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Micellization of Bovine β-Casein Studied by Isothermal Titration Microcalorimetry and Cryogenic Transmission Electron Microscopy

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The association behavior, critical micellization concentration (CMC), and enthalpy of demicellization (ΔH_{demic}) of bovine β-casein were studied, for the first time by isothermal titration calorimetry, in a pH 7.0 phosphate buffer with 0.1 ionic strength and in pure water. In the buffer solutions, the CMC decreased asymptotically from 0.15 to 0.006 mM as the temperature was raised from 16 to 45 °C. ΔH_{demic} decreased with increasing temperature between 16 and 28 °C but increased from 28 to 45 °C. Thermodynamic analysis below 30 °C is consistent with the Kegeles shell model, which suggests a stepwise association process. At higher temperatures, this model exhibits limitations, and the micellization becomes much more cooperative. The CMC values in water, measured between 17 and 28 °C, decreased with increasing temperature and, expectedly, were higher than those found in the buffer solutions. β-Casein micelles were visualized and characterized, for the first time in their hydrated state, using advanced digital-imaging cryogenic transmission electron microscopy. The images revealed small, oblate micelles, about ∼13 nm in diameter. The micelles shape and dimensions remained nearly constant in the temperature range of 24−35 °C.

KEYWORDS: β-Casein; ITC; cryo-TEM; self-association; micellization

INTRODUCTION

β-Casein (∼38% of bovine caseins, ∼24 kDa, 209 amino acids) is a highly amphiphilic calcium-sensitive phosphoprotein, displaying a pronounced self-association behavior. β-Casein is characterized by a highly polar, negatively charged N-terminal domain containing its five phosphoseryl groups and a highly nonpolar C-terminal domain (*1*, *2*). The amphiphilic nature of β-casein is apparent also from what is currently known about its tertiary structure. It is accepted that it has a somewhat flexible open structure, which may be found in any one of several energetically favorable conformations in solution, i.e., a "rheomorphic" structure (*3*−*5*). At low temperatures, the protein is monomeric, with a reported Stokes radius of 3.7 nm (*6*) and a radius of gyration, R_g, of 4.6 nm (*6*, *7*). The molecule is somewhat elongated and highly hydrated (6−8 g H_2O/g) (*2*). Because of its amphiphilic nature, β-casein self-organizes into micelles in a manner strongly dependent on temperature and solvent composition (*4*, *8*−*11*). The critical micellization concentration (CMC), i.e., the concentration above which micelle formation is favorable, ranges between 0.05 and 0.2% w/v, depending on temperature, pH, and ionic strength (*6*, *12*−*15*). The micelles contain 15−60 molecules (*6*, *13*−*15*), and their R_g values range between 7.3 and 13.5 nm (*7*, *16*, *17*).

Most of the early studies of β-casein self-organization adopted the "all-or-none" or "closed-association" micellization mechanism (*12*, *18*, *19*). This type of micellization involves highly cooperative association into monodispersed aggregates and is characterized by a CMC and by an association number. An alternative model for the association behavior entitled "the shell model" was proposed by Kegeles (*20*−*22*). In this model, also referred to as "the consecutive (stepwise) micellization model", each consecutive addition step is characterized by an association constant *K*. Only the first step, dimerization, is assumed to be a "nucleation" step having a smaller association constant (*10*). Once a dimer forms, growth by association of additional monomers becomes easier, expressing the cooperativity of the association. The association constant for the first step is expressed as $f \cdot K$, where *f*, the cooperativity factor, is smaller than 1. Kegeles (*20*−*23*) considered the micellization as a series of consecutive reactions of the general type:

$$A_0 + A_{i-1} \leftrightarrow A_i$$

in which A_0 represents the monomers that form a series of

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micelles, which contain i monomers; $1 < i < n$, where n is an upper limit of the degree of association. The model assumed that n is limited, e.g., by steric factors due to space limitation on a "shell-like" surface; otherwise, the system would phase separate macroscopically. Both the closed-association model and the shell model assume a two-state system, i.e., monomers and micelles, with the occurrence of a CMC (*11*, *20*, *21*). However, the shell model also accounts for a micellar size distribution. Recently, the Kegeles model received experimental support from static and dynamic light scattering studies and differential scanning microcalorimetry (DSC) (*8*, *10*, *11*, *24*). Using high-sensitivity DSC, Mikheeva et al. (*10*) evaluated the effects of protein concentration, temperature, and addition of cosolutes on the thermodynamic parameters of β-casein micellization. Their results strongly support the applicability of the stepwise model for this process. Evans et al. (*12*) studied enthalpy changes using calorimetery; however, their experimental setup did not allow direct CMC measurements.

