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Self-Assembly of Bovine β-Casein below the Isoelectric pH

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β-Casein is an intrinsically unstructured amphiphilic protein that self-assembles into micelles at neutral pH. This paper reports that β-casein self-organizes into micelles also under acidic conditions. The protein association behavior and micelle characteristics at pH 2.6, well below the p*I*, are presented. The pH was found to strongly affect the micelle shape and dimensions. Cryogenic transmission electron microscopy (cryo-TEM) experiments revealed disk-like micelles of 20–25 nm in length and ∼3.5 nm in height in acidic conditions. An aggregation number of 6 was determined by sedimentation equilibrium under these conditions. Isothermal titration calorimetry experiments verified the association below the p*I* and allowed determination of the micellization enthalpy, the critical micellar concentration, and the micellization relative cooperativity (MR). Small-angle X-ray scattering results at concentrations below the critical micellization concentration (CMC) suggest that the monomeric protein is likely in a premolten globule state at low pH. Calculations of the protein charge at acidic and neutral pH reveal a similar high net charge but considerable differences in the charge distribution along the protein backbone. Overall the results show that β-casein is amphiphilic at low pH, but the distribution of charge along the protein chain creates packing constraints that affect the micelle organization, leading at concentrations above the CMC to the formation of disk micelles.

KEYWORDS: β-casein; micellization; self-assembly; acidic pH; cryo-TEM; ITC

INTRODUCTION

β-Casein is a 24 kDa calcium-sensitive phosphoprotein displaying self-assembly behavior. Recently, it has been classified as an "intrinsically unstructured/disordered" protein (IUP) (*1*). Members of this family are characterized by a distinct amino acid composition. High charge and low hydrophobicity lead to an open conformation and lack of tertiary structure. High proline content further promotes extended conformations by being incompatible with protein secondary structures and decreasing the degrees of freedom of the polypeptide backbone. Disulfide bonds, which cross-link and constrain the polypeptide, are generally absent (*2*).

Whereas most IUPs lack hydrophobic regions, above the isoelectric pH (p*I*) β-casein contains a large, hydrophobic C-terminal domain. Because it also has a polar, negatively charged N-terminal domain, β-casein is amphiphilic (*3*). Therefore, in contrast to typical IUPs that remain in a monomeric state in solution, β-casein self-assembles at physiological conditions into micelles.

Studies related to caseins have generally been performed under physiological conditions, where stable milk casein micelles exert biological functions (*1, 4–11*). Similarly, the self-assembly of β-casein into micelles was mostly investigated above the p*I*, in the pH range of 6.5–7.0. The association mechanism and its mode of cooperativity, as well as the structure of the protein monomers and the morphology of the micelles, were studied by numerous spectroscopic, scattering, and microscopy techniques (*4, 5, 11–14*) and found to be strongly affected by temperature and, to a lesser degree, by pH, concentration, ionic strength, and solvent composition (*6, 9, 11*).

This paper examines, for the first time, the physicochemical characteristics of β-casein at low pH and its association behavior into stable micelles in acidic environment. The study aims to achieve more insight into the distinct properties of β-casein as a self-assembling unstructured protein.

Our study shows that β-casein associates into stable micelles at low pH. The self-assembly process and structural characteristics of the monomers and the micelles in acidic conditions, below the protein p*I*, were studied by several techniques. The shape and dimensions of the micelles were characterized by

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Table 1. Parameters of β-Casein Micellization as a Function of pH and Ionic Strength at 24 °C

system characteristics			CMC			
pH	ionic strength	ΔH_{demic}, kJ/mol	mM	mg/mL	MR, mM	N_{agg}
2.6	0.002	−17.9	0.079	1.89	0.14	∼6
7.0	0.02	−18.58	0.094	2.26	0.11	ND[a]
7.0	0.1	−40.53	0.039	0.98	0.06	∼20

[a] Not determined.

cryogenic transmission electron microscopy (cryo-TEM). The critical micellar concentration (CMC), the relative cooperativity of the association process, and the enthalpy of micellization were determined by isothermal titration calorimetery (ITC). The micellar aggregation number (N_{agg}) was calculated from sedimentation equilibrium experiments, and the monomer conformation was determined from small-angle X-ray scattering (SAXS) measurements. The experiments were performed at 24 °C in dilute lactic acid solution or aqueous HCl solution at pH 2.6 and a low ionic strength (IS) of 0.002. Our findings at low pH were compared with the characteristic behavior of β-casein under physiological conditions (neutral pH). The effect of ionic strength on the protein assembly process at neutral pH is also studied.

EXPERIMENTAL PROCEDURES

Materials. Bovine β-casein (>99%; Sigma-Aldrich) was dissolved in diluted lactic acid solution and the pH was adjusted to 2.6. Additional solutions were prepared in pH 7.0 phosphate buffer containing 5.65 mM Na_2HPO_4 and 3.05 mM NaH_2PO_4 (buffer A) and in buffer A containing 0.08 M NaCl (buffer B). All compounds were from Merck. The lactic acid solution and buffer A were characterized by low ionic strengths of 0.002 and 0.02, respectively. The ionic strength of buffer B was 0.1 (**Table 1**). Each protein solution was filtered through a porous membrane of 0.45 μm to avoid large protein aggregates. Protein solutions were prepared at concentrations ranging from 0.1 to 20 mg/mL (0.0041−0.83 mM). The protein concentration was determined from absorbance at 280 nm using an Ultrospec 2000 UV−visible spectrophotometer (Pharmacia Biotech), using an extinction coefficient of $4.6_{(1\%)}$ (*9*).

