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Thermodynamics of Micellization of Bovine β-Casein Studied by High-Sensitivity Differential Scanning Calorimetry

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High-sensitivity differential scanning calorimetry was applied for the first time to study the micellization of bovine β-casein in aqueous solutions, complemented by analytical ultracentrifugation data. The micellization was described as a single endothermic heat capacity peak located in the temperature range of 0−50 °C. The transition originated at temperatures very close to 0 °C. The position of the heat capacity peak was strongly dependent on the protein concentration and the solvent composition. Thermodynamic parameters of the micellization, the transition temperature and enthalpy, were determined over a wide range of protein concentration. The observed thermal behavior of β-casein was well fitted by the thermodynamic shell model of micellization proposed by Kegeles (*J. Phys. Chem.* **1979**, *83*, 1728). The effects of different cosolutes (inorganic salts, urea, Tris, and ethanol) on the thermodynamic parameters of micellization of β-casein underline the hydrophobic character of the transition.

1. Introduction

β-Casein is one of the principal components (about 35%) of the casein fraction of bovine milk proteins. In view of the bioavailability of milk proteins as important nutrients with significant functional properties, casein micelles and submicelles of the individual caseins, both human and bovine, have been studied intensively and their physicochemical properties have been characterized and reviewed many times.[1−10] Nevertheless, knowledge of the mode of association and the structure of both the monomer and the association products of caseins is still insufficient for full understanding of milk as a system.

β-Casein belongs to the Ca^{2+}-sensitive phosphoproteins. In bovine milk, it exists in the pentaphosphorylated form. The primary structure of bovine β-casein is proline-rich. It consists of 209 amino acid residues, including five phosphorylated serines. The molecular mass of the constituent chain (protomer) of β-casein is of about 24 kDa.[11−13] Bovine β-casein as well as β-caseins from caprine and ovine milk demonstrate genetic polymorphism.[14,15]

At least two distinctive structural features of β-casein important for its functionality are particularly noteworthy. First, β-casein is one of only a few proteins lacking tertiary structure in its native state. Second, the primary sequence of the β-casein suggests an amphiphilic nature of the protein. Indeed, the content of hydrophobic amino acids in β-casein is relatively high, and the great majority of these amino acids is grouped at the C-terminus. Conversely, the N-terminal region of the polypeptide chain is rich in polar and negatively charged amino acid residues including all five phosphate groups attached to seryl residues located at positions 15,17, 18, 19, and 35.[11,13−16] The pronounced amphiphilic character of the primary structure of β-casein imparts many unique properties to the protein, approximating those of low-molecular-weight surfactants. Similar to them β-casein tends to self-associate under appropriate conditions with the formation of stable micelle-like structures.[2,17] Both hydrophobic interaction and electrostatic repulsion are suggested to be important for the micellization of β-casein. Recently, Farrell et al.[62] have explicitly studied changes in the secondary structure of β-casein in the course of micelli-

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zation by FTIR and CD spectroscopy. In particular, they found that a convenient deconvolution of the CD spectra reveals no changes in the secondary structure of β-casein over the temperature range 5−70 °C. However, a more careful inspection of the data allowed them to detect a number of weakly cooperative conformational transitions of the protein upon heating. Integrally, these transitions resulted in accumulation of polyproline II-like structural elements in the secondary structure of β-casein.

At very low ionic strengths, low temperatures (below 5−15 °C)[17−21] and relatively low protein concentrations (less than 1−5 mg mL^{-1})[17,20,22,23] β-casein seems to exist in aqueous solutions as primary particles in a random coil conformation.[2,18−20,24,25] The tendency of the β-casein primary particles to associate is strongly promoted by increasing temperature and/or protein concentration. The association is characterized by the critical values of both temperature and protein concentration, at which micelles of β-casein appear in the system.[20,21,23,26,27] The micellization is an equilibrium and reversible process. In the first place it is under control of temperature and protein concentration. The transition is also affected by ionic strength, electrolyte composition, pH, and various cosolutes.[17,21,28−30]

The micellization and recognition of the micellar structure of β-casein have attracted considerable interest for a long time and have been studied by various methods, primarily providing information about the aggregation state, shape, and size of the particles (electron microscopy,[17,20,26,27] analytical ultracentrifugation,[18,19,21,22,26,28−33] viscosimetry,[18,19,23,24,26] gel permeation chromatography,[20,23,26] static and dynamic light scattering,[17,22,28,29,34,35,66] SANS,[34,64,65] SAXS,[20,36] and spectral methods[22,25,26,28,30,35,37−40,65]). Despite all these studies, there is no general point of view on mechanism of the micellization of β-casein and structure of its micelles.[19,20,26,27,33,34,36,41−43,64−66]

In particular, one of the main questions requiring further investigation is whether the micellization of β-casein is a highly cooperative process, in which the monomer coexists with a single species of micellar particles or involves a series of consecutive association steps. The thermodynamic approach to study the micellization of β-casein might help to solve this problem. An attempt to do this was undertaken by Evans et al.[44] using isothermal mixing calorimetry.

In the present study, the high-sensitivity differential scanning calorimetry is used for the first time to study the micellization of β-casein. This method is widely used to study conformational transitions of proteins.[45,46] From the calorimetric experiments, the temperature dependence of the partial heat capacity of a protein can be obtained. Analysis of this function yields the fundamental thermodynamic parameters of a thermally induced transition: transition temperature, enthalpy, and heat capacity increment. Consequently, the driving forces and the mechanism of the transition can be evaluated.

Effects of protein concentration and some cosolutes (inorganic salts, urea, Tris, and ethanol) on the thermodynamic parameters of micellization of β-casein are evaluated. The results of calorimetric measurements are supplemented by the data from velocity sedimentation experiments performed at different temperatures. We show that the thermodynamics of micellization of β-casein is in agreement with the shell model of Kegeles[41] that considers a micellization as a series of consecutive additions of primary particles to a growing micelle.