Isothermal titration microcalorimetry (ITC) is a powerful technique for obtaining thermodynamic parameters of micellization. It has been successfully applied in recent years in a growing number of studies of small surfactant micellization (*25*−*29*) but not yet to study micellization of amphiphilic proteins. The main advantage of ITC for micellization studies is the ability to perform direct measurements of both the heat of demicellization ($\Delta H_{mic} = -\Delta H_{demic}$) and the CMC, in a single isothermal titration experiment.

Cryogenic transmission electron microscopy (cryo-TEM) is a powerful technique for visualizing nanometric aggregates and has been successfully used to visualize surfactant and block copolymers micelles (*30*−*32*) and protein assemblies (*33*). The technique directly reveals structural details such as size, shape, and spatial organization at a high resolution of ∼1 nm, which is comparable with the micelles smallest dimension. Moreover, cryo-TEM involves ultrarapid fixation that preserves the structures in their native (hydrated) conditions; thus, structures are seen close to their state in solution (*34*, *35*). The application of cryo-TEM in the study of molecular assemblies has increased in recent years, with the development of digital recording and advanced imaging procedures (*36*) that have considerably improved the efficiency of image recording and the quality of the structural details that can be resolved.

This study had two main objectives. Our first goal was to use the high sensitivity of ITC to measure the effect of temperature on the thermodynamics of micellization of β-casein, to accurately obtain the CMC, ΔH_{mic}, and ΔH^{VH} (van't Hoff), and to better understand the association process. Our second objective was to characterize the shape, size, and morphology of the β-casein micelles as close as possible to the native state, by utilizing a state of the art cryo-TEM set up. To the best of our knowledge, both techniques were applied here for the first time to analyze the association and structure of β-casein micelles.

EXPERIMENTAL PROCEDURES

Materials. Bovine β-casein (>99%; Sigma-Aldrich, Germany) was dissolved in pH 7.0 phosphate buffer containing 80 mM NaCl, 5.65 mM Na_2HPO_4, and 3.05 mM NaH_2PO_4 (all compounds from Merck, Germany) with an ionic strength of 0.1. Each protein solution was filtered through a porous membrane of 0.45 μm, to avoid large protein aggregates. Then, it was dialyzed against this buffer for 24 h at 4 °C, using 1 mM ethylenediaminetetraacetic acid (EDTA) (Fluka, Switzerland) in the first part of the dialysis, to avoid a Ca^{2+} bridging effect. The protein concentration after dialysis was determined from the absorbance at 280 nm by an Ultrospec 2000 UV/visible Spectrophotometer (Pharmacia Biotech, England) and adjusted to 40 mg/mL (1.67 mM) using an extinction coefficient of $4.6_{(1\%)}$ (*10*). For studies at low ionic strength, the protein was dissolved in double-distilled water (pH 6.8) and filtered through the porous membrane, and then, the concentration was determined as above and adjusted to 40 mg/mL. The highest concentration of β-casein was used for injections in the temperature range between 16 and 20 °C. In the experiments performed above 20 °C, the injected β-casein concentrations were reduced to 20, 10, and 5 mg/mL, in the temperature ranges of 22−26, 28−35, and 40−45 °C, respectively.

