high in α_{s1}-casein	0	2.6 ± 0.3	0	2.6 ± 0.3
	100	−4.0 ± 0.2	100	1.5 ± 0.3
	200	−6.5 ± 0.1	200	1.2 ± 0.2
	300	−5.6 ± 0.1	300	−3.1 ± 0.2
low in α_{s1}-casein	0	2.7 ± 0.6	0	2.7 ± 0.6
	100	0.5 ± 0.4	100	−1.6 ± 0.3
	200	0.4 ± 0.2	200	−3.5 ± 0.2
	300	−1.7 ± 0.2	300	−7.1 ± 0.2

[a] The protein concentration was in grams of protein per milliliter of solvent.

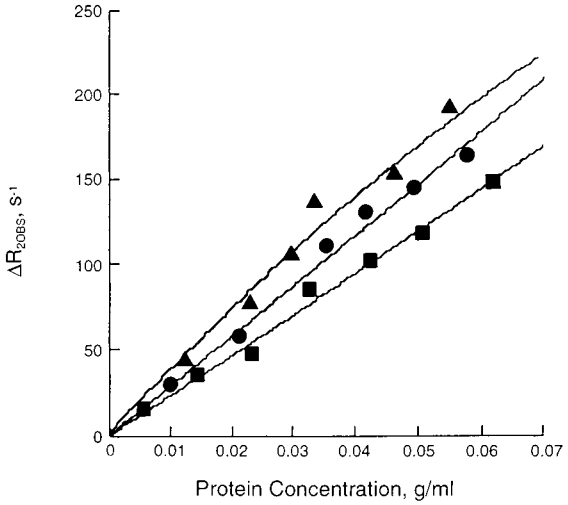

Figure 3. Dependence of the oxygen-17 NMR transverse relaxation rates, ΔR_2 (s^{-1}), on protein concentration (g/mL) for caprine casein low in α_{s1}-casein in the presence of sucrose at pD 7.20 and 21 ± 1 °C: 300 mM sucrose (▲); 200 mM sucrose (●); 100 mM sucrose (■). Data were fitted by eq 4. Results are in Tables 2 and 4.

associate as a function of concentration, these negatively charged molecules should exhibit increased charge repulsion.

To obtain a better understanding of effects on protein−solvent and protein−protein interactions of bovine and caprine caseins, the data were evaluated by nonlinear regression analysis. In the protein concentration range investigated [0−7% (w/v)], all sets of data were found to require only the B_0 virial coefficient of eq 4 (Table 2). In the absence of sugars all three caseins showed positive B_0 values (charge repulsion as a result of well described self-associations) in accord with previously published data at neutral pH/pD in the absence of salts (Mora-Gutierrez et al., 1995).

Figure 3 shows the effect of 300 mM sucrose on the oxygen-17 NMR transverse relaxation rates as a function of concentration for caprine casein low in α_{s1}-casein. Compared to the original data (Figure 2, in the absence of sucrose) the data exhibit a downward curvature, indicative of a change from repulsive electrostatic effects toward attractive electrostatic effects. Similar results

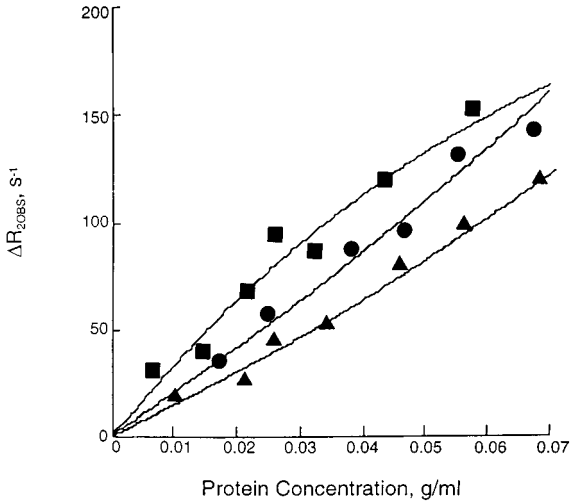

Figure 4. Dependence of the oxygen-17 NMR transverse relaxation rates, ΔR_2 (s^{-1}), on protein concentration (g/mL) for caprine casein high in α_{s1}-casein in the presence of lactose at pD 7.20 and 21 ± 1 °C: 300 mM lactose (■); 200 mM lactose (●); 100 mM lactose (▲). Data were fitted by eq 4. Results are in Tables 2 and 4.

