

Small-angle X-ray scattering investigation of the micellar and submicellar forms of bovine casein

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SUMMARY. Small-angle X-ray scattering was performed on whole casein under submicellar (Ca^{2+} removed) and micellar (Ca^{2+} re-added) conditions. Submicellar scattering curves showed two Gaussian components which were interpreted in terms of a spherical particle with two concentric regions of different electron density, a relatively compact core of higher electron density and a looser shell. Normalized scattering curves and calculated distance distribution functions were consistent with this picture. Micellar scattering data, which can yield only cross-sectional information related to a window of scattered intensities, could be analysed by a sum of three Gaussians with no residual function. The two Gaussians with the lower radii of gyration were again taken to indicate the two concentric regions of different electron density of inhomogeneous spherical particles; the third Gaussian was shown to reflect the packing number of these particles within a cross-sectional portion of the micelle, which was 3:1 for this system. These results are a strong indication that submicellar inhomogeneous particles containing hydrophobically stabilized inner cores exist within the colloidal micelle.

The caseins occur in bovine milk as colloidal complexes of protein and salts, commonly called casein micelles. Removal of Ca is thought to result in the dissociation of this micellar structure into noncolloidal protein complexes called submicelles (Schmidt, 1982). These consist of four proteins, α_{s1} , α_{s2} , β , and κ -casein, in the ratios of 4:1:4:1 (Davies & Law, 1980). All are phosphorylated to various extents, have an average monomer molecular weight of 23300, and are considered to have few specific secondary structural features (Farrell & Thompson, 1988). The isolated fractions exhibit varying degrees of self-association, believed to be mostly hydrophobically driven (Schmidt, 1982). Little work has been done on the tertiary and quaternary structure of these proteins in mixed associations in their native state. However, there is hydrodynamic evidence that, in the absence of Ca, casein associates form aggregates with an apparent upper limit of 9.4 nm for the Stokes radius (submicellar form) (Pepper & Farrell, 1982).

It has long been hypothesized that, upon the addition of Ca, these hydrophobically stabilized, self-associated casein submicelles further self-associate via Ca-protein side-chain salt bridges to the colloidal micelles with average radii of 65 nm, as determined by electron microscopy (Schmidt, 1982; Farrell & Thompson, 1988). 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 (Walstra, 1979; Farrell & Thompson, 1988), and to a newer model of a homogeneous sphere with a 'hairy' outer layer (Holt & Dalgleish, 1986).

With this as background, small-angle X-ray scattering (SAXS) was undertaken on whole bovine casein in the absence of Ca to ascertain whether limiting polymers (submicellar structures) exist, and in the presence of Ca to determine if the colloidal micelle consists of discrete submicellar particles with a particular packing structure or of a non-specific, unordered, gel-like structure.

MATERIALS AND METHODS

Sample preparation

Warm milk (2 l) was obtained from the whole milk of an individual healthy Jersey cow in mid-lactation. Phenylmethylsulphonyl fluoride (0.1 g/l) was added immediately to retard proteolysis. The milk was transported to the laboratory and skimmed twice by centrifugation at 4000 g for 10 min at room temperature. A portion (500 ml) was diluted with an equal volume of distilled water and warmed to 37 °C. Casein was precipitated by careful addition of 1 M-HCl to pH 4.6. The precipitate was homogenized with a Polytron ST-10 homogenizer at low speed and dissolved by addition of NaOH to yield a solution of pH 7.0. (Reference to brand or firm name here and in the following does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.) The casein was reprecipitated, washed, and then resuspended. The sodium caseinate was subsequently cooled to 4 °C, centrifuged at 10000 g for 30 min to remove residual fat, and then freeze dried. Alkaline urea gel electrophoresis with standard caseins of known structure (Thompson, 1964) showed the following genotype of the animal: α_{s1} -BB, β -AA, and κ -BB.

