NMR of Casein Hydration and Activity in Solutions with Ions

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SUMMARY. Oxygen-17 NMR studies of caseins isolated from fresh milk of several species were carried out in concentrated electrolyte solutions as a function of both protein and electrolyte concentration. Molecular dynamics simulations of ion and water binding to caseins are compared with the experimental observations by O-17 NMR of caseins in solutions with ions. Protein activities are also determined by utilizing Wyman's theory of linked functions with a detailed model of ion binding to casein.

Introduction

Casein micelles, which are highly hydrated colloidal complexes of protein and salts, function biologically to transport protein, calcium, andphosphorus to the neonate[1)]. In the absence of calcium, the micellar structure dissociates into casein submicelles, consisting of α_{s1}-, α_{s2}-, β-, and κ-CN, which have significantly lower hydration[2)]. Addition of calcium results in the reformation of model casein micelles from the hydrophobically associated casein submicelles through the calcium-protein side chain of salt bridges[1)]. These calcium-protein interactions have been thought to involve both phosphate and carboxylate groups and can be influenced by ionic species such as K^+ and Na^+ [3)].

The hydration properties of the casein micelles of cow and goat milks are determined largely by the physical-chemical properties of α_s-, κ-and β-caseins and their interactions, among themselves and with their aqueous environment. κ-Casein in all species acts to stabilize the system against precipitation by Ca^{2+} ion, but the proportions of α_{s1}- and α_{s2}-casein appear to vary greatly among individual goats[4,5)] offering greater possibilities for diversity. Thus, investigation of protein-water interactions of whole caseins of cow and goat milk may be useful reporters on the protein-protein interactions between α_s- and κ-caseins. For this work, NMR relaxation measurements of water were made with electrolytes i.e. KCl and NaCl under both submicellar (no Ca^{2+}) and micellar (added Ca^{2+}) proteins.

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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

Materials and methods

Preparation of Whole Caseins

The bovine whole casein was obtained from the milk of a Jersey cow. The caprine whole caseins characterized by high and low levels of the α_{s1}-casein component[1)] were obtained from the milk of an Anglo-Nubian and a French-Alpine goat, respectively. Whole caseins were isolated from milk by methods described by Mora-Gutierrez *et al.*[3)]. The compositions of the bovine and caprine caseins used in this study are given in Table 1.

The lyophilized caseins were dissolved in D_2O, pD 7.10, containing 110 mM KCl or NaCl. To produce micellar casein[1,2)], a stock solution of $CaCl_2$ was added for a final concentration of 10 mM with a concomitant reduction in KCl or NaCl to maintain constant ionic strength.

Nuclear Magnetic Resonance (NMR) Measurements

Oxygen-17 NMR measurements were performed as described by Mora-Gutierrez *et al.*[6)].

Data Analysis

The dependence of the observed Oxygen-17 NMR relaxation rates was fitted with an iterative nonlinear regression program on a Macintosh II microcomputer[6,7,8,9)].

Results and discussion

The changes in transverse relaxation rates (ΔR_2) of water were measured by oxygen-17 NMR for bovine, and two caprine caseins (one low and one high in α_{s1}-casein) as a function of protein concentration (Figure 1). Calculations of B_0 for these data (Tables 2 and 3) show

Table 2. Calculated Hydration Products $n_H\Delta R$ and Virial Coefficients B_o from Nonlinear Regression Analysis of Oxygen-17 NMR Transverse Relaxation Data for Bovine and Caprine Casein Submicelles and Micelles in D_2O-KCl (pD 7.10) at 24 °C.

	submicelles (+K)		micelles (+K)	
casein	$n_H\Delta R^a$	$B_o{}^b$	$n_H\Delta R^a$	$B_o{}^b$
bovine	1.06 ± 0.028	0.015 ± 0.001	1.99 ± 0.016	0.008 ± 0.001
caprine high in α_{s1}-casein	0.54 ± 0.015	0.011 ± 0.001	1.02 ± 0.020	0.007 ± 0.001
caprine low in α_{s1}-casein	1.84 ± 0.019	0.006 ± 0.001	1.46 ± 0.026	0.008 ± 0.001

[a] mL mg^{-1} s^{-1}. The protein concentration was in mg of protein/mL of solvent.
[b] mL/mg.

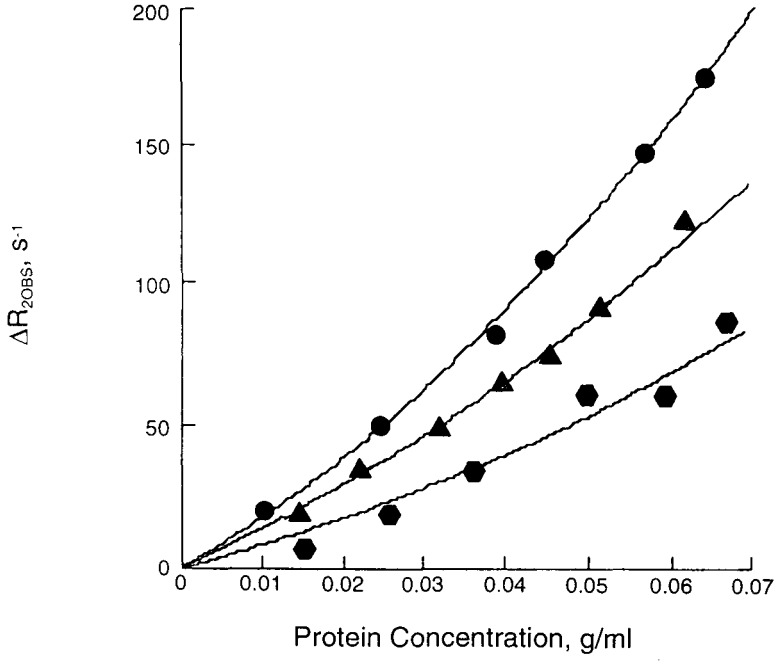

Figure 1. Dependence of the oxygen-17 NMR transverse relaxation rates, ΔR_2 (s^{-1}), on protein concentration (g/mL) for bovine casein (●), caprine casein low in α_{s1}-casein (▲) and caprine casein high in α_{s1}-casein (solid hexagon) submicelles in D_2O-KCl at pD 7.10 and at 24 ± 1 °C. Results are in Tables 2 and 3.