2. Experimental Section

Materials. Bovine β-casein (>90%) from "Eurial" (Rennes, France) was used without additional purification. β-Casein was dissolved in one of the buffers of pH 7.0 (their compositions are

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(63) The assumed relation between sedimentation coefficients and molecular masses for β-casein micelles is close to that for a large polymer globule, which consists of a dense homogeneous core and a relatively thin fringe-like surface layer (Grosberg, A. Yu.; Khokhlov, A. R. *Statistical Physics of Macromolecules*, AIP Press: New York, 1994; pp 130−131). There is a relation between radius and molecular mass of the polymer globule: $R \propto M^{1/3}$. As $s \propto M/f$ where f is a frictional coefficient and $f \propto R$ for spherelike particles, in the case of the polymer globule $s \propto M^{2/3}$.
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given below) or in bidistilled water at pH 7.0 to prepare the 5% stock protein solution. The solution was filtered through a porous (o.d. 0.45 μm) membrane to avoid large protein aggregates. Then it was dialyzed against the appropriate solvent (water or buffer, pH 7.0, correspondingly) for 24 h at 4 °C, maintaining an external solvent-to-sample solution ratio of about 1:200 v/v with two additional replacements of the external solvent. To avoid the Ca^{2+}-bridging effect, 1 mM EDTA solution was used as a first solvent portion, followed by a dialysis in pure water to remove the EDTA−Ca complex from the protein solution. Protein concentrations after dialysis were spectrophotometrically determined at 280 nm using the extinction coefficient $E_{1cm}^{1\%} = 4.6$.[47] As a rule, sodium phosphate buffers of pH 7.0 and ionic strength 0.1 were used: 80 mM NaCl, 5.65 mM Na_2HPO_4, 3.05 mM NaH_2PO_4 (buffer **A**); 80 mM NaCl, 5.65 mM Na_2HPO_4, 3.05 mM NaH_2PO_4, 1 M urea (buffer **B**); 28.25 mM Na_2HPO_4, 15.25 mM NaH_2PO_4 (buffer **C**). In addition, several experiments were performed using 10−100 mM Tris-HCl buffers, pH 7.0. Bidistilled water was used for preparation of the buffer solutions. All chemicals were of analytical grade quality and were used without further purification.

Methods. *High-Sensitivity Differential Scanning Calorimetry (HS—DSC).* Calorimetric measurements were carried out with a differential adiabatic scanning microcalorimeter DASM-4A (NPO BIOPRIBOR, Pushchino, Russia). Thermograms were obtained within the temperature range 0−100 °C and at an excess pressure of 2 bar.

The most experiments were done at the heating rate 1.0 °C/min since it provides the maximal sensitivity, precision and reproducibility of the calorimetric measurements. Variations in the heating rate were strongly limited because of a small enthalpy of the transition of β-casein. Any decrease in the heating rate was unpractical as it resulted in a proportional decrease in the heat capacity output of the instrument. For this reason we could only perform additional experiments at the maximal heating rate of 2.0 °C/min, which is available with the instrument used. No effect of the heating rate on the transition parameters was observed.

The protein concentration in the calorimetric experiments was 2−40 mg mL^{-1}. The observed transitions were characterized by the midpoint transition temperature (T_t) or by the heat capacity peak temperature (T_m). The transition enthalpy ($\Delta_t H$) was determined as the area under the excess heat capacity function of the transition. Processing of the calorimetric data was done by the software package "Nairta" (Institute of Biochemical Physics, Moscow). The "SigmaPlot 3.06" software was used for fitting of models to the experimental results.

Analytical Ultracentrifugation. Velocity sedimentation experiments were performed with the analytical ultracentrifuge 3170 B (MOM) equipped with a Schlieren optical system, at 50 000 rpm. The experiments were carried out with 10 mg mL^{-1} β-casein solutions in buffer **A** at different temperatures from 3 to 50 °C fixed with accuracy of ±1 °C. Before each experiment, the centrifuge rotor and cells as well as the protein solution and the reference buffer were equilibrated at the required temperature for 2−4 h. Experimental sedimentation patterns were taken, enlarged, and digitized with a Genitizer GT-01212B digitizer. Sedimentation velocities of the components were determined from the time-dependent displacement of the peaks on the Schlieren curves. Sedimentation coefficients were reduced to the standard conditions ($s_{20,w}$) using the standard correction procedure.[48] The experimental sedimentation curves were deconvoluted in Gaussians using the "PeakFit 4.0" software. Weight fractions of the sedimenting components (primary particles and micelles) were evaluated from the areas under the Gaussians neglecting the radial dilution effect.

3. Results

High-Sensitivity Differential Scanning Calorimetry. A typical thermogram of bovine β-casein is presented in Figure 1A (curve 1). Evidently, β-casein undergoes a cooperative transition upon heating. It manifests itself as a single considerable endothermic peak of heat capacity within a relatively wide temperature range. This transition is completely reversible and reproducible during the second and subsequent heating scans. It indicates the absence of any irreversible or kinetically complicated changes accompanying the transition.

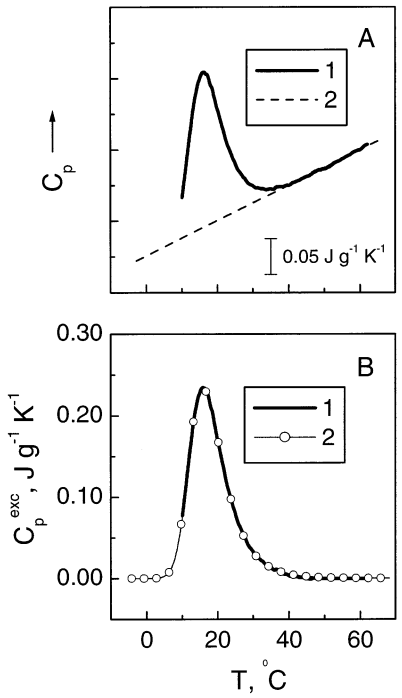

Figure 1. Typical HS−DSC data for the β-casein micellization. Panel A: apparent partial heat capacity of the protein (1), transition baseline (2). Panel B: experimental excess heat capacity function of the transition (1) and its approximation by a Pearson distribution (2). A part of the calculated points is omitted for clarity. Protein concentration 9.2 mg mL^{-1}, buffer **A**; heating rate 1.0 °C min^{-1}.