Methods. *Isothermal Titration Calorimetry*. ITC measurements were performed with a VP-ITC calorimeter (Microcal, MA) in the temperature range of 16−45 °C. The reaction cell ($V = 1.43$ mL) was filled with degassed phosphate buffer (pH 7.0). The injector−stirrer syringe (289 μL) was loaded with β-casein micellar solution ($c \gg$ CMC). 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 10 s, and the equilibration time between consecutive injections was 3 min. Such an interval was sufficient to equilibrate the reaction cell after each injection. Each experiment was performed at least three times. Calorimetric data analysis was carried out with ORIGIN 5.0 software (Microcal).

Cryo-TEM. Specimens were prepared in a homemade controlled environment vitrification system (CEVS) at controlled temperature and humidity to avoid loss of volatiles. The examined solutions (at concentrations ranging between 0.8 and 1.7 mM) were brought to a desired temperature (24 and 35 °C) and allowed to equilibrate in the CEVS for an hour. Then, a 7.0 μL drop of each solution was placed on a TEM copper grid covered with a perforated carbon film and blotted with a filter paper to form a thin liquid film of the sample (100−200 nm thick). The thinned sample was immediately 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 until examination. The vitrified specimens were examined in a Philips CM120 TEM (Philips, The Netherlands) operating at an accelerating voltage of 120 kV. We used an Oxford CT3500 cryo-specimen holder (Oxford Instruments, United Kingdom) that maintained the vitrified specimens below −175 °C during sample transfer and observation. Specimens were recorded digitally on a cooled Gatan MultiScan 791 CCD camera (Gatan, United Kingdom) using the Digital Micrograph 3.1 software (Gatan, United Kingdom) (*36*), in the low-dose imaging mode to minimize beam exposure and electron beam radiation damage.

RESULTS AND DISCUSSION

ITC. Buffered micellar β-casein solutions (1.67 mM) were titrated into phosphate buffer placed in the ITC cell. A typical titration curve obtained at 18 °C is presented in **Figure 1A**. The large exothermic enthalpy changes observed for the initial injections are associated mainly with micelle dilution, demicellization, and dilution of individual β-casein molecules, while the small enthalpy changes of the final injections are attributed to micelle dilution only (*29*).

The heat of reaction, obtained by integrating the peaks of the individual injections in **Figure 1A**, is shown in **Figure 1B**, plotted against β-casein concentration in the cell. The figure reveals that at 18 °C there is a gradual change in the reaction enthalpy, suggesting that the association of β-casein into micelles under these conditions is a gradual process, taking place over a certain concentration range. Such a transition region seems compatible with a stepwise association process and consistent with the shell model described by Kegeles for β-casein association (*20*, *21*). O'Connell et al. (*11*) and Horne (*9*) also concluded from their experiments that β-casein micellization is a stepwise process of successive association of primary particles.

Figure 1B also presents the micellization region, designated MR, and the heat of demicellization, ΔH_{demic}. ΔH_{demic} is equal

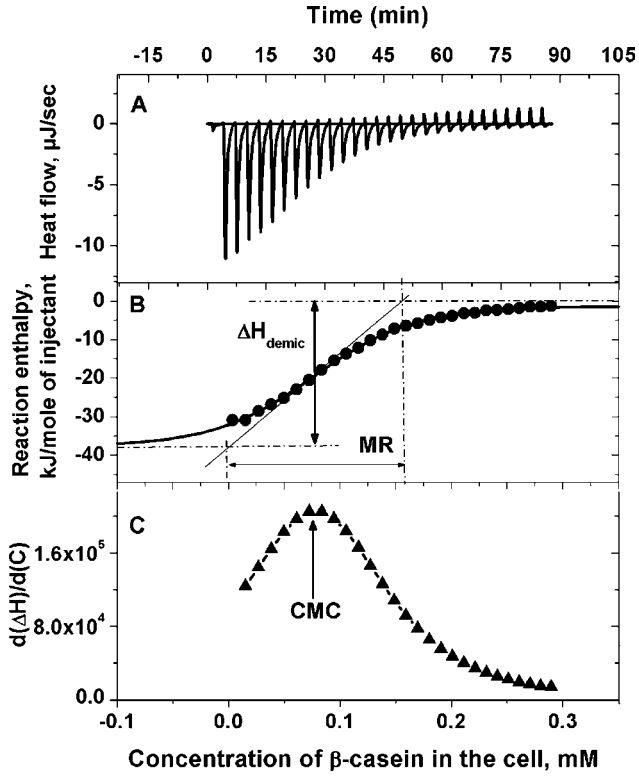

Figure 1. Titration of micellar β-casein solution (1.67 mM) into phosphate buffer, pH 7.0, at 18 °C: (**A**) calorimetric traces, (**B**) reaction enthalpy vs β-casein concentration in the cell, and (**C**) first derivative of curve B calculated from the interpolated value.

