

Water Interactions with Varying Molecular States of Bovine Casein: ^2H NMR Relaxation Studies

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The caseins occur in milk as spherical colloidal complexes of protein and salts with an average diameter of 1200 Å, the casein micelles. Removal of Ca^{2+} is thought to result in their dissociation into smaller protein complexes stabilized by hydrophobic interactions and called submicelles. Whether these submicelles actually occur within the micelles as discrete particles interconnected by calcium phosphate salt bridges has been the subject of much controversy. A variety of physical measurements have shown that casein micelles contain an inordinately high amount of trapped water (2 to 7 g H_2O /g protein). With this in mind it was of interest to determine if NMR relaxation measurements could detect the presence of this trapped water within the micelles, and to evaluate whether it is a continuum with picosecond correlation times or is associated in part with discrete submicellar structures with nanosecond motions. For this purpose the variations in ^2H NMR longitudinal and transverse relaxation rates of water with protein concentration were determined for bovine casein at various temperatures, under both submicellar and micellar conditions. D_2O was used instead of H_2O to eliminate cross-relaxation effects. From the protein concentration dependence of the relaxation rates, the second virial coefficient of the protein was obtained by nonlinear regression analysis. Using either an isotropic tumbling or an intermediate asymmetry model, degrees of hydration, \bar{v} , and correlation times, τ_c , were calculated for the caseins; from the latter parameter the Stokes radius, r , was obtained. Next, estimates of molecular weights were obtained from r and the partial specific volume. Values were in the range of those published from other methodologies for the submicelles. Temperature dependences of the hydration and Stokes radius of the casein submicelles were consistent with the hypothesis that hydrophobic interactions represent the predominant forces responsible for the aggregation leading to a submicellar structure. The same temperature dependence of r and \bar{v} was found for casein under micellar conditions; here, the absolute values of both the Stokes radii and hydrations were significantly greater than those obtained under submicellar conditions, even though τ_c values corresponding to the great size of the entire micelle would result in relaxation rates too fast to be observed by these NMR measurements. The existence of a substantial amount of trapped water within the casein micelle is, therefore, corroborated, and the concept that this water is in part associated with submicelles of nanosecond motion is supported by the results of this study. © 1987

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Previous reports from this laboratory have shown that when the hydration of

proteins is evaluated from the NMR relaxation of water, either in the longitudinal or transverse relaxation mode, the relaxation rate must be obtained at various pro-

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tein concentrations (1,2). The nonlinear portion of this concentration-dependent function was found to be related to the activity coefficient or its equivalent, the second virial coefficient of the protein. Using a two-state isotropic tumbling model, it was demonstrated that the linear portion is a product of the degree of hydration and the relaxation rate of the bound water in the absence of cross relaxation. The hydration and relaxation rate of the bound water can be obtained separately from the linear portion of longitudinal and transverse protein-dependent relaxation rates through use of the Kubo and Tomita (3) and Solomon equations (4); from these relationships the correlation time, τ_c , of the bound water also can be evaluated.

These relationships had been tested earlier (1) on β -lactoglobulin A (β -Lg A)² at pH 5.2, where the protein exists as a dimer of M_r 36,200, and at pH 4.65, where β -Lg A undergoes a temperature-dependent dimer-to-octamer self-association involving hydrophilic groups (5-9). Values of τ_c obtained at pH 5.2 were in agreement with structural information derived from small-angle X-ray scattering (SAXS) (10). At pH 4.65, τ_c and hydration values increased with decreasing temperature in direct correlation with increased octamer formation. Calculations using a three-state model and a β -Lg A octamer structure derived from SAXS measurements (10) showed quantitative agreement between NMR number-average hydration and τ_c values. These increased values indicated the existence of trapped water in the internal cavity of the β -Lg A octamer. Hence, it would appear that hydrophilic self-association of proteins can increase hydration values through changes in quaternary structure.

