Further Observations on the Size, Shape, and Hydration of Casein Micelles from Novel Analytical Ultracentrifuge and Capillary Viscometry Approaches

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The size, shape, and hydration of casein micelles were estimated using a combination of sedimentation velocity (time-derivative analysis) in the analytical ultracentrifuge and capillary viscometry applied to skimmed milk. On the basis of sedimentation time-derivative and Wales−van Holde analyses the casein micelles appear as large spherical molecules of $s^0_{T,b} = 845S$, $M_w \sim 2.8 \times 10^8$, hydrodynamic radius ∼77.8 nm, and $k_s/[\eta] = 1.6$. The molecular hydration (i.e., the extent of chemically bound and physically entrained solvent) was calculated to be 3.4 g/g. These results appear to be in good agreement with comparable results from electron microscopy and dynamic light scattering.

Introduction

Casein is the major protein component of bovine milk at ∼2.8 ± 0.3%.[1] The casein component is a complex mixture of the four most common caseins—α_{s1}, α_{s2}, β, and κ in ratios of approximately 4:1:4:1,[2] respectively.

A large proportion of casein in milk is in the form of casein micelles, which can be thought of as a complex of calcium caseinate−calcium phosphate (citrate).[1] The casein micelle is usually thought of as a hydrated sphere, with the "hydration", δ (i.e. the amount of solvent associated with the protein either chemically or physically entrained expressed as mass of water per unit mass of protein), previously estimated on the basis of dynamic light scattering[3] to be 3.7 ± 0.5. The colloidal calcium phosphate is essential for micellular stability.[4] Removal of Ca^{2+} ions results in a smaller complex—the casein "submicelle". κ-Casein is usually found on the exterior of the casein submicelle and therefore important to submicellular and micellular stability. The κ-casein is thought to coat the hydrophobic core of the submicelle and is important in casein/polysaccharide interactions due to having a positively charged region available for electrostatic bonding.[5] Other micelle models have been suggested including continuous gellike casein micelle model with exterior "hairy" κ-casein favored by Holt[6] and now at least partially supported by Walstra.[7] The size distribution of casein micelles is very broad (20−250 nm in diameter), and this is at least partially due to the availability of κ-casein.[1]

Milk proteins have been extensively studied using the analytical ultracentrifuge over the last 70 years although most researchers have concentrated on fractionated samples[8,9] or on the individual caseins.[10−25] During the 1970s, Dewan[26,27] subsequently made studies on the intact micelle, measuring both the sedimentation coefficient and diffusion coefficient at very low concentration and applying the Svedberg equation (see eq 10), and an estimate for molecular weight of ∼2.5 × 10^8 was obtained.

In this study we reexamine the question of size, shape and hydration of casein micelles, using a combination of modern analytical ultracentrifugation and capillary viscometry. Advantage will be taken of some recent advances in sedimentation velocity analysis, namely the time-derivative procedure.[28] The method is particularly useful for quantitatively assaying the heterogeneity of a preparation without the need for a so-called separation medium (as required by chromatographic procedures). The time-derivative or $g^*(s^*)$ (usually simplified to $g(s^*)$) allows the real time computation of sedimentation coefficient distributions by the dc/dt method, i.e., the change in concentration with time. The subtraction of pairs of data sets allows the averaging of $g(s^*)$ curves and therefore reduces baseline contributions. The concentration dependence of the sedimentation coefficient, k_s (mL/g) is also a very useful parameter.[29] When combined with the intrinsic viscosity, $[\eta]$ this provides an estimate for particle shape without assumptions over hydration as described in previous articles.[30−34] Once the expected spherical shape has been confirmed for the casein micelles, the intrinsic viscosity data can be re-interpreted to provide a value for the molecular hydration.

Materials and Methods

Reconstituted Skimmed Milk. Skimmed milk powder was dissolved in distilled water 1:10 w/v. The resulting dispersion was of the following composition: protein, 35.6 g/L (of which 28.0 g/L is casein); sugar (mainly lactose), 50.4 g/L; fat, 0.6 g/L; sodium, 0.6 g/L; and calcium, 1.3 g/L. (This represents a concentration typical of some modern food applications.) Dilutions were subsequently made for hydrodynamic analyses.

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Sedimentation Velocity. There are four main optical systems, which can be used, in the analytical ultracentrifuge—Schlieren, absorption, turbidity, and interference.[35] It was found however that Schlieren and turbidity studies were only valid at high concentrations and absorption at low concentrations. The interference system on the Beckman Optima XLI (Beckman Instruments, Palo Alto, CA), which was sensitive over the concentration range of interest, was therefore employed. Rotor speeds of 12 600 rpm and a 4 mm column length in a 12 mm path length double sector cell (one sector for solution, the other for solvent) were used together with an accurately controlled temperature of 20.0 °C. A weighted average partial specific volume, $\bar{v}$ of 0.733 ± 0.002 mL/g was calculated from the individual casein amino acid sequences.[36] As casein micelles are 4:1:4:1 mixture of the four main caseins, α_{s1}, α_{s2}, β, and κ^2, the partial specific volume of the casein micelle is therefore the weighted average of individual caseins. The $g(s^*)$ (sedimentation time-derivative) method was used to determine apparent sedimentation coefficients at each concentration by the procedure described in ref 28.