to the enthalpy difference between the two asymptotes (*29*) of the sigmoidal fit (obtained with the Origin software), of the reaction enthalpy dependence on β-casein concentration. The figure shows that at 18 °C the MR spans a range of ~0.16 mM and ΔH_{demic} is ~ −38 kJ/mol.

The CMC is reached during the increase in the reaction enthalpy (curve 1B), corresponding to the concentration at which the first derivative of the reaction heat with respect to the β-casein concentration in the cell displays an extreme value (*26*, *29*, *37*, *38*). This is presented in **Figure 1C**, where the CMC of β-casein at 18 °C was determined to be ~0.08 mM. Above the CMC, the heat from further addition of micelles to the cell evolved from dilution of the micelles (*29*).

Micellization of β-casein is believed to be controlled by both hydrophobic interactions and electrostatic repulsion forces (*10*, *11*). The effect of temperature on the enthalpy change measured for each injection as a function of β-casein concentration in the cell, at temperatures ranging from 16 to 45 °C, is presented in **Figure 2**. Clearly, as the temperature is raised, the micellization process begins and ends at lower protein concentrations, which is characteristic of increased hydrophobic interactions (*38*). Indeed, at temperatures higher than 22 °C, the initial injections with the 40 mg/mL (1.67 mM) β-casein micellar solution produced a concentration in the cell that was near or exceeding the CMC, so that ΔH_{demic}, the transition region, and the inflection point, were difficult, if not impossible, to determine. Thus, in the experiments performed above 20 °C, the injected concentrations were reduced to 20, 10, and 5 mg/mL, in the temperature ranges of 22−26, 28−35, and 40−45 °C, respectively. By comparing results at constant temperature and different injected concentrations (not shown), we have verified that this change in the concentration of the injected protein had a negligible effect on the results.

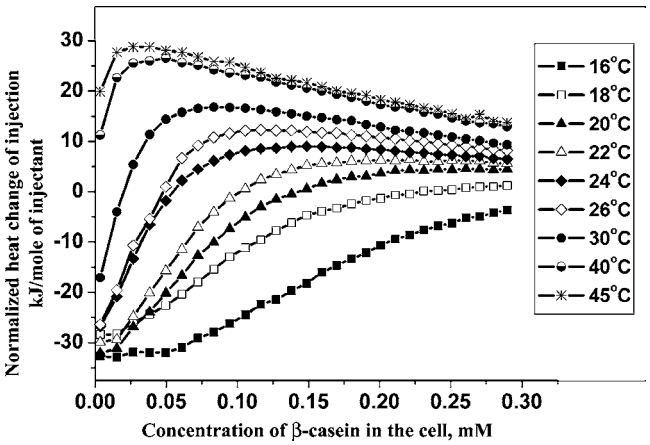

Figure 2. Dependence of the reaction enthalpy vs β-casein concentration on the temperature. Experiments were done by titrating a micellar β-casein solution (40 mg/mL, 1.67 mM) into phosphate buffer (pH 7.0).

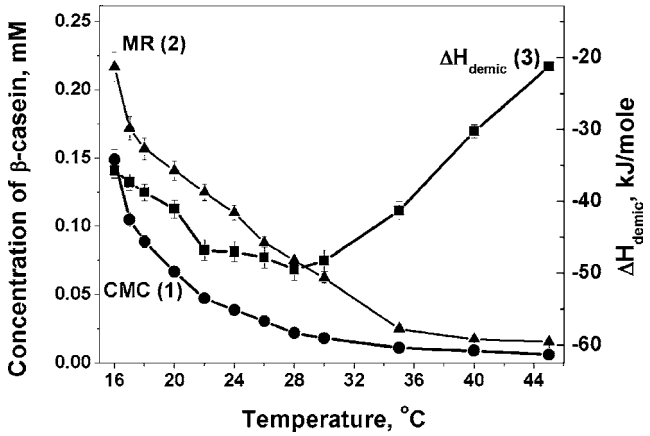

Figure 3. Temperature dependence of β-casein micellization: 1, CMC; 2, region of micellar transition "MR"; and 3, ΔH_{demic}.