The lyophilized caseinate was dissolved in PIPES-KCl buffer (25 mM piperazine-N-N'-bis(2-ethanesulphonic acid), pH 6.75, made to be 80 mM in KCl). Dithiothreitol (0.1 mM) was added to promote self-association of κ -casein (Pepper & Farrell, 1982). The samples were stirred for 3 to 5 min, then passed through a 0.44 μ m filter; blanks were treated in the same fashion. To produce casein micelles, CaCl₂ was added to a final concentration of 10 mM. Alternatively, to produce submicelles, KCl was added to a final concentration of 30 mM (ionic strength comparable to the added CaCl₂). Casein concentrations were determined spectrophotometrically on samples diluted 1/50 to 1/100 in 0.1 M-NaOH; an absorptivity of 0.850 ml mg⁻¹ cm⁻¹ at 280 nm was used for whole casein (Pepper & Farrell, 1982). Protein concentrations were 19.38, 10.77, and 9.19 mg/ml for the submicellar form and 16.4, 12.2, and 4.68 mg/ml for the micellar form.

SAXS measurements

All SAXS measurements were made on a Kratky camera (Anton Paar, Graz) with Ni-filtered Cu-K α radiation (Pilz *et al.* 1979), equipped with a one-dimensional position-sensitive detection system with pulse-height discrimination (Technology for Energy Corporation, Knoxville). Absolute intensities were obtained by means of a standard Lupolen (polyethylene) platelet sample of known scattering power as a secondary standard. A Paar sample cell with mica windows and a 1 mm path length was used throughout. All measurements were made at room temperature.

Data evaluation

The working equations and the notation used have been previously described (Luzzati *et al.* 1961*a*; Pessen *et al.* 1973). Here, ρ_1 , the electron density of the solvent, was calculated as $0.355 \text{ e}^-/\text{\AA}^3$; ψ_2 , the electron partial specific volume of the solute, equals $\bar{v}/q = 2.329 \text{ \AA}^3/\text{e}^-$, where \bar{v} is the partial specific volume of the protein, 0.736 ml/g (Farrell & Thompson, 1988), and q is the number of electrons per g of particle, 0.316×10^{24} , both calculated from the amino acid composition (Eigel *et al.* 1984). Tabulated parameters, when derived from concentration-dependent variables, were obtained by extrapolation to zero concentration. Slit-smear curves were deconvoluted by the use of a computer program due to Lake (1967).

The customary Guinier plot (Guinier & Fournet, 1955) was not used for calculation of the SAXS results because of the nonlinearity of the plot for the casein micelle. To avoid the need for personal judgment regarding the linear portions of a plot, all data were fitted by multiple Gaussian functions by the use of a Gauss-Newton nonlinear-regression computer program developed at this laboratory. The rationale for the use of nonlinear regressions and for their analysis by the criterion of randomness of residual plots has been thoroughly explored by Meites (1979).

RESULTS

The shapes of the scattering curves when plotted in the usual Guinier form as $\ln(\text{scattered intensity}) \nu. (\text{scattering angle})^2$ show qualitative differences between the submicellar and the micellar protein. The curve for the submicelle shows two linear regions, that for the micelle three. Fitting of these linear portions is a matter for which good objective criteria are lacking. For this reason, the data were analysed by nonlinear regression using multiple Gaussian functions. The submicellar casein data could best be fitted by a sum of two Gaussian functions, as seen in Fig. 1*a*. Also plotted on the same graph is the contribution of each of the two Gaussians. The micellar casein data, on the other hand, required a sum of three Gaussians (Fig. 1*b*) for the best fit. The quality of the analysis is indicated by the standard error for each parameter in Table 1, by the root-mean-square for the fit, and by the randomness of residuals. Residual plots for a single Gaussian (not shown), on the other hand, were not random. The smallest Gaussian, labelled 'A' in each instance, is so broad as to resemble a straight line, but serves to eliminate the need for a baseline which would be incompatible with the requirement that scattered intensities must approach zero for large angles.