As an extension of this work, a study of the manner in which protein-protein interactions involving hydrophobic and electrostatic groups influence the hydration of proteins, again as evaluated by NMR re-

laxation measurements was undertaken. The model system chosen was casein, a family of phosphoproteins which are the major components of milk (11, 12). Casein monomers undergo hydrophobic self-associations at pH 7 which increase with increasing temperature (13, 14). The associated state is commonly referred to as the submicellar form (11, 14). It has been hypothesized that, upon addition of calcium, casein polymers further associate via calcium phosphate salt bridges into a colloidal state referred to as the micellar form (11, 14). However, the exact supramolecular structure of the casein micelle has remained controversial. Models presented have ranged from those having discrete submicelles to those having the structure of a loose porous gel (15). Indeed, a variety of physical techniques have yielded hydration values for casein micelles from 2 to 7 g H₂O/g protein (15). Since most proteins have hydrations of about 0.3 g/g, this has been taken to indicate the existence of trapped water within the micelle. The nature of this trapped water could thus vary from a continuum with picosecond correlation times to water associated with discrete submicelles with nanosecond correlation times. For these reasons, NMR relaxation measurements of water were made with varying concentrations and temperatures of casein under both submicellar and micellar conditions.

MATERIALS AND METHODS

Sample preparation. Casein micelles were isolated from 2 liters of fresh warm milk to which 1 g of phenylmethylsulfonyl fluoride had been added to retard proteolysis. The milk was centrifuged at 4000g for 10 min to remove the cream fraction. This skim milk (400 ml) was centrifuged for 1 h at 88,000g (37°C). The pellets were washed twice in D₂O containing 25 mM 1,4-piperazinediethanesulfonic acid (Pipes) (pH 6.75), 20 mM CaCl₂, and 80 mM KCl. The final protein concentration was fixed at about 100 mg/ml (total volume of 5 ml). Subsequent dilutions were made with the same buffer. To produce submicelles, sodium caseinate prepared from the same skim milk was dialyzed and lyophilized at pH 7.2; the lyophilized protein was dissolved in D₂O, in the same Pipes-KCl buffer without CaCl₂, but with added dithiothreitol to promote self-

² Abbreviations used: β -Lg A, β -lactoglobulin A; SAXS, small-angle X-ray scattering; Pipes, 1,4-piperazinediethanesulfonic acid.

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association of κ -casein (13). These procedures were designed to minimize the concentration of H_2O in the D_2O solutions and thus to eliminate any significant contribution from deuterium exchange to the relaxation rates. Casein concentrations were determined spectrophotometrically on samples diluted 1/50 to 1/100 in 0.1 N NaOH; an absorptivity of 0.850 ml mg^{-1} cm^{-1} at 280 nm was used for whole casein (12).

Relaxation measurements. Deuteron NMR spectra were obtained by Fourier transform spectroscopy with a JEOL FX60Q spectrometer³ operating at a nominal frequency of 60 MHz. The frequency of observation was 9.17 MHz. Raw data were in the form of relative intensities as calculated by the JEOL 980B computer.

Since the high concentration of water in a dilute solution produces an intense signal, a single accumulation at the particular sample temperature (2, 15, or $30 \pm 1^\circ C$) was sufficient for each spectrum. Even then, care was necessary to avoid exceeding the dynamic range of the computer with consequent truncation. To this end, small sample volumes were employed by use of a microcell assembly with an expandable 35- μ l sample bulb, available from Wilmad Glass Co., Inc. The protein solution was introduced very slowly into the spherical bulb by means of a fine-gauge syringe needle inserted through its capillary neck, to avoid the inclusion of any air bubbles. Bubbles trapped below the neck could lead to vortex formation in the spinning sample bulb, vitiating the necessary assumption of a spherical sample geometry. The bulb, suspended by its neck from a chuck attached to a plastic cap, was positioned snugly inside a precision 5-mm o.d. sample tube which, initially, contained also the lock-signal solvent. The small amount of this solvent in the residual annular space outside the bulb was not always sufficient to assure maintenance of the lock; occasional failure of the lock during a lengthy series of automatic measurements resulted in loss of usable data. A second arrangement was then used in which the 5-mm tube, containing the sample bulb but no solvent, was positioned by means of fluorocarbon plastic spacers concentrically within a precision 10-mm o.d. sample tube accommodating a much larger quantity of lock-signal solvent. Incidental advantages of this arrangement were that the outside of the sample bulb was thus kept dry, and that the solvent could be sealed within the annular space between the two tubes and so kept from contamination for a greatly extended time. Except for these advantages, either arrangement resulted in the same measurements. The cell assembly, in either case, was positioned in the JEOL FX600 10-mm $^1H/^{13}C$ dual probe insert.

³ Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Longitudinal relaxation rates, R_1 , were measured by the inversion-recovery method (16), where the repetition time T in the pulse sequence [$\dots T \dots \pi \dots \tau \dots \pi/2 \dots$] was chosen to be at least five times T_1 ($\equiv R_1^{-1}$) and the values of the variable delay time τ ranged from 10 ms to 3 s, for a total of between 5 and 20 τ values, depending on the detail desired. Under the conditions of this method, the relation of the peak intensity A , to the pulse delay time τ becomes

$$A_\tau = A_\infty[1 - 2\exp(-R_1\tau)], \quad [1]$$

where A_∞ is the limiting peak intensity for $\tau \rightarrow \infty$. Independent measurement of A_∞ , a source of irreducible error, can be dispensed with, and the problem of weighting the data points in the conventional linear plot of the logarithm of a function of relative peak heights can be eliminated, by fitting directly to the data points (τ, A_τ) by least squares an exponential of the form of Eq. [1], from which the two parameters A_∞ and R_1 can be obtained (Fig. 1, curve A). The fitting of this two-parameter exponential was carried out by computer by means of an iterative program. For each sample R_1 was determined at least four times, and the results were averaged; standard errors amounted to 1-2%. This procedure was repeated at each concentration; a minimum of six concentrations were used under each set of conditions of temperature at which resonance relaxation was examined.

Transverse relaxation rates, R_2 , were determined by spin-locking measurement (17) of R_{1p} , the longitudinal relaxation rate in the rotating frame. R_{1p} ,

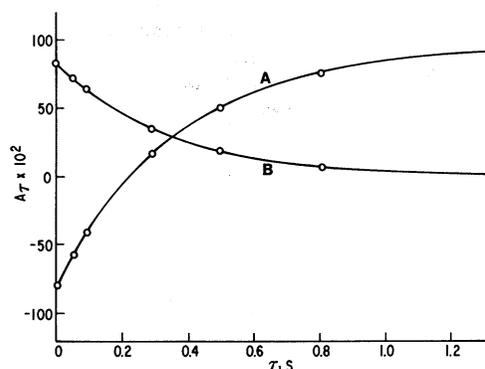


FIG. 1. Deuteron resonance peak intensities, A_τ , as a function of time, τ , for casein micelle solution at pH 6.75, $15^\circ C$, in D_2O (78.5 g/ml) and in Pipes-KCl-CaCl₂ buffer. (A) Spin-lattice relaxation measurements from inversion-recovery method. (B) Spin-spin relaxation measurements from spin-locking measurements of T_{1p} . Solid lines are best fits (F test) of corresponding equations in text (Eqs. [1] and [2]) to experimental intensities by nonlinear regression.

equals R_2 in dilute solutions of low viscosity whenever the magnitude of R_{1p} is independent of H_{1p} , the spin-locking radiofrequency field in the rotating frame (17); this was the case, within the limits of experimental error, in the present work. R_2 was evaluated as described above for R_1 , except that the relation between peak intensity A_r and decay time τ in this case becomes

$$A_r = A_0 \exp(-R_2\tau), \quad [2]$$

where the initial intensity A_0 replaces A_∞ as the maximum peak intensity. Again, a least-squares two-parameter exponential fit to the data points was performed by an iterative computer program, from which A_0 and R_2 were obtained (Fig. 1, curve B).

For each sample, R_2 was determined with the same number of replications as R_1 ; standard errors amounted to 2–3%. Measurements of one mode of relaxation were made on the identical sample and immediately following the completion of measurements of the other mode, or at latest the next day. Measurements for deuteron relaxation at 9.17 MHz were made at pH 7.0 and at 2, 15, and $30 \pm 1^\circ\text{C}$, respectively. These rates were measured in D_2O to eliminate cross-relaxation effects between water and protein protons, such as observed by Edzes and Samulski (18), and by Koenig *et al.* (19).