The most important problem in interpretation of HS−DSC data for β-casein was related to the determination of a baseline of the transition. We shall consider this point in details. Since freezing of aqueous solutions in the calorimetric cells causes their irreversible damage, the starting temperature of any experiment with a HS−DSC instrument must be higher than the sample freezing temperature by a few tenths of a degree centigrade (∼0.5 °C). Depending on solvent composition, the freezing temperature in our experiments changed from ∼0 °C (diluted buffers) up to ∼−4 °C (1.0 M NaCl). In addition, when the instrument is switched to the heating mode, a transient condition preceded measurements of heat capacity. Its duration corresponds to an increase in temperature by ∼10 °C at a heating rate of 1 °C/min. It could be shortened by decreasing the heating rate. However, this resulted in the proportional decrease in the instrument sensitivity. Thus, in the case of samples with marginal heat effects similar to β-casein, this expedient was not appropriate.

In all cases studied, the starting point of the micellization transition of β-casein and a pretransition part of the heat capacity function were located within the temperature range corresponding to the transient regime of the instrument (that is out of the measurement range). In the presence of urea, the transition temperature was shifted to higher temperature (∼30 °C). At the same time, the transition enthalpy was noticeably reduced; the heat capacity peak was significantly broadened to lower temperatures, and thus its onset was still masked by the transient condition zone.

The discussed above features of the β-casein transition forced us to search an approximated procedure for

Table 1. Effects of Some Cosolutes on Thermodynamic Parameters of the Micellization of β-Casein at pH 7.0[a]

		cosolutes														
		[NaCl], M							[Tris], M			[ethanol], vol. %				
transition params	without cosolutes	0.01	0.02	0.05	0.1	0.2	0.5	1.0	0.01	0.05	0.1	1	2	5	10	20
T_m, °C	13.3	13.5	13.9	13.3	13.3	13.1	9.9	4.9	15.0	16.1	16.9	13.1	15.5	15.1	n.d.	n.d.
$\Delta_t H$, J g^{-1}	1.8	2.8	3.0	3.2	3.2	3.3	~3	~2	2.2	2.2	2.7	3.3	4.5	1.0	n.d.	n.d.

[a] Protein concentration is 10 mg mL^{-1}. T_m is the heat capacity peak temperature. No transition was detected at ethanol concentrations 10 vol % and above.

calculations of the excess heat capacity of the transition. The only possibility to carry out such calculations was an appropriate extrapolation of the post-transition branch of the measured heat capacity and of the experimental excess heat capacity function to lower temperatures inaccessible for direct calorimetric measurements. The extrapolation procedure was based on the following assumptions.

First, the transition heat capacity increment was assumed to be negligibly small. Then the baseline of the transition was defined as a straight line extrapolating the linear post-transition part of the experimental thermogram, where the calorimetric measurements were produced with a reliable precision. This line was extrapolated to lower temperatures as it is plotted in Figure 1A by the dashed line (curve 2). The excess heat capacity function was calculated by subtraction of the baseline from the experimental thermogram. This function is presented in Figure 1B as a thick line (curve 1). It was then approximated by a Pearson distribution, which is known for its high fitting performance. In addition, the Pearson function resembles the differential transition curve for the model of cooperative association with respect to the specific nonsymmetry: a steep ascending branch vs a flat descending branch.[67] A thin line in Figure 1B (curve 2) gives the result of such a fitting. As a rule, the quality of the fitting was very high (standard fit error about ±0.002 J g^{-1} K^{-1}). This allowed us to suggest that the calculated Pearson distributions reproduce well the excess heat capacity functions of the β-casein transition in a temperature range wide enough for appropriate estimation of the transition parameters: T_m, T_t, and $\Delta_t H$.

The effect of the protein concentration was studied in the range of concentrations of β-casein from 2 to 40 mg mL^{-1} for buffers **A** (pH 7.0) and **B** (pH 7.0, 1 M urea). The transition was not detected at concentrations less than 2 and 5 mg mL^{-1} for buffers **A** and **B**, respectively. In both buffers, it was observed that with increased concentration of β-casein the transition heat capacity peak shifts to lower temperatures without significant changes in its profile. The transition temperature decreases nonlinearly and the transition enthalpy remains practically constant when the protein concentration increases (Figure 2). Importantly, urea induces an increase in the transition temperature (10 ± 0.5 °C) and a decrease in the transition enthalpy (0.6 ± 0.2 J g^{-1}) independent of protein concentration. Note that our estimates of the transition enthalpy of β-casein are comparable with the result of Evans et al.[44] ($\Delta_t H = 2.8 \pm 0.1$ J g^{-1}) obtained by isothermal dilution calorimetry.

It is well-known that the micellization of β-casein is drastically affected by a variety of low-molecular-weight cosolutes.[28−30,49] To illustrate such effects thermodynamically we performed calorimetric experiments at different concentrations of NaCl, Tris−HCl, and ethanol. Results of these experiments are presented in Table 1.

