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ORIGINAL CONTRIBUTION

Effect of temperature and pH on the aggregation and the surface hydrophobicity of bovine κ-casein

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Abstract κ-Casein (κ-CN) aggregation by heating has been studied at pH 7.2 and 5.2 using UV-visible spectrophotometry, sodium dodecyl sulfate polyacrylamide gel electrophoresis, spectrofluorometric study of the 1–8 aniline naphtalene sulfonate (ANS)–κ-CN binding and circular dichroism (CD) spectroscopy. The aggregation process to form aggregates like micelles or submicelles and the structural characteristics of these aggregates were pH dependent. Far-UV CD showed that the aggregates obtained by heating presented changes in the κ-CN secondary structure. Near-UV CD spectra showed a certain degree of tertiary organization in the Tyr environment for the protein heated or unheated, only at pH 5.2. ANS binding at both pH was quite different and depends on the self-association process. Heating produced exposition of hydrophobic binding sites only at pH 7.2, including those in the neighborhood of the κ-CN Trp residue.

Keywords Bovine κ-casein · Aggregation · Surface hydrophobicity · ANS · Structure

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Introduction

The κ-casein (κ-CN) is a specific milk phospho-glycoprotein which presents only a phosphorserine residue per molecule [1]. It has 169 amino acids with a protein monomer molecular weight of 19,000, and it is naturally found as a mixture of protein polymeric forms produced by the linkage of monomers through disulfide covalent bonds [2].

κ-CN is associated with αS_1-, αS_2-, and β-casein in milk naturally in the presence of colloidal calcium phosphate forming spherical colloidal particles known as casein micelles (CM) [3]. The preferential location of κ-CN in the CM surface accounts for its capacity to stabilize CM suspensions by protruding its hydrophilic moiety to the CM environment, contributing in that way to the colloidal particle stabilization against coagulation through steric and electrostatic effects [4–7].

Although the abundance of proline in the caseins normally inhibits the formation of ordered helical structures, κ-CN presents some organized regions in the form of short α-helix and β-sheet fragments [8], conformation which has been compared with that of proteins in the molten globule state. The concept of tensegrity has been recently used as an attempt to understand the structure of the caseins, particularly as regards their dynamic arrangements. This model proposes a structure with rigid domains (i.e., groups of β-sheets and β-turns centered in the proline residues) which are bound by more flexible elements: α-helix and loops [9, 10]. This arrangement supports an open structure rheomorphic in nature, which is stabilized by a balance between tension and compression, that is to say a completely dynamic structure free to vary due to the shear strengths exerted by the environment, which causes the protrusion of hydrophobic zones to the solvent [11, 12].

κ-CN molecules interact strongly with each other, and the proteins exist in solution as aggregates of about six to seven molecules, probably associated trough hydrophobic interactions. They also exist as free micelles or submicelles not bound to the CM in milk [13]. These submicelles contained on average 30 κ-CN monomers, and their critical micelle concentration is about 0.4% by mass [14, 15, 16]. Some authors suggest that the interaction site of κ-casein with the others casein molecules could be located at rigid domains [17]. The molecular basis for this association and the κ-CN self-association process still remains without a clear explanation [9].

The κ-CN from bovine milk contains two cysteine residues (Cys^{11} and Cys^{88}). If a κ-CN solution is heated, disulfide bonds will be produced by β-elimination. This fact gives place to heterogeneous aggregates formed by a mixture of monomers and polymers of different molecular weight or a semisolid structure similar to a gel, depending on the solution concentration [18].

Since caseinates in general and κ-CN among them have wide applications in the food industry and other technological areas, the study of the basic structure of κ-CN could be interesting in order to expand the field of its knowledge [9, 19, 20].

Other authors have worked with κ-CN samples in the presence of reducer agents and enzymes [16, 21]. In this work, we investigated the effects of heat and pH on the aggregation and the surface hydrophobicity of κ-CN samples, properties closely linked to its functionality.

Experimental sections

Materials and methods

Samples

κ-CN and 1–8 aniline naphtalene sulfonate (ANS) as ammonium salt were purchased from Sigma Chemical Co. and used without further purification. Stock solutions nearly 20 mM for ANS and 1.5% (*w*/*v*) for κ-CN were prepared in distilled water, sodium azide was added at 0.01% (*w*/*v*) as a preservative, and the solutions were stored in the dark at 4 °C. The concentration of the stock solution of ANS was determined by absorbance measurement, using a molar extinction coefficient (ε)=4,950 M^{-1} cm^{-1} at 350 nm. The κ-CN concentration used in several of experiments was below the critical micelle concentration of κ-CN (which is about 0.4% by mass) [19, 20]. Dilutions of the κ-CN or ANS suspensions were prepared in 10 mM phosphate buffer, pH 7.2 or 5.2.

A Jasco V-550 was used for absorbance measurements, and relative fluorescence intensity (FI) measurements were made on an Aminco Bowman Serie 2 spectrofluorometer. These instruments were equipped with thermostatically controlled jacketed cuvette holder.

The measurements of pH were carried out either on digital pH meters Orion 720 or Horiba serie D, both equipped with proton-selective glass membrane electrodes combined with saturated calomel reference electrodes.

Spectrophotometric study of κ-CN aggregation by heating

The effects of thermal treatments on κ-CN were monitored through spectrophotometry with the aim of following the protein aggregation by heating at different temperatures, as well as to detecting absorption spectra shifts related to possible protein conformational changes.