Analysis. For a two-state model (bound and free water), Kumosinski and Pessen (1) have shown that for the change in R_{obs} , the observed longitudinal or transverse relaxation rate of water in the presence of varying protein concentration, c ,

$$R_{\text{obs}} - R_f = (R_b - R_f)\bar{v}_w a_p / W, \quad [3]$$

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, and a_p is the activity of the protein. \bar{v}_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{v}_w differs from n , the number of available binding sites per 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{v}_w and n disappears. In the following we will, for simplicity and convenience, use the expression "hydration" for short to indicate the quantity \bar{v}_w in units of grams per gram. Also,

$$a_p = c \exp(2B_0c + \dots), \quad [4]$$

where B_0 is the second virial coefficient of the protein.

Data points of the observed relaxation rate (lon-

gitudinal or transverse) vs protein concentration were fitted with a combined function of Eqs. [3] and [4] via an iterative Gauss-Newton nonlinear regression program developed at this laboratory. Analysis by this program produced values for B_0 , $(R_b - R_f)\bar{v}_w$, and R_f . R_{1b} or R_{2b} , \bar{v}_w , and τ_c values were obtained by simultaneous solution of the Kubo and Tomita (3) and Solomon equations (4),

$$R_{1b} = 2K\tau_c[(1 + \omega_0^2\tau_c^2)^{-1} + 4(1 + 4\omega_0^2\tau_c^2)^{-1}] \quad [5]$$

and

$$R_{2b} = K\tau_c[3 + 5(1 + \omega_0^2\tau_c^2)^{-1} + 2(1 + 4\omega_0^2\tau_c^2)^{-1}], \quad [6]$$

where R_{1b} and R_{2b} are the longitudinal and transverse relaxation rates and τ_c is the correlation time of the bound water, respectively; ν_0 or $\omega_0 = 2\pi\nu_0$ is the nuclear angular precession frequency (Larmor frequency) in Hertz or in radians per second, respectively; and K is a measure of the strength of the nuclear interaction, i.e.,

$$K = (3/80)(e^2qQ/h)^2(\eta^2/3 + 1)^{-1}S^2. \quad [7]$$

Here e is the electronic charge, 1.6022×10^{-19} coulomb, q is the electric field gradient, Q is the nuclear electric quadrupole moment, h is Planck's constant divided by 2π , 1.056×10^{-27} erg · s, η is a dimensionless parameter measuring the deviation from axial symmetry (20), and S is the order parameter for intermediate asymmetry of the motion of the bound water (21). Hence, this thermodynamic theory can be used whether isotropic ($S = 1$) or anisotropic motion ($S < 1$) is hypothesized, where in the latter case the "bound" should be understood in the sense of "hydrodynamically influenced layers" or "surface-induced probability distribution of water molecules." For these experiments η is assumed to be zero, $\nu_0 = 9.17$ MHz, and $e^2qQ/h = 215.6$ kHz (22).

RESULTS AND DISCUSSION

Bovine casein is composed of four major proteins, α_{s1} -, α_{s2} -, β -, and κ -casein, in the approximate ratio of 4:1:4:1 (23). α_{s1} -casein contains 8 phosphoserines, while β -casein contains 5 phosphoserines; κ -casein contains on the average 1 phosphoserine, while α_{s2} is variable, containing 8–11 (12). All caseins are largely considered to have little or no ordered secondary structure and to contain a large number of hydrophobic residues. With the above assumptions, the average molecular weight of monomeric casein is estimated to be 23,300; the average partial specific volume, \bar{v} , is 0.736 ml/g; and

the weight-average number of phosphate groups is 6.6 per 23,300-Da monomer unit.