Methods. *Cryo-TEM.* Specimens were prepared in the controlled environment vitrification system (CEVS) (*15*) at 24 °C and 100% relative humidity to avoid loss of volatiles. First, the solutions were incubated in the CEVS at the desired temperature for 1 h. Then, a 7 μL drop of each solution was placed on a TEM copper grid covered with a perforated carbon film (Pelco International) and blotted with filter paper to form a thin liquid film of the sample (100–200 nm thick). The thinned sample was plunged into liquid ethane at its freezing temperature (−183 °C) to form a vitrified specimen and then transferred to liquid nitrogen (−196 °C) for storage. The vitrified specimens were examined in a Philips CM120 transmission electron microscope operating at an accelerating voltage of 120 kV. We used an Oxford CT3500 (Oxford Instruments) cryoholder that maintained the specimens below −175 °C during sample transfer and observation. Images were recorded digitally on a cooled Gatan MultiScan 791 CCD camera using DigitalMicrograph 3.1 software (Gatan) in the low-dose imaging mode to minimize beam exposure and electron-beam radiation damage (*16*).

ITC. ITC measurements were performed with a VP-ITC calorimeter (MicroCal) at a temperature of 24 °C. The reaction cell (V = 1.43 mL) was filled with degassed solvent (lactic acid at pH 2.6, or phosphate buffer at pH 7.0). The injector-stirrer syringe (289 μL) was loaded with a β-casein micellar solution (20 mg/mL). The micellar solution was injected into the reaction cell in 28 steps of 10 μL aliquots each, and the heat flow was measured. During the titration, the stirring speed was 310 rpm. The duration of each injection was 20 s, and the equilibration time between consecutive injections was 3 min. Such an interval was sufficient to equilibrate the reaction cell after every injection. Each experiment was performed at least three times. Calorimetric data analysis was carried out using Origin 5.0 software (MicroCal).

Analytical Ultracentrifugation. Sedimentation equilibrium experiments were performed at 24 °C using a Beckman Optima XL-A (Palo Alto, CA) analytical centrifuge at 6000, 10000, and 12000 rpm for the low-pH solutions and at 4000, 6000, and 8000 rpm for the pH 7.0 solutions. Data were collected at 280 nm. The β-casein solutions were studied at concentrations ranging from 0.2 to 10.0 mg/mL at pH 2.6 and from 0.2 to 2 mg/mL at pH 7.0 and an ionic strength of 0.1. Past studies showed that the protein self-assembly is not affected by pressure and, therefore, it is not speed-dependent (*7, 17*).

The average apparent molecular weight of the micelles $\bar{M}_{w,app}$ at the various protein concentrations was calculated from the expression

$$\overline{M}_{w,app} = \frac{d\ln(c)}{dr^2}\frac{2RT}{\omega^2(1-\bar{\nu}\rho)} \quad (1)$$

where c is the concentration (assumed to be proportional to absorbance) at radius r, ω the angular velocity (radians), T the temperature in K, and R the gas constant (g cm^2/gmol min^2 K). The partial specific volume $\bar{\nu}$ of the solute was taken to be 0.742 cm^3/g^3, and a solution density ρ of 1.0044 g/cm^3 was measured. At β-casein concentrations of 2 mg/mL and above under low pH conditions, the plot of the natural logarithm of the measured absorbance versus the square of the radius from the axis of rotation was not linear. To estimate N_{agg}, the limiting slope toward the outer edge of the sample cell was used to provide $d\ln(c)/dr^2$. The molecular weight calculated using this slope was divided by the monomer molecular weight calculated from the β-casein amino acid sequence (24000).

SAXS. SAXS data were obtained using a slit collimated Kratky camera (A. Paar, Graz, Austria) with a one-dimensional sensitive detector (Ni-filtered, Cu Kα radiation, operating at 40 kV and 25 mA). The wave vector h is defined as

$$h = \frac{4\pi \sin\theta}{\lambda} \quad (2)$$

where λ is the wavelength (λ = 0.154 nm) and 2θ the scattering angle. Samples were placed in the camera within a ∼2 mm glass capillary. Experiments were performed in a vacuum of 5×10^{-2} Torr at 24 ± 0.1 °C.

The scattering intensity $I(h)$ was normalized with regard to time, solid angle, first beam intensity, capillary thickness, transmission, and Thompson factor, and the scattering from the solvent and empty capillary were subtracted. Long exposures of 44 h were needed to obtain accurate data and good statistics.

Statistical Analysis. For each of the methods applied here, a statistical analysis of the data was performed, based on at least three separate replicate experiments. The standard error of the ITC data was found to be no more than 5% for the CMC and MR values and no more than 3% for ΔH_{demic}. The standard error of the analytical ultracentrifugation data is 5%, and that of the Rg is 4%. The analysis supports the statistical significance and validity of the results.

