κ-Carrageenan Interaction with Bovine and Caprine Caseins As Shown by Sedimentation and Nuclear Magnetic Resonance Spectroscopic Techniques

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The solubility and hydration characteristics of κ-carrageenan−casein systems from bovine and caprine milk with incorporated salt (NaCl) were determined by means of sedimentation and ^{17}O nuclear magnetic resonance (NMR) experiments. Relative salt interaction parameters for both caseins alone and caseins in mixtures with κ-carrageenan were assessed by nonlinear regression analysis from the characteristics of solubilization of the systems. The κ-carrageenan−casein interactions appear to depend largely on the ratios of κ- to α_{s1}-casein and possibly α_{s2}-casein. Second virial coefficients (B_0 values) and hydration products derived from ^{17}O NMR data suggest that while soluble at high salt, the caprine casein mixtures exhibit strong interactions, whereas the bovine counterparts do not. At lower salt concentrations the solubility data and the ^{17}O NMR data are in agreement. Thus, a structural dependence upon protein components in salt-containing κ-carrageenan−casein solutions from bovine and caprine milk has been demonstrated.

Keywords: *Solubility; NMR, ^{17}O; salt binding; water binding; κ-carrageenan; bovine casein; caprine casein; caprine α_{s1}-casein*

INTRODUCTION

One of the most important properties of polysaccharide hydrocolloids in food systems (e.g., carrageenans, dextrans, starches) is their ability to complex protein to form modified food structures. In model systems complex formation has been observed, and although both polymers carry a net negative charge, the interaction has been generally recognized to be electrostatic in nature (Sasaki and Noguchi, 1959; Mathews, 1965; Öbrink and Wasteson, 1971). In milk systems κ-carrageenan is an important determiner of sensory texture, rheological properties, and functional properties (Andersen, 1962; Payens, 1972).

Salt cations and/or anions may not only affect protein−hydrocolloid electrostatic interactions but may also alter water binding in carrageenan systems (Rey and Labuza, 1981). The usage of salts to improve the textural properties of such products as imitation cheese structures is due to their water-holding capacity. In all likelihood, the carrageenan−protein interaction is a combination of several mechanisms and should be thoroughly understood to aid in the development of protein-based food systems.

Snoeren et al. (1975) demonstrated that, at the pH and ionic strength prevailing in milk, it is mainly the casein micelles (and perhaps κ-casein in particular) that are involved in κ-carrageenan−protein interactions. The amino acid sequence of the κ-casein molecule suggests that in addition to the highly negatively charged macropeptide it has also areas of "net" positive charge, which have been speculated to be on the surface of the casein micelle (Mercier et al., 1973). Such an accumulation of positive charges is thought to be lacking in α_{s1}- and β-casein, where positive and negative amino acid side chains appear to be evenly distributed along the polypeptide chain (Mercier et al., 1971; Ribadeu-Dumas et al., 1972). Of all the protein fractions in milk, κ-casein is the most reactive through normal food processing (Morr, 1974).

Caprine caseins, in contrast to bovine caseins, vary considerably in the types of casein present; some are poor in α_{s1}-casein, and some are richer in α_{s2}-casein (Mora-Gutierrez et al., 1997). The α_{s2}-casein has a primary structure quite different from those of α_{s1}- and β-caseins. In a linear array the α_{s2}-casein displays a cluster of net positive charge for residues 170−207 at the C-terminal end (Farrell, 1988). Physical−chemical studies of α_{s2}-casein by Snoeren et al. (1980) suggested a model in which this positively charged tail participates in the isodesmic self-association of the protein; this argues for a surface position for the positively charged cluster. Thus, caprine caseins rich in α_{s2}-casein may offer enhanced sites for interactions with κ-carrageenan, provided that this positive cluster is on the surface in associated whole casein.

Moreover, sodium caseinates from bovine and caprine milks containing various casein components have not been characterized comparatively relative to their interaction with κ-carrageenan. This is especially true with regard to the elevated α_{s2}-casein content of some caprine milks. Therefore, the objective of this work is to examine the effect of NaCl on solubility and the hydration behavior of bovine casein and two caprine caseins of known casein distribution following complex

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

casein type	bovine	caprine casein high in α_{s1}-casein	caprine 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

formation with κ-carrageenan in the absence of calcium ions by use of sedimentation and ^{17}O nuclear magnetic resonance (NMR) techniques.

MATERIALS AND METHODS

Materials. All reagents used were of analytical grade or ACS certified from Aldrich, Baker, Sigma (St. Louis, MO). κ-Carrageenan was obtained from Sigma Chemical Co. Deuterium oxide (99.8%; D_2O) was obtained from Sigma.

κ-Carrageenan was exhaustively dialyzed against deionized water that had been adjusted to pH 7.0 with 0.5 M sodium hydroxide and then lyophilized.

Preparation of Bovine and Caprine Caseins. 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 were obtained from the milk of an Anglo-Nubian and a French-Alpine goat, respectively (Mora-Gutierrez et al., 1991). 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 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.

Solubility Measurements. Solubility of pure caseins and κ-carrageenan−casein mixtures at 21 °C was determined as follows.

(1) Caseins (~20 mg/mL) were dissolved in 0.005 M EDTA solution, adjusted to pH 7.0 by addition of 0.1 N NaOH. To study their interaction, κ-carrageenan and caseins were mixed at concentrations of 0.028% and 2%, respectively. The solutions were heated at 80 °C for 5 min to disrupt possible aggregates and subsequently cooled to room temperature. (2) To 2 mL of pure protein and κ-carrageenan−protein solutions (in thick-walled centrifuge tubes) was added 2 mL of NaCl solutions. The tube was inverted and left to stand at 21 °C for 30 min. (3) Tubes were centrifuged for 15 min at 91082g_{max} at 21 °C in an ultracentrifuge (model L-7; Beckman Instruments, Palo Alto, CA) with an SW 60 Ti swinging bucket rotor. (4) One milliliter of supernatant was transferred to a 10-mL volumetric flask containing a few milliliters of deionized water and made up to volume with deionized water. Concentrations were determined in 1-cm cuvettes at 280 nm; an absorptivity of 0.850 mL/(mg cm) at 280 nm was used for whole casein (Pepper and Farrell, 1982).

