Interactions in Micellar Solutions of β-Casein

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β-casein is a flexible amphiphilic milk protein which forms spherical micelles in very dilute solution. The magnitude of the weight-average interactions between the solute particles has been inferred from small-angle neutron scattering experiments. At relatively high protein concentrations the interactions between micelles are repulsive, whatever the temperature. At lower concentration these interactions vanish and become more and more attractive when the critical micelle concentration is approached. Although indispensable for micelle formation, this fact seems to have not been previously reported. [S0031-9007(96)02001-7]

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Amphiphilic molecules comprise both solvophilic and solvophobic moieties. To segregate their solvophobic regions from the solvent, these molecules can form a variety of aggregates known as micelles. Schematically, at very low concentrations, the molecules (monomers) are individually solvated. When the concentration approaches the so-called critical micelle concentration (CMC) the molecules begin to aggregate, and a mixture of free monomers and aggregates of different sizes is present in the solution. At higher concentrations, nearly monodisperse micelles coexist with single molecules. Generally, the micelles are spherical. However, at still higher concentrations, their shape can change and the solution can also separate into two phases.

Such systems have been widely studied, both experimentally and theoretically [1,2]. Nevertheless, little attention has been paid to the interactions existing between the different structural species present in the solution. Most of the experimental work concerns large-micelle systems, where free monomers contribute only very little to the overall properties of the solution. This is probably due to the fact that the size of the usual amphiphilic molecules is too small for allowing scattering techniques to give significant structural information on very dilute micellar solutions. On a theoretical point of view the problem has been recognized. However, it is extremely difficult to solve it, especially without any experimental information [3,4]. The purpose of this Letter is to provide such information, at least partially. To this end, large amphiphilic molecules are required. β-casein was chosen as a model of such molecules, and the micelles they form were studied by means of small-angle neutron scattering (SANS) at concentrations relatively close to the CMC.

Casein is the main protein of milk. The four genetic types of casein (α_{S1}, α_{S2}, β, and κ) possess very little secondary structure. In milk, they form very large spherical complexes trapping inorganic material, especially calcium phosphate. These colloidal particles of casein, usually termed micelles, are typical of milk structure. About 35% of the total casein in milk is β-casein. This 24 kg/mol protein is devoid of any cystenyl residue and consists of a few irregular alternating hydrophilic and hydrophobic sequences [5] whose total length would be about 720 Å in extended β conformation. In particular, starting from the *N*-terminus, the first 50 out of the 209 amino acids are hydrophilic, whereas the rest are mainly hydrophobic. As a result, β-casein forms micelles in the solution [6]. The monomer is too flexible to be crystallized, and its structure in solution is not yet known. As a matter of fact, the low value of the CMC ($<1.5 \times 10^{-3}$ g/cm^3 in heavy water at 4.5 °C) has prevented neutron scattering spectra from free monomers to be measured with sufficient accuracy. However, this seems possible now, and we plan to do it in the near future. In any case, a β-casein monomer may resemble a random coil, possibly with little local secondary structure allowing some hydrophobic residues to be protected from water [5].

The present study was conducted on β-casein in a deuterated 0.1 M phosphate buffer of pH 7, containing 0.1 M NaCl to reduce electrostatic interactions. The protein was obtained from the skimmed milk of a single cow and purified according to the method of Mercier *et al.* [7]. SANS experiments were carried out at protein concentrations c, ranging from 1.25×10^{-3} to 10^{-2} g/cm^3 and at different temperatures T, between 4.5 and 70 °C. Scattering spectra $I(q)$ were recorded with the PAXE spectrometer for $7 \times 10^{-3} \leq q \leq 7 \times 10^{-2}$ Å^{-1} and sometimes up to about 5×10^{-1} Å^{-1}. The wave number transfer is defined as $q = (4\pi/\lambda)\sin(\theta/2)$, where λ is the neutron wavelength and θ the scattering angle. The data were processed as usual [8], and the small-q regions of the coherent scattering spectra of the solute were first described by means of the Guinier approximation

$$I(q, c) \cong I(0, c) \exp[-q^2 R_g'^2(c)/3], \qquad (1)$$

where $I(0, c)$ is the forward scattered intensity and $R_g'(c)$ the apparent radius of gyration of the scatterers. The forward scattered intensity can be written

$$I(0, c) = KcM_w(c)/[1 + B_w(c)M_w(c)c], \qquad (2)$$

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where K is a constant depending on both the experimental apparatus and the sample. Different methods were used to calibrate the spectrometer [8]. They all led to $K = (9.7 \pm 0.5) \times 10^{-4}\,\mathrm{mol\,cm^2/g^2}$.