Table 1. Effect of Ionic Strength and Temperature on the CMC of β-Casein[a]

T (°C)	17	22	28
low ionic strength, β-casein in double-distilled water, pH 6.8	0.128	0.091	0.057
ionic strength of 0.1, β-casein in phosphate buffer, pH 7.0	0.105	0.047	0.022

[a] The concentration is presented in millimolar.

The values of the CMC and the MR as a function of temperature are displayed in curves 1 and 2 of **Figure 3**, respectively. The CMC was found to drop with increasing temperature, in the whole range of studied temperatures, from 0.15 mM at 16 °C to 0.006 mM at 45 °C. The rate of decrease was especially rapid up to 22 °C and became moderate at higher temperatures.

To evaluate the effect of ionic strength on the β-casein micellization, ITC experiments with β-casein solution in water (low ionic strength, pH 6.8) were also performed. A comparison between the CMC values obtained for solutions with different ionic strength at 17, 22, and 28 °C is shown in **Table 1**. As expected, at a given temperature, the CMC values of β-casein in water were higher than those in the buffer solutions, since increased electrostatic repulsion forces under low ionic strength required higher protein concentrations to initiate micellization. For example, at 28 °C, the CMC in water was more than twice the value found in the buffer solution. A CMC value of 0.05%

w/v was reported by O'Connell et al. (*11*) at 40 °C, pH 6.5, in water. At this temperature, we have determined a value of 0.02% w/v (0.0083 mM) in the buffer solution. The results are in reasonable agreement, and we attribute the difference in the values primarily to the differences in the ionic strength. The pH difference, of 6.5 vs 7.0, is believed to have a small influence on the CMC values.

The β-casein MR decreased by about an order of magnitude, when the temperature was raised from 16 to 45 °C (**Figure 3**, curve 2). Curve 3 in **Figure 3** shows the experimental values of ΔH_{demic} of β-casein in the studied temperature range. A nonlinear dependence of ΔH_{demic} on temperature was observed with ΔH_{demic} declining up to 28 °C and then rising quite linearly. According to Jelesarov and Bosshard (*39*) who reviewed protein−protein associations, large conformational rearrangements of the protein molecules and the preexistence of temperature-dependent conformational equilibria of proteins can cause deviations from linearity in the plots of ΔH vs T. Thus, evidently, the nonlinear changes of ΔH with temperature in the case of β-casein association suggest the occurrence of possible conformational rearrangements of the protein molecules as the temperature is raised. The change of the demicellization enthalpy with temperature defines the heat capacity change:

$$\frac{\partial \Delta H_{\text{demic}}}{\partial T} = \Delta C_{\text{Pdemic}} = -\Delta C_{\text{Pmic}}$$

The parameter ΔC_{Pdemic} varies sharply from a negative value between 16 and ~28 °C to a positive value above 28 °C (slope of curve 3 in **Figure 3**). Therefore, ΔC_{Pmic} changes inversely, from positive to negative. A positive ΔC_{Pmic} in protein solutions is a good indication of hydrophobic hydration (*40*). Hence, the negative ΔC_{Pmic} observed in our system above 30 °C suggests the possibility that dehydration of the hydrophobic regions of the protein molecules becomes a dominant factor, as they associate to form micelles at this temperature range.

ITC studies of detergents micellization, which is believed to obey the "all or none" classical model much more closely, showed a nearly linear increase of ΔH_{demic} with temperature, implying negative ΔC_{Pmic} of detergent micellization throughout the entire studied temperature range (*29*, *41*). Thus, overall, our results support a continuous build up of protein aggregates that takes place up to ~30 °C, as proposed by the shell model, and that above this temperature either the kinetics of micellization increases in rate or the association mechanism changes.