were obtained with 300 mM lactose and high α_{s1}-caprine casein (Figure 4). Indeed, calculations of B_0 for these data (Table 2) show that the virial coefficient has changed to a negative sign. While this set is the most dramatic for the effect of lactose, both of the caprine casein samples show a net incremental decrease in B_0 as a function of added lactose with the exception of bovine casein, which appears to reach a minimum between 100 and 300 mM lactose. When lactose was replaced by sucrose, similar changes in B_0 occurred, but with this sugar the most dramatic change was caprine casein high in α_{s1}-content.

Data for the sucrose−casein and lactose−casein mixtures in Figures 2−4 show that, compared to the caseins alone, the presence of sucrose and lactose resulted in higher relaxation rate increments (ΔR_2 values). The general increase in the ΔR_2 relaxation rates, after the addition of sucrose or lactose, could be caused by two phenomena: an increase in the solution viscosity as a result of sugar added and/or changes in solution viscosity as a result of alterations in protein−protein aggregation. These two phenomena are probably involved in the observed increase of ΔR_2 values with sugar concentration. Clearly, increases in viscosity lead to slower protein tumbling, an increased τ_c, and accompanying increased ΔR_2. Such changes are seen at lower protein concentrations by comparison of Figures 2 and 3. However, at elevated protein concentrations, individual protein samples diverge considerably from each other. A significant measure of this divergence is the B_0 values of Table 2. Note that, in our set of B_0 virial coefficients (Table 2) for caprine caseins at high sucrose or lactose, the B_0 coefficients change in sign. The observed change in sign of B_0 values is caused by the presence of both repulsive (positive values) and attractive (negative values) interactions among bovine and caprine caseins.

At neutral pH the carboxylate and phosphate groups of the caseins would be largely unprotonated. This pH condition favors the formation of colloidal aggregates (micelles) of caseins (Kumosinski and Farrell, 1991). It has been shown that the negatively charged phosphate and carboxylic acid residues play a key role in controlling the colloidal stability properties of bovine and caprine casein micelles (Mora-Gutierrez et al., 1993b).

Table 3. Calculated Preferential Binding Term ($\partial g_s/\partial g_p$) of Bovine and Caprine Caseins in Deuterated Water Solutions of Sucrose and Lactose at pD 7.20 and 21 ± 1 °C Using Equation 5

casein	sucrose (mM)	$-(\partial g_s/\partial g_p)^a$	$-(\partial g_s/\partial g_p)^b$	lactose (mM)	$-(\partial g_s/\partial g_p)^a$	$-(\partial g_s/\partial g_p)^b$
bovine	100	0.0460		100	0.0521	
	200	0.0495		200	0.0522	
	300	0.0526		300	0.0484	
caprine						
high in α_{s1}-casein	100	0.0605	0.0492	100	0.0505	0.0363
	200	0.0744	0.0655	200	0.0495	0.0348
	300	0.0798	0.0716	300	0.0698	0.0602
low in α_{s1}-casein	100	0.0525	0.0390	100	0.0563	0.0440
	200	0.0526	0.0391	200	0.0658	0.0556
	300	0.0635	0.0528	300	0.0853	0.0654

[a] Grams of sugar per gram of protein. Calculated with B_0 values from Table 2; M_p and Z were chosen as 23 300 Da and −16.1 esu, respectively (Eigel, 1984), and a $\bar{v}_p$ of 0.736 (Kumosinski et al., 1987) was taken for an average partial specific volume of the bovine caseins. [b] Grams of sugar per gram of protein. Calculated with B_0 values from Table 2, as above, except Z was chosen as −12.0 for the caprine caseins on the basis of their composition (Table 1).