$$M_w(c) = \sum c_i M_i / c \tag{3}$$

is the weight average of the solute molecular weight. c_i is the concentration and M_i the molecular weight of micelles comprising i monomers. $c = \sum c_i$ is the total protein concentration.

$$B_w(c) = \sum n A_{n,w}(c) c^{n-2}, \quad \text{for } n \geq 2 \tag{4}$$

represents a virial expansion, where $A_{n,w}(c)$ is the weight average of the n^{th} virial coefficient. The weight average of 2^d virial coefficient writes [9]

$$A_{2,w}(c) = \sum\sum c_i c_j M_i M_j A_{2,ij} \Big/ \sum\sum c_i c_j M_i M_j \,. \tag{5}$$

where $A_{2,ij}$ is the second virial coefficient associated with micelles of types i and j.

Guinier plots of the SANS spectra obtained at 4.5 °C are shown in Fig. 1. In this representation $[R_g'^2(c)/3]$ is the slope of the scattering curve at small q values and $I(0, c)$ the intercept. At other temperatures the scattering profiles have similar features: Except for particular values of c and T, $R_g'(c)$ and $I(0, c)$ are found to depend on the range of q used to fit Eq. (1) with the spectra. However, when only the data corresponding to $1 \leq qR_g'(c) \leq 2$ are taken into account, $R_g'(c)$ values become almost independent of concentration at each temperature. In contrast, the data such that $qR_g'(c) \leq 1$ generally lead to different $R_g'(c)$ and $I(0, c)$ values which are higher at the lowest concentrations and temperatures, and lower at the highest concentrations, whatever the temperature. This result indicates that the mean interactions between the solute particles are attractive in the first case and repulsive in the second one. It is worth noting that the q-range variations of the apparent value of $R_g'(c)$ are significant. For instance, the data obtained at 4.5 °C for $q < 1.4 \times 10^{-2}\,\text{Å}^{-1}$ show that the apparent value of $R_g'(c)$ decreases from about 115 Å at $c = 1.25 \times 10^{-3}$ g/cm^3 to 45 Å at $c = 10^{-2}$ g/cm^3. In contrast, all the data corresponding to $1 \leq qR_g'(c) \leq 2$ give $R_g'(c) = (71.5 \pm 1.1)$ Å. This can be regarded as the actual value of the radius of gyration of the micelles. These features are clearly shown in Fig. 1.

However the validity of the Guinier approximation is limited to small values of $qR_g'(c)$. For spherical and homogeneous scatterers the upper limit can be estimated to be $qR_g'(c) \leq 1.3$. For random coils this limit is still lower ($\leq$0.7). Therefore it is necessary to have a suitable model to depict the form of the micelles at larger q values in order to confirm the results of the previous analysis. The scattering form factor of block copolymer micelles of Pedersen and Gerstenberg [10] has been found to well describe all the spectra up to about $q = 0.3\,\text{Å}^{-1}$. However, for $q \leq 6 \times 10^{-2}\,\text{Å}^{-1}$, the scattering profiles are not very sensitive to the internal scattering-length fluctuations, and the form factor becomes almost equivalent to the one of a relatively dense spherical core surrounded by a spherical corona of lower density. This simple model was found to well describe all the spectra in the q range previously defined. Various examples are given in Fig. 2. This is consistent with the usual picture of micelle structure inferred from experiments on other systems [11] and with the conformation of β-casein at the air-water interface [12]. The core of the β-casein aggregates is not compact: In the ranges of c