Statistical analysis of the data presented in **Figure 3** was based on at least three separate replicate experiments, using the Origin software. It shows that the standard error was no more than 5%, for the CMC and MR values, and no more than 3% for ΔH_{demic} (error bars are shown in **Figure 3**). The analysis supports the statistical significance and validity of the results.

Using a high-sensitivity DSC system Mikheeva et al. (*10*) demonstrated the existence of a linear correlation between the reciprocal micellization (transition) temperature and the natural logarithm of the total β-casein concentration and showed that such relationship is in good agreement with the shell model predictions. An analogous linear relationship between the reciprocal temperature and the natural logarithm of the CMC, which is based on the ITC measurements in the temperature range of 17−30 °C, is shown in **Figure 4**. The slope of the line is $8.391 \pm 0.192 \times 10^{-5}$ K^{-1}, $r = 0.9984$, SD = 0.003, and $P < 0.0001$. This analysis further supports the applicability of the shell model at low and moderate temperatures. The deviation of the plot from linearity above 30 °C suggests decreased compliance of β-casein micellization with the shell model at the higher temperatures.

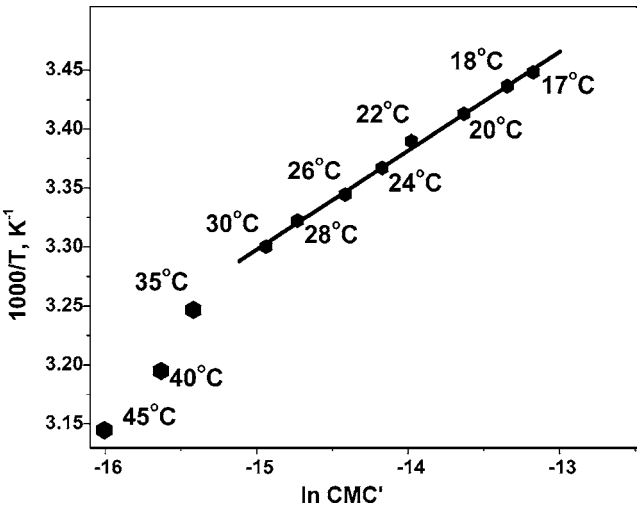

Figure 4. Correlation between the CMC (CMC expressed in mole fraction) of β-casein and the absolute temperature, in the range of 17–30 °C.

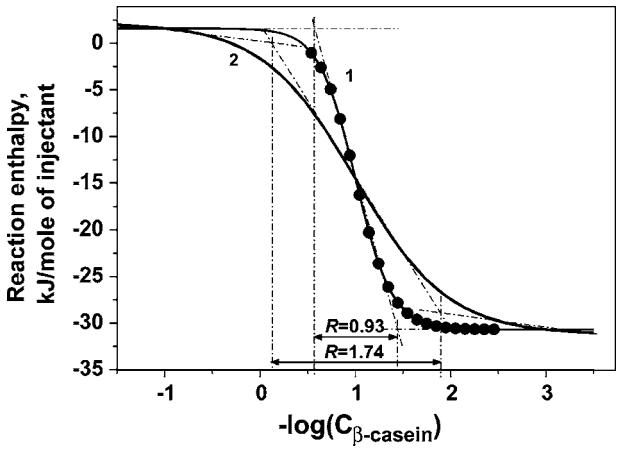

Figure 5. Graphical presentation of the micellization cooperativity parameter R. Plot of the reaction enthalpy vs the negative log of β-casein concentration (mM) at 18 °C. Curve 1: experimental curve, according to **Figure 1**. Curve 2: theoretical curve for a noncooperative process.