Solubility Theory and Data Analysis. Wyman's concept for linked functions (Wyman, 1964) was useful for the treatment of the sequential precipitation (salting-out) and resolubilization (salting-in) of individual bovine and caprine casein components as a function of added calcium (Farrell and Kumosinski, 1988; Farrell et al., 1988; Mora-Gutierrez et al., 1993a−c). Here, we have extended that theory to assume that the triphasic sequential changes in solubility (e.g., Figure 1C) which occur with increasing NaCl concentration are also thermodynamically linked to salt concentrations. The mechanism of precipitation could be salt binding followed by charge neutralization (Farrell et al., 1988) or bulk salt−solvent surface tension incremental effects, that is, change in solvent−solute interactions by added cosolute, namely, salt (Melander and Horvath, 1977a,b). Therefore, the application of linked functions as developed by Wyman (1964) can be used to treat these processes if the following equilibria are assumed:

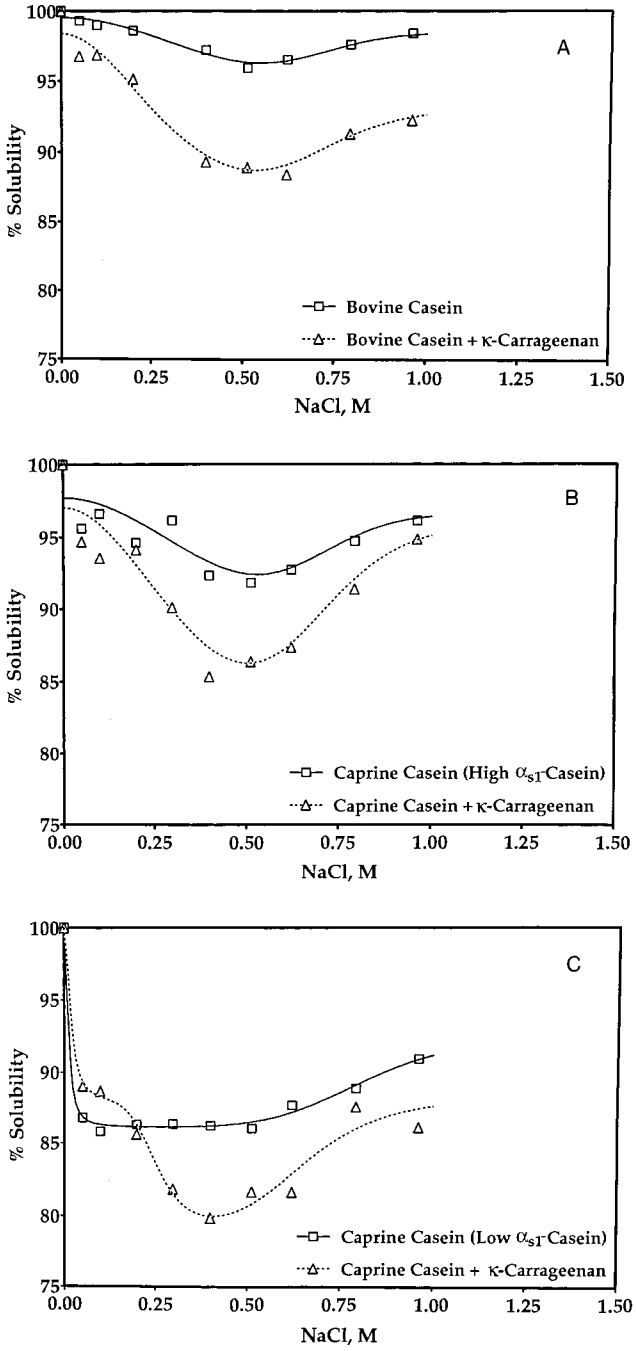

Figure 1. Solubility at 21 °C of casein and κ-carrageenan−casein as a function of NaCl concentration: (A) bovine casein; (B) caprine casein high in α_{s1}-casein; (C) caprine casein low in α_{s1}-casein. Solutions were buffered at pH 7.0, 0.005 M EDTA. Data represent the average of triplicate determinations and were fitted by eq 4. Results are given in Table 2.

$$\begin{array}{ccccc} (S_o) & & (S_1) & & (S_2) \\ p + nX & \overset{k_1}{\rightleftharpoons} & PX_n + mx & \overset{k_2}{\rightleftharpoons} & PX_mX_n \\ & & + qx & & + qx \\ & & \Big\Updownarrow k_1' & & \Big\Updownarrow k_1' \\ & & PX_nX_q + mx & \overset{k_2}{\rightleftharpoons} & PX_mX_nX_q + mx \\ & & (S_2') & & (S_1') \end{array} \quad (1)$$

where p is the unbound protein; x is the free salt; n, m, and q are the apparent numbers of moles of X bound to species PX_n, PX_nX_m, and $PX_nX_mX_q$; and S_0, S_1, S_1', and S_2' are the solubilities of the species indicated. The mathematical relationship representing this stoichiometry can be represented according to

$$S_{app} = S_0\, f(p) + S_1\, f(PX_n) + (S_2 - S_1)\, f(PX_n\, X_m) + S_1'\, f(PX_nX_mX_q) \quad (2)$$

where S_{app} is the apparent protein solubility at a given salt concentration (X_T), $f(i)$ are the protein fractional components of species i, and the S's are the solubilities of each species. For this study S_1 and S_2 will be relative to S_0. Incorporation of these constants as defined by eq 1 into eq 2 leads to

$$S_{app} = \frac{S_0 p}{p + k_1^{\,n} p x^n} + \frac{S_1 k_1^{\,n} p x^n}{p + k_1^{\,n} p x^n} + \frac{(S_2 - S_1) k_2^{\,m} p\, x^m}{p + k_2^{\,m} p x^m + k_1'^{q} p x^q + k_2^{\,m} k_1'^{q} p x^m x^q} + \frac{S_1' k_2^{\,m} k_1'^{q} p x^m x^q}{(p + k_2^{\,m} p x^m)(p + k_1'^{q} p x^q)} \quad (3)$$

where p is the concentration in percent of the unbound protein and x is the concentration of unbound salt. Cancellation of common terms yields

$$S_{app} = \frac{S_0}{1 + k_1^{\,n} x^n} + \frac{S_1 k_1^{\,n} x^n}{1 + k_1^{\,n} x^n} + \frac{(S_2 - S_1)\, k_2^{\,m} x^m}{1 + k_2^{\,m} x^m + k_1'^{q} x^q + k_2^{\,m} k_1'^{q} x^m x^q} + \frac{S_1' k_2^{\,m} x^m k_1'^{q} x^q}{(1 + k_2^{\,m} x^m)(1 + k_1'^{q} x^q)} \quad (4)$$

Equation 4 represents sequential binding (i.e., $k_1 > k_2 > k_1'$, where n sites saturate prior to the binding of m sites on the protein). Also, for n, m, or $q > 1$, k_1, k_2, and k_1' represent an average value for each of the n, m, or q binding sites. In reality, n, m, or q mol of salt will bind with only one equilibrium constant (k_1) (i.e., $k_1 = k_1^{\,n}$; $k_2 = k_2^{\,m}$; and $k_1' = k_1'^{q}$). Finally, protein−κ-carrageenan complexes have very high molecular weight, so that the molar concentrations of salt (up to 0.9 M) far exceed the molarity of the complexes. Therefore, total salt may be used rather than "free" salt when binding is implied. This is analogous with enzyme kinetics where substrate concentration [S] ≫ enzyme concentration [E].