Submicelles

The submicellar data were interpreted by means of a model in which the particle has two regions of different electron densities with the same scattering centre. In this model, the scattered amplitudes rather than the intensities of the two regions must be added because of interference effects of the scattered radiation. Luzzati *et al.* (1961*b*) have developed a series of expressions for calculating the molecular and structural parameters of such a particle. In the following, subscripts C and L pertain to the parameters for the higher and lower electron density regions, respectively, while subscript 2 designates the particle including both regions. With these expressions, the molecular and structural parameters for casein under submicellar condition were evaluated for all three concentrations of protein used in this study, as listed in Table 1.

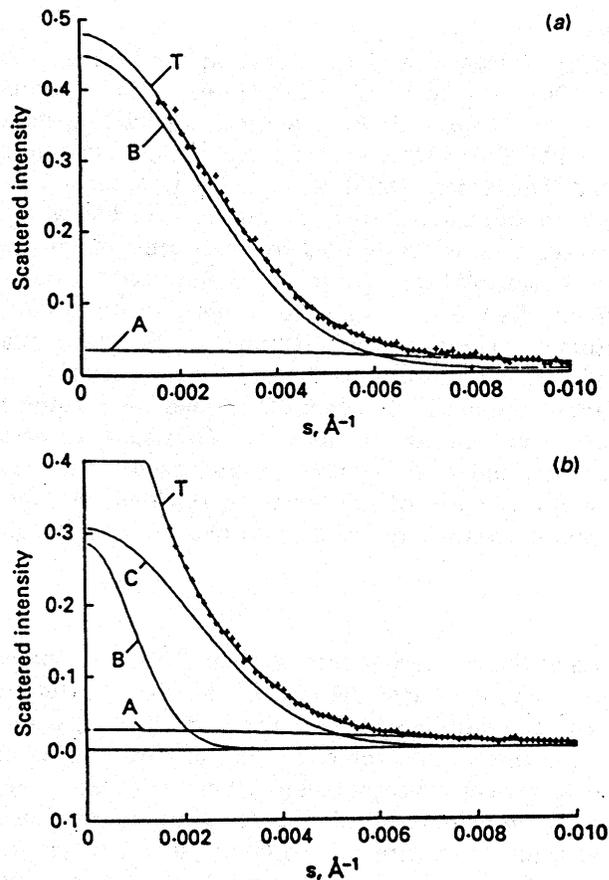


Fig. 1. (a) SAXS of submicelles. +, Smeared SAXS at 19.38 mg/ml; curve T, best fit for sum of two Gaussians by nonlinear regression; curves A and B, two separate Gaussians. (b) SAXS of micelles. +, Smeared SAXS at 16.4 mg/ml; curve T, best fit for sum of three Gaussians by nonlinear regression; curves A, B, and C, three separate Gaussians.

Micelles

For the SAXS results on the casein micelle solutions, i.e. with the addition of 10 mM-CaCl₂, the same procedure was used for analysing the two Gaussians having the lower radii of gyration; these constitute the contribution of the submicellar structure to the SAXS results. The third Gaussian, which has the highest radius of gyration, reflects the total number of submicellar particles within the cross-sectional scattering profile. Here, at zero angle, the intensity of the larger Gaussian contribution can be simply added to the intensity of submicellar contribution. A new parameter, k_2 , the ratio of the mass of the submicelles to the total observed mass ascribable to a cross section, can be expressed in terms of the radii of gyration and the zero-angle intercepts for the three Gaussians. The packing number, the reciprocal of k_2 , is the number of submicellar particles found within a micellar cross section. The meaning to be ascribed to the cross section in this context will be discussed further below.

The resulting parameters for the micelle are listed in Table 1, where subscript 2 now designates the corresponding parameters for a submicellar particle when incorporated in the micelle, and unsubscripted parameters refer to the total cross section of the colloidal particle.