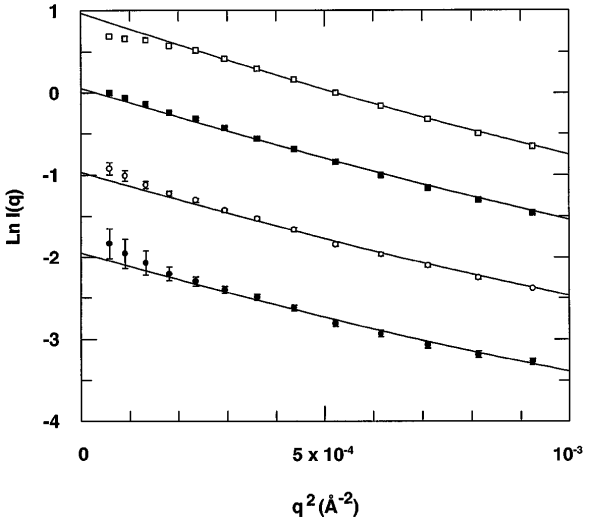

FIG. 1. Guinier plots of the neutron scattering spectra $I(q)$, of β-casein at 4.5 °C. The protein concentrations are 1.25 mg/cm^3 (●), 2.5 mg/cm^3 (○), 5 mg/cm^3 (■), and 10 mg/cm^3 (□). Full lines represent the results of the fit to the model described in the text.

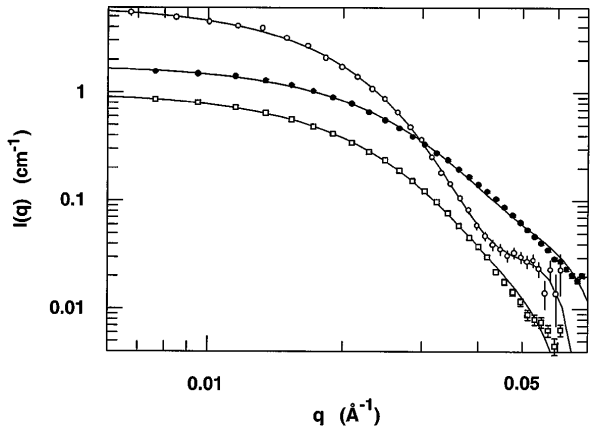

FIG. 2. Examples of scattering spectra $I(q)$, obtained in various conditions: $c = 1.25$ mg/cm^3 and $T = 23$ °C (□), $c = 5$ mg/cm^3 and $T = 9.6$ °C (●), and $c = 3.85$ mg/cm^3 and $T = 58.4$ °C (○). These spectra are compared to those given by a model consisting of a relatively dense spherical core surrounded by a spherical corona of lower density. This simple model is fitted to the data (full lines) and found to well describe all the other spectra for $q \leq 6 \times 10^{-2}$ Å^{-1}.

and T investigated, the core density varies between 0.4 and 0.9 g/cm^3, whereas the density of a globular protein is close to 1.35 g/cm^3. The density of the outer shell is much lower, between 0.025 and 0.14 g/cm^3, respectively. The external radius of the micelles is about (130 ± 10) Å and is almost independent of c and T up to 70 °C. The radius of the core increases with the average weight of the micelles, from about 45 Å at 4.5 °C to 70 Å at 70 °C.

This two-shell model was used to infer from each spectrum the quantity $[KcM_w(c)]$ in Eq. (2). This quantity is the value the forward scattered intensity would have if $B_w(c) = 0$. As already explained, the actual value of the forward scattered intensity was deduced from the spectra by means of the Guinier approximation for $q < 1.4\,\text{Å}^{-1}$. Using Eq. (2), these two values allow the interaction coefficient $B_w(c)M_w(c)$ to be evaluated. The results are shown in Fig. 3. In spite of the uncertainties, they clearly demonstrate that at low protein concentrations, the interactions are strongly attractive and concentration dependent, whereas they become weakly repulsive and almost constant when the protein concentration or the temperature increases. It is noteworthy that the first analysis using the Guinier approximation led to similar results.