The van't Hoff micellization enthalpy of β-casein, ΔH^{VH}, was calculated from the inverse of the slope of the linear curve in **Figure 4** according to ref *10*

$$\left[\frac{\partial(1/T)}{\partial(\ln \text{CMC}')}\right] = \frac{R}{\Delta H^{\text{VH}}}$$

In the temperature range between 17 and 30 °C, the van't Hoff enthalpy is $\Delta H^{\text{VH}} = 99 \pm 2$ kJ/mol in the phosphate buffer solutions. The values reported by Evans et al. (*12*) and by Mikheeva et al. (*10*) were 66.0 ± 2.6 and 112 ± 6 kJ/mol, respectively, demonstrating a reasonable agreement of our result with these literature reports.

The micellization profile of β-casein is presented in **Figure 5** as the change in the reaction enthalpy vs negative log of β-casein concentration. One can practically attribute the center of this experimental profile (**Figure 5**, curve 1) to the CMC. Shinitzky and Fridkin (*42*) proposed this type of a general graphical method to characterize simple titrations or simple binding interactions, namely, concentration-dependent association processes (the vertical axis is of a parameter that quantifies the extent of the association). Denoting by R the difference

Table 2. Temperature Dependence of the Cooperativity Parameter Y of β-Casein Self-Association

T (°C)	16	17	18	20	22	24	26	28	30	35	40	45
R	1.10	0.97	0.93	0.92	0.86	0.84	0.81	0.79	0.73	0.70	0.63	0.60
Y	0.91	1.03	1.08	1.09	1.16	1.19	1.23	1.27	1.37	1.43	1.59	1.67

between the negative logarithms of the initial and final concentrations of the free species over which the transition takes place, these authors showed that a value of R of 1.74 is characteristic of simple binding [i.e., lack of cooperativity (*43*) (**Figure 5**, curve 2)]. Taking it a step further, this implies that for concentration-dependent transitions, $R < 1.74$ and $R > 1.74$ would indicate positive and negative cooperativity, respectively. One can therefore define a cooperativity parameter: $Y \equiv 1/R$, for which the borderline of cooperativity would be $1/1.74 = 0.575$. Likewise, values of $Y > 0.575$ and $Y < 0.575$ would indicate association processes of positive and negative cooperativity, respectively. Because in our ITC experiments it was not possible to determine the concentration of free protein during an experiment, we plot the results as a function of the total protein concentration; therefore, the cooperativity parameter determined this way is always underestimated (this is so, because the "free" concentration is always smaller than the "total" concentration). For β-casein, at 18 °C, an R value of 0.93 was obtained, i.e., $Y = 1.08$ (>0.575), suggesting that the transition is of moderate cooperativity. The cooperativity parameter values as a function of temperature (**Table 2**) increased throughout the studied temperature range, gradually moving away from the value of 0.91 at 16 °C to 1.67 at 45 °C, indicating quantitatively the increase in the cooperativity with increasing temperature.

The changes observed above ~30 °C, explicitly, the shrinkage of the MR (**Figure 3**, curve 2), the abrupt rise of ΔH_{demic} (**Figure 3**, curve 3), the deviation from linearity in **Figure 4**, and also the increase in the cooperativity with increasing temperature (**Table 2**), suggest a decreased compliance of β-casein association with the shell model above ~30 °C. This interpretation is in agreement with the O'Connell et al. (*11*) findings that polydispersity of β-casein micelles, one of the important characteristics of the shell model, decreases as the temperature is raised.

Cryo-TEM. To directly reveal the shape and dimensions of the micelles at high-resolution, β-casein solutions were studied by cryo-TEM. At 24 °C, small micelles with a characteristic oblate shape and a diameter of about 13 nm were observed (**Figure 6**). Because the micelles are randomly oriented in the vitrified ice matrix, several projections are visualized. The contrast along the β-casein micelles varies, and they display dark and light regions that suggest nonuniform packing. The dark regions represent denser or thicker parts of the micelle, while the lighter areas reflect regions that are thinner or of lower density. In the case of β-casein, this probably reflects packing constraints due to a structure, which deviates from a simplified hydrophilic−hydrophobic structure of classical low molecular weight surfactants. Because the exact tertiary structure of β-casein is unknown and is accepted to be rheomorphic, i.e., not a fixed structure but rather an open and a somewhat flexible one, it is difficult to predict the packing arrangement of these molecules. Kumosinski et al. (*4*) described the protein as having a crablike shape, with the hydrophobic C-terminal domain forming the "body", and the hydrophilic N-terminal domain forming the "head and two arms". Thus, in an aqueous environment, such crablike molecules would pack into oblate