Table 1. Molecular and structural parameters* (desmeared SAXS)

Parameter	Submicelle	Micelle**
M	—	882000 ± 28000
k_2	—	0.308 ± 0.005 (3.2:1)
M_2	285000 ± 14600	276000 ± 18000
k	0.212 ± 0.028	0.216 ± 0.003
M_C	60000 ± 5650	56400 ± 3700
M_L	225000 ± 18500	220000 ± 18700
$\Delta\rho$, e ⁻ /nm ³	—	8.1 ± 0.4
$\Delta\rho_2$, e ⁻ /nm ³	9.9 ± 0.4	7.3 ± 0.5
$\Delta\rho_C$, e ⁻ /nm ³	14.8 ± 1.4	12.8 ± 0.7
$\Delta\rho_L$, e ⁻ /nm ³	9.1 ± 0.3	6.5 ± 0.3
H , g _{water} /g _{protein}	—	7.92 ± 0.42
H_2 , g _{water} /g _{protein}	6.31 ± 0.30	8.98 ± 0.44
H_C , g _{water} /g _{protein}	3.97 ± 0.48	4.70 ± 0.31
H_L , g _{water} /g _{protein}	6.90 ± 0.64	9.95 ± 0.58
V , nm ³	—	12720 ± 250
V_2 , nm ³	3330 ± 260	4440 ± 160
V_C , nm ³	467 ± 2	529 ± 3
V_L , nm ³	2860 ± 400	3910 ± 30
R_2 , nm	8.024 ± 0.039	9.057 ± 0.003
R_C , nm	3.798 ± 0.001	3.962 ± 0.001
R_L , nm	8.822 ± 0.082	10.019 ± 0.009

* Averages of three concentrations (see under Materials and Methods).

** For caution in the interpretation of some of these values, see Discussion.

DISCUSSION AND CONCLUSIONS

Submicelles

The molecular weight found for the submicellar particle, M_2 , was 285 000 ± 14 600. Both M_2 and k , the mass fraction of the denser or 'core' region, were independent of protein concentration, ruling out an explanation of the multiple Gaussian character of the scattering as due to extreme particle size polydispersity (Kratky, 1963; Pessen *et al.* 1973). Extreme particle asymmetry (e.g. rods) can be ruled out from electron-microscopic, hydrodynamic, and light scattering evidence indicating approximately spherical particles (Schmidt & Payens, 1976; Schmidt, 1982). Hence, the molecular parameters given in Table 1 are a measure of the limiting aggregate of the hydrophobically driven self-association of the mixed caseins in the absence of Ca. The molecular weight of this limiting polymer, M_2 , is in agreement with those found by a variety of techniques (Schmidt, 1982), i.e. 200 000–300 000. The value of 285 000 is also consistent with the value of 300 000 obtained from small-angle neutron scattering (Stohtart & Cebula, 1982).

In the latter work, the data were analysed on the basis of a model consisting of a homogeneous limiting aggregate. Here, we have found a heterogeneous particle consisting of two regions of differing electron density, with the mass fraction of the higher electron density region, k , equal to 0.212 ± 0.028. This denser region, moreover, has an electron density difference, $\Delta\rho_c$, of 14.8 ± 1.4 e⁻/nm³, a hydration, H_c , of 3.97 ± 0.48 g water/g protein, and a molecular weight, M_c , of 60 100 ± 5650 (see Table 1). The region of higher electron density most likely results from the intermolecular hydrophobically driven self-association of the casein monomer units (Schmidt, 1982), since a hydrophobic inner core would be protected from interactions with water (Tanford, 1961; Kuntz & Kauzmann, 1974) by a less electron-dense region presumably consisting mainly of hydrophilic groups. The hydration value formally ascribed to this core is likely to arise from the packing density (Richards,

1974; Lumry & Rosenberg 1975) of the hydrophobic side chains rather than the actual amount of water within this region. The electron density of the denser region is still exceedingly low ($14.8 \text{ e}^-/\text{nm}^3$) compared to compact globular proteins (Pessen *et al.* 1988), such as lysozyme ($78 \text{ e}^-/\text{nm}^3$), α -lactalbumin ($67 \text{ e}^-/\text{nm}^3$), ribonuclease ($71 \text{ e}^-/\text{nm}^3$), or even the phosphoglycoprotein, riboflavin-binding protein ($56 \text{ e}^-/\text{nm}^3$, in its acid-denatured form), emphasizing the consequences of the random conformation of the casein polypeptide chains.