The α_{s1}-casein−Ca^{2+} interaction appears to be a strong interaction that contributes to the formation *in vitro* of the casein micelle. The predominant effect of the binding of Ca^{2+} to the caseins is to reduce considerably the charge on the protein molecules, which allows formation of the larger colloidal aggregates.

The casein submicelle, casein aggregates that occur in the absence of Ca^{2+} (referred to simply as casein in this work), is a good candidate for the study of solute− and solvent−protein interactions because the removal of calcium may decrease its stability as a result of increased net negative charge. In the absence of Ca^{2+}, the proteins are maintained in solution by a balance of hydrophobic forces, hydrophilic interactions between protein and solvent, and repulsions between proteins as a result of the charge (Farrell, 1988). Nevertheless, the magnitude of such repulsive interactions may become significant if these negative charges are partially or completely buried inside the protein. In such cases, each of the buried charges will destabilize the native protein structure to the order of 20 kcal/mol (Lumry and Biltonen, 1969).

The positive sign of the B_0 virial coefficients suggests that under the experimental conditions (0 mM sugar, pD 7.20, 21 °C) the bovine and caprine casein molecules are negatively charged (Table 2). These electrostatic interactions, which increase with increasing protein concentration, may destabilize the bovine and caprine casein structures, but binding of counterions may decrease these repulsive forces and provide stability to the casein structure.

Preferential Interactions. The effect of sugars on the stability of proteins in water has been interpreted in terms of changes in protein conformation, state of aggregation of the proteins, and changes in the interactions between protein and solvent components (Lee et al., 1975; Lee and Timasheff, 1981; Arakawa and Timasheff, 1982a). The overall decreases in B_0 seen in this work indicate a general decrease in electrostatic repulsions as the sugar concentrations are increased. The best interpretation of these data would be that sugars cause protein stabilization most likely through preferential hydration. That is, the water layer around the protein is enriched in water relative to the sugar−water solvent, and as the concentration of the sugar increases, the preferential hydration increases as well.

Although the relaxation increment (ΔR_2) is influenced by viscosity, it is of interest to estimate the magnitude of the preferential interaction term ($\partial g_s/\partial g_p$) from eq 5. The problem with this equation is that the preferential term occurs as a square so that its solution is not exact and yields a ± sign. However, as the overwhelming evidence argues, the preferential interaction term here (Arakawa and Timasheff, 1982a) will be considered negative, describing preferential hydration (a + sign would indicate preferential binding of sugar). The calculated values for the preferential interaction terms are given in Table 3. The values calculated from the oxygen-17 NMR data for the preferential hydration of caseins by sugars are in excellent agreement with those calculated by densimetry for globular proteins (Arakawa and Timasheff, 1982a). Additionally, the increase in magnitude of the term with increasing sugar follows the trends previously reported for globular proteins (Arakawa and Timasheff, 1982a) and for tubulin (Na and Timasheff, 1981). Thus, the oxygen-17 NMR estimates of B_0 and ($\partial g_s/\partial g_p$) appear to yield good approximations of the terms even though the effect of viscosity on τ_c is not taken into account. Bearing this caution in mind, the average number in Table 3 is 0.0590 g of sugar excluded/g of protein. This translates to 43 mol of sugar excluded per 250 000, which is the approximate molecular weight of the average submicelle. It is not hard to visualize this number if we consider the view shown in Figure 5 which represents a working 3D model for a putative bovine casein submicelle of 250 000 MW (Kumosinski et al., 1994). This figure contains 2823 water molecules representing the number to be in accord with small-angle X-ray calculations for "internal" water. It does not include casein-"influenced" water near the surface.