RESULTS AND DISCUSSION

Cryo-TEM. Cryo-TEM is becoming a central technique in the study of micellar assemblies and nanoparticles in solution because it provides the morphology and dimensions of the particles, directly, at high resolution, and in their native (hydrated) state. In the present study, cryo-TEM provided two major findings. First, it revealed that β-casein self-assembles into micelles below the p*I*. Second, it showed that these micelles have a disk-like shape, a morphology that is rather unique among self-assembling amphiphiles, whether surfactants, block-copolymers, or proteins. As an example, **Figure 1** shows the micelles in diluted lactic acid solution at pH 2.6. These micelles have a round cross-section 20–25 nm in diameter and a width of 3–4 nm. The vitrified ice thickness (150–200 nm) in the field of view is an order of magnitude larger than the micelle size;

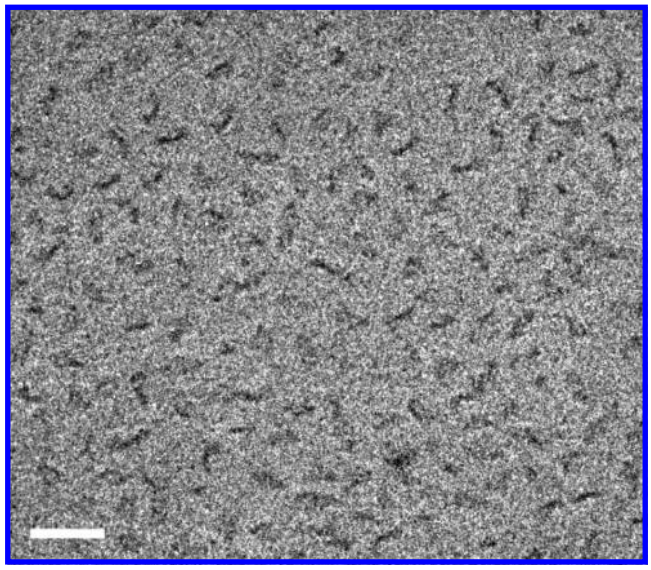

Figure 1. Cryo-TEM image of 20 mg/mL β-casein micelles at 24 °C, pH 2.6, and low ionic strength in lactic acid solution. Bar = 50 nm.

hence, the micelles are distributed in the film randomly, without a preferred orientation. From the many projections seen in the image, it is clear that the micelles are uniform. We further found that their shape and dimensions, at least within the range of concentration studied (10−40 mg/mL), are independent of concentration. Flat disk assemblies of similar dimensions also form by β-casein in HCl solution at the same temperature and pH in the absence of salt (low IS, not shown), suggesting that the anion type and its interaction with the protein do not significantly affect the morphology of the micelles or their dimensions.

As mentioned above, the self-organization of β-casein into micelles under physiological conditions has been widely studied. Indeed, scattering techniques (*13, 14*) and cryo-TEM (*18*) showed that at room temperature in the pH range of 6.5–7.0, in phosphate buffer as well as in water, β-casein self-assembles into oblate micelles with a diameter of ∼13 nm. We further showed by cryo-TEM that under those conditions the micelles have a nonuniform packing (*18*), in contrast with the uniform appearance of the micelles in the acidic environment (**Figure 1**). To better understand the organization of β-casein at low pH into disk-like assemblies, we calculated the net charge and its distribution along the protein backbone at pH 2.6. We also studied the monomer conformation and the self-organization characteristics (CMC, mode of self-assembly, cooperativity) of β-casein by ITC, analytical ultracentrifugation, and SAXS, as described below.

ITC. ITC is a sensitive method, directly providing both the heat of demicellization ($\Delta H_{mic} = -\Delta H_{demic}$) and the CMC in a single experiment (*18, 19*). We previously used this technique to characterize the ΔH_{mic} and the CMC of β-casein in phosphate buffer (pH 7.0) and ionic strength of 0.1. Data analysis also provided the relative cooperativity, MR, which defines the protein concentration range over which the micellization process takes place (*18*). MR depends on temperature, pH, and ionic strength. Its increase and decrease are indicative of decrease and increase of the cooperativity, respectively.

In a typical demicellization experiment, a micellar solution is titrated into buffer placed in the ITC cell, and the heat flow is measured as a function of time. Such an experiment, performed on β-casein at pH 2.6, is presented in **Figure 2A**.

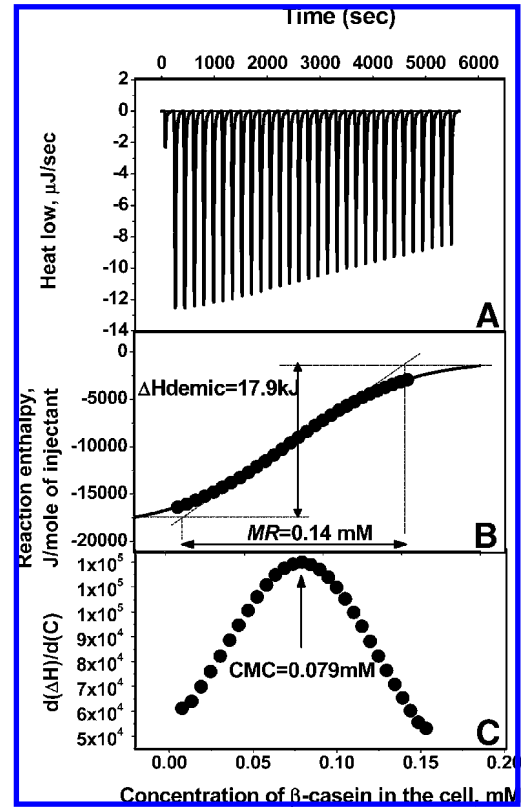

Figure 2. Titration of micellar (20 mg/mL) β-casein solution in diluted lactic acid (pH 2.6) and very low ionic strength (0.002) into lactic acid solution, having the same pH and ionic strength, at 24 °C: (**A**) calorimetric traces; (**B**) reaction enthalpy versus β-casein concentration in the cell; (**C**) first derivative of curve B calculated from the interpolated values.