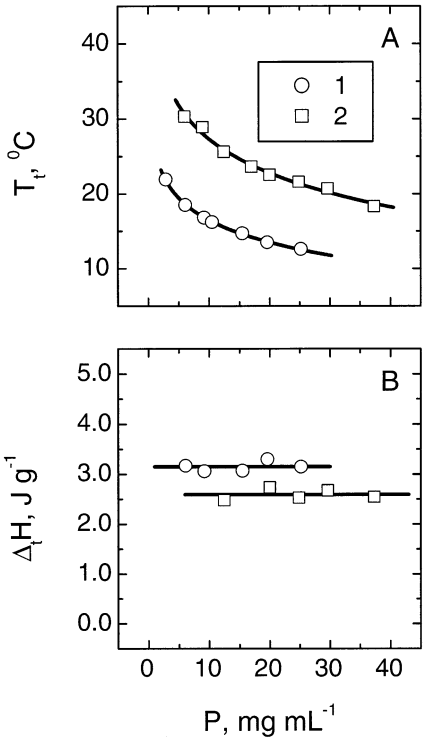

Figure 2. Midpoint temperature (A) and specific enthalpy of the micellization (B) vs β-casein concentration (1, buffer **A**; 2, buffer **B**).

It can be seen that ionic strength (NaCl concentration) has a noticeable effect on the transition. An increase in ionic strength from 0 up to 0.1−0.2 results in the gradual increase in the transition enthalpy. Within this selected range of ionic strengths the transition temperature remains practically constant. Upon a further increase of the salt concentration, the transition curves are progressively shifted to lower temperatures. The transition temperature linearly decreases with the salt concentration. However, the reliable estimation of the transition enthalpy was difficult because a significant initial part of the transition curve was inaccessible for measurements, as it was apparently located in temperatures lower than the start-up temperature of the calorimeter. One could only suggest that the transition enthalpy does not change significantly at high ionic strengths, remaining about 2−3 J g^{-1}.

The effect of Tris−HCl looks less pronounced in comparison with that of NaCl. In Tris−HCl buffer the transition temperature and enthalpy gently increase with increasing buffer concentration.

In the presence of ethanol (1−20 vol %), a noticeable decrease in the intensity of the heat capacity peak and its broadening were found, without significant changes in the position of the peak. Apparently, the transition enthalpy vs ethanol concentration passes through a

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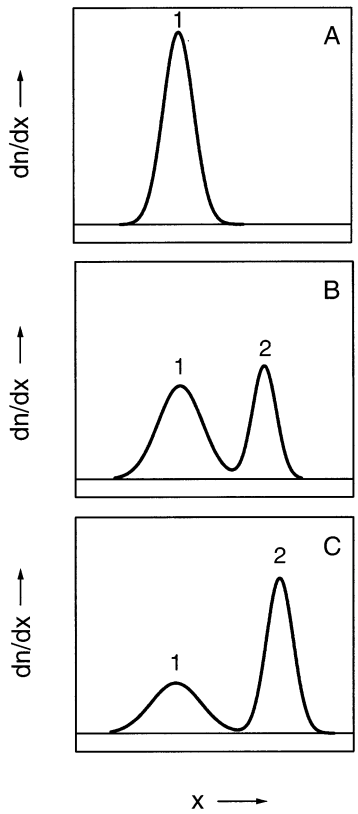

Figure 3. Velocity sedimentation patterns of β-casein at different temperatures, °C: 3 (A), 15 (B), and 50 (C). Primary particles and micelles are presented by peaks 1 and 2, respectively. Protein concentration 10 mg mL^{-1}, buffer **A**.

maximum at 2 vol % and vanishes at ethanol concentrations more than 10 vol %.

Velocity Sedimentation. The sedimentation behavior of β-casein was studied in buffer **A** at a protein concentration of 10 mg mL^{-1} within the temperature range 3−50 °C. Figure 3 shows a few typical sedimentation patterns of β-casein solutions obtained at different temperatures.

A single symmetric peak associated with β-casein primary particles has been detected on the sedimentogram at temperatures 3−5 °C (Figure 3A). The micellization seems to start at temperatures above 5 °C. Under these conditions a second faster migrating peak appears in addition to the peak of primary particles. It has been attributed to β-casein micelles. Note that this peak was quite symmetric. Its width is likely not to change appreciably with increasing temperature. The obtained data are in agreement with results of analytical ultracentrifugation,[2,10,18,21,29,38,44,50] electron microscopy,[1,26,27] and gel permeation chromatography,[20,23,26,51] which revealed bimodal distribution of the β-casein particles at room and higher temperatures and at β-casein concentrations near 5 mg mL^{-1}. Remarkably, the obtained bimodal sedimentograms are perfectly deconvoluted in two Gaussians. Thus, we suggest that the size distributions of micelles are close to Gaussians.

The peaks observed on the sedimentograms were resolved quickly and more completely with a rise in temperature due to an evidently increasing difference in the sedimentation coefficients of the primary particles and micelles. Figure 4 shows sedimentation coefficients ($s_{20,w}$) of the primary particles (1) and micelles (2) of β-casein as a function of temperature.

The sedimentation coefficient of the primary particles was found to be temperature invariant and equal to $s_{20,w}$ = 1.54 ± 0.21 S. This result is in agreement with the published data.[2,10,18,38,44,50,52,53] The sedimentation coefficient of the micelles increases markedly with temperature. It is of about 3- and 15-fold of $s_{20,w}$ for the monomer at 8 and 50 °C, respectively. A well-defined temperature dependence of the sedimentation coefficient of β-casein micelles suggests a dramatic reorganization of the micellar structure with temperature.

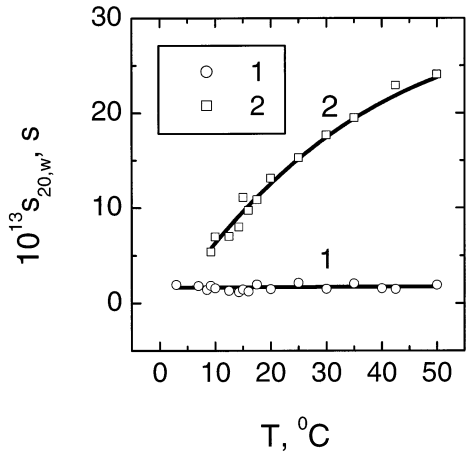

Figure 4. Sedimentation coefficients of primary particles (1) and micelles (2) of β-casein vs temperature. Protein concentration 10 mg mL^{-1}, buffer **A**.