At pH 6.75 with no calcium present, studies of the individual caseins have shown that they undergo mainly hydrophobically driven self-associations which increase with increasing temperature and ionic strength (14). Studies on whole casein are limited but show similar results (14). Figure 2A shows one proposed structure of this limiting polymer, commonly referred to as the submicellar form of casein. Here, the hydrophobic core is considered to be composed mostly of the hydrophobic portions of α_s - and β -caseins, while κ -casein resides mostly at the surface because of its ability to keep α_s - and β -caseins from precipitating at 37°C in the presence of calcium. All charged groups, including the serine phosphates, are located on the surface of the submicellar structure. In this model the κ -casein content of the submicelles is variable.

Upon addition of calcium, these submicellar spherical particles are thought to self-associate, due to calcium phosphate salt bridges, to form a large colloidal spherical particle of approximate radius of 650 Å, the micellar form of casein (see Fig. 2B). As noted above, a controversy exists as to whether or not the integrity of the submicelle is preserved upon the addition of calcium (15). While it is widely accepted that trapped water exists within the micellar structure, the nature of this water

is uncertain (15, 24). The characteristic of κ -casein to be predominantly on the surface of the micelle has been shown by electron microscopy coupled with gold-labeled κ -casein (14), or with ferritin conjugate and anti- κ -casein (25).

For the above reasons ^2H NMR relaxation measurements, both longitudinal, R_1 , and transverse, R_2 , of D_2O with varying concentrations of casein were performed with casein, with and without calcium, at 30, 15, and 2°C. Figure 3 shows R_1 and R_2 measurements at 15°C under submicellar and micellar conditions. All data were fitted by Eqs. [3] and [4] using a Gauss-Newton nonlinear regression program developed at this laboratory. The experimental data and the data calculated from the model employed are in excellent agreement, as shown by the solid line in Fig. 3. Under these and all other conditions the nonlinear portion of the curves yielded a virial coefficient of 0.0032 ± 0.0003 ml/mg, indicating the consistency of the experimental results. The linear portions of the curves were evaluated with a propagated standard error of about 4%; they contain the product of the relaxation rate of the bound water, the hydration, and finally the asymmetry parameter, S . These will be separated and each will be discussed in the following.

Hydration and dynamics: Isotropic model. From the linear portion of the longitudinal and transverse relaxation results, Eq. [3], and the Kubo-Tomita-Solomon equations, Eqs. [5] and [6], the following parameters were calculated at the various environmental conditions of the caseins: correlation times, τ_c , hydration values, \bar{v}_w , for an isotropic tumbling model ($S = 1$), and the relaxation rates of the bound water, R_{1b} and R_{2b} . The results are shown in Table I. Here, \bar{v}_w values increased from 0.00652 to 0.01201 g water/g protein and τ_c values decreased from 38.9 to 29.8 ns as the temperature decreased from 30 to 2°C for casein in the submicellar form; propagated standard errors were about 8% for τ_c and 6% for \bar{v}_w . The same temperature dependence of τ_c and \bar{v}_w was exhibited under micellar conditions, although at all temperatures

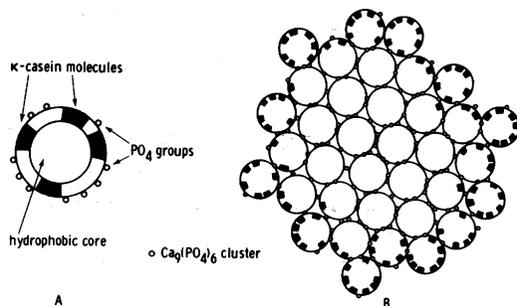


FIG. 2. Quaternary structural forms of casein. (A) Submicellar form; (B) micellar form upon addition of calcium. Reproduced, by permission of the publisher, from Ref. (14).