Figure 4 shows how $M_w(c)$ varies with concentration at four different temperatures. Above the CMC, which is about 1.4×10^{-3} g/cm^3 at 4.5 °C and lower at higher temperatures, $M_w(c)$ first increases with increasing concentration and then remains constant. This feature indicates that the micelles are nearly monodisperse at concentrations sufficiently high with respect to the CMC. In these conditions the behavior of β-casein solutions can be approximated well by a simple two-state chemical equilibrium ($n\beta \Leftrightarrow \beta_n$). Closer to the CMC, the micelles are probably polydisperse. However, it is impossible to obtain any information about the relative variance of the micellar size distribution because it is no longer proportional to $[d \ln M_w(c)/d \ln c]$ when concentration dependent interactions are present.

The increase of the scattered intensity corresponding to negative values of $B_w(c)$ could also be explained by the presence of relatively long-range concentration fluctuations. These fluctuations might be regarded as appearing or disappearing aggregates whose structure is looser than that of full-grown micelles observed at higher concentrations. The scattering spectra obtained at $T = 4.5$ °C and $c \leq 1.25 \times 10^{-3}$ g/cm^3 suggest that this actually happens. The radius of gyration corresponding to the increase of scattered intensity observed at small q values is indeed very large. It is comparable to the one of the heaviest micelles observed at 70 °C. In this particular case the micelles comprise about 70 monomers, and their radius of gyration is close to 100 Å. At high protein concentrations the extent of the spatial correlations corresponds well to what is expected from the size of the micelles, as explained below.

As shown in Fig. 3 the interactions become weakly repulsive when the protein concentration or the temperature is high enough. Furthermore, $B_w(c)$ is nearly constant so that the 2^d virial coefficient is sufficient to account for the interactions. In these conditions the concentration and the weight of the micelles are large, and their contribution to the scattering intensity prevails over the one of monomers. If the system is described by a simple ($n\beta \Leftrightarrow \beta_n$) chemical equilibrium, then, according to Eq. (5), $A_{2,w}(c)M_w(c) \cong A_{2,nn}M_n = N_A v_{nn}/2M_n$, where N_A is Avogadro's number and v_{nn} the pair excluded volume associated with micelles comprising n monomers [3]. The data of Fig. 3 show that $A_{2,nn}M_n = (10 \pm 5)$ cm^3/g at $c = 10$ mg/cm^3, whatever the weight of micelles. Other data obtained at higher temperatures confirm this result. As suggested by the variations of density of the external shell, this could be ascribed to the fact that β-casein micelles interact as soft spheres which become

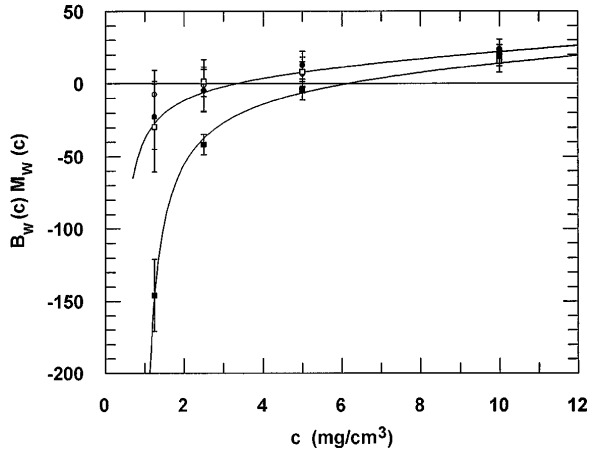

FIG. 3. Variation of the interaction parameter $B_2(c)\ M_w(c)$ with the protein concentration c. $B_2(c)\ M_w(c)$ is defined by Eqs. (2) and (4). The temperatures are 4.5 °C (■), 9.6 °C (□), 15 °C (●), and 23 °C (○). The solid lines are guides for the eye.