Measurements at increasing temperature were made at 600 nm from 35 to 100 °C with a heating rate of 0.5 °C per minute. The equipment used was a Jasco V-550 double-bean spectrophotometer equipped with a cuvette holder heated by Peltier effect, controlled by a programmable unit. The cuvette was filled with a 5-g L^{-1} κ-CN solution in buffer at pH 7.2 or 5.2 up to a final volume of 2.5 mL and sealed with a teflon stopper to avoid evaporation during each experiment. The spectrophotometer compartment was continuously purged with nitrogen to prevent the condensation of water vapor on the cuvette walls.

On the other hand, the production of κ-CN conformational changes at distinct temperature was monitored as a function of time, with the same equipment. To do this, absorption spectra were taken every 5 min during 1 h keeping the cuvette at the temperature chosen. The temperatures were 40, 57, and 95 °C for samples in buffer at pH 7.2 and 40, 70, and 95 °C for samples at pH 5.2. The wavelength range employed was from 260 to 600 nm, and the protein concentration and the technical cares were the same as those described in the previous paragraph.

The absorbance measurements were corrected for the turbidity (τ) contribution using the wavelength (λ) dependence of τ in the 400- to 700-nm range, where the absorption due to the protein chromophores is negligible. In this wavelength range, the plots of log τ as function of log λ were linear. From them, we calculated the τ contribution for λ below 400 nm and subtracted these values from the absorbance measurements.

κ-CN aggregation degree study by SDS-PAGE

Samples of κ-CN with and without aggregation were prepared by combining the effects of pH and temperature on the protein, according to Farrell et al. [18].

The state of these different samples was analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel elctrophoresis (PAGE) using a vertical gel system, according to the method

of Laemmli [22]. Adequate amounts of protein samples were dissolved in 1 mL of buffer containing 0.066 M Tris–HCl, pH 6.8, 2% (*w*/*v*) SDS, 20% (*v*/*v*) glycerol, and 0.01% (*w*/*v*) Bromophenol blue or Coomassie brillant blue R250, with or without 1% (*v*/*v*) β-mercaptoethanol. The resolving gel was composed of 11.68% (*w*/*v*) acrylamide and 0.32% (*w*/*v*) bis-acrylamide solubilized in 1.5 M Tris–HCl buffer pH 8.8, containing 0.1% (*v*/*v*) SDS. The stacking gel was composed of 3.8% (*w*/*v*) acrylamide–0.2% (*w*/*v*) bis-acrylamide solubilized in 0.5 M Tris–HCl buffer pH 6.8, containing 0.1% (*w*/*v*) SDS. The polymerization reaction was started by adding 50 μL of 1% (*w*/*v*) ammonium persulfate (freshly prepared) and 5 μL of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine.

The running time was about 45 min, at 25 °C, and the constant intensity was 11 mA for the stacking gel and 23 mA for the resolving gel. Proteins were stained with Coomassie brillant blue R250 staining solution and destained with 10% (*v*/*v*) methanol–10% (*v*/*v*) acetic acid destaining solution. The relative intensity of the stained bands was determined by scanning of the stained gels and analysis of the pixel densities of the digitalized protein bands, using software specially designed for this purpose (X-GEL), including deconvolution of the scanning pattern curves, when necessary. The protein bands were identified using molecular weight markers (Sigma Chemical Co.).

Spectrofluorometric study

Study of the κ-CN-ANS binding

Fluorescent markers have been satisfactorily used to determine the hydrophobic or hydrophilic nature of proteins [23]. In this work, the interaction of the anionic fluorescent marker ANS with κ-CN polymerized in different degrees has been studied.

Previously, fluorescence excitation and emission spectra of ANS, κ-CN, and their mixtures were obtained, in order to detect spectral shifts or changes in the relative FI. Excitation (λ_{ex}) and emission (λ_{em}) wavelengths and the range of concentration at which the inner filter effect was negligible were determined for the systems under study.

Working at constant κ-CN concentration and varying ligand concentration and assuming a single type binding site model, the values of the apparent association constant (K) and the amount of marker moles bound at saturation per protein mole (n) were obtained, by means of nonlinear fitting of ΔF vs. l experimental curves, according to:

$$\Delta F = \frac{\Delta F_M}{2\,Kmn}\left\{1 + Kl + Kmn - \left[(1 + Kl + Kmn)^2 - 4K^2lmn\right]^{1/2}\right\} \quad (1)$$

where ΔF is the difference between the relative FI of the ligand–κ-CN complex (FI_c) and the relative FI of the sole ligand (FI_0), at a given ANS concentration l and a given temperature (T); ΔF_M is the value of ΔF when all the protein particles are saturated by the ligand; and m is the protein concentration. K and n were determined at two temperatures and the standard thermodynamic functions ΔG^0, ΔH^0, and ΔS^0 were calculated as:

$$\Delta G^0 = -RT\ln K \quad (2)$$

where T is constant,

$$\Delta H^0 = \frac{R\ln\frac{K_1}{K_2}}{\frac{1}{T_2} - \frac{1}{T_1}} \quad (3)$$

being K_1 and K_2 the apparent association constants at T_1 and T_2, respectively, supposed ΔH^0 constant in the range of temperatures employed, and

$$\Delta S^0 = \frac{\Delta H^0 - \Delta G^0}{T} \quad (4)$$

obtained as an average value in the range of temperature used.