Capillary Viscometry. Solutions and reference solvents were analyzed using a 2 mL automatic Schott-Geräte Ostwald viscometer, under precise temperature control (25.04 ± 0.01 °C). The relative viscosity, η_{rel}, was calculated from the standard equation[37]

$$\eta_{rel} = (t/t_0)(\rho/\rho_0) \quad (1)$$

where t is the flow time for the solution, t_0 is the flow time for the solvent, 82.63 ± 0.01 s. (ρ/ρ_0) is assumed to be unity (as the density of the solvent and the sample are approximately equal at low concentrations). A plot reduced specific viscosity $\eta_{red} = (\eta_{rel} - 1)/c$ vs concentration, c yields the intrinsic viscosity, $[\eta]$, at the intercept, and slope is related to the Huggins constant,[38] K_H, or the related concentration dependence regression coefficient, k_η.[33]

$$\eta_{red} = [\eta]\,(1 + K_H[\eta]c) \quad (2)$$

$$\eta_{red} = [\eta]\,(1 + k_\eta c) \quad (3)$$

Results and Discussion

Sedimentation Velocity. The sedimentation profile of casein micelles results in a broad peak, i.e., large size distribution, and shows a large concentration dependence (Figure 1). Apparent mode average sedimentation coefficients were calculated at various concentrations and extrapolated to zero concentration using the standard equation.[39]

$$s = s^0(1 - k_s c) \quad (4)$$

where the Gralen parameter,[9] k_s, is a measure of concentration dependence (Figure 2). The mode average sedimentation coefficient, $s^0_{T,b}$, is 845 ± 2 S, and k_s = (16.9 ± 0.1)mL/g, which is characteristic of a large extremely hydrated spherical molecule.

Capillary Viscometry. Results indicate a classical positive dependence of reduced viscosity on concentration (Huggins) (Figure 3). The relatively large intrinsic viscosity (for a protein) would also suggest a rather hydrated molecule.

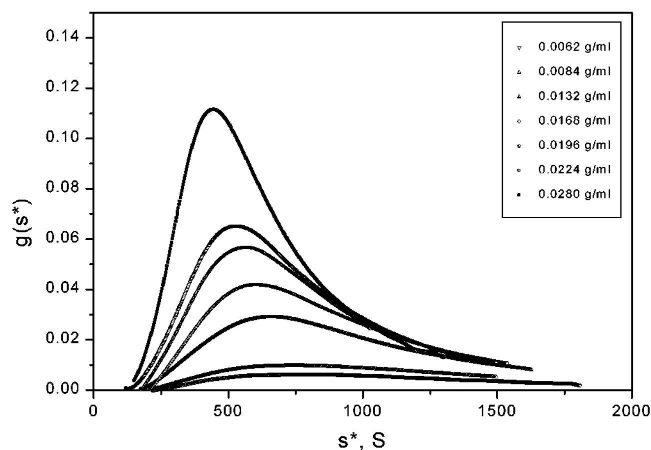

Figure 1. $g(s^*)$ profiles for casein micelles at differing concentrations.

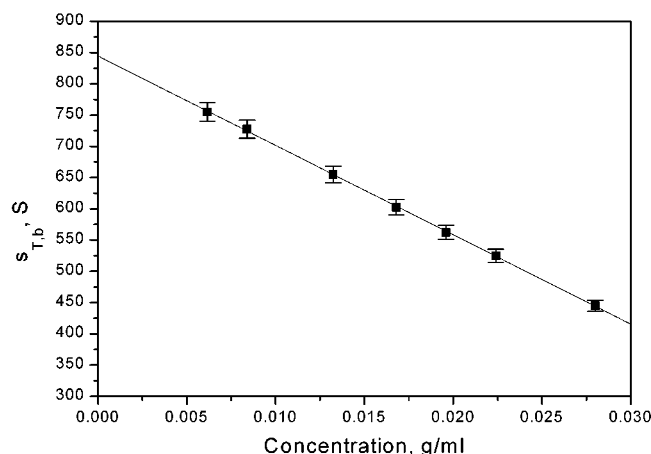

Figure 2. Concentration dependence of sedimentation. From the graph above $s^0_{T,b}$ = 845 ± 2 S and k_s = 16.9 ± 0.1 mL/g.

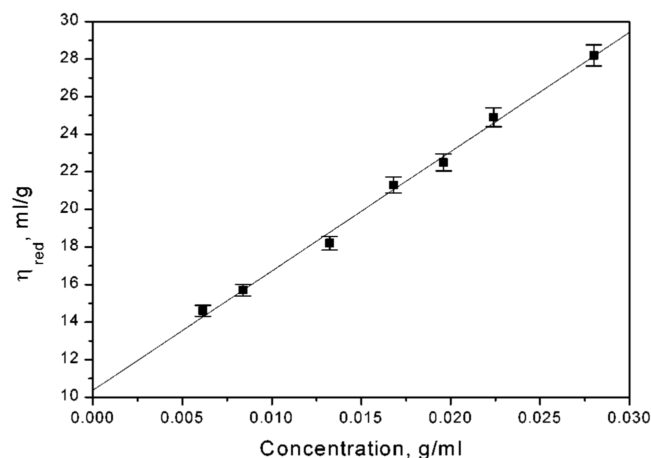

Figure 3. Concentration dependence of reduced viscosity (Huggins plot). From the graph above $[\eta]$ = 10.4 ± 0.4 mL/g, k_η = 61 ± 4 mL/g, and k_H = 5.9 ± 0.5.