Three factors contribute to the exothermic enthalpy changes observed at the initial injections: micelle dilution, demicellization, and dilution of individual β-casein molecules. The enthalpy changes decrease in magnitude as more protein is added and the concentration in the ITC cell increases. Eventually (final injections), the concentration in the cell exceeds the CMC and only micelle dilution contributes to the heat flow (*18, 19*).

In **Figure 2B** the heat of the reaction, obtained by integrating the peaks of the individual injections given in **Figure 2A**, is plotted against the β-casein concentration in the cell. A slow increase in the reaction enthalpy is observed, resulting in MR of 0.14 mM (**Figure 2** and **Table 1**), which is more than twice than the value found at pH 7.0 and IS of 0.1 (**Table 1**) (*18*). **Figure 2B** also presents the heat of demicellization, ΔH_{demic}, which equals the enthalpy difference between the two asymptotes (*19*) of the sigmoid fit of the experimental data (obtained by using the Origin software). It is shown that at 24 °C ΔH_{demic} is ∼−17.9 kJ/mol (**Table 1**), relatively small compared with the −40.53 kJ/mol found at pH 7.0 and IS of 0.1 (*18*).

In **Figure 2***C* we present the first derivative of the reaction enthalpy versus the β-casein concentration in the cell. The CMC, obtained from the concentration at which the first derivative of the reaction heat displays a maximum (*19–22*), was determined to be 1.89 mg/mL (**Figure 2C** and **Table 1**) at pH 2.6. This value is approximately twice the CMC found at pH 7 and ionic strength 0.1 (**Table 1**) (*18*).

The ITC measurements presented in **Figure 2** support the cryo-TEM findings; that is, they confirm the self-assembly of β-casein into micelles at acidic pH (2.6) and low ionic strength (0.002). However, the small ΔH_{demic}, the high CMC, and the large MR indicate that the driving forces for micellization under

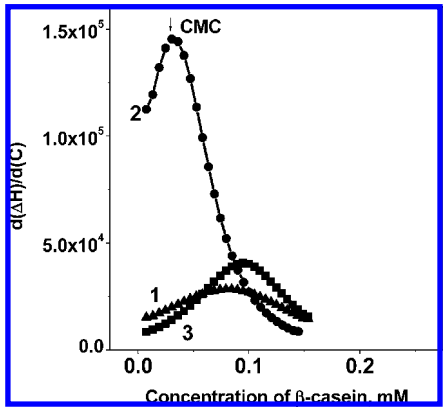

Figure 3. CMC of β-casein at different pH values and ionic strengths: 1, pH 2.6, ionic strength 0.002; 2, pH 7, ionic strength 0.1; 3, pH 7, ionic strength 0.02.

Table 2. Analytical Ultracentrifugation Results of β-Casein Solutions at pH 2.6 and Ionic Strength 0.002[a]

	population a		population b	
concn, mg/mL	$\bar{M}_a$	N_{agg}	$\bar{M}_b$	N_{agg}
0.2	16800	~1	16800	~1
0.7	16200	~1	16200	~1
1.0	14200	~1	26400	~1
2.0	25900	~1	76700	~3
5.0	24000	~1	142600	~6
10.0	26600	~1	135100	~6

[a] The micelle aggregation numbers were obtained by dividing the measured aggregate molecular weight by the monomer molecular weight (24000) calculated from the β-casein amino acid sequence.

acidic conditions are reduced compared with those at physiological pH and high IS (*18*).

To separate the effect of pH from that of IS, we also studied β-casein solutions at pH 7.0 and low ionic strength (0.02). In **Figure 3** we plot the first derivatives of the reaction enthalpies versus the protein concentration for the three compositions studied (see also **Table 1**). From these graphs the CMC values are evaluated. The positions and magnitudes of the peaks of the two low ionic strength solutions (pH 2.6 and 7.0) are almost identical and wider than the peak of the high IS solution. Additionally, their ΔH_{demic} are of similar magnitudes and are lower relative to the sample with high ionic strength. Likewise, the CMC values of the two low ionic strength samples are similar and about twice as high as the CMC of the sample with IS 0.1.

The differences in the micellization parameters of β-casein solutions between low (0.02) and high (0.1) ionic strength (at 24 °C and 20 mg/mL) can be explained by the screening of electrostatic repulsion forces at high IS, which lowers the repulsions between head-groups, thereby enabling micellization at a lower concentration. Therefore, at low IS, ΔH_{demic} has a lower magnitude than at high IS, whereas the CMC and MR are significantly larger. Thus, the larger value of MR at low IS signifies decreased micellization cooperativity.