Samples for spectra determination or FI measurement were prepared by adding the adequate volume of 1 mM ANS solution to 3 mL of dilution 1/100 *v*/*v* of κ-CN (0.15 g L^{-1}) in buffer at pH 7.2 or 5.2.

These samples were transferred to a 1-cm quartz fluorescence cuvette in a thermostatically controlled jacketed cuvette holder maintained at the desired temperature. The FI of the mixtures was followed using 380 nm as λ_{ex} and 468 nm as λ_{em}.

Moreover, the existence of an energy transfer process was studied by using 286 nm as λ_{ex} of the protein and, measuring the emission at 348 and 468 nm, the λ_{em} of κ-CN and the ANS–protein complex, respectively.

Determination of κ-CN surface hydrophobicity

The surface hydrophobicity (S_0) was estimated according to the method of Kato and Nakai [24, 25], using ANS as a hydrophobic fluorescent marker. The FI of samples containing a fixed volume of 6 mM ANS and consecutive aggregates of 1.5% *w*/*v* κ-CN with different treatments (FI_b), as well as protein alone in the correspondent buffer at the same concentrations (FI_p), were measured using 380 and 468 nm as λ_{ex} and λ_{em}, respectively, at 25 °C. Net FI was calculated as $FI_b - FI_p$, and S_0 was measured as the initial slope of a net FI versus κ-CN concentration plot.

Circular dichroism

Circular dichroism (CD) spectra in the far-UV have been widely used to estimate the relative proportions of the different kinds of secondary structure present in a protein. Since the secondary structure known as α-helix shows a

characteristic depression near the 222 nm in the CD spectra, the β-sheet does the same at 210 nm, and so does the structure called random coil near the 200 nm, it is possibly to detect changes in the secondary structure of a protein submitted to different treatments by comparing these spectral zones [26, 27]. On the other hand, near-UV CD can also be a useful technique in determining the role of aromatic side chains groups in protein aggregation. Particularly, the near-UV CD spectra of Tyr and Trp residues are dependent upon the side chain conformation and environment and can be used to monitor changes in the protein structure [28].

CD spectra were obtained at 25 °C employing a spectropolarimeter Jasco J-810. Samples of 0.25 g L^{-1} κ-CN in buffer at pH 7.2 or 5.2, either with or without previous heating, were poured in a quartz cuvette of 1 cm path length (far-CD) or 0.1 cm path length (near-CD) with the cell temperature controlled by a circulating water bath. The CD spectra (average of three runs) were obtained in the far-UV (190–240 nm) and near-UV (240–340 nm) at 100 nm/min, employing 1-s-like time constant, with a sensibility of 20 millidegrees in the first case and 5 millidegrees in the second one. Spectra were corrected from solvent contribution and were expressed in ellipticity (θ) units (millidegrees) vs. wavelength.

Statistical analysis

Data were reported as mean value±standard deviations for all data points. All the experiments were carried out three times at least. Standard ANOVA analysis of variance was used to determine significant differences between variables.

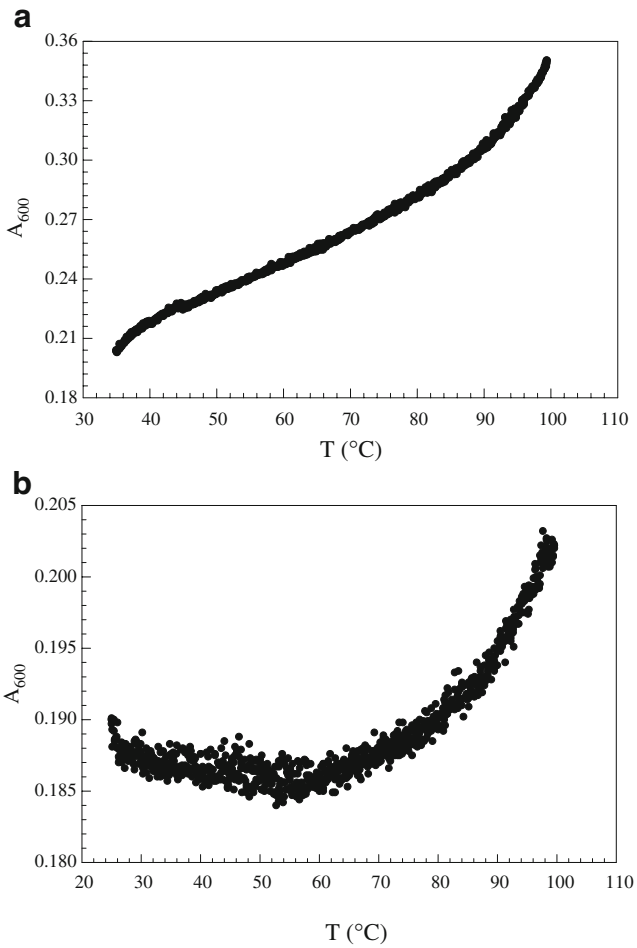

Fig. 1 Absorbance (±0.001) at 600 nm of κ-CN in buffer phosphate 10 mM as function of temperature. **a** pH 5.2, **b** pH 7.2. Protein concentration: 5 g L^{-1}