Along with improving resolution of the sedimentation peaks, the increase in temperature induced monotonic changes in areas under the peaks indicating the redistribution of the protein between primary particles and micelles. Thus, the content of primary particles decreased progressively, whereas the content of micelles increased. This behavior clearly demonstrates the shift of the transition equilibrium toward the micellization upon heating.

4. Discussion

Thermodynamic Mechanism of the Transition. As a rule, the micellization of β-casein has been considered as a two-state or "all-or-none" transition.[2,17,19−21,26,29,32,39,44] However, this model seems to contradict a relatively large width of the transition (Figure 1B) at a rather high degree of association of the protein in micelles (of the order of several tens of β-casein molecules)[2,9,19,21,27,34,36] and the significant increase in the sedimentation coefficient of the micelles upon increasing temperature (Figure 4). At the same time, both features seem to be provided by the shell model of micellization elaborated by Kegeles.[41] Therefore, we will discuss our experimental results in terms of the shell model.

The main output results of our experiments, which we will use in the analysis, are as follows: (i) the specific transition enthalpy, $\Delta_t H = 3.2 \pm 0.2$ J g^{-1} (buffer **A**) and $\Delta_t H = 2.6 \pm 0.1$ J g^{-1} (buffer **B**), independent of protein concentration; (ii) the dependence of the transition temperature, T_t, on protein concentration, P; (iii) the temperature dependence of the apparent degree of association of the protein in micelles, N_{app}, calculated from the velocity sedimentation data via an empirical correlation between sedimentation coefficients and molecular mass of globular proteins and related biological structures.

According to the shell model, the micellization is considered as a series of consecutive reactions of the general type:

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

where A_0 represents primary particles (monomers) participating in the micellization; $1 \le i \le n$; n is an upper limit of the degree of association defined, e.g., by steric factors. The degree of association of the ith cluster is $(i + 1)$.

The rate constant of addition of a monomer to the $(i - 1)$th cluster is suggested to be

$$k_{i-1,i} = ik_f\frac{n-(i-1)}{n} \tag{1a}$$

while the rate constant of release of the monomer from the ith cluster can be defined as

$$k_{i,i-1} = (i+1)k_b \tag{1b}$$

where k_f and k_b are the intrinsic association and dissociation rate constants for a monomer−cluster interactions.

All steps at $i > 1$ are characterized by the same equilibrium constant, K. However, the dimerization constant ($i = 1$) is equal to fK where $f \le 1$ is a cooperativity parameter. It identifies the dimerization as a nucleation step of the transition. When the parameter f is decreasing the transition becomes more cooperative.

The main parameter of the shell model is the product $K[A_0]$, where $[A_0]$ is a concentration of primary particles. At $K[A_0] < 1$, only primary particles are present in the system. At $K[A_0] > 1$, along with the primary particles the system involves also a population of clusters with a rather narrow size distribution:

$$(i+1)\frac{[A_i]}{[A_0]} = f\frac{n!}{(n-i)!}\left(\frac{K[A]_0}{n}\right)^i \tag{2}$$

With increasing $K[A_0]$ the distribution shifts to larger degrees of association. It tends to be centered at about $n/2$ at $K[A_0] \sim 2$.

The shell model was successfully used for description of the sedimentation behavior of β-casein as a function of its concentration as well as for the kinetics of micellization of this protein.[33,54] We will show that this model can also give an adequate interpretation of the HS−DSC data.

In terms of the shell model (1), the mass conservation condition for primary particles can be written in the form:

$$[A_0] + \sum_{i=1}^{n}(i+1)[A_i] = P \tag{3}$$

where P is the total concentration of primary particles. Introducing the fraction of primary particles, $\beta = [A_0]/P$, as a new variable and taking into account eq 2, one gets the following equation for the integral transition curve, $\beta(T)$:

$$\beta(T)\sum_{i=1}^{n} f\frac{n!}{(n-i)!}\left(\frac{PK(T)\beta(T)}{n}\right)^i + \beta(T) - 1 = 0 \tag{4}$$

The temperature dependence of the equilibrium constant is given by the Van't Hoff equation:[55]

$$K(T) = \exp\left[-\frac{\Delta_t H^{VH}}{R}\left(\frac{1}{T} - \frac{1}{T^*}\right)\right] \tag{5}$$

where $\Delta_t H^{VH}$ is the van't Hoff enthalpy of transition per double mole of primary particles and T^* is a characteristic temperature corresponding to the condition $K(T^*) = 1$.

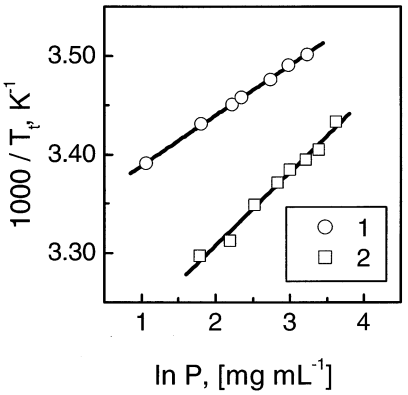

Figure 5. Correlation between the transition temperature and total concentration of β-casein according to the shell model by eq 9 for buffer **A** (1, $r = 0.9997$; the slope of the line is $(5.11 \pm 0.06) \times 10^{-5}$ K^{-1}) and for buffer **B** (2, $r = 0.9930$; the slope of the line is $(7.41 \pm 0.36) \times 10^{-5}$ K^{-1}).