Axial ratios calculated from the appropriate parameters for the submicelle as well as for its denser core confirm the approximate spherical symmetry of the casein submicelle predicted from electron microscopy and other physical techniques (Schmidt, 1982).

Micelles

As previously reported (Schmidt, 1982; Farrell & Thompson, 1988), addition of 10 mM-CaCl₂ to casein submicelles causes aggregation of the protein to colloidal particles called casein micelles. Whether the integrity of the submicellar structure is maintained within the colloidal micelle has been a subject of much controversy (Walstra, 1979). To address this problem, the scattering of whole casein solutions with 10 mM-CaCl₂, but without phosphate buffer to compete with the protein-Ca binding sites, was studied. As seen in Table 1, k_2 for casein micelles was 0.308 ± 0.005 , and the packing number, its reciprocal, was 3.2. Because of the large average radius of the micelles (65 nm), information pertaining to the total particle cannot be obtained from SAXS, since for a particle of this size sufficiently low scattering angles are not experimentally accessible, so that one can observe only a cross-sectional portion of the colloid, with molecular weight, M , of 882000 ± 28000 , an electron density difference, $\Delta\rho$, of $8.1 \pm 0.4 \text{ e}^-/\text{nm}^3$, a hydration, H , of $7.92 \pm 0.42 \text{ g water/g protein}$, and a volume, V , of $(12.72 \pm 0.25) \times 10^3/\text{nm}^3$. Since, by contrast, molecular weights of whole casein micelles have been reported to range from 0.5 to 1×10^9 (Schmidt & Payens, 1976), of the cross-sectional parameters only the hydration can be compared with literature values. Our result of 7.92 is somewhat larger than the largest value reported by small-angle neutron scattering (4.0–5.5, Stothart & Cebula, 1982). Other reported values have ranged from 2 to 7, depending upon the method employed (Walstra, 1979).

A note of caution is in order regarding the use of the micellar parameters, other than the hydration. As already mentioned, these do not refer to the entire particle but only to a sample portion which is restricted in size by a window of scattered intensities bounded by the lower small-angle limit of observation. They do not bear a readily defined relationship to the corresponding, but inaccessible, parameters applicable to the entire micellar particle, and therefore cannot be used to derive values for the latter. Nonetheless, the cross-sectional parameters are of value in affording an internal view of the micellar structure, which was the aim of this investigation.

The crucial comparison is between the molecular and structural parameters of the casein submicellar structure by itself (column two, Table 1) and within the casein micelle (column three). Within experimental error, M_2 , k , M_C and M_L are the same, but a substantial decrease is observed in $\Delta\rho_2$ and $\Delta\rho_L$. H_2 , H_L , V_2 , V_L , R_2 , and R_L increase; $\Delta\rho_C$ and V_C increase slightly; and H_C and R_C for independent submicelles and for submicelles within the micellar structure are essentially the same. From the changes in these parameters it appears likely that the large swelling and hydration in the loose region is due to Ca²⁺ binding to protein electrostatic groups within this

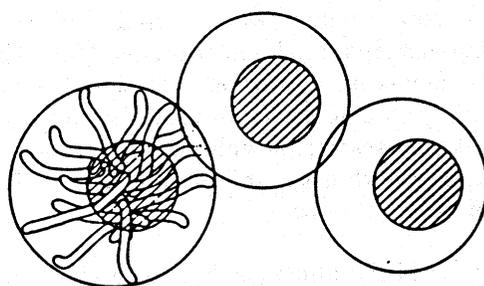
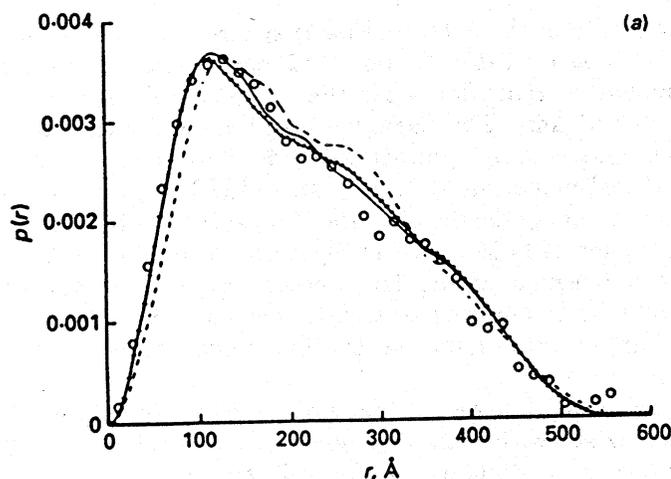