Sedimentation Equilibrium. To determine the aggregation number of the micelles at pH 2.6, analytical ultracentrifugation experiments were conducted at various protein concentrations (see **Table 2**) and at pH 7.0 and IS of 0.1. Sample data are plotted in **Figure 4**. At concentrations lower than the CMC, determined by ITC to be 1.89 mg/mL (0.079 mM, **Table 1**), a straight line was obtained. The aggregation numbers calculated from the slope of this line and eq 1 confirmed that the protein

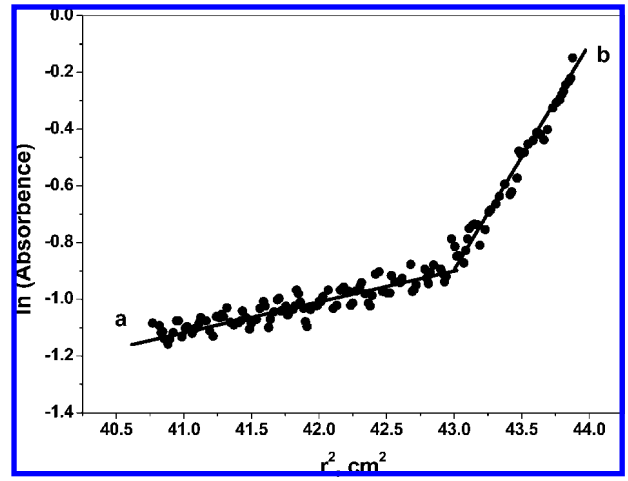

Figure 4. Determination of β-casein aggregation number from the ultracentrifugation data, following eq 1: pH 2.6, protein concentration 5 mg/mL, 0.002 IS.

is monomeric at these concentrations (see **Table 2**). At concentrations higher than the CMC, two regions could be defined, indicating the presence of two protein populations: monomers at relatively short radii (i.e., in **Figure 4** at $r < 6.5$ or $r^2 < 43$) and assemblies at large radii. The two limiting slopes toward the inner and outer edges of the sample cell were used to calculate the minimal and maximal apparent molecular weights, respectively. The micelles at pH 2.6 are characterized by a small aggregation number of 3 around the CMC and 6 at higher concentrations (**Table 2**). In contrast, using the same technique we measured N_{agg} of 20 at pH 7.0 and ionic strength 0.1, and values of 17–23 were reported in the literature by Evans et al. (*12*) and Mikheeva et al. (*9*) at comparable temperature, pH, and ionic strength. Thus, compared with assembly at neutral pH, assembly at low pH is characterized by two special features: the micelles are flat and disk-like in shape, and they have a low molecular weight. To understand the origin of these properties, we calculated the charge and charge distribution at pH 2.6 and 7.0.

Evaluation of the Net Charge and Charge Distribution of β-Casein at pH 2.6. As mentioned, β-casein has been characterized as an intrinsically unstructured protein. IUPs can exist in a random coil conformation, resembling denatured globular proteins, or in a premolten globule state exhibiting some secondary structure (*2, 23*). Indeed, β-casein is thought to have an open rheomorphic structure at low concentrations. However, in contrast to other IUPs, β-casein is amphiphilic and hence self-assembles into micelles. Micellization processes are driven primarily by hydrophobic interactions, whereas electrostatic and steric repulsive forces oppose the association process and stabilize the formed structures (*9, 11*). In β-casein, close to neutral pH, a high negative charge of −14.8 is concentrated mainly in the first 50 amino acids of the N-terminal domain (**Figure 5A**) (*7, 24, 25*), whereas the C terminus has only a few charged groups and a small net charge and is rich in hydrophobic groups (*7, 24, 25*).

To estimate the net charge and the charge distribution of β-casein at pH 2.6, we used a procedure similar to that reported by Ribadeau et al. (*25*). The degree of protonation, $\theta_{i,A}$ of the individual titratable sites was calculated from the Henderson–Hasselbach equation (*26*). This equation is not valid when strong electrostatic interactions exist between titratable sites but can be used when the protein is in an unfolded state (*27*). Because β-casein is intrinsically unstructured, the equation is appropriate for calculating its net charge at low pH.