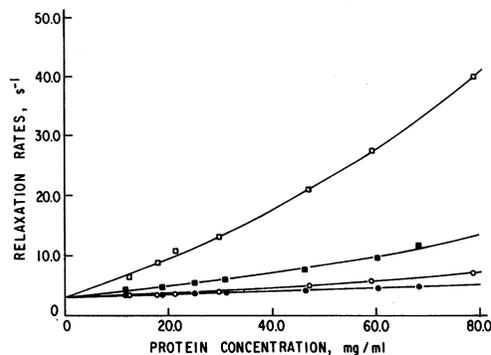


FIG. 3. Dependence of deuteron relaxation rates of water on casein concentrations in D_2O at pH 6.75 in 0.2 M Pipes-KCl buffer at $15^\circ C$. (●) R_1 measurements, submicellar form; (■) R_2 measurements, submicellar form; (○) R_1 measurements, micellar form; (□) R_2 measurements, micellar form.

their absolute values were larger for the micellar form than for the submicellar form.

At this point it would be appropriate to note that although the caseins are self-associating, we need to consider here only the aggregated form. The concentrations used were high enough so that the association equilibrium favors polymer formation (14). For both micelles and submicelles, no significant differences in hydration would result from protein concentration-dependent dissociation effects at $30^\circ C$. (At lower temperatures, this must be qualified as discussed below.) Also, these \bar{v}_w values will in all probability show only a fraction of the total hydration, since at 9.17 MHz any

bound water with $\tau_c < 6$ ns would have a R_2/R_1 ratio of unity and would not be observable by this methodology.

Since it has been shown previously that the τ_c values derived from NMR relaxation results are those for the unhydrated rather than the hydrated form of the protein (1, 2), the Stokes radius, r , calculated from τ_c values using the Stokes-Einstein relationship would indeed be a representation of the quaternary structure for the unhydrated protein. Such r values were calculated from all τ_c results and are listed in Table II for the caseins.

A Stokes radius of 36.4 \AA (Table II) found at $30^\circ C$ is at the lower limit of radii reported for submicelles, the sizes of which range from 40 to 90 \AA depending on the method of measurement (13, 14, 26, 27). (It should be recalled that direct comparison between Stokes radii derived by this NMR method and those calculated from hydrodynamic or small-angle scattering data would be inappropriate because these latter include all water of hydration, whereas the NMR values (1, 2) pertain to the anhydrous protein. Nevertheless, changes in this parameter accompanying protein structural changes are of interest in helping to establish structural information.) Results for the submicelles show a decreasing hydration value (Table I) and an increase in the Stokes radius (Table II) with increasing temperature. This suggests that hydrophobic interactions are involved in the formation of the submicelle, since, as the temperature is raised, water is ex-

TABLE I
HYDRATION AND DYNAMICS OF BOUND WATER

	Temperature ($^\circ C$)	τ_c (ns)	\bar{v}_w (g H_2O /g protein)	R_{1b} (s^{-1})	R_{2b} (s^{-1})
Submicelle	30	38.9	0.00652	1904	10,510
	15	34.7	0.00824	2080	9,840
	2	29.8	0.01201	2323	9,070
Micelle	30	63.6	0.0165	1249	14,790
	15	51.1	0.0225	1515	12,570
	2	45.1	0.0282	1689	11,530

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TABLE II

MOLECULAR PARAMETERS OF CASEINS DERIVED FROM DATA OF TABLE I

	Temperature (°C)	r (Å)	M_r	$(\bar{v}_w)_r$	S	$(\bar{v}_w)_s = 0.237$
Submicelle	30	36.4	165,000			0.116
	15	30.5	97,200			0.147
	2	25.5	56,800			0.214
Micelle	30	42.9	270,700	0.469	0.188	0.294
	15	34.8	144,500	0.357	0.251	0.400
	2	29.3	86,200	0.380	0.272	0.502

cluded from the hydrophobic interface during an association process.

Although the absolute value of the Stokes radius calculated for the micelle is on the same order of magnitude as that of the submicelle, it was not as large as would be expected, because of instrumental limitations. These limitations are due to the large size of the casein micelle ($r = 650$ Å), which would result in a τ_c value of nearly 200 μ s. Such a slow motion would yield a transverse relaxation rate too large to be seen by these NMR experiments at 9.17 MHz. In one sense, what the data may show is the average hydration of the caseins within the micelle, since the fastest motions dominate relaxation data. The micelle exhibits the same temperature dependence as the submicelle, showing hydrophobic interactions, in agreement also with previous investigators who theorized that micelles are formed by aggregation, via Ca^{2+} salt bridges, of submicelles (11, 14). The slight increase in r from submicelle to micelle is probably due to a gradual increase in internal hydration (trapped water) as the submicelle is incorporated into the micelle. This is also in agreement with the extraordinary hydration (2 to 7 g $\text{H}_2\text{O}/\text{g}$ protein) found by hydrodynamic measurements (15). In the course of micelle formation, it is likely that the electrostatic forces involving Ca^{2+} and phosphate or carboxyl groups on the exterior of the submicelle are in competition with, and finally outweigh, the hydrophobic effects within the submicelle.