Fig. 2. (a) Distance distribution of micelles. \circ , $p(r)$ v. r for micellar casein at 16.4 mg/ml and 10 mM- CaCl_2 ; ticked line, theoretical for three inhomogeneous spheres at coordinates (0, 0), (350, 0) and (180, 100) with same outer radii (10.2 nm), and inner radii (5.0 nm); dashed line, theoretical for three inhomogeneous spheres with same outer radii in a symmetrical triangular arrangement; solid line, theoretical for three inhomogeneous spheres with two different outer radii at coordinates (0, 0; 12.5 nm), (350, 0; 10.2 nm) and (180, 0; 10.2 nm.) Theoretical curves were calculated by the method of Glatter (1980). (b) Schematic representation of submicelles in micellar cross section, corresponding to solid line in Fig. 2a. Cross-hatched area, approximate region of higher concentration of hydrophobic side chains and higher electron density: in lower left particle, a few representative monomer chains are indicated.

region. The relatively small changes in the density and radius of gyration of the internal core region on addition of calcium suggest strongly that this region consists mainly of a hydrophobically rich environment. Thus, the binding of Ca^{2+} to submicelles and the subsequent incorporation of submicelles into micelles does not produce more compact structures; the low electron density of the independent submicelle persists or decreases in the micelle.

To ascertain the spatial arrangement of the three spheres within the observed cross-sectional scattering volume, the distance distribution function, $p(r)$, was calculated from the SAXS data for casein micelles as shown in Fig. 2a. Calculation of the radius of gyration from the second moment of the $p(r)$ data (Pilz *et al.* 1979; Glatter, 1980) in Fig. 2a, to the maximum diam of 51.2 nm, yielded a value of 17.52 nm.

The experimental $p(r)$ results in Fig. 2a were then compared with theoretical curves calculated by the method by Glatter (1980), using various geometrical

models. For these, the radii of the outer and inner spheres, calculated from V_2 and V_C values of column three of Table 1, were 10.2 and 5.0 nm, respectively. The equilateral or symmetrical triangular arrangement gave the poorest fit to the experimental data (dashed line). The Cartesian coordinates for the centres of the three inhomogeneous spheres most compatible with the experimental data were found at non-symmetrical values of (0, 0), (35, 0), and (18, 10) (ticked line), but a better fit resulted from changing the radius of the (0, 0) sphere to 12.5 nm (solid line). In fact, a radius of gyration of 17.45 nm is calculated from the theoretical $p(r)$ curve for this inhomogeneous, irregular, triangular structure, in excellent agreement with the value of 17.52 nm found from the experimental $p(r)$ data. It is notable that these best-fit coordinates imply interdigitation of the 'loose' regions of the three submicelles (Fig. 2b).

It may be concluded from these results that a discrete hydrophobically stabilized submicellar structure exists within the colloidal casein micelle, arguing against models predicting either a continuous, porous gel structure or an impenetrable homogeneous sphere with a 'hairy surface' (Holt & Dalgleish, 1986; Walstra, 1979). The submicellar particles consist of an inner, spherically symmetrical, hydrophobic, and relatively electron-dense core, surrounded by a hydrophilic and less electron-dense region, both much less dense than globular proteins. Upon the addition of Ca, the loose region swells with increased hydration and significantly lower electron density, which may be caused by Ca binding to hydrophilic groups within this region. Calculations of the cross-sectional scattering volume support a packing density of about .3 to 1 for the submicelles within the micelle and indicate some interaction between the loose regions of adjacent submicelles.

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