The model in eq 4 was applied in the present study to the Na^+-induced solubility profiles of bovine and caprine whole caseins in the absence and in the presence of κ-carrageenan. These solubility profiles were analyzed using an iteractive nonlinear regression program (NLLSQ in BASIC) on an microcomputer that employed the Marquardt algorithm. This program minimizes the standard deviation (SD) of the experimental points from the curve, also known as the root-mean-square (RMS), where the RMS is defined as

$$RMS = SS/(NO - NP + NX) \quad (5)$$

The SS is the sum of the squares of the differences ($YC - Y$) between the calculated and observed Y values, NO is the number of data points, NP is the number of parameters, and NX is the number of excluded parameters. All solubility profiles were analyzed by fixing the values of n, m, and q and calculating the best least-squares fit for the optimum evaluated k_1, k_2, and k_1' values. The n, m, and q values were then fixed to new values, and the whole procedure was repeated. The n, m, and q values, which yielded the minimum RMS value for the analysis, were then reported.

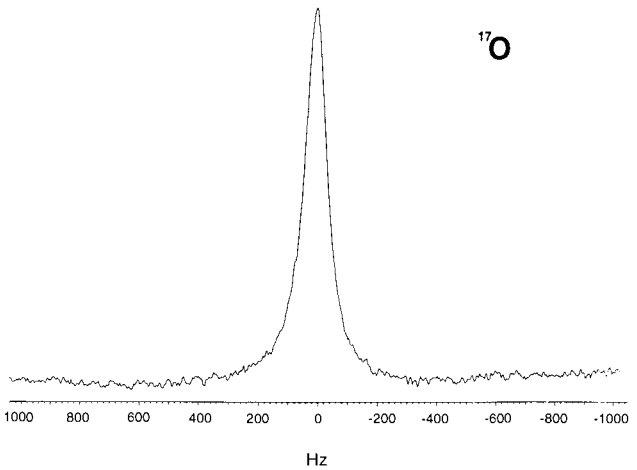

Figure 2. 27.1 Hz ^{17}O NMR spectrum of D_2O in a 4.17.% (w/v) caprine casein low in α_{s1}-casein solution in the presence of 0.0078% κ-carrageenan and 0.2 M NaCl at pD 7.2 and 21 ± 1 °C.

Preparation of Samples for NMR Measurements. A set of bovine and caprine casein solutions was mixed with 0.0078% (w/v) κ-carrageenan. A second set of samples was prepared by adding incremental amounts of casein from 0 to 6% (w/v) to a constant ratio of κ-carrageenan and salt to water. After mixing, the solutions were heated to 80 °C for 5 min to prevent local aggregation, subsequently cooled to room temperature, and then allowed to equilibrate at 4 °C in ice, prior to the ^{17}O NMR measurements. The pD was 7.2 for all samples. pD was calculated from the equation pD = pH + 0.4 (Covington et al., 1968).

About 4 mL of well-dispersed and thoroughly mixed pure casein and κ-carrageenan−casein in D_2O solutions was transferred to 10-mm high-resolution NMR tubes (Wilmad, Buena, NJ). In all experiments two independent series of NMR measurements were conducted at 21 ± 1 °C.

^{17}O NMR Transverse Relaxation Rate Measurements. An XL-200 multinuclear spectrometer (Varian Associates, Palo Alto, CA) was used for the ^{17}O NMR R_2 relaxation measurements. Natural abundance ^{17}O (3.7×10^{-2}%) measurements were made in D_2O. Single-pulse experiments were done at 21 ± 1 °C, at a resonance frequency of 27.1 MHz. The samples were spun at 12 ± 1 Hz. Other conditions were as follows: 90°, pulse width of 19 μs, acquisition time of 0.50 s, and a spectral width of 5 kHz. The number of scans for adequate signal-to-noise (>100:1) was ~1000. Fourier transforms were carried out on line with a Varian 4000 series data system computer with Pascal software (v. 6.3). Spectra were stored in an 8K point array, which provided adequate resolution.

Figure 2 shows the ^{17}O NMR Fourier transform spectrum of a 4.17% solution of caprine casein low in α_{s1}-casein in the presence of 0.0078% κ-carrageenan and 0.2 M NaCl in D_2O. The line width (υ_{obs}) at half-height of each spectrum was obtained by using the computer line fit routine available on the XL-200 Varian computer software (Varian Associates). The ^{17}O NMR transverse relaxation rate (R_2, s^{-1}) was then calculated from the line width by (Dwek, 1973)

$$T_2^{-1} = R_2\,(s^{-1}) = \pi \Delta \upsilon_{obs}\,(s^{-1}) \quad (6)$$

where $\Delta\upsilon_{obs}$ is the line width at half-height for the ^{17}O NMR peak of the sample, after correction for a small inhomogeneity broadening.

The net or differential transverse relaxation rate ΔR_2 (s^{-1}) was calculated as

$$\Delta R_2\ (s^{-1}) = \pi(\Delta \upsilon_{obs} - \Delta_{free}) \quad (7)$$

where Δ_{free} is the line width at half-height for D_2O without added protein.