Influence of Sugars on Hydration Product and "Bound Water". Table 4 shows that the average hydration product, which includes the relaxation rate for bound water and the apparent number of "isotropically bound" water molecules, increases when sugar is added. It is seen that the effect of sucrose and lactose on the hydration product $n_H\Delta R$ (Table 4) strongly depends on both the sugar and the casein; the latter reflects the relative distributions of the α_{s1}-, α_{s2}-, β-, and κ- fractions of the individual caseins (Table 1). Although the abrupt change in the hydration product $n_H\Delta R$ (Table 4) of caprine casein high in α_{s1}-casein solutions containing sucrose and that of caprine casein low in α_{s1}-casein solutions containing lactose could not be definitely attributed to a conformational change of protein molecules, a plausible interpretation implies that these caprine caseins are internally stabilized by the hydra-

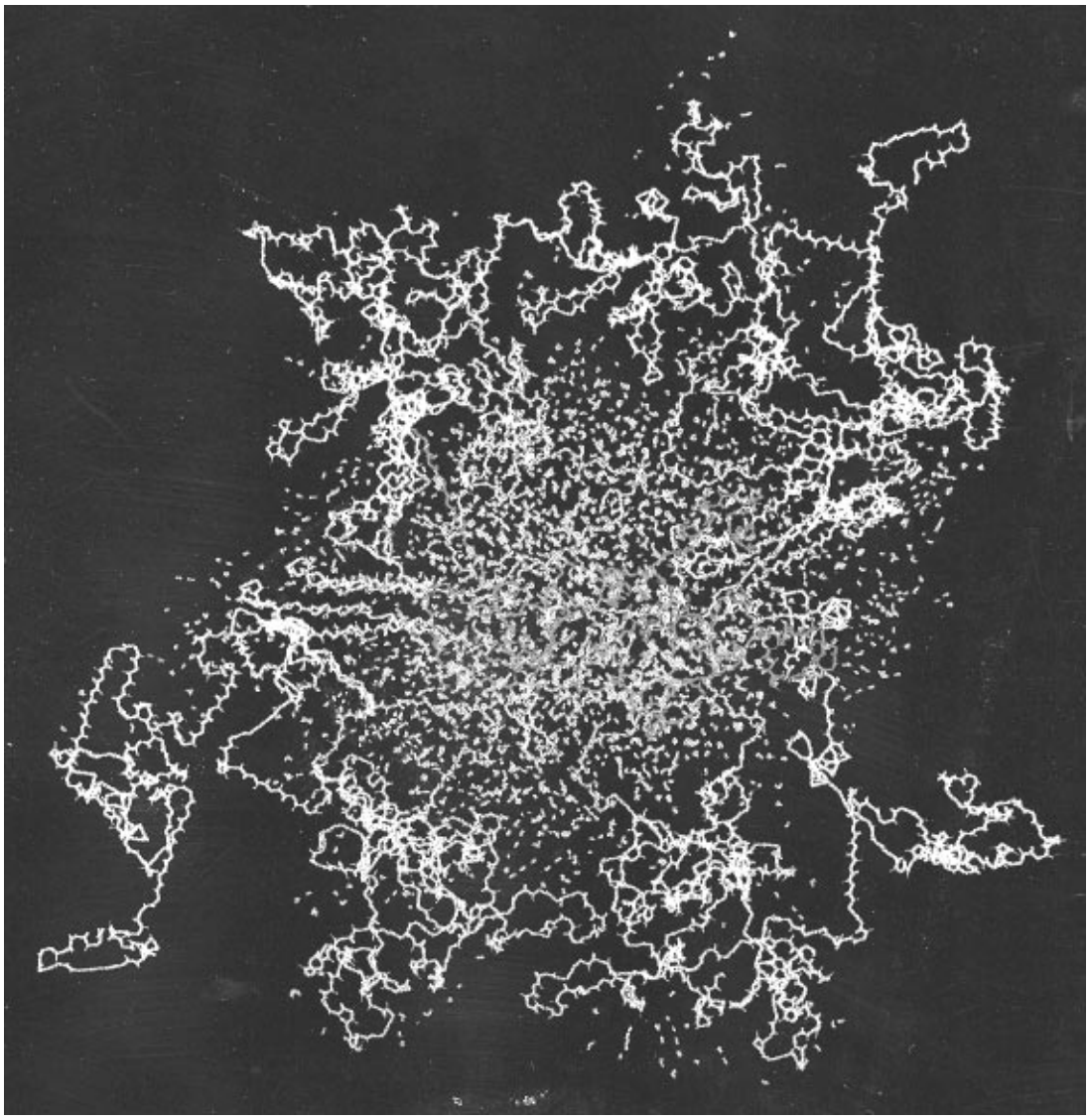

Figure 5. Backbone asymmetric structure of casein submicelle with waters from droplet algorithm, i.e., 2823 water molecules: κ-casein in blue, α_{s1}-casein in red, β-casein in magenta, oxygen from droplet waters in cyan (Kumosinski et al., 1994).

Table 4. Calculated Hydration Products $n_H\Delta R^a$ from Nonlinear Regression Analysis of Oxygen-17 NMR Transverse Relaxation Data for Bovine and Caprine Caseins in Deuterated Water Solutions of Sucrose and Lactose at pD 7.20 and 21 ± 1 °C Using Equation 4

casein	sucrose (mM)	$n_H\Delta R$	lactose (mM)	$n_H\Delta R$
bovine	0	1668.3 ± 26.4	0	1668.3 ± 26.4
	100	1986.4 ± 19.2	100	2600.5 ± 30.9
	200	2545.5 ± 23.7	200	3101.6 ± 26.8
	300	3231.9 ± 25.4	300	3582.7 ± 26.4
caprine	0	842.8 ± 16.7	0	842.8 ± 16.7
high in α_{s1}-casein	100	3437.0 ± 33.8	100	1406.5 ± 27.9
	200	5178.4 ± 38.3	200	1934.7 ± 24.7