Results and discussion

Spectrophotometric study of κ-CN aggregation by heating

Preliminary observations of the effect of heating on κ-CN aggregation were made by UV-visible spectrophotometry, monitoring the absorbance of 5 g L^{-1} κ-CN solution samples at 600 nm during their heating from 35 to 100 °C at a rate of 0.5 °C/min. κ-CN samples were dissolved in buffer either at pH 7.2 or 5.2, in order to take consider the effects of pH. The continuous increase in the absorbance at the wavelength used, where the chromophore groups of the protein do not show any absorption, can be attributed entirely to a turbidity increase due to the aggregation of the κ-CN to form aggregates or micelles of increasing size (Fig. 1a,b). At pH 5.2 (Fig. 1a), the shape of the curve was slightly sigmoid, with a small shoulder near 40 °C, showing perhaps a transition in the aggregation process. At pH 7.2 (Fig. 1b), the shape of the curve suggested that the aggregation process is quite different. Indeed, an initial decrease in absorbance can be observed from 35 to 60 °C, indicating perhaps a structural change preceding the aggregation and the correspondent absorbance increase. The maximal absorbance value reached at 100 °C was clearly lower than the value reached at pH 5.2 at the same temperature, suggesting a lower aggregate final size.

The κ-CN spectra (not shown) recorded every 5 min during 1 h at different constant temperatures and at the two pH values, corrected for the turbidity contribution, showed an increasing red shift for the protein band at increasing temperature only in the case of pH 5.2. This behavior could be indicating that the Trp residues of the aggregated protein were increasingly exposed to the medium at this pH during the κ-CN aggregation process.

κ-CN aggregation degree study by SDS-PAGE

The SDS-PAGE patterns of the κ-CN samples obtained at different pH values and temperatures corresponding to the

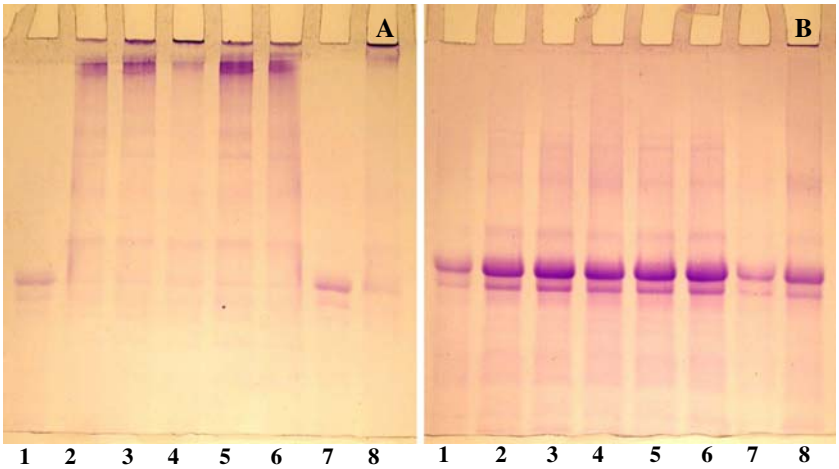

Fig. 2 SDS-PAGE of κ-CN samples (**a** unreduced, **b** reduced) at pH 7.2 (*lanes 1*, *5–8*) and 5.2 (*lanes 2–4*) heated at different temperatures: 30 (*lanes 1*, *2*, *7*), 40 (*5*), 57 (*6*), 70 (*3*), and 95 °C (*4*, *8*). *Lane 7*: standard κ-CN monomer

different regions of the curves in Fig. 1 can be observed in Fig. 2, either in the absence (Fig. 2a) or in the presence (Fig. 2b) of β-mercaptoethanol. In case A, only the lane of the sample at pH 7.2, maintained at 30 °C, showed the presence of κ-CN monomer clearly identified by comparison with the standard in lane 7. On the contrary, all the others samples contained only vestiges of the monomer, showing the presence of high molecular weight aggregates, including protein that could not enter the stacking gel because of its excessively high molecular weight [18]. This was evident even in the case of the samples at pH 5.2 maintained at 30 °C. These results contributed also to show that the pH value of the solution plays an important role in the aggregation of κ-CN and that heating of the protein solutions at temperatures as low as 40 °C was enough to produce extensive κ-CN aggregation. When the samples were run in reducing conditions, all of them showed that they contain almost exclusively the κ-CN monomer, with very low amounts of aggregates of different aggregation degree. This behavior indicates that sulfhydryl disulfide interactions are involved primarily in aggregate formation [18]. The fact that the aggregation was higher at the lower pH, at which the formation of disulfide bridges would be less favored, could suggest the possibility of the presence of other kind of bonds, such as hydrophobic interactions participating in the κ-CN aggregation at this pH value. In effect, at this pH value, close to the protein isoelectric point, the net charge was lower diminishing the electrostatic repulsion between κ-CN particles.

Spectrofluorometric studies of the κ-CN-ANS binding

The fluorescence emission spectra of ANS at a given concentration obtained by exciting the fluorescence probe λ_{ex} (380 nm), either in absence or in presence of κ-CN, allowed us to determine λ_{em} values of 468 nm for the ANS–protein complex and 520 nm for the ANS alone, at pH 7.2 (Fig. 3). There was a clear blue shift of the ANS fluorescence emission as consequence of the presence of the protein.