NMR Hydration Theory and Data Analysis. Interpretation of NMR relaxation 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 water−macromolecule interactions has been applied to several polymer systems (Child and Pryce, 1972; Cooke and Wien, 1973; Oakes, 1976; Hansen, 1978; Derbyshire, 1982; Mora-Gutierrez and Baianu, 1990). The relaxation behavior, when the exchange time between states is short in relation to the NMR relaxation times of each state, shows a single relaxation time

$$T_{obs}^{-1} = \sum_i P_i T_i^{-1} \quad (8)$$

where T_{obs}^{-1} is the observed relaxation time, T_i^{-1} is the relaxation time of the ith state, and P_i is the probability that the nucleus is found in that state (Cooke and Kuntz, 1974). Now, if we assume that there are fundamentally two possible water states, bound and free, eq 8 simplifies to the standard two-state model, with fast-exchange (Derbyshire, 1982)

$$T_{obs}^{-1} = P_B T_B^{-1} + P_F T_F^{-1} \quad (9)$$

where T_B^{-1} is the relaxation rate of bound water, P_B is the probability of water being bound, T_F^{-1} the relaxation rate of the free water, and P_F is the probability of water being free. Because $P_F = (1 - P_B)$, we can cast eq 9 into a linear form:

$$T_{obs}^{-1} = P_B(T_B^{-1} - T_F^{-1}) + T_F^{-1} \quad (10)$$

Thus, the two-state model, with fast exchange, predicts a linear relationship between the observed relaxation rate (T_{obs}^{-1}) and the probability of the water being in the bound state (P_B), where P_B can be related to the concentration of substance in solution. This model predicts a linear relationship between the observed relaxation times of water (T_{obs}^{-1}) 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 (Halle et al., 1981), and the derivation of equations for a nonlinear three component system using relaxation techniques has been given in detail by Kumosinski and Pessen (1982). Pessen and Kumosinski (1985) attribute the nonlinearity to charge repulsion or charge fluctuations as predicted by the Kirkwood−Shumaker (1952) theory. The major application of the Pessen and Kumosinski (1985) work is the use of activities in place of concentrations when dealing with systems strongly deviating from ideality.

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 varying protein concentration, C_p, is

$$R_{obs} - R_{free} = (R_b - R_f)\bar{\nu}_w a_p / W \quad (11)$$

where R_f is the appropriate 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 disappears. 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:

$$a_p = \gamma C_p \quad (12)$$

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

$$\mathrm{d} \ln \gamma / \mathrm{d} C_p = 2B_0 + 3B_2 C_p + \ldots \quad (13)$$

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. (1990), and Mora-Gutierrez et al. (1995, 1996a,b).

By applying the protein activity concept, as discussed in a previous paper (Mora-Gutierrez et al., 1997), in conjunction with the two-site model with fast exchange model, one obtains

$$R_{2obs} - R_{2F} = n_H(R_{2B} - R_{2F})C_p \exp[2B_0 C_p + 2B_{0.5} C_p^{0.5} + 0.667 B_{1.5} C_p^{1.5} + 1.5 B_2 C_p^{2} + \ldots] \quad (14)$$

where R_{2obs} is the measured transverse relaxation rate corrected for inhomogeinity broadening; the subscripts B and F stand for bound and free water, respectively; n_H is the hydration number (i.e., the average number of water molecules bound per molecule of dry protein); C_p is the varying protein concentration; and B_i are the virial coefficients. The virial coefficients are a measure of the various intermolecular interactions (Tanford, 1963; Pessen and Kumosinski, 1985). 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 (Mora-Gutierrez et al., 1995, 1996a,b, 1997).

Our ^{17}O NMR data were analyzed with a quasi-Newton nonlinear regression program as described in a previous paper (Mora-Gutierrez et al., 1997). Root-mean-square (RMS) values were normalized to be within at least 5% error of the fit for all the data.

RESULTS AND DISCUSSION

Solubility Characteristics. Neutral salts have a profound effect on the solubility of proteins, on the structure of hydrocolloids, and on protein−hydrocolloid electrostatic interactions. Therefore, the solubility of bovine casein and two caprine caseins with high and low levels of α_{s1}-casein was determined at 90000g to test the effect of NaCl treatment on their solubility and on their interactions with κ-carrageenan.

In these experiments no gels were formed because of the low concentration of κ-carrageenan and the high

Table 2. Salt-Induced Insolubility and Solubility of Bovine and Caprine Caseins (2%) and κ-Carrageenan (0.028%) Mixtures at 21 °C and 90000*g*[a,b]

casein type	k_1, L/mol	S_1, %	k_1', L/mol	S_2', %	k_2, L/mol	S_2, %
			Casein			
bovine	1.5 ± 1.3	89.4 ± 12.7			1.3 ± 0.1	96.4 ± 5.8
caprine						
high in α_{s1}-casein	1.7 ± 1.3	82.6 ± 18.4			1.4 ± 0.3	94.0 ± 9.3
low in α_{s1}-casein	83.7 ± 29.0	86.1 ± 0.2			1.2 ± 0.1	92.7 ± 1.2
			κ-Carrageenan−Casein			
bovine	2.9 ± 1.4	82.9 ± 7.0			1.4 ± 0.3	92.2 ± 4.7
caprine						
high in α_{s1}-casein	2.4 ± 1.9	75.8 ± 19.3			1.4 ± 0.3	94.3 ± 11.9
low in α_{s1}-casein	54.3 ± 18.2	87.9 ± 2.1	3.9 ± 0.8	79.0 ± 2.1	1.5 ± 0.3	88.3 ± 2.8[c]

[a] Solutions buffered at pH 7.0, 0.005 M EDTA. [b] $n = 2$ and $m = 6$ for all calculations; $q = 6$. [c] S_1' of eq 1.

concentration of casein (Drohan et al., 1997). Figure 1A shows the solubility of whole bovine casein as a function of NaCl concentration. It is interesting to note that a dip occurs in the profile at ~0.5 M NaCl. For the individual bovine caseins, κ- and β-caseins are quite soluble in this region, but α_{s1}-casein is not (Waugh et al., 1970; Snoeren, 1976). Figure 1A is reminiscent of the stabilization of α_{s1}-casein by κ-casein in the presence of Ca^{2+}, where a similar dip is observed (Mora-Gutierrez et al., 1993b). Alternatively, the salting out of α_{s1}-casein, above 0.6 M, can be prevented by complexation with β-casein (Waugh et al., 1970). Therefore, either κ-casein or β-caseins can help stabilize α_{s1}-casein from precipitation in solution in the presence of NaCl.