	300	5061.1 ± 36.7	300	3583.6 ± 31.7
low in α_{s1}-casein	0	1335.2 ± 35.6	0	1335.2 ± 35.6
	100	2265.8 ± 36.9	100	3114.4 ± 49.7
	200	2850.4 ± 28.4	200	4327.7 ± 41.2
	300	3840.9 ± 37.8	300	7093.9 ± 51.8

[a] ln mL g^{-1} s^{-1}. The protein concentration was in grams of protein per milliliter of solvent.

tion occurring in the presence of these two sugars (Table 4). From the values of the preferential binding term $\partial g_s/\partial g_p$ calculated at different sugar concentrations (Table 3) it seems that sucrose and lactose foster preferential hydration of these two caprine caseins. For bovine casein in sucrose− and lactose−casein mixtures, a lower preferential hydration is observed (Table 3).

By making assumptions regarding the nature of the "bound" water, R_{2B} can be calculated and used to dissect the hydration product to yield an apparent isotropically bound hydration number (Mora-Gutierrez et al., 1995). The calculated hydration from oxygen-17 NMR data given in Table 5 shows the effect of increasing sugar concentrations. The hydration of all of the caseins increases with increasing sugar concentration. These values represent estimates of bound water because the water sensed by the oxygen-17 nucleus in the sugar−casein systems is exchangeable water "trapped" within the casein complexes and not water merely influenced by the caseins. This conclusion is borne out by the recent ^{17}O NMR experiments by Denisov and Halle (1995). The latter workers were able to mathematically separate the contributions to relaxation of ^{17}O by various classes of exchangeable water through field dependency studies. For bovine pancreatic trypsin inhibitor (BPTI) the classes of water molecules included 4 internal (trapped) water molecules, 250 surface water molecules, and 2500 protein-influenced bulk water molecules. However, the four internal water molecules