Apart from this strong blue shift, a high enhancement of the FI in the presence of κ-CN can also be observed in the emission spectra illustrated in Fig. 3. These are results that have been generally considered as indicating the existence of an ANS–protein interaction, with insertion of the fluorescence probe into low polarity regions of the κ-CN [29].

Another characteristic which can be observed in the fluorescence behavior of ANS–κ-CN mixtures is an important overlapping of the ANS absorption spectrum with the protein emission one (Fig. 4), a fact that can be due to the presence of an energy transfer process between the fluorophores of the protein and the bound ANS.

On the other hand, the emission spectra obtained at a given κ-CN concentration for different ANS concentrations, exciting the protein fluorophores at 286 nm (Fig. 5), allowed us to verify the existence of an isoemissive point at

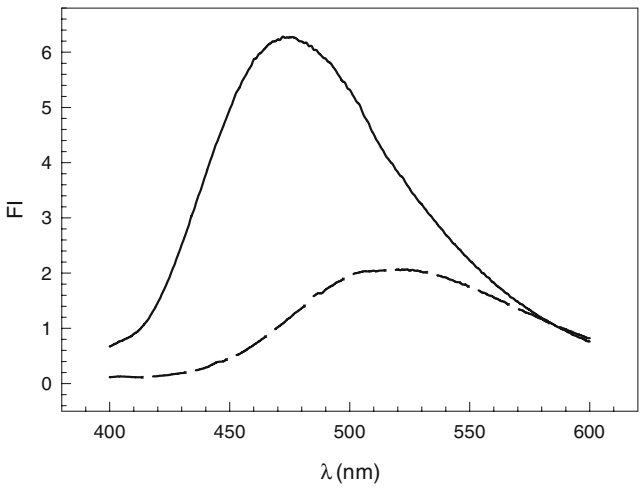

Fig. 3 ANS fluorescence emission spectra in presence (*solid line*) or in absence of κ-CN (dashed line). λ_{ex}=380 nm, [ANS]=0.14 mM, [κ-CN]=0.15 g L^{-1}, buffer phosphate 10 mM, pH 7.2, *T*=27 °C

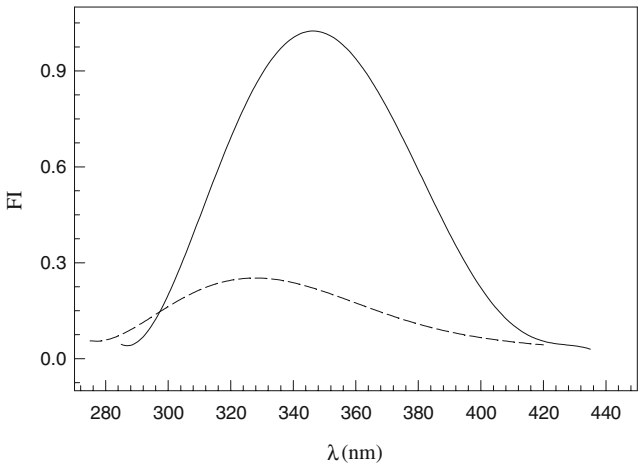

Fig. 4 Overlapping between the absorption spectrum of 0.14 mM ANS (*dashed line*) and the emission spectrum of 0.03 g L^{-1} κ-CN, λ_{ex} 286 nm (*solid line*). Buffer phosphate 10 mM, pH 7.2, *T*=27 °C

417 nm. This fact has been interpreted as evidence that the bound ANS molecules, which are acting as acceptor in the energy transfer process, present the same fluorescence quantum yield over the range of ANS concentrations used [29]. Furthermore, no shifts were detected in the protein emission band during the binding, a behavior that suggests the presence of only one kind of energy donors, the Trp residue of κ-CN, which takes part in the energy transfer to ANS molecules bound in the neighborhood of it. Similar results (not shown) have been obtained working at pH 5.2.

The binding of ANS to κ-CN was then studied in an ANS concentration range in which the ANS FI varied linearly with the ANS concentration, in order to avoid the inner filter effect in the measure of the FI of the samples. The study was made at two temperatures, 33 and 40 °C, either at pH 7.2 or 5.2, and for different κ-CN aggregation degrees. The ΔF values of the samples were obtained with excitation at both 286 and 380 nm, using 468 nm as λ_{em}. The ΔF vs. ANS total concentration curves obtained in all the cases were well fitted by Eq. 1, corresponding to a binding model with only one type of binding site, as can be seen in the example shown in Fig. 6.

The K and n values obtained at 33 and 40 °C, respectively, by fitting the binding curves with Eq. 1 are shown on Table 1. The values of the thermodynamic functions (ΔH^0 and ΔS^0) at 33 °C, obtained by calculation with Eqs. 2 to 4, are reported on Table 2.

The results obtained for the ANS binding to nonheated κ-CN at pH 7.2 (Table 1) showed that n values for 33 and 40 °C did not present significant differences between them. This fact could indicate that the conformational change induced by this temperature increase, suggested by a turbidity decrease in κ-CN solutions (Fig. 1b), was not able to vary the number of κ-CN-binding sites for ANS. The effect of the temperature increase could then be due to an expansion of the already unfolded protein structure.

On the other hand, none of the K values determined at both temperatures presented any difference. The lack of response of the affinity constant to temperature variations indicated that the ANS–κ-CN interaction had an enthalpy change near zero and a positive entropy change (Table 2). The binding appeared then as a process driven by entropy, with loss of structured water associated with the binding sites [30].