For caprine casein high in α_{s1}-casein a similar profile is obtained (Figure 1B), but the caprine casein low in α_{s1}-casein gives a different profile (Figure 1C). It should be noted that this latter caseinate, although low in α_{s1}-casein, is relatively high in α_{s2}-casein (Table 1). Curiously, the degree of aggregation of bovine α_{s2}-casein increases on going from 0.02 to 0.2 M NaCl; it then decreases to a dimer ~0.6 M NaCl (Snoeren et al., 1980), so α_{s2}-casein shows no insolubility over this range of salt concentration. The reason for the behavior of the caprine casein low in α_{s1}-casein must somehow lie in casein−casein interactions peculiar to this mixture. Table 2 summarizes quantitative differences in relevant parameters of pure caseins derived from the solubility data (Figure 1) by nonlinear regression analysis using eq 4. The salting-out constant, k_1, for the caprine casein low in α_{s1}-casein is significantly higher than those of bovine and caprine casein high in α_{s1}-casein (Table 2). This suggests that the solubility of low caprine casein is much more sensitive to Na^+ ions. Nevertheless, the predicted maximal amounts of casein salted out (S_1) are all within the experimental error (86.07% ± 3.4 average and SD for all three).

Values of $1/k_1$ represent the salt concentrations for half-precipitation of the caseins. These are 0.66, 0.59, and 0.011 M for bovine, caprine high in α_{s1}-, and caprine low in α_{s1}-casein, respectively. The first two values represent concentrations of salt near the incipient precipitation point of α_{s1}-casein (0.6 M; Farrell, 1988). However, the extremely low value of 11 mM for caprine casein low in α_{s1}-casein is anomalous. This effect occurs at salt concentrations far too low for surface increment effects on hydrophobicity (Melander and Horvath, 1977a,b) to be operative. Taken with the fact that micelles of caprine casein low in α_{s1}-casein are destabilized by lower concentrations of added Ca^{2+} (Mora-Gutierrez et al., 1993b), this type of casein may contain fairly selective cation binding sites that lead to destabilization (precipitation).

As incipient precipitation occurs, salt binding to lower affinity sites may in turn lead to resolubilization of these milk proteins at higher salt concentrations ($1/k_2$ average = 0.77 M). The values for the salting-in constants for all caseins are nearly equivalent, an indication that a common mechanism, perhaps salt binding to the proteins, is at work.

The n and m values are the same; that is, $n = 2$ and $m = 6$ for the bovine and caprine caseins in the absence of κ-carrageenan. The relatively low values of 2 and 6 for n and m, respectively, should not be interpreted literally as only a simple binding site, because it is well-known that multiple binding sites with exactly the same equilibrium constant yield only a single isotherm (Tanford, 1961). Hence, a value of n or m represents a class of protein binding sites rather than a single binding site linked to the solubility change of the protein.

For κ-carrageenan alone, Snoeren (1976) demonstrated that the molecule is 35−40% salted out under the conditions used here. In studying the isolated bovine caseins, only κ-casein and not β- or α_{s1}-casein could reverse this precipitation (Snoeren, 1976). As is evident from Figure 1, the addition of κ-carrageenan decreased solubility of the caseins, apparently due to the formation of κ-carrageenan−casein aggregates. A comparison of the solubility curves of the κ-carrageenan−casein mixtures shows that the extent of the aggregates formed is much greater in the caprine caseins than in the bovine casein, particularly the caprine casein low in α_{s1}-casein. The reason for such differences is probably associated with either their higher κ-casein content or increased percent of α_{s2}-casein, in the case of the low α_{s1}-casein (Table 1). It is well established that κ-carrageenan specifically interacts with κ-casein (Grindrod and Nickerson, 1968; Payens, 1972; Snoeren et al., 1975), but α_{s2}-casein has not been studied in this regard and its positively charged C-terminal tail (Snoeren et al., 1980) may facilitate this interaction.

The solubility curves for the κ-carrageenan−casein systems of caprine caseins can be better understood quantitatively from the nonlinear regression analysis results (Table 2). As NaCl is added to both κ-carrageenan−casein mixtures of caprine caseins, salting-out occurs (S_1; Table 3). However, for the caprine casein low in α_{s1}-casein the addition of κ-carrageenan in the presence of NaCl induces a second drop in solubility (S_2') that affects both n and k_1' (Table 2). Equilibrium dialysis studies (Parker and Dalgleish, 1981) have shown that NaCl can alter both n and K_a in isolated α_{s1}-casein, and light scattering and optical rotation studies have revealed that in the presence of NaCl κ-carrageenan undergoes a conformational change (Sno-

Table 3. Calculated Hydration Products $n_H\Delta R^a$ and Virial Coefficients $B_0{}^b$ from Nonlinear Regression Analysis of Oxygen-17 NMR Transverse Relaxation Data for Bovine and Caprine Casein Deuterated Solutions in the Presence of 0.0078% κ-Carrageenan at 21 ± 1 °C and pD 7.2 Using Equation 14

	bovine casein				caprine casein high in α_{s1}-casein				caprine casein low in α_{s1}-casein			
NaCl (M)	$n_H\Delta R$	$n_H\Delta R^c$	B_0	$B_0{}^d$	$n_H\Delta R$	$n_H\Delta R^c$	B_0	$B_0{}^d$	$n_H\Delta R$	$n_H\Delta R^c$	B_0	$B_0{}^d$
0.0	1786 ± 49.9	2595	8.2 ± 0.7	3.6	1100 ± 38.9	3358	5.2 ± 0.9	0.8	1339 ± 21.5	1806	5.9 ± 0.3	4.8
0.2	1037 ± 25.3	2487	13.6 ± 0.7	4.3	190 ± 8.3	3069	23.9 ± 0.9	1.5	291 ± 11.7	1828	26.5 ± 1.0	3.8
0.5	1207 ± 33.2		5.1 ± 0.6		378 ± 19.4		12.7 ± 1.0		358 ± 14.5		18.5 ± 0.9	
0.9	2179 ± 12.7		0.3 ± 0.1		525 ± 22.3		11.1 ± 0.8		679 ± 28.6		14.1 ± 1.1	

[a] mL g^{-1} s^{-1}. The protein concentration was in g of protein/mL of solvent. [b] mL/g. [c] $n_H\Delta R$ values obtained under similar conditions but without κ-carrageenan (Mora-Gutierrez et al., 1995). [d] B_0 values obtained under similar conditions but without κ-carrageenan (Mora-Gutierrez et al., 1995).

eren, 1976). The implication of this work is that these interactions described for the κ-carrageenan molecule (Rees, 1969; Rees et al., 1982) and the individual casein components (Parker and Dalgleish, 1981) may carry over to the κ-carrageenan−casein complex. In these studies, κ-carrageenan is dissolved completely at high temperature (see Materials and Methods), and this polysaccharide becomes insoluble at 90000*g* on cooling. Thus, the formation of random linkages between the κ-carrageenan molecules and casein molecules may be expected. This would be particularly true in the 0.2−0.6 M salt region. Here the casein components may be near incipient precipitation (aggregated) and the κ-carrageenan is undergoing a conformational change. As a consequence of its higher content of α_{s2}-casein, the caprine casein low in α_{s1}-casein may be more likely to interact with κ-carrageenan. For the caprine casein high in α_{s1}-casein, which also has a high κ-casein content but with a lower α_{s2}-casein content, gross solubility changes were not apparent because the *n* value was not altered and no additional k_1' value was added to fit the data. Moreover, no detectable gross solubility change is observed for κ-carrageenan−casein complexes of bovine casein (Table 2).