Table 5. Hydration Estimates of Bovine and Caprine Caseins[a]

casein	sucrose (mM)	hydration[b] (g of water/g of protein)	lactose (mM)	hydration[b] (g of water/g of protein)
bovine	0	0.00452	0	0.00452
	100	0.00428	100	0.00687
	200	0.00498	200	0.00813
	300	0.00605	300	0.00830
caprine	0	0.00097	0	0.00097
high in α_{s1}-casein	100	0.00527	100	0.00281
	200	0.00528	200	0.00586
	300	0.00673	300	0.00772
	0	0.00342	0	0.00342
low in α_{s1}-casein	100	0.00442	100	0.00919
	200	0.00534	200	0.00996
	300	0.00656	300	0.01151

[a] From oxygen-17 NMR data at 21 ± 1 °C and at pD 7.20, according to a two-state, isotropic model (Mora-Gutierrez et al., 1995). [b] Assuming $\tau_c = 56$ ns for bovine casein submicelles (Kakalis et al., 1990b).

contribute disproportionately to the relaxation. Although we also observe a weighted average for the relaxation and are studying a more complex protein system in our work, internal or trapped water undoubtedly dominates our experiments as well. This dominance is true for the casein aggregates, which provide many internal cavities and surface pockets for water (Figure 5). Therefore, our experiments report changes undergone by this trapped water and not water transiently bound to surface charges. Kumosinski et al. (1994) proposed an apparent molecular mechanism that postulates internal cavities in the casein to account for the nature of the trapped water. This theory was derived by comparison of casein 3D models with small-angle X-ray scattering data (Figure 5). Regardless of the exact positioning of this trapped water, the ^{17}O NMR data describe changes with environmental conditions.

Nevertheless, in the presence of sugars the average hydration value of Table 5 is 0.0057 g of water/g of protein; in the absence of sugar this value is 0.0030. This translates to about 40 more water molecules, which accounts for only a small fraction of those depicted in Figure 5. Thus, increased bound water represents a small fraction of the water molecules associated with highly porous caseins. It can be shown that the preferential hydration caused by exclusion of 43 sugar molecules is significant. Using the equations of Arakawa and Timasheff (1982a), it can be calculated that in 200 mM lactose a value of $(\partial g_s/\partial g_p)$ of 0.0522, as given in Table 3 for bovine casein, leads to a value of 0.7250 for $(\partial g_w/\partial g_p)$. This represents an enormous increase in solvation of the casein. The net result is quite dramatic in that these forces enhance casein stabilization as judged by favorable changes in B_0 and in the curvature of the raw data shown in Figures 2−4.

Interpretation of the results in terms of increased state of aggregation of the caseins is feasible. However, the porous nature of the casein submicelles (Figure 5) might yield the reverse effect. The presence of the sugars could disaggregate the highly rugose submicelles and lead to smaller more compact structures with greater stability and lower surface area. Alternatively, the submicelles may merely become more compact in response to the presence of the sugars. No experimental evidence is apparently available on the effects of sugars on the size and/or shape of submicellar caseins. However, Dewan et al. (1973) noted that for colloidal calcium caseinates (micelles) 10% sucrose decreased the voluminosity of the micelles by 20%. In addition, Mozerski et al. (1991) were also able to detect a significant decrease in MW of reformed micellar protein aggregates of bovine whole caseins in the presence of sucrose and lactose by sedimentation field flow fractionation. In analogy with globular proteins, both sugars are expected to favor association of molecular dispersed caseins (Lee and Timasheff, 1981; Arakawa and Timasheff, 1982a). In this context, the values of the B_0 virial coefficients in the presence of sugars are in magnitude smaller than the values estimated for bovine and caprine caseins in the absence of these two sugars (Table 2). This indicates that it is likely that sucrose and lactose stabilize bovine and caprine caseins perhaps by causing preferential hydration and thus decreasing electrostatic interactions between oppositely charged groups of these milk proteins. Thus, the mechanism of bulk surface tension changes may be at work in these studies, as suggested by Arakawa and Timasheff (1982a), as small changes in hydration relate to large changes in the macromolecule. Since a large and positive B_0 term may be interpreted as an indication of increased charge repulsions due to protein aggregation (Pessen and Kumosinski, 1985), it is demonstrated by this experiment that the reduction in B_0 predicts increases in the stability of the caprine caseins with sugar concentration (Table 2). For our systems it is obvious that the functionality of the casein aggregates formed by the attractive interaction depends on the types of the caseins as well as the concentration of the sugar present as previously reported for re-formed micelles (Mozerski et al., 1991).