κ-CN heated at 95 °C, at pH 7.2, bound per molecule a number of ANS molecules higher than that determined for

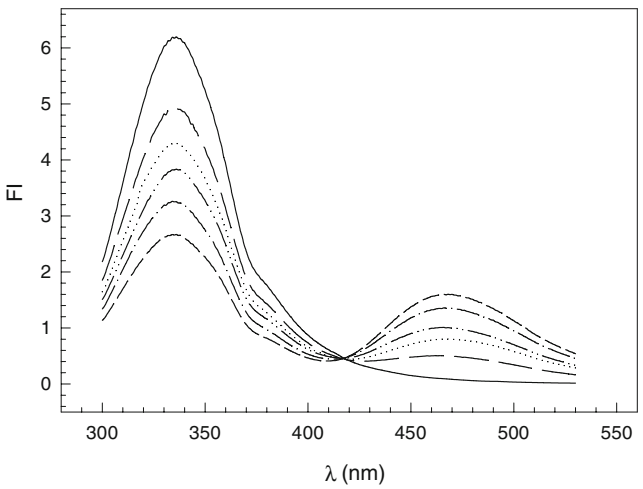

Fig. 5 Fluorescence emission spectra of κ-CN (0.15 g L^{-1}) in the absence (*solid line*) or in presence of different ANS concentrations: 6.62 (*long dashed line*), 13.16 (*dotted line*), 19.61 (*dash-dot-dot-dash line*), 32.26 (*dash-dot-dash line*), and 44.59 μM (*short dashed line*), λ_{ex}=286 nm. Buffer phosphate 10 mM, pH 7.2, *T*=27 °C

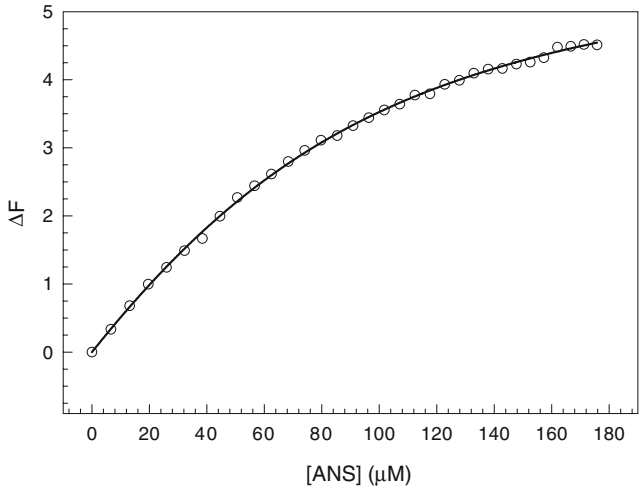

Fig. 6 ΔF of ANS in the presence of κ-CN (0.15 g L^{-1}) as function of ANS total concentration (*empty circles*) and curve calculated using Eq. 1 (*solid line*). Buffer phosphate 10 mM, pH 7.2, λ_{ex}=380 nm, λ_{em}=468 nm, *T*=27 °C. Each experimental point is the average of four determinations

Table 1 Apparent association constant (K) and moles of ligand bound at saturation per mole of κ-CN (n) for ANS–κ-CN interactions, determined by fluorescence intensity measurements

Samples	λ_{ex} (nm)	T (°C)	n (mol/mol)	K (mM^{-1})
Unheated	380	33	8.5±0.8[a]	20±3
pH 7.2	380	40	9.6±0.9	21±3
	286	33	5.3±0.3	230±50
Heated	380	33	12.3±0.2	34±1
pH 7.2	286	33	6.7±0.2	180±10
Unheated	380	33	7.0±0.6	28±4
pH 5.2	380	40	5.1±0.8	18±2
	286	33	2.8±0.4	80±20
Heated	380	33	7.4±0.4	25±0.7
pH 5.2	286	33	4.6±0.3	90±5

κ-CN (0.15 g L^{-1}) in 10 mM phosphate buffer at pH 7.2 or 5.2
[a] Standard variations were obtained by fitting of experimental dates with Eq. 1

the unheated protein. An important increase was also observed for the average affinity constant (Table 1). The increase in the κ-CN aggregation degree by heating appeared then as producing a further exposition of sites or regions able to bind ANS and with higher energy than the sites of the unheated protein. This behavior could be related to the capacity of caseins to generate quaternary structures without a previous folding into more compact tertiary ones, since compaction could very likely lead to hiding of hydrophobic regions by formation of hydrophobic interactions in the folding process and to a diminution of the number of ANS-binding sites. This could also be the case if the aggregation was produced mainly by hydrophobic interactions. On the other hand, aggregation by heating at pH 7.2, which is produced essentially by formation of S–S bridges, could thus result in more open and probably more hydrated structures.

The study of ANS binding at pH 7.2 at the protein λ_{ex} showed that almost half of the bound ANS was located in the neighborhood of Trp residues, with average affinity constants of one order of magnitude higher than the values obtained with excitation at the ligand λ_{ex} (Table 1). The difference found in the average affinity constant, however, was not enough to produce bimodal binding curves. As regards to this, it is interesting to realize that there are nine Tyr, one Trp, and, at both pH studied, positively charged Lys residues, which represents an ideal configuration for the binding of an amphiphylic ligand such as ANS, which has a strong negatively charged polar group [3].