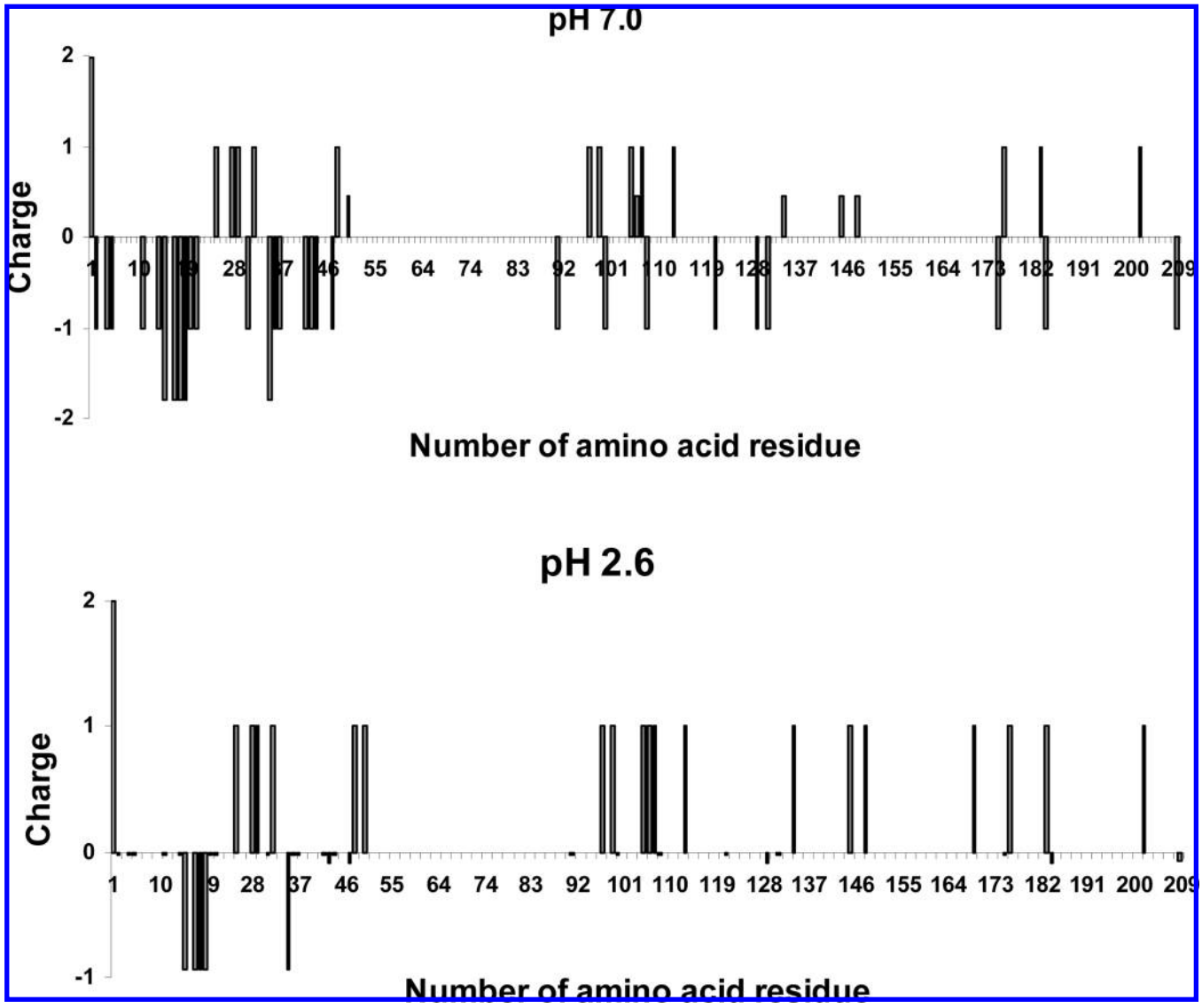

Figure 5. Charge distribution along the β-casein backbone at pH 7 (**A**, top) and pH 2.6 (**B**, bottom).

The average pK_i^A values of the titratable groups (side chains, terminal amino, and carboxyl groups) were taken from Matthews (*27*). **Figure 5** shows that lowering the pH from 7.0 to 2.6 exerts profound changes on the distribution of charges along the backbone of β-casein (**Figure 5B**) and, therefore, on the hydrophobicity and hydrophilicity of the molecule. The net charge at pH 2.6 is +15.8, slightly higher than the absolute value of the net charge at physiological conditions (−14.8). However, in the N-terminal region [amino acids 1–50 (*24*)], there is a drastic lost of net charge at low pH and, hence, considerable decrease in hydrophilicity. Moreover, although the number of charged groups along the large C-terminal region [amino acids 51–209 (*24*)] is decreased at low pH, the overall net charge is higher. Thus, overall, the positive charge at low pH is mainly distributed along the last 100 amino acids, whereas at neutral pH it is concentrated primarily along the first 50 residues.

The increased hydrophobicity of the C-terminal part of β-casein at pH 2.6 favors intermolecular association, whereas the increased net positive charge favors repulsion. These two contradicting forces apparently balance each other when considering the CMC and ΔH_{demic}, but they affect the morphology of the micelles and the aggregation number (**Figure 1** and **Table 1**). Nonetheless, one cannot rule out the possibility that clusters of opposing charges at the N-terminal end at the low pH participate in stabilizing the micelles via electrostatic interactions.

SAXS Measurements. We used SAXS to determine the monomer conformation in the acidic conditions. In this method, the scattered intensity is sensitive to the size and conformation of proteins in solution and is thus well suited to investigate flexible, extended proteins with low compactness such as β-casein (*23, 28*). SAXS experiments were conducted below the CMC at a concentration of 1.5 mg/mL (pH 2.6 and low IS).

The smeared scattering intensity $I(h)$ versus the scattering vector h is shown in **Figure 6A**. A typical exponential decrease was observed at the low h regime. To obtain the structural characteristics of a monomer chain in solution and distinguish between the compact, globular structures of folded proteins and the expanded state of a random coil, a Kratky chart was plotted, presenting the normalized data of $I(h) \times h$ versus the scattering vector, h (*29*). This representation emphasizes the signal at higher scattering angles. In a characteristic Kratky plot, the tightly packed core of a globular protein yields a bell shape with a clear maximum, whereas that of a random coil results in a monotonically increasing curve followed by a plateau (*23*).