Derived molecular parameters of the protein. Since it has been shown that the Stokes radius of the bound water derived from NMR relaxation results can be related to the anhydrous volume (1, 2), a molecular weight of the caseins can be calculated from

$$M_r = 4/3\pi r^3 N/\bar{v}_p, \quad [8]$$

where r is the Stokes radius (Table II), N is Avogadro's number, and \bar{v}_p is the average partial specific volume of the caseins, taken here to be 0.736. The results are presented in Table II. Here the increase in M_r for both the submicelle and the micelle, as the temperature is increased, is a qualitative indication of hydrophobic self-association not only for the submicelle but also within the micelle structure itself.

To quantitate this temperature-depen-

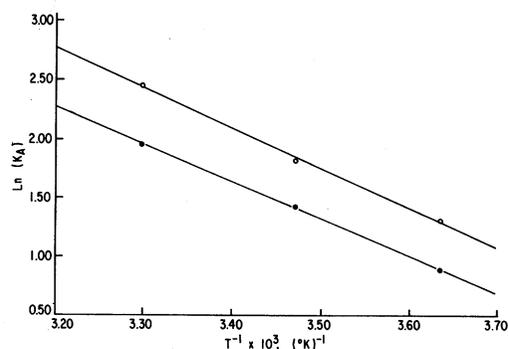


FIG. 4. Van't Hoff plots for temperature dependence of the self-association of casein. (●) Submicellar form; (○) micellar form.

dent variation of M_r , apparent equilibrium constants K_A were calculated from $K_A = M_r/23,300$ where 23,300 is the average monomer molecular weight of casein. (This relationship is reasonable since the measurements were performed at high concentrations of casein, where the equilibrium is driven nearly completely toward the aggregated form.) The $\ln K_A$ and the inverse temperature ($1/T$) were then used with the van't Hoff expression (see Fig. 4) to calculate the apparent enthalpy of self-association, ΔH , for submicellar and micellar casein. As can be seen in Fig. 4, the van't Hoff plots for the two forms of casein are essentially parallel. In fact, ΔH values for the submicelle formation were calculated to be 6.34 ± 0.11 kcal and only slightly higher at 6.81 ± 0.28 kcal for the self-association to the micelle. These values are in good agreement with ΔH of 4.67 found for the association of purified α_{s1} - and κ -caseins (28). This quantitation of the temperature variation of the self-association strongly suggests that the integrity of the submicelle is at least partly preserved when it is incorporated into the micellar form by Ca-phosphate salt bridges. Moreover, extrapolation of the van't Hoff plot to 37°C yields an apparent M_r of 210,000 which is in agreement with results from other investigations (14, 26, 29).

Finally, an average charge Z per average monomer molecular weight $M_r = 23,300$ can be evaluated from the virial coefficient B_0 of $0.0032 \text{ ml}^2/\text{g}^2$ using the following expression (30):

$$2B_0 = \frac{Z^2}{4m_s M_r} + \bar{v}_p/1000, \quad [9]$$

where m_s is the molarity of salt used. The value of ± 8.1 , calculated from the NMR relaxation measurements, is not in good agreement with the average value of -16.1 , derived from the amino-acid sequence (12), average pK values from the literature, and the assumption that at pH 6.75, where these experiments were performed, the serine phosphates have a charge of -2 . However, Arakawa and Timasheff (31) have shown that another term must be