Table 2 Thermodynamic functions (ΔH^0 and ΔS^0) at 33 °C for ANS–κ-CN interactions

pH	ΔH^0 (Kcal/mol)	ΔS^0 (cal/mol K)
7.2	~0	23.72
5.2	−12.473	−20.53

In the case of heated κ-CN at pH 7.2, the energy transfer process was similar, with a number of bound ANS molecules close to the Trp residues higher than for the case of the unheated protein (Table 1). This result could be explained if the aggregation by heating were accompanied by a conformational change which would offer more binding sites for ANS near to the Trp residue. The K values found in this case were clearly lower than the values determined for the unheated protein (Table 1), a fact that could be coherent with the presence of such conformational change in the Trp neighborhood.

The ANS binding to unheated κ-CN at pH 5.2 presented some differences with the binding at pH 7.2. The number of binding sites by protein molecule was slightly lower at 33 °C and tended to decrease when the temperature increased from 33 to 40 °C. This loss of ANS-binding sites could be related to a change in the aggregation process introduced by heating, which is consistent with the features of the absorbance curve of heating of κ-CN, which showed a shoulder at temperatures near 40 °C (Fig. 1a). Although the K values were of the same order of magnitude as those determined at pH 7.2, at pH 5.2, they showed a decrease for the temperature increase (Table 1). Negative enthalpy and entropy changes were then calculated (Table 2), suggesting a higher participation of electrostatic interactions in the binding at pH 5.2. This can be explained if we take into account that at pH 5.2, the negative residues of the proteins are protonized in a higher degree and the negative net charge of the κ-CN will be lower than at pH 7.2. The negative moiety of the ligand will be less rejected, and its anionic sulfonate group could reach the positive residues of the κ-CN more easily to give interactions mainly electrostatic.

The number of ANS molecules bound in the Trp neighborhood, determined exciting at the protein λ_{ex}, was lower at pH 5.2 than at pH 7.2, either for not heated or heated κ-CN, as was the case of the affinity constants for this kind of binding sites (Table 1). Since at pH 5.2, κ-CN showed a high degree of aggregation even at relatively low temperatures, it is possible to suppose that the mechanism of this aggregation differs from that produced at pH 7.2, leading to a higher participation of apolar regions, in intra- or intermolecular hydrophobic interactions, hiding them to the possibility to interact with the hydrophobic moiety of ANS. This hypothesis would be coherent with the predominance of the electrostatic component in the ANS binding showed by the sign of the thermodynamic parameters of the interaction.

Surface hydrophobicity measurements

The S_0 values determined for heated and unheated κ-CN, either at pH 7.2 or 5.2, are shown on Table 3.

Table 3 Hydrophobicity surface (S_0) for heated and unheated κ-CN (T=25 °C)

Samples	S_0 (% w/v^{-1})
Unheated pH 7.2	59[a]±1
Heated pH 7.2	80±1
Unheated pH 5.2	77.7±0.7
Heated pH 5.2	175±2

[a] Each value is the average of at least three determinations.

At pH 7.2, S_0 was higher for the κ-CN heated at 95 °C, which is consistent with the results obtained for the ANS binding in such conditions, thus confirming in that way that a higher exposition of hydrophobic binding sites was produced as an effect of the increase in the aggregation degree by heating.

The S_0 values obtained at pH 5.2 were higher than at pH 7.2, either in the case of the heated or the unheated protein. Comparison with the results for the number of ANS-binding sites at this pH suggested that, under the conditions of S_0 determination, that is to say in a high excess of ANS, important amounts of the fluorescent probe interact with the κ-CN occupying hydrophobic regions that cannot be filled in the binding experiments. Furthermore, in such excess by ligand, the possibility of ANS aggregation or hemimicelle formation on the molecules already bound to the protein and also favored by the lower repulsion between ANS and κ-CN could not be discarded.

Anyway, the S_0 increase observed for the heated protein pointed to the opening of the κ-CN aggregated by heating, consistent with the experimental results of UV-visible spectroscopy, ANS binding, and far-UV CD determinations.

Circular dichroism studies

Far-UV CD measurements (190–240 nm) of heated and unheated κ-CN, either in buffer at pH 7.2 or 5.2, showed spectra with a minimum of ellipticity around 200 nm and a secondary negative between 210 and 230 nm (Fig. 7), similar in their general features to the already observed by Griffin et al. under similar conditions [31]. Structural predictions from these spectra using the CONTIN procedure of Provencher and Glöckner [32] and the CDsstr program (variable selection method) of Johnson [33], using a set of standard proteins chosen by the CLUSTER program of Venyaminov and Vassilenko [34], were almost coincident, showing a very low helix content but with significant amounts of β-sheet for all of them (Table 4).

The CD spectra obtained at each of the pH values were similar for the heated as well as for the unheated protein. Comparison of such spectra showed that heating produced a clear increase in the unspecific disordered structures and a low increase in α-helix at the expense of a β-sheet decrease, independently of the pH of the medium.

Near-UV spectra of κ-CN submitted to different heating treatments either in buffer at pH 7.2 or 5.2 can be observed on Fig. 8. In the case of samples at pH 7.2, either heated or unheated, the CD signal remained nearly zero in all the wavelength range screened. Such could be the behavior in the case of protein molecules poorly structured at the tertiary level.