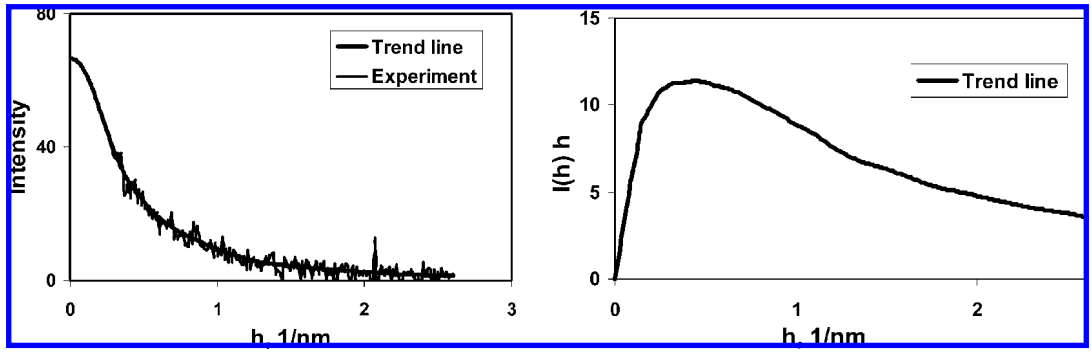

Figure 6. Normalized SAXS $I(h)$ (right) and the corresponding Kratky plot, $I(h) \times h$ (left) versus the wave vector h: protein concentration, 1.5 mg/mL; pH 2.6; and low ionic strength.

A monotonic increase in $I(h) \times h$ versus h followed by a peak and a mild decrease for the 1.5 mg/mL solution (pH 2.6) are shown in **Figure 6B**. According to Longhi and co-workers (*28*), who studied the C-terminal domain of the measles virus nucleoprotein, this intermediate shape between the two characteristic plots of globular and random coil states suggests the presence of an intermediate premolten globule conformation with some residual structure. This interpretation is also in line with the studies of Farrell and co-workers (*30, 31*), which suggested the existence of limited but defined secondary structures in β-casein. Thus, our SAXS findings suggest that under acidic conditions β-casein exists in a premolten globule state.

SAXS measurements can also be used to evaluate the radius of gyration, R_g, estimated from the Guinier approximation (*32*):

$$I(h) = I(0)\, e^{h^2 R_g^2/3} \qquad (3)$$

To increase the accuracy, we evaluated the radius of gyration from the smeared SAXS data using the ITR program package developed by Glatter (*33*).

R_g of β-casein monomers (1.5 mg/mL, pH 2.6, IS 0.002) was found to be 5.50 ± 0.5 nm. This value is nearly identical to the value 5.40 ± 0.3 nm reported at pH 7.0 and IS of 0.1 by Evans et al. (*12*) and only slightly smaller than the value of 5.87 ± 0.2 nm reported in the presence of 4 M guanidyl chloride (at the same pH and IS), which represents the denaturated state of β-casein (*24*). Thus, we find that β-casein at low pH, as in neutral pH, is in a premolten globule conformation and has an R_g close in value to that of the fully denatured state of the protein.

Why Do Disk-Shaped Micelles with Lower Aggregation Number Form in Acidic pH? Berry and Creamer (*34*) confirmed that the main driving force for the endothermic self-assembly of β-casein at pH ∼7 is the hydrophobic C-terminal region. They showed that removal of the last 20 amino acids from the C terminus (β-casein 1–189) destroyed the ability of the protein to associate into micelles at physiological conditions, whereas deletion of segments from the N terminus (β-casein 29–209) did not decrease the ability of the modified protein to self-assemble.

We show that below the p*I*, at pH 2.6, the charge of the N-terminal domain decreases and that of the C-terminal region increases, but at the same time the overall number of charged groups in the C-terminal region decreases (see **Figure 5B**). These contradicting contributions balance each other, leading to association at concentration and enthalpy of similar magnitude as in neutral pH at low IS (**Table 1**). However, the high charge density along the C-terminal portion and the electrostatic repulsion between the monomers (and likely increase in protein backbone rigidity) do not enable changes in the size or shape of the micelles or in the monomer conformation during the early stages of the assembly process as the change proposed by Farrell and co-workers (*7*) at neutral pH.

Conclusions. β-Casein is an intrinsically unstructured protein that, in contrast to other IUPs, self-assembles into detergent-like micelles at physiological conditions. We show here that the ability of β-casein to self-organize into micelles is preserved even at low pH. The charge and its distribution along the protein backbone vary significantly with the pH and strongly affect the protein amphiphilicity and as a result the micelle morphology, dimensions, and aggregation number. Cryo-TEM shows that flat, disk-like micelles form at pH 2.6, compared with the spheroidal micelles that exist at physiological pH. Analytical ultracentrifugation experiments indicate on only 6 monomers per micelle at the low pH, whereas about 20 monomers constitute a micelle at neutral pH. The formation of disk-like micelles can be explained by packing constraints that result from strong intra- and intermolecular repulsion forces between the unscreened charges along the β-casein backbone.

The decrease in the protein amphiphilicity and the spread of charges along the protein backbone lead us to question the validity of applying the block copolymer micellization model [the dual theory by Horne (*8*)] to describe the aggregation process at low pH. Also interesting is the effect of ionic strength on the assembly process and the protein characteristics. We found that the CMC and heat of demicellization were hardly affected by the pH when the IS was low, but they were significantly altered when the IS was increased under pH ∼7. Future studies will test the effect of IS on the assembly.