We consider some general properties of eq 4 those allow one to use the equation for analysis of experimental data, in particular data of HS−DSC. First of all, we are interested in a dependence of the transition midpoint temperature, T_t, at which $\beta = 0.5$, on the total protein concentration, P. Differentiating $K(T_t)$ in eq 4 with respect to P as an implicit function of P, at $\beta = 0.5$, we find that

$$[K(T_t) + PK']\sum_{i=1}^{n} A(i)i[PK(T_t)]^{i-1} = 0 \tag{6}$$

where

$$K' = \left(\frac{\partial K}{\partial P}\right)_{T=T_t}$$

and $A(i)$ is a function independent of P and T. The sum in eq 6 is always positive. Hence, a solution of eq 6 requires that

$$[K(T_t) + PK'] = 0 \tag{7a}$$

or

$$P\left(\frac{\partial K}{\partial P}\right)_{T=T_t} = \left(\frac{\partial K}{\partial(\ln P)}\right)_{T=T_t} = -K(T_t) \tag{7b}$$

Alternatively, from eq 5 it follows that

$$\left(\frac{\partial K}{\partial(\ln P)}\right)_{T=T_t} = -\frac{\Delta_t H^{VH}}{R}K(T_t)\left[\frac{\partial(1/T_t)}{\partial(\ln P)}\right] \tag{8}$$

Therefore, from eqs 7b and 8, we conclude that in terms of the shell model the reciprocal of transition temperature is a linear function of the logarithm of the total protein concentration with the slope

$$\left[\frac{\partial(1/T_t)}{\partial(\ln P)}\right] = \frac{R}{\Delta_t H^{VH}} \tag{9}$$

Interestingly, this result coincides with a prediction of the two-state model of micellization for large association degrees ($n \gg 1$).[56]

Figure 5 presents data on $1/T_t$ vs ln P for β-casein in water (buffer **A**, curve 1) and in 1 M urea (buffer **B**, curve 2). Both dependences are actually linear, in agreement with the prediction of the shell model. From their slope,

the van't Hoff enthalpy per double mole of primary particles of the protein, could be calculated: $\Delta_t H^{VH} = 163 \pm 2$ kJ mol^{-1} (buffer **A**) and $\Delta_t H^{VH} = 112 \pm 6$ kJ mol^{-1} (buffer **B**). According to the calorimetric data obtained at different protein concentrations, the average molar transition enthalpy of β-casein per double mole of the protein monomer is $\Delta_t H = 157 \pm 5$ kJ mol^{-1} for buffer **A** and $\Delta_t H = 125 \pm 6$ kJ mol^{-1} for buffer **B**. Hence the ratio of the experimental enthalpy to the van't Hoff one is very close to 1 in both cases (0.97 ± 0.02 and 1.1 ± 0.1, respectively). Thus, it can be concluded that the predictions of the shell model on the concentration dependence of the transition temperature are in good agreement with the experimental data for β-casein.

The next useful property of the shell model is a limiting behavior of the integral transition curve, $\beta(T)$, at small β, i.e., at high temperatures. We now calculate the derivative of β with respect to temperature considering $\beta(T)$ as an implicit function according to eq 4 and taking into account eq 5:

$$\left(\frac{\partial\beta}{\partial T}\right)_P = -\frac{\Delta_t H^{VH}}{RT^2}\frac{\beta F_1(\beta,T)}{1 + F_1(\beta,T) + F_2(\beta,T)} \qquad (10)$$

where

$$F_1(\beta,T) = \sum_{i=1}^{n} f\frac{i(n!)}{(n-i)!}\left[\frac{PK(T)}{n}\right]^i \beta^i$$

and

$$F_2(\beta,T) = \sum_{i=1}^{n} f\frac{n!}{(n-i)!}\left[\frac{PK(T)}{n}\right]^i \beta^i$$

At high temperatures the apparent monomer fraction β approaches 0 and the function F_1 becomes much more than 1 and F_2. For this reason

$$-\frac{T^2}{\beta}\left(\frac{\partial\beta}{\partial T}\right)_P = \left(\frac{\partial \ln\beta}{\partial 1/T}\right)_P \rightarrow \frac{\Delta_t H^{VH}}{R} \qquad (11)$$

Thus, at high temperatures the integral transition curve presented as $\ln\beta$ against $1/T$ approaches a straight line with the slope $\Delta_t H^{VH}/R$.

The apparent monomer fraction β as a function of temperature can be calculated from HS−DSC data. It is evident that

$$\beta(T) = 1 - \alpha(T) \qquad (12)$$

where $\alpha(T)$ is the extent of the transition on the primary particle basis. On the other hand, there is a well-known thermochemical relation between the extent and enthalpy of a transition:

$$\Delta_t H(T) = \alpha(T)\Delta_t H \qquad (13)$$

where $\Delta_t H(T)$ and $\Delta_t H$ are the transition enthalpy at a current temperature and the total transition enthalpy, respectively. Applying eqs 12 and 13 to HS−DSC data, it is possible to obtain the integral curve of a transition.

As an example, the integral transition curve of β-casein at the total protein concentration of 10 mg mL^{-1} in buffer **A** is presented as a function of $\ln\beta$ vs $1/T$ in Figure 6. It illustrates the limiting behavior of the shell model at high temperatures using the relation in (11). Actually, in the range of high temperatures, the experimental dependence

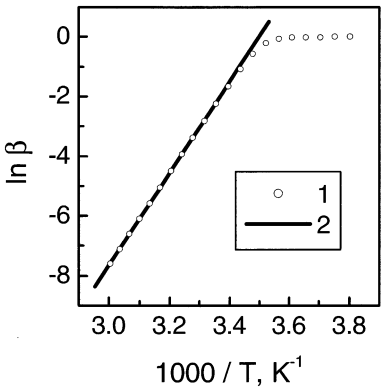

Figure 6. Apparent fraction of primary particles of β-casein vs temperature: 1, experimental from HS−DSC data; 2, linear approximation of the experimental curve at high temperatures ($r = 0.9999$; the line slope is 15234 ± 11 K). A part of the experimental points is omitted for clarity. Protein concentration 10 mg mL^{-1}, buffer **A**.

of $\ln\beta$ on $1/T$ can be approximated by a straight line with the slope corresponding to $\Delta_t H^{VH} \sim 130$ kJ mol^{-1}. This is rather close to the estimate of the van't Hoff enthalpy made from the analysis of the concentration dependence of the transition temperature. Therefore, a profile of the transition curve seems to agree with the predictions of the shell model.