The observed increase in the solubility of all κ-carrageenan−casein complexes of bovine and caprine caseins (S_2; Table 2) at high salt concentrations is due to screening-out of basic macromolecular charge−charge interactions negating the casein−carrageenan interaction. However, the extent of casein solubilization as a result of increased salt content seems to depend on the ratio of nonpolar surface to charge density of the κ-carrageenan−casein complex. In this respect the salting-in values of k_2 and S_2 (Table 2) reflect the interplay of electrostatic and hydrophobic forces involved in the solubility behavior of κ-carrageenan−casein complexes of bovine and caprine whole caseins in the presence of high concentrations of NaCl. The solubility data, however, disclose no information regarding the presence or nature of interactions at high salt.

Hydration Characteristics. From the point of view of concentration, proteins are often the most important and most reactive ingredients that can be added to a structured/textured carrageenan food system. However, the exact physical state of the majority of water within the carrageenan−protein complex is not well understood, as the water may be partially inmobilized or trapped. ^{17}O NMR water relaxation experiments may be used to report upon the water in such systems (Kumosinski and Pessen, 1982; Mora-Gutierrez et al., 1996a,b). Because salt also influences water−macromolecule interactions, the hydration characteristics of κ-carrageenan−casein complexes of bovine and caprine caseins were determined in the absence and in the presence of NaCl. The ^{17}O NMR relaxation (R_2) results are given in Figures 3−5.

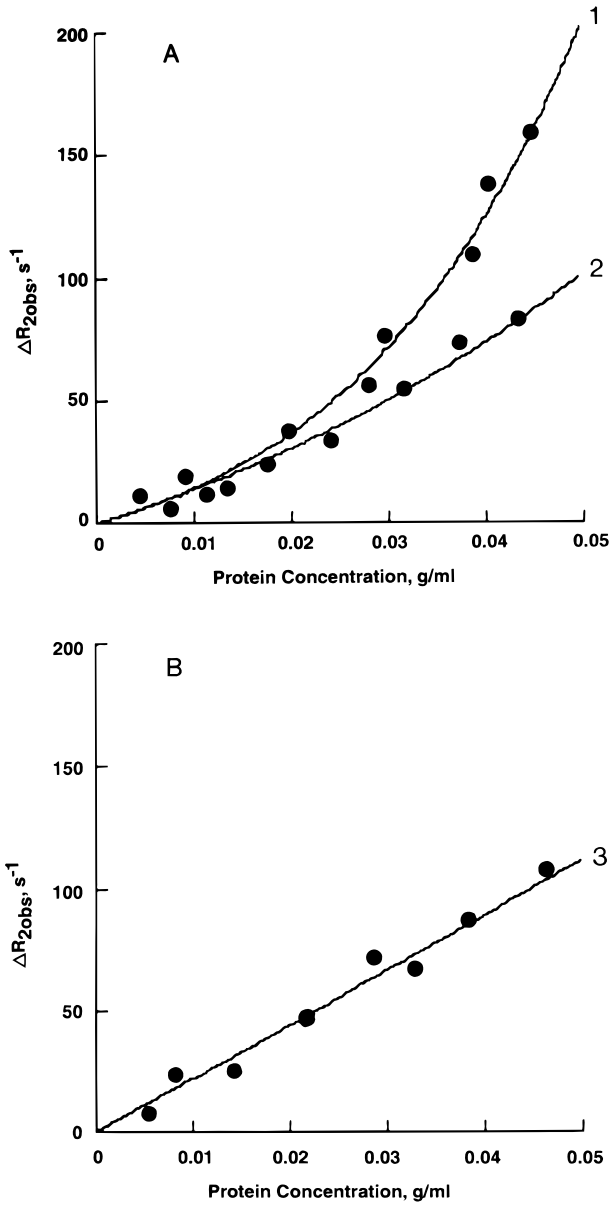

Figure 3. Dependence of the oxygen-17 NMR transverse relaxation rates, ΔR_2 (s^{-1}), on protein concentration (g/mL) for bovine casein in the presence of 0.0078% κ-carrageenan at pD 7.2 and 21 ± 1 °C: (A) 0.2 M NaCl (1), 0.5 M NaCl (2); (B) 0.9 M NaCl (3). Data were fitted by eq 14. Results are in Table 3.

As seen in Figures 3−5, the ^{17}O NMR transverse relaxation rates increased nonlinearly with milk protein concentration. Under ideal conditions this relationship is linear. The marked deviations from linearity at