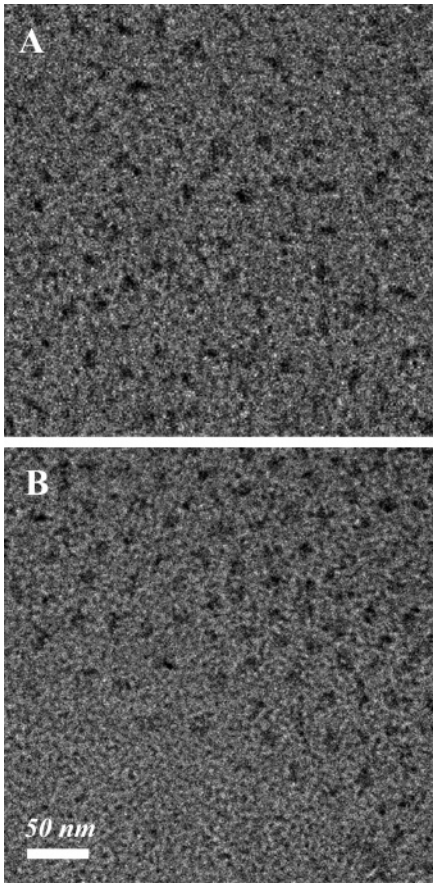

Figure 6. Cryo-TEM images of β-casein micelles in phosphate buffer solutions at 24 (**A**) and 35 °C (**B**). The concentration of β-casein is 1.5 mM.

or even spherical micelles by pointing the "body" parts inward and the highly charged "crab arms" outward. While the core would mainly be stabilized by hydrophobic and van der Waals interactions, the highly charged hydrophilic "arms" may also be interconnected by divalent ions when those are present.

Horne (*9*) suggested an analogy between the amphiphilic structure of β-casein and amphiphilic diblock copolymers, because they share some aspects of behavior in solution as the formation of micellar aggregates. The hydrophobic regions of β-casein interact intermolecularly in solution, rather than compact themselves into a folded form. Such intermolecular hydrophobic interactions naturally lead to a detergent-like micellar structure with a central hydrophobic core and charged hydrophilic regions forming a hedgehoglike external "coating". The appearance and dimensions of the micelles are in line with the amphiphilic "block copolymer-like" nature of β-casein (*44*, *45*).

The effect of temperature on the micelles shape and dimensions was also studied by cryo-TEM. As shown in **Figure 6A**,**B**, no significant changes are observed as the temperature is raised from 24 to 35 °C, and the micelles remain rather round and small. No indication of significant micellar growth due to an increase in temperature has been found under the conditions studied (1.5 mM β-casein). These results are in good agreement with those of O'Connell et al. (*11*), which indicated that the size of the micelles remained rather constant between 25 and 35 °C, despite a linear increase in the aggregation number with temperature.

Conclusions. The self-association of β-casein was studied by ITC and cryo-TEM. Employing ITC, the temperature

dependence of the CMC, the MR, and the degree of the micellization cooperativity, *Y*, were calculated from the reaction enthalpy. CMC values were expectedly higher at lower ionic strength, due to the lower screening effect, causing higher electrostatic repulsion that opposes micellization. All together, our results suggest that a continuous build up of β-casein aggregates takes place up to 28 °C, providing further support for the shell mechanism proposed by Kegeles. Above ~30 °C, the association process becomes highly cooperative. It is intriguing whether at this temperature range the system converges into a behavior compatible with the all-or-none micellization model or that the association rate increases to a point where the behavior is indistinguishable from that described by the all-or-none model.

The β-casein micelles were visualized directly, for the first time in their native state, using cryo-TEM. A population of rounded, oblate micelles of ~13 nm in diameter was observed. In the concentration range studied, which was significantly above the CMC (0.8−1.7 mM protein), the micelles remained nearly unchanged between 24 and 35 °C.

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