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LITERATURE CITED

(1) Tompa, P. *Trends Biochem. Sci.* **2002**, *27* (10), 527–533.
(2) Uversky, V. N. *Eur. J. Biochem.* **2002**, *269* (1), 2–12.
(3) Swaisgood, H. E. In *Advanced Dairy Chemistry*; Fox, P., McSweeney, P. L. H., Eds.; Kluwer Academic/Plenum: New York, 2003; pp 139–201.
(4) Arima, S.; Niki, R.; Takase, K. *J. Dairy Res.* **1979**, *46* (2), 281–282.
(5) Buchheim, W.; Schmidt, D. G. *J. Dairy Res.* **1979**, *46* (2), 277–280.
(6) de Kruif, C. G.; Grinberg, V. Y. *Colloid Surf. A: Physicochem. Eng. Asp.* **2002**, *210* (2–3), 183–190.

(7) Farrell, H. M.; Wickham, E. D.; Unruh, J. J.; Qi, P. X.; Hoagland, P. D. *Food Hydrocolloids* **2001**, *15* (4–6), 341–354.
(8) Horne, D. S. *Curr. Opin. Colloid Interface Sci.* **2002**, *7* (5), 456–461.
(9) Mikheeva, L. M.; Grinberg, N. V.; Grinberg, V. Y.; Khokhlov, A. R.; de Kruif, C. G. *Langmuir* **2003**, *19* (7), 2913–2921.
(10) Niki, V. R.; Takase, K.; Arima, S. *Milchwiss.−Milk Sci. Int.* **1977**, *32*, 577–582.
(11) O'Connell, J. E.; Grinberg, V. Y.; de Kruif, C. G. *J. Colloid Interface Sci.* **2003**, *258* (1), 33–39.
(12) Evans, M. T. A.; Phillips, M. C.; Jones, M. N. *Biopolymers* **1979**, *18* (5), 1123–1140.
(13) Kajiwara, K.; Niki, R.; Urakawa, H.; Hiragi, Y.; Donkai, N.; Nagura, M. *BBA−Proteins Struct. Mol. Enzymol.* **1988**, *955* (2), 128–134.
(14) Thurn, A.; Burchard, W.; Niki, R. *Colloid Polym. Sci.* **1987**, *265* (8), 653–666.
(15) Bellare, J. R.; Davis, H. T.; Scriven, L. E.; Talmon, Y. *J. Electron Microsc. Technique* **1988**, *10* (1), 87–111.
(16) Danino, D.; Bernheim-Groswasser, A.; Talmon, Y. *Colloid Surf. A−Physicochem. Eng. Asp.* **2001**, *183*, 113–122.
(17) Payens, T. A. J.; Brinkhui, J. A.; Vanmarkw, B. W. *BBA−Protein Struct.* **1969**, *175* (2), 434–437.
(18) Portnaya, I.; Cogan, U.; Livney, Y. D.; Ramon, O.; Shimoni, K.; Rosenberg, M.; Danino, D. *J. Agric. Food Chem.* **2006**, *54*, 5555–5561.
(19) Paula, S.; Sus, W.; Tuchtenhagen, J.; Blume, A. *J. Phys. Chem.* **1995**, *99*, 11742–11751.
(20) Dai, S.; Tam, K. C. *Langmuir* **2004**, *20*, 2177–2183.
(21) Moroi, Y. *Micelles: Theoretical and Applied Aspects*; Moroi, Y., Ed.; Plenum: New York, 1992; pp 47–50.
(22) Tanford, C. *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*, 2nd ed.; Wiley: New York, 1980.
(23) Receveur-Brechot, V.; Bourhis, J. M.; Uversky, V. N.; Canard, B.; Longhi, S. *Proteins* **2006**, *62* (1), 24–45.
(24) Aschi, A.; Gharbi, A.; Daoud, M.; Douillard, R.; Calmettes, P. *Polym. Int.* **2007**, *56* (5), 606–612.
(25) Ribadeau Dumas, B.; Brignon, G.; Grosclaude, F.; Mercier, J. C. *Eur. J. Biochem.* **1972**, *25* (3), 505–514.
(26) Kundrotas, P. J.; Karshikoff, A. *Protein Sci.* **2002**, *11* (7), 1681–1686.
(27) Matthews, H. R. In *Biochemistry: A Short Course*; Miesfeld, R. L., Freedland, R. A., Eds.; Wiley: 1997; p 505.
(28) Longhi, S.; Receveur-Brechot, V.; Karlin, D.; Johansson, K.; Darbon, H.; Bhella, D.; Yeo, R.; Finet, S.; Canard, B. *J. Biol. Chem.* **2003**, *278* (20), 18638–18648.
(29) Glatter, O.; Kratky, O. In *Small Angle X-ray Scattering*; Glatter, O., Ed.; Academic Press: London, U.K., 1982; p 515.
(30) Qi, P. X.; Wickham, E. D.; Farrell, H. M. *Protein J.* **2004**, *23* (6), 389–402.
(31) Qi, P. X.; Wickham, E. D.; Piotrowski, E. G.; Fagerquist, C. K.; Farrell, H. M. *Protein J.* **2005**, *24* (7–8), 431–444.
(32) Guinier, A., Fournet, G., Eds. *Small Angle Scattering of X-rays*; Wiley: New York, 1955.
(33) Glatter, O.; Gruber, K. *J. Appl. Crystallogr.* **1993**, *26*, 512–518.
(34) Berry, G. P.; Creamer, L. K. *Biochemistry* **1975**, *14*, 3542–3545.

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