The Wales–van Holde ratio,[30] R, a combination of the Gralen parameter, k_s, and the intrinsic viscosity, $[\eta]$, can give an approximate concentration independent indication of macromolecular shape, which is independent of an assumed value of the hydration, δ.

$$R = k_s/[\eta] \quad (5)$$

A value of R = 1.6 is typical for a sphere.[40] From the data

Table 1. Experimental Hydrodynamic Parameters for Casein Micelles

parameter	value
$s^0_{T,b}$, S	845 ± 2
k_s, mL/g	16.9 ± 0.1
$[\eta]$, mL/g	10.4 ± 0.4
k_η, mL/g	61.0 ± 2.0
K_H	5.9 ± 0.5
$k_s/[\eta]$	1.63 ± 0.07
k_η/k_s	~3.6

above $R = 1.63 \pm 0.07$ (Table 1, experimental hydrodynamic parameters). Since the theoretical value for a sphere is 1.6,[30−34] we can reasonably say that casein micelles are spherical. Having confirmed the spherical shape it is possible to calculate the hydration using the Einstein viscosity increment, ν, where ν is 2.5 for a sphere[40,41]

$$[\eta] = \nu v_s \quad (6)$$

$$v_s = (\bar{v} + \delta/\rho_0) \quad (7)$$

where v_s is the swollen volume, δ is the hydration, and ρ_0 is the density of solvent. Equation 6 yields a value of 4.2 mL/g for the swollen specific volume. If we approximate ρ_0 to the density of water, eq 7 then results in a hydration, δ, of 3.4 ± 0.5 g of solvent/g of protein, which is in good agreement with a previously estimated value of 3.7 ± 0.5[3,23] from dynamic light scattering. A further "ballpark" estimate for the swollen specific volume can be made from the following approximate relationship.[43]

$$k_\eta/k_s \sim v_s/\bar{v} \quad (8)$$

This yields a value of ~3 mL/g for v_s, which is in general agreement with the value from eq 6. (A typical protein v_s is usually on the order of ~1 mL/g.)

It is also possible to estimate the molecular weight from the sedimentation coefficient, $s^0_{T,b}$, the Gralen parameter, k_s, and the swollen volume, v_s.[33]

$$M_w = N_A[6\pi\eta s^0_{T,b}/(1 - \bar{v}\rho)]^{3/2}[(3\bar{v}/4\pi)(k_s/2\bar{v} - v_s/\bar{v})]^{1/2} \quad (9)$$

From eq 9 a molecular weight, M_w of 2.8×10^8 can be calculated from the mode average sedimentation coefficient of 845 S. Further simple mathematical manipulation of the data can yield the corresponding diffusion coefficient, $D^0_{T,b}$ and hydrodynamic radius, r_H, via rearrangements of the Svedberg[42] and Stokes−Einstein[43] relationships. (Table 2, calculated parameters).

$$D^0_{T,b} = [s^0_{T,b}/M_w]\,[RT/(1 - \bar{v}\rho)] \quad (10)$$

$$r_H = k_B T/(6\pi\eta D^0_{T,b}) \quad (11)$$

where R is the universal gas constant (8.314×10^7 erg K^{-1} mol^{-1}) and k_B is the Stefan−Bolztmann constant (1.381×10^{-16} erg K^{-1} mol^{-1}). The calculated $D^0_{T,b} = 2.8 \times 10^{-8}$ cm^2/s and $r_H = 78$ nm are also in good agreement with the previously published values from comparable techniques.[26,27]

Conclusions

The results obtained using the novel sedimentation velocity (time-derivative)−capillary viscometry approach are clearly in good agreement with the results of the more widely used methods, for example, dynamic light scattering.[3,26] The qualitative estimation of shape from the Wales−van Holde[30−34,40] ratio requires no prior knowledge of hydration and is model independent. The parameters discussed are all mode average values, which will have a broad distribution, as in Figure 1.

It is therefore clear that this combined hydrodynamic approach could in the future be used to define the state of the casein micelle under a variety of physiological conditions such as pH, ionic strength, and soluble solid concentration and could be of particular use in mixed biopolymer systems.

Table 2. Calculated Hydrodynamic Parameters for Casein Micelles

parameter	value
a/b	1.0
δ (eqs 6 and 7)	3.4
v_s, mL/g (eq 6)	4.2
v_s, mL/g (eq 8)	~3.0
M_w, g/mol	2.8×10^8
$D^0_{T,b}$ cm^2/s	2.8×10^{-8}
r_H, nm	78

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