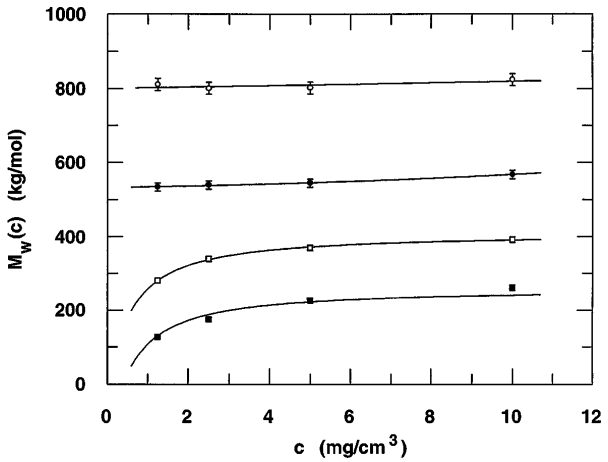

FIG. 4. Concentration dependence of the weight average of the micelle molecular weight $M_w(c)$ at four different temperatures: 4.5 °C (■), 9.6 °C (□), 15 °C (●), and 23 °C (○). The solid lines are guides for the eye.

harder with increasing values of M_n. Such soft spheres can be regarded as having an impenetrable spherical core of radius $R_n = (3\upsilon_{nn}/4\pi)^{1/3}$, depending on the density of the outer shell. In any case it is found that R_n is larger than the radius of the dense hydrophobic core and smaller than the one of the whole micelle. This indicates that the positive values of $B_w(c)$ are correctly estimated and that full-grown β-casein micelles can actually be regarded as interacting as soft spheres.

In conclusion, it has been shown that interactions are very important in micellar systems. Close to the CMC, the weight-average interactions have been found to be strongly attractive. This result is satisfactory because it explains well why micelles can form. At higher concentrations or temperatures the interactions are repulsive, and full-grown micelles interact like soft spheres. This is in fair agreement with their particular structure consisting of a relatively dense hydrophobic core surrounded by a hydrophilic shell of much lower density. A careful analysis of the scattering spectra strongly suggests that both attractive and repulsive interactions are present whatever the protein concentration. Finally, it is worth noting that the presence of interactions alters the equilibrium between free monomers and micelles. This point can no longer be neglected. Consequently, further experimental and theoretical work is necessary to collect more structural data close to the CMC, and to explain them.

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[1] Surfactants in Solution, edited by K. L. Mittal and B. Lindman (Plenum, New York, 1984), Vol. 1, 2, and 3.
[2] Proceedings of the International School of Physics Enrico Fermi—Physics of Amphiphiles: Micelles, Vesicles and Microemulsions, edited by V. Degiorgio and M. Corti (North-Holland, The Netherlands, 1985).
[3] W. M. Gelbart, in *Micelles, Membranes, Microemulsions and Monolayers*, edited by W. M. Gelbart, A. Ben-Shaul, and D. Roux (Springer-Verlag, New York, 1994), pp. 1–104.
[4] D. Blankschtein, G. M. Thurston, and G. B. Benedek, J. Chem. Phys. **85**, 7268 (1986).
[5] H. E. Swaisgood, in *Development in Dairy Chemistry*, edited by P. F. Fox (Elsevier, London, 1982), Vol. 1, pp. 1–58.
[6] D. G. Schmidt and T. A. J. Payens, J. Colloid Interface Sci. **39**, 655 (1972).
[7] J. C. Mercier, J. L. Maubois, S. Poznanski, and B. Ribadeau-Dumas, Bull. Soc. Chim. Biol. **50**, 521 (1968).
[8] J. P. Cotton, in *Neutron, X-Ray and Light Scattering*, edited by P. Lindner and T. Zemb (Elsevier, New York, 1991), pp. 3–31.
[9] J. G. Kirkwood and R. J. Goldberg, J. Chem. Phys. **18**, 54 (1950).
[10] J. S. Pedersen and M. C. Gerstenberg, Macromolecules **19**, 1363 (1996).
[11] J. B. Hayter in Ref. [2], pp. 59–93.
[12] P. J. Atkinson, E. Dickinson, D. S. Horne, and R. M. Richardson, J. Chem. Soc. Faraday Trans. **91**, 2847 (1995).