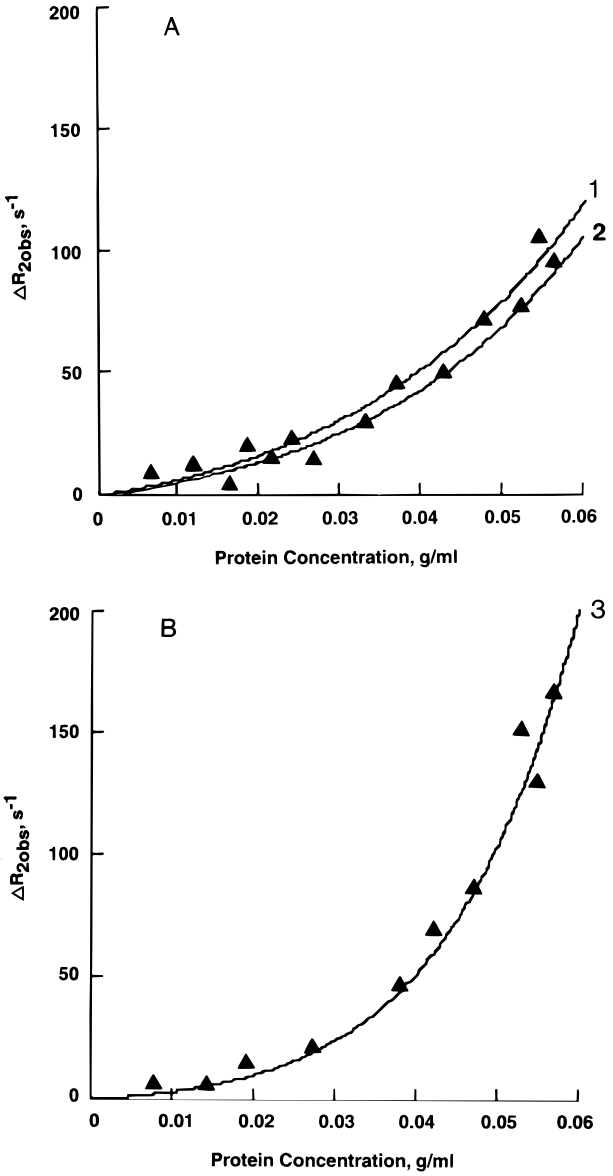

Figure 4. Dependence of the oxygen-17 NMR transverse relaxation rates, ΔR_2 (s^{-1}), on protein concentration (g/mL) for caprine casein high in α_{s1}-casein in the presence of 0.0078% κ-carrageenan at pD 7.2 and 21 ± 1 °C: (A) 0.9 M NaCl (1), 0.5 M NaCl (2); (B) 0.2 M NaCl (3). Data were fitted by eq 14. Results are in Table 3.

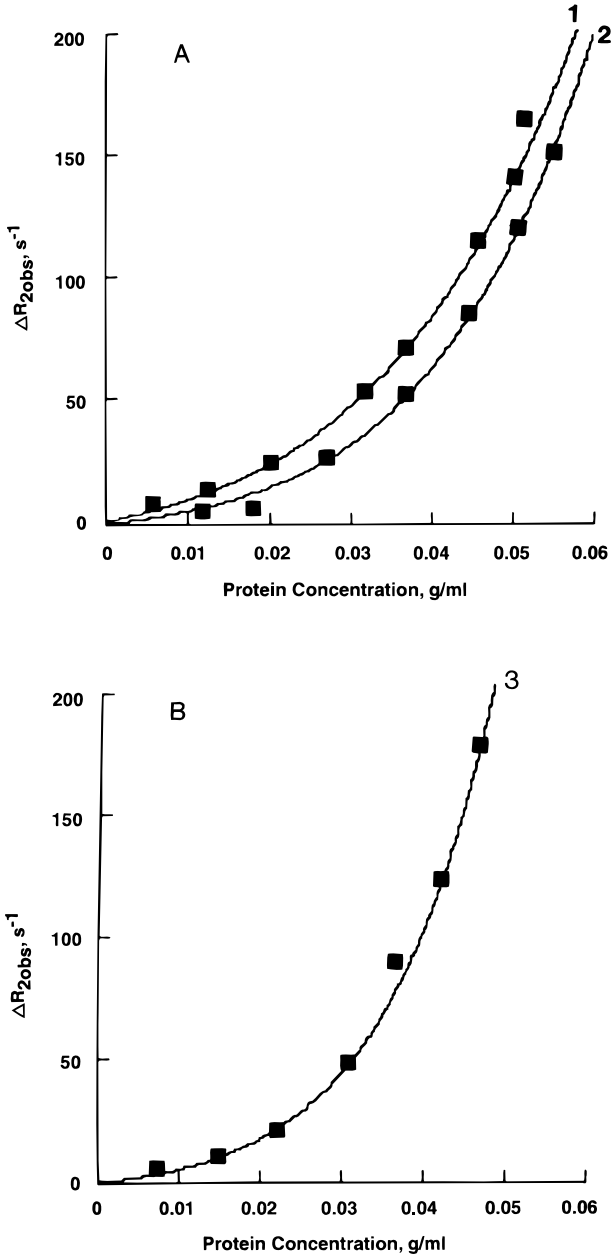

Figure 5. Dependence of the oxygen-17 NMR transverse relaxation rates, ΔR_2 (s^{-1}), on protein concentration (g/mL) for caprine casein low in α_{s1}-casein in the presence of 0.0078% κ-carrageenan at pD 7.2 and 21 ± 1 °C: (A) 0.9 M NaCl (1), 0.5 M NaCl (2); (B) 0.2 M NaCl (3). Data were fitted by eq 14. Results are in Table 3.

higher protein concentrations have been postulated to be due to protein−protein interactions (Pessen and Kumosinski, 1985). The results obtained from the nonlinear regression analysis of the ^{17}O NMR relaxation data according to eq 14 are presented in Table 3. In the investigated protein concentration range (0−7% w/v), the use of virial coefficients other than B_0 was not necessary. The positive sign of the second-order virial coefficient B_0 indicates that charge−charge repulsive interactions are present and increase in all of the κ-carrageenan−casein systems, as the protein concentration increases at constant concentration of κ-carrageenan (0.0078%). The B_0 values of Table 3 clearly indicate that at pH 7.2 and in the presence or absence of NaCl, all of the κ-carrageenan−casein complexes (except bovine casein at 0.9 M) exhibit larger deviations from ideal behavior than do the caseins alone. Therefore, B_0 can be taken as a measure of interaction if we assume large B_0 values are induced by enhanced nonideal conditions due to casein−carrageenan interactions.

In aqueous solutions at 1.0% concentration κ-carrageenan molecules undergo a sol/gel transition as the temperature is lowered from 80 to 5 °C. The transition point is highly dependent on salt concentration (Snoeren, 1976). Moreover, as the transition occurs, there is an accompanying coil/helix transition. In the experiments conducted here the sol/gel transition is inhibited by the lowered concentration of κ-carrageenan and the presence of casein (Drohan et al., 1997). As predicted by Drohan et al. (1997), casein−κ-carrageenan interactions should predominate in our experiments. However, the salt-dependent conformational change in the κ-carrageenan may not initially occur as electron micro-

graphs of κ-casein−κ-carrageenan complexes, at 0.07 M NaCl, reflect the thickened (coiled) state for κ-carrageenan (Snoeren, 1976). Thus, at low ionic strength the coiled hydrocolloid and casein would be predicted to have minimum degree of interaction. The B_0 values of Table 3, however, show interaction even with no added NaCl. Moreover, the hydration products ($n_H\Delta R$), which measure indirectly the motion and hydration of the macromolecules, are greatly decreased. Thus, the NMR relaxation experiments are detecting strong protein−hydrocolloid interactions under these conditions. The relaxation of the κ-carrageenan alone is reflected in the ordinant values of Figures 3−5 and is minimal.