Table 3. Calculated Hydration Products $n_H\Delta R$ and Virial Coefficients B_O from Nonlinear Regression Analysis of Oxygen-17 NMR Transverse Relaxation Data for Bovine and Caprine Casein Submicelles and Micelles in D_2O-NaCl (pD 7.10) at 24 °C.

	submicelles (+Na)		micelles (+Na)	
casein	$n_H\Delta R$[a]	B_O[b]	$n_H\Delta R$[a]	B_O[b]
bovine	0.60 ± 0.018	0.023 ± 0.001	2.36 ± 0.050	0.010 ± 0.001
caprine high in α_{s1}-casein	0.50 ± 0.017	0.011 ± 0.001	1.23 ± 0.029	0.006 ± 0.001
caprine low in α_{s1}-casein	2.13 ± 0.029	0.002 ± 0.001	1.22 ± 0.024	0.009 ± 0.001

[a] mL mg^{-1} s^{-1}. The protein concentration was in mg of protein/mL of solvent.
[b] mL/mg.

that the virial coefficient of protein activity has a positive sign under both submicellar and micellar conditions. This has been attributted to the progressive association of the caseins, resulting in an increasingly net negative charge on the protein and an electrostatic repulsion leading to increasingly positive values of B_O. The net average charge of the caseins can be screened by the low concentrations of salt used here (0.11 M); under these conditions would be anticipated to be small. The effect of the protein excluded volume would make a second order contribution to the value of B_O. Thus under submicellar conditions, in the absence of Ca^{2+}, the replacement of Na^+, which has an ionic radius of 0.97Å, with K^+, which has a radius of 1.3Å, results in a surprising decrease of electrostatic repulsion for the bovine casein, indicating a difference in cation binding as chloride cocentration is constant. The caprine casein high in α_{s1}-casein is unsensitive to a change in electrolyte whereas the low α_{s1}-casein shows a slight increase in electrostatic repulsion. Moreover, the apparent binding of K^+ (and its attendant hydration shell) over Na^+ seems to yield a more hydrated structure for the submicellar forms of bovine and caprine casein low in α_{s1}-casein (Table 4).

Colloidal calcium caseinate is also negatively charged (Tables 2 and 3). This charge might help to maintain the calcium caseinate as a physically stable colloid. As Ca^{2+} is added to casein systems, the aggregation of the submicellar units to colloidal dimensions occurs mainly through Ca^{2+} binding. Ca^{2+} binding to surface casein phosphates of α_{s1}-casein and β-casein may also alter the pattern of interaction between the casein constituents. Thus, the stability of all three caseins seems to be nearly equal in the presence of K^+, as inferred by the

Table 4. Hydration Estimates of Bovine and Caprine Caseins.

Casein	submicelles (+K)	micelles (+K)	submicelles (+Na)	micelles (+Na)
bovine	0.004057	0.007308	0.003722	0.006796
caprine high in α_{s1}-casein	0.001388	0.003749	0.001644	0.003613
caprine low in α_{s1}-casein	0.003705	0.004070	0.003523	0.004375

[a] g of water/g of protein. (Assuming τ_c = 56 ns for bovine casein submicelles[10]).
[b] From oxygen-17 NMR data at 24 ± 1 °C and at pD 7.10, according to a two-state, isotropic model[6].

similar values of their virial coefficients (B_o; Table 2)· Charge repulsions among the submicellar components are now relieved in the formation of the colloid. Moreover, in the presence of K^+, the values of hydration were significantly greater for the bovine and the caprine casein high in α_{s1}-casein than those obtained under submicellar conditions (Table 4). It is clear that the interaction between the bovine casein and K^+ ions contribute to a more hydrated micellar casein structure as well.

The hydration results once more indicate that the caprine casein low in α_{s1}-casein is less sensitive to all changes in electrolyte when going from K^+ to Na^+ or from submicellar to micellar forms (Table 4). Furthermore, this system contains proportionately greater α_{s2}-casein (Table 1), which has a higher net negative charge than α_{s1}-casein, showed an increase in electrostatic repulsion when Ca^{2+} is added in the presence of both electrolytes (Tables 2 and 3). It seems apparent, then, that the submicelle-micelle equilibria observed here does not follow the pattern of those caseins 'richer' in α_{s1}-casein.

Evidently, a combination of hydrophobic and electrostatic interactions are involved in natural micelle formation, probably with the production of salt bridges of Ca^{2+} and phosphate ion between submicelles. Note that in ruminant milks, the ratio of K^+ to Na^+ is usually 3:1 [1]. Thus, the interaction of K^+ ions with the casein micelle suggests that this monovalent ion might be responsible for a less compact and more flexible structure which is essential for their biological role, i.e., the efficient transport and delivery of protein, calcium and phosphorus to the neonate.

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