Using eqs 10 and 12 and taking into account the general definition of the transition excess heat capacity in the first thermochemical approximation ($\Delta_t H \neq f(T)$):

$$C_p(T) = \Delta_t H\left(\frac{\partial\alpha}{\partial T}\right)_p \qquad (14)$$

we can derive an expression for the molar excess heat capacity function of the micellization in terms of the shell model

$$C_p(T) = \frac{(\Delta_t H^{VH})^2}{RT^2} \times \frac{\beta F_1(\beta,T)}{1 + F_1(\beta,T) + F_2(\beta,T)} \qquad (15)$$

where the heat capacity is expressed per double mole of the protein monomer.

Equation 15 can be used for the quantitative analysis of HS−DSC data on micellization. Though, it needs to be noted that the limiting association degree, n, cannot apparently be considered as an adjusting parameter in any fitting procedure. It should be obtained from the independent data on the apparent association degree of the micelles as a function of temperature at the protein concentration for which the HS−DSC data are obtained. Such information could be extracted from the velocity sedimentation results if we assume that β-casein micelles are hydrodynamically similar to globular proteins and related to hard-sphere-like biological particles. For these proteins and supramolecular structures there is an empirical correlation between sedimentation constants and molecular masses,[57] $s_{20,w} = AM^B$, where $A = 0.312 \pm 0.011$ and $B = 0.635 \pm 0.006$ with a correlation coefficient of 0.996. It is valid for the ranges 2 S $\leq s_{20,w} \leq$ 4000 S and 10 kDa $\leq M \leq 20 \times 10^6$ kDa. We have used this correlation to convert the sedimentation coefficients of β-casein micelles to their apparent association degrees expressed as a number of monomers per micelle, N_{app}.[63] These values vary from 4 to 40 over the temperature range 10−50 °C. On the basis of the results of the preliminary simulations by the shell model, we have found that the similar range of the weight-average association degree can be achieved

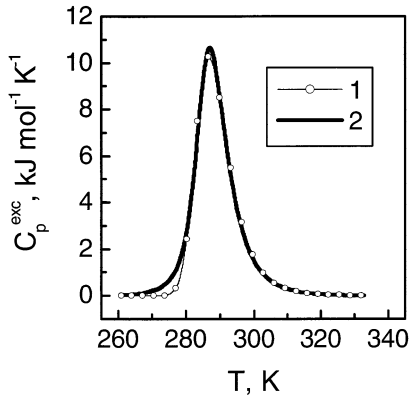

Figure 7. Experimental excess heat capacity function (1) of the micellization of β-casein at protein concentration 10 mg mL^{-1} (buffer **A**) and its approximation (2) in terms of the shell model by eq 15: $n = 175$, $\Delta_t H^{VH} = 132 \pm 1$ kJ mol^{-1}, $f = 10^{-0.83 \pm 0.03}$, and $T_t = 288.25 \pm 0.03$ K. The standard fit error is $\pm$ 0.28 kJ mol^{-1} K^{-1}; the ratio $\Delta_t H/\Delta_t H^{VH} = 1.02$. A part of the experimental points is omitted for clarity. The heat capacity is expressed per double mole of the monomer.

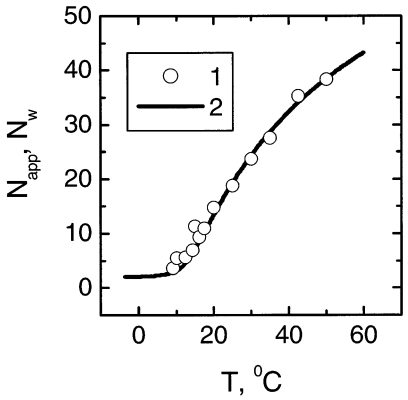

Figure 8. Comparison of the apparent association degree of β-casein micelles determined from velocity sedimentation data (N_{app}, 1) and the weight-average association degree of micelles calculated in terms of the shell model by eq 16 (N_w, 2). Both parameters are expressed in monomer number per micelle. The calculations were performed using most probable values of the model parameters obtained as a result of the fitting of the shell model to the experimental excess heat capacity function of the micellization of β-casein (see Figure 7).

in the temperature range studied if one assumes that $n = 175$. For this condition, we fitted eq 15 as a function of experimental variables T and β to the experimental molar excess heat capacity function of the micellization at the protein concentration 10 mg mL^{-1} (buffer **A**). In that way $\Delta_t H^{VH}$, f, and T_t were used as adjusting parameters. In the course of the fitting, the parameter T^* was calculated via eq 5 using a polynomial approximation of the function $PK(T_t)$, which is a solution of eq 4 for $\beta = 0.5$, against log f. Results of the fitting are given in Figure 7. A good agreement between the theory and the experimental data (standard fit error is $\pm$ 0.28 kJ mol^{-1} K^{-1}) was found at the following values of the parameters: $\Delta_t H^{VH} = 132 \pm 1$ kJ mol^{-1}, $f = 10^{-0.83 \pm 0.03}$ and $T_t = 288.25 \pm 0.03$ K. Note that the ratio $\Delta_t H/\Delta_t H^{VH} = 1.02$, which practically does not differ from 1. This assessment corresponds quite well with the results of the analysis of the concentration dependence of the transition temperature and the integral transition profile. The estimate of the cooperativity parameter f is rather unreliable, since this parameter is mainly defined by the start section of the experimental curve that was determined with lowest precision. Just in this region the maximal deviation of the experimental curve from the calculated one is observed. Note that Tai and Kegeles[33] estimated $f \sim 10^{-4}$ for β-casein from a dependence of the apparent molecular mass of micelles on protein concentration at a constant temperature. Sources of this disagreement are still unknown. However, it should be noted that for $f \sim 10^{-4}$ the micellization transition of β-casein would be much more cooperative and the ascending part of its excess heat capacity function would be practically perpendicular to the T-axis.