It is shown that under the experimental conditions the κ-carrageenan−casein complex of bovine casein is more "hydrated" ($n_H\Delta R$ = 1786 mL g^{-1} s^{-1}) than the κ-carrageenan−casein complex of caprine caseins characterized by high and low contents of the α_{s1}-casein component ($n_H\Delta R$ = 1100 and 1339 g^{-1} s^{-1}, respectively), but, again, without the polysaccharide these values are much higher for all caseins.

At an ionic strength of 0.2 M NaCl, the thickness of the electrostatic double layer increases and could lead to increased repulsion due to increased negative charges on both polymers, which in turn would lead to decreased complex formation. However, at the same time, the caseins are aggregating and becoming less soluble (Figure 1) and while the coil/helix transition of the κ-carrageenan is facilitated, complexation increases. At ionic strengths >0.2 M, salt addition leads to a more effective screening of the charges on the polymers, which results in the suppression of the electrostatic interaction, increases in helix−helix interaction for the hydrocolloid (Snoeren, 1976), and solubilization of casein. This is particularly true of bovine casein as B_0 (Table 3) at 0.5 M salt approaches the values obtained in the absence of κ-carrageenan. This is not true for either caprine casein.

Because for κ-carrageenan−NaCl solutions conformational changes as detected by optical rotation have been observed (Snoeren, 1976), the magnitude of B_0 values might also be a consequence of the coil/helix transition of κ-carrageenan in casein mixtures, particularly for those combinations in which casein is on the verge of precipitation. When going from the coil to the helix, the charge density of the κ-carrageenan−casein mixtures increases and the hydrocolloid forms more aggregated helixes, as seen in electron microscopy (Snoeren, 1976). It is seen that in the salt concentration range of 0.5 M and above, at which the κ-carrageenan coil/double-helix transition is facilitated, casein mixtures of caprine caseins, especially that low in α_{s1}-casein (high in α_{s2}-casein), exhibit considerably higher B_0 values than the corresponding estimates for the bovine casein−κ-carrageenan mixtures. The difference is most probably explained by first a lower α_{s1}-casein content in caprine and second by the fact that strong interactions of free κ-carrageenan and κ-casein and possibly α_{s2}-casein from caprine casein low in α_{s1}-casein (κ-casein = 14.4% of total casein, α_{s2}-casein = 29.2% of total casein) are involved in this process. This electrostatic complex formation with purified κ-casein is maximal at an ionic strength of ~0.2 M NaCl (Snoeren, 1976), as are our B_0 and "hydration" data (Table 3). This conclusion is consistent with observations from sedimentation studies on mixtures of κ-carrageenan and κ-casein, which supported a maxima at about I = 0.2 (Snoeren, 1976). Clearly such studies on α_{s2}-casein and κ-carrageenan are warranted as the positive tail on this protein postulated by Snoeren et al. (1980) could play a role in the interactions of this protein and κ-carrageenan. However, both caprine caseins exhibit higher deviations from ideality in the presence of κ-carrageenan.

The hydrophobic nature of the casein fractions and the temperature dependence of aggregation reactions (Schmidt, 1982) strongly suggest that the structure of κ-carrageenan−casein complexes may also be dependent upon hydrophobic interactions. Carbohydrates do exhibit some hydrophobic forces. There appears to be a relationship between complex formation and the second-order virial coefficient B_0 of protein activity for 0.0078% κ-carrageenan mixed with the bovine and the caprine caseins, as seen in Table 3. Thus, the nonidealities of κ-carrageenan−casein mixtures of caprine caseins in the absence of NaCl is considerably less than those of carrageenan mixtures of bovine casein, as indicated by B_0 values (Table 3). However, all are greater than those observed in the absence of κ-carrageenan. As noted above, this would argue for complex formation. Also as the NaCl is increased to 0.9 M, then B_0 values fall dramatically for bovine casein but not for the two caprine caseins, which on the average have a higher content of β-casein than bovine casein (Table 1). The β-casein molecule itself possesses a large hydrophobicity (Farrell, 1988). A similar result is seen for the hydration product, which again increases for bovine casein at high salt but not for the caprine caseins (Table 3). Thus, at 0.9 M salt, carrageenan−casein mixtures of bovine casein may represent casein particles tightly self-packed, no longer interacting with κ-carrageenan, and with nearly normal solvation and B_0 (Table 3).

A decrease in the hydration product $n_H\Delta R$ (Table 3) is seen for all κ-carrageenan−casein systems of bovine and caprine caseins relative to the casein-only system. Table 3 also shows the effect of ionic strength on the hydration product $n_H\Delta R$ of bovine and caprine caseins. The hydration product of the three caseins decreased with NaCl concentrations of 0.2 M but showed somewhat higher values at 0.9 M. As described above, if the interaction of the carrageenan−protein complex with NaCl is due to changes in the electrostatic interactions with increased ionic strength, the hydration product $n_H\Delta R$ should at first decrease with increased interactions and then return to normal values as interactions are abolished at high salt. Although this occurred for bovine casein, this tendency was not observed for caprine caseins (Table 3). Thus, the importance of interactions was suggested in the hydration characteristics of caprine caseins and their κ-carrageenan−casein mixtures, whereas Table 2 reports no significant differences in S_2. Thus, solubility studies suggest limited interactions in solution at high salt, but the NMR studies point to extensive interactions for the caprine caseins but lesser for the bovine casein.

Conclusions. The solubility and hydration properties of the salt-containing κ-carrageenan−casein system from bovine and caprine milk reflected milk protein composition, in particular κ-, α_{s2}-, and α_{s1}-casein. The results reported here indicate that caprine caseins may undergo significant interactions with κ-carrageenan and with NaCl. Although the amount of κ-carrageenan used in this study (0.0078% w/v) does not generate enough three-dimensional structure to form gels, the increased interactions with caprine caseins, particularly that low

in α_{s1}-casein (high in α_{s2}-casein), suggest that a positive tail proposed by Snoeren et al. (1980) for α_{s2}-casein could enhance κ-carrageenan binding and electrostatic complex formation for this protein system. Finally, ^{17}O NMR spectroscopy offers a sensitive means for probing the interactions of κ-carrageenan with caseins by sensing the motion and hydration of complex formation not available from simple solubility studies that result from variations in ionic strength (NaCl) and from molecular and ionic interactions.

ABBREVIATIONS USED

NMR, nuclear magnetic resonance; SD, standard deviation; RMS, root mean square; D_2O, deuterium oxide; EDTA, ethylenediaminetetraacetic acid.

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