added to Eq. [9] to take into account the preferential interactions of salt and water at the protein interface, namely the quantity $-(\partial g_s/\partial g_p)^2/m_s$, where $(\partial g_s/\partial g_p)$ is the preferential binding term. If this quantity is added to Eq. [9], with Z chosen as -16.1 , and $B_0 = 0.0032 \text{ ml}^2/\text{g}^2$, a value of $0.046 \text{ g salt/g protein}$ can be calculated for the preferential binding term. For micellar casein this value is reasonable, if we translate Ca^{2+} binding of $0.043 \text{ g salt/g protein}$ into $8.5 \text{ moles Ca}^{2+}$ per mole of $23,300\text{-Da}$ protein. This is consistent with the notion of Ca^{2+} binding to phosphate groups since, as stated above, the weight-average value for all the caseins is 6.6 phosphates per monomer.

Hydration: Anisotropic tumbling model. Up to this point we have calculated all hydration values using an isotropic motion mechanism of the bound water ($S = 1$). However, the motion of the bound water may, in fact, be anisotropic ($S < 1$) if the correlation times are long with respect to the Larmor frequency used in these experiments. Such may be the case for the casein micelles where water may be trapped at the surfaces of submicelles as they self-associate via calcium-phosphate salt bridges into micelles. Here an attempt will be made to estimate the asymmetry factor S for casein.

In Table II, r values at all temperatures are somewhat larger for the micellar form of casein than that for the submicellar form. This consistently larger Stokes radius (also represented by the molecular weight, M_r , in Table II) could be due to increased hydration either by weakening of hydrophobic interactions within the submicellar form, caused by calcium-phosphate salt bridge formation, or to trapped water at the surfaces of the submicelles as they are incorporated into the micelle. This increased hydration value, $(\bar{v}_w)_r$, may easily be calculated from the Stokes radii of the micelle, r_m , and submicelle, r_{sm} , by

$$(\bar{v}_w)_r = \bar{v}_p \left(\frac{4/3\pi r_m^3}{4/3\pi r_{sm}^3} - 1 \right), \quad [10]$$

where $\bar{v}_p = 0.736$ for an average partial specific volume of the caseins. These results

are presented in Table II and range between 0.357 and 0.469 g water/g protein at these temperatures. Asymmetry values, S , can now be calculated from $S = (\bar{v}_w/(\bar{v}_w)_r)^{1/2}$ and the \bar{v}_w and $(\bar{v}_w)_r$ values from Tables I and II, respectively. These S values are listed in Table II and average 0.237 ± 0.033 , which is in good agreement with the value of 0.23 predicted by Walmsley and Shporer (32). Now, new hydrations $(\bar{v}_w)_{S=0.237}$ can be calculated for an anisotropic motion mechanism using the \bar{v}_w values of Table I and the average S of 0.237. These are listed in the last column of Table II. The absolute values of these new hydrations, ranging from 0.116 to 0.502 g water/g protein for submicellar and micellar casein, are closer to the expected hydration values derived by other methods (1, 2).

It should be stressed that, although the above calculation is not proof of the existence of water with anisotropic motion bound to casein, it does furnish significant information. What is important here is the variation of hydrations with quaternary structural changes of the casein rather than their absolute values. It may be that the absolute value of the hydration derived from NMR relaxation results will be obtained only at some future time when the controversy regarding the isotropic vs anisotropic nature of water binding to proteins in solution is resolved. At any rate, water bound at the surface of the micelles or influenced by the slow motion of the large particle would not be sensed at the frequencies used here.

Here we have found that the hydration value, no matter what absolute value is used, decreases and that r increases for submicellar casein with increasing temperature, in agreement with the our general notion of hydrophobic protein self-association. Moreover, this same temperature-dependent variation of \bar{v}_w and r exists for micellar casein, indicating preservation of the submicelle structure within the micelle. The consistent increase in r and \bar{v}_w for the micellar form over the submicellar form clearly demonstrates that trapped water exists in the micellar form of casein, as had been conjectured by Kuntz and

Kauzmann (24), and, further, that this trapped water is to a significant extent not a continuum with picosecond motion but is associated in part with hydrophobically stabilized submicelles which exist within the micellar structure.

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