Using the results of the fitting and eqs 2 and 4, it is possible to calculate the weight-average association degree of micelles of β-casein as a function of temperature:

$$N_w(T) = \frac{\sum_{i=1}^{n}(i+1)\frac{n!}{(n-i)!}\left(\frac{PK(T)\beta(T)}{n}\right)^i}{\sum_{i=1}^{n}\frac{n!}{(n-i)!}\cdot\left(\frac{PK(T)\beta(T)}{n}\right)^i} \qquad (16)$$

Results of these calculations, $N_w(T)$, are compared with the experimental data, N_{app} against temperature, in Figure 8. We see that in general the shell model correctly reproduces the micelle size with increasing temperature.

Unfortunately, it was not possible to measure the heat capacity of β-casein solutions at subzero temperatures and in the range 0−10 °C with adequate accuracy using the DASM-4 instrument working only in the heating mode. Measurements in a cooling mode would allow a more precise determination of the cooperativity parameter f as well as to obtain valuable information on the micelle structure via determination of the transition heat capacity increment.

Cosolute Effects on the Transition. There is a distinct similarity between the thermotropic conformational transition of β-casein and the micellization of low-molecular-weight surfactants. The main driving force of micellization in aqueous solutions is the hydrophobic effect,[58] so all factors promoting hydrophobic interactions are expected to facilitate the micelle formation. When temperature increases, hydrophobic interactions become more favorable as water aims to keep its structure intact by substituting water−hydrophobic solute contacts with water−water ones. Salts with the structure-making effect on water structure increase the hydrophobic effect and, thus, promote micellization, as was observed, e.g., for buffer **C**. The replacement of "lyotropically" neutral NaCl by sodium phosphates brings about a decrease in the transition temperature.

Some other additives have a destabilizing effect on the micelles of β-casein. Buffer **B**, containing urea, which has a structure-breaking effect on water, is a better solvent for β-casein monomers. It is necessary to increase temperature and/or concentration of the protein in order to shift the equilibrium toward micellization. At the same time, in the presence of urea the micellization is accompanied by a decrease in the transition enthalpy. Sood et al.[50] have shown that in 3.3 M urea the intrinsic viscosity of the human β-casein has only a weak dependence on temperature. Under these conditions the protein conserves its monomeric state in a wider temperature range. In the present study, where the urea concentration was more than three times lower, the micellization of bovine β-casein still occurs at the increased transition temperature and decreased transition enthalpy. The fact that eq 9 is validated for buffer **B** (Figure 5, curve 2) makes it possible

to suggest that the mechanism of the micellization does not change principally in the presence of urea.

Tris buffer has a similar but weaker effect on the transition temperature. It may also be attributed to the specific nature of the buffer. It is possible that micelles of β-casein, like those of low-molecular-weight surfactants, are capable of solubilization of various hydrophobic and amphiphilic organic compounds into their hydrophobic core.[58] Such a solubilization could, in some cases, lead to weakening of the attractive interaction between the surfactant molecules in the micelles. This effect could be compensated by an increase in temperature. In the present experiments it was observed as an increase of the transition temperature.

The effect of ethanol is quite different. It promotes association of the protein even at low temperatures, however the trend in changes of the transition enthalpy caused by ethanol is likely to indicate that the mechanism of the micellization in mixed water−ethanol solvent differs significantly from that in water. It may involve specific interactions of the protein with ethanol, which result in the precipitation of the protein at high ethanol concentrations. Moreover, the addition of ethanol to water decreases the dielectric constant of the solvent, so the role of electrostatic interactions increases.

Although the main driving force of micellization of amphiphilic compounds in aqueous solutions is the hydrophobic effect, in the case of charged surfactants the electrostatic interactions also play an important role. Indeed, electrostatic repulsion between likely charged β-casein monomers will weaken or prevent the micellization. The net charge of bovine β-casein is about −12 at pH 7.0.[2,59,60] Increasing the ionic strength or strong specific ion binding to the charged groups of the protein could diminish the electrostatic repulsion and favor self-association of the protein monomers.

In the present study, the ionic strength effect was displayed as a significant increase in the transition enthalpy with increasing ionic strength up to 0.1−0.2. It can be attributed to the screening of the electrostatic repulsion between negatively charged side chains of interacting β-casein monomers. It is remarkable that the transition temperature remains constant within this range of ionic strength assuming the hydrophobic interactions are invariable. Evidently, the decrease in the transition temperature at high salt concentrations results from the strengthening of the hydrophobic effect due to the influence of the salt on the water structure. At NaCl concentrations above 0.2 M, the salting-out effect manifests itself. The magnitude of this effect in general strongly depends on the chemical nature and the concentration of a salt (and/or other cosolutes) presented.[58,61]

5. Conclusions

The predictions of the shell model agree with the experimental data on thermodynamics and structure of β-casein micelles. Therefore, the micellization transition of β-casein can be considered as a successive association of primary particles. The first stage of this process is thermodynamically hindered. It is a nucleation step. Existence of the nucleation imparts cooperativity to the transition.

Effects of the low-molecular-weight cosolutes (salts, urea, and ethanol) on the thermodynamic parameters of the micellization of β-casein are typical of the lyotropic phenomena.[68] It underlines a leading role of hydrophobic interaction in the mechanism of the transition.

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