Apparently the preferential effect in sugar−casein mixtures is more predominant among caprine caseins, and this ability seems to be related to their distinct compositional characteristics (Grosclaude et al., 1987; Mora-Gutierrez et al., 1991) and resulting influences on physicochemical properties (Mora-Gutierrez et al., 1993a−c, 1995, 1996a,b). In this context, it is possible that the high content of β-casein of the caprine casein preparations (Table 1) favors the formation of highly hydrated casein aggregates. The β-casein molecule itself possesses a large hydrophobicity (Farrell, 1988). In addition, the β-casein molecule exhibits a high voluminosity, which has been ascribed to water entrapped in the spatial structure (Le Meste et al., 1990; Kumosinski et al., 1993). The above results clearly indicate that the preferential binding parameter $(\partial g_s/\partial g_p)$, the hydration product $n_H\Delta R$, and protein hydration (Tables 3, 4, and 5, respectively) are strong functions of the chemical nature of the protein composition in both the sucrose and lactose systems.

It may be suggested that at subzero temperatures, the osmotic shock that occurs as ice is formed and other destabilizing conditions that may arise during the frozen storage of proteins (Soliman and van den Berg, 1971; Fishbein and Winkert, 1979) can be viewed simply as other types of solution-induced perturbations. As Timasheff and his colleagues have proposed for such perturbations in unfrozen solutions, we suggest that sucrose and lactose serve to protect against these forces in the frozen state. This conclusion should not be surprising since the interactions of both destabilizing and stabilizing solutes with proteins during freeze−thawing actually occur in the aqueous phase. Furthermore, as stressed by Franks (1985), the basic thermodynamic principles governing protein stability in frozen

systems should not be different from those that have been defined from the unfrozen state.

Since stabilization in sugar-casein systems appears to be primarily due to alteration of protein associations, the relative contents of individual casein components (α_{s1}-, α_{s2}-, β-, κ-caseins), the concentration of sugars, and the kind of disaccharides are important in the preservation of milk products in solution and, by extension, during freezing. The results offer explanations for the differing cryoprotective influences of sucrose and lactose on the stability of whole casein in bovine and caprine milk-based products. Determination of virial coefficients (B_0 values) could serve as guiding parameters in studies designed to test the efficacy of various carbohydrates as cryoprotectants for casein in milk. Oxygen-17 NMR transverse relaxation rates of water in food systems provide a rapid and convenient means of ascertaining the preponderant interactions under a variety of experimental conditions.

CONCLUDING REMARKS

It has been shown in this work that sucrose and lactose strongly affect bovine and caprine caseins and result in increased protein hydration by two mechanisms. This was strongly supported by the preferential binding term $-(\partial g_s/\partial g_p)$ derived from the virial activity coefficient B_0 for caseins in sugar solutions, which suggests that the caseins, in particular the caprine caseins, are preferentially hydrated, i.e., there is an excess of water in the domain of these milk proteins that occurs as sugar concentration increases. In addition, the NMR sensitive internal trapped water increased as well for all samples.

Results of this work serve as strong evidence that the effects of sucrose and lactose on the stability of caseins are produced mainly by preferential interactions leading to increased overall casein hydration and reduced electrostatic repulsions. It is suggested that the differences observed between casein samples may reflect primarily the differences between bovine and caprine caseins with respect to the relative β-casein contents.

ABBREVIATIONS USED

NMR, nuclear magnetic resonance; SDS, sodium dodecyl sulfate; SD, standard deviation; RMS, root mean square; BPTI, bovine pancreatic trypsin inhibitor.

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