On the contrary, in the samples at pH 5.2, either heated or unheated, a small negative band was found in the wavelength region between 260 and 290 nm, with its peak located near 275 nm. The presence of this band could be associated with an effect of the solvent on the protein structure, producing differences in the environment of the aromatic chromophores, especially Tyr (characteristic signal between 270 and 290 nm), with a higher degree of tertiary organization for the samples at pH 5.2 than those at pH 7.2 [18]. These results, combined with that of the amount of ANS binding decrease at pH 5.2, could be indicated that the aggregation region would be near to the ANS-binding sites. This region could form a β-sheet conformation during the self association, blocking the binding of ANS.

On the other hand, if we take in account that κ-CN is a phosphoglycoprotein and, at high temperatures, the hydrolysis of sugar groups occurs, the presence of these groups would influence the self-association behavior.

Conclusions

The ability to aggregate and form micelles with the participation of different kind of interactions: hydrophobics,

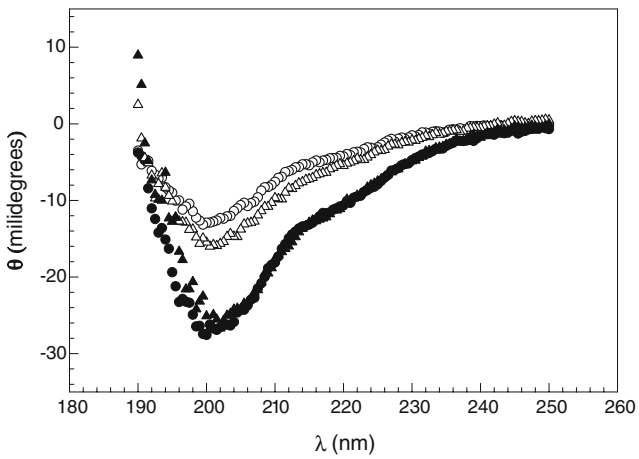

Fig. 7 Far-UV CD spectra, as ellipticity (θ) vs. λ, for κ-CN in 10 mM phosphate buffer pH 7.2 unheated (*empty circles*) or heated (*filled circles*) or pH 5.2 unheated (*empty triangles*) or heated (*filled triangles*). κ-CN concentration 0.25 g L^{-1}, temperature=25 °C. Each point is the average of two independent determinations

Table 4 Structure predictions from far-CD spectra using the CONTIN and the CDsstr programs

Samples	CONTIN				CDsstr			
	α-Helix	β-Sheet	Turns	Unordered	α-Helix	β-Sheet	Turns	Unordered
Heated pH 5.2	8.4	23.5	15.6	56.1	5.4	23.2	16.4	54.5
Heated pH 7.2	7.9	21	15.2	56	5.9	19.5	14.3	60.1
Unheated pH 5.2	3.9	3.06	16	49.4	2.6	30.4	16.7	48.8
Unheated pH 7.2	3.4	21.9	15.4	50	3.1	30.9	17.2	46.9

In all the cases, the standard deviations were below 4%.

electrostatics, and formation of disulfide bridges, is a well known property of κ-CN [2, 19, 35, 36]. The results of our work showed that the pH of the medium plays an important role in such aggregation, being this more considerable at the lower pH and in the structural characteristics of the resultant aggregates. For example, although the aggregates appeared always as produced mainly through disulfide bridges, at pH 5.2, the aggregation occurred at lower temperature than at pH 7.2, and the aggregates' final size was higher. The effect of the pH medium on the net κ-CN charge would indubitably be an important factor to be taken into account as regards to the differences in the aggregation process. Lower negative net charge and the resultant lower electrostatic repulsion at pH 5.2 would increase the number of collisions between κ-CN molecules per time unit effective to lead to protein–protein interactions, which could be hydrophobics, which are favored by the increase in temperature.

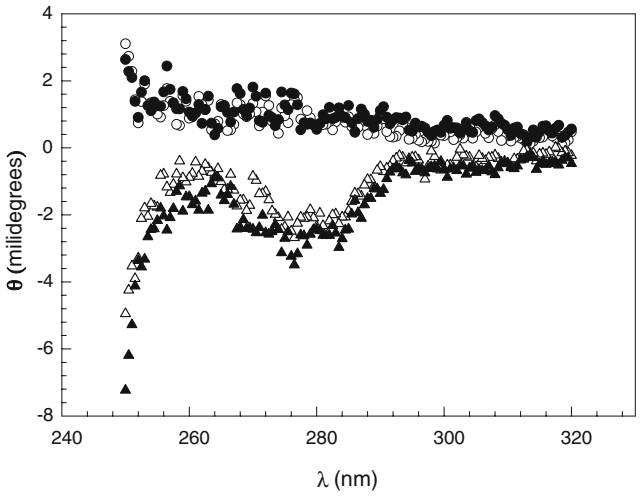

Fig. 8 Near-UV CD spectra, as ellipticity (θ) vs. λ, for κ-CN in 10 mM phosphate buffer pH 7.2 unheated (*empty circles*) or heated (*filled circles*) or pH 5.2 unheated (*empty triangles*) or heated (*filled triangles*). κ-CN concentration 0.25 g L^{-1}, temperature=25 °C. Each point is the average of two independent determinations

Different aggregation processes at different pH values gave place to aggregates which presented some structural differences. On the other hand, the effects of heating on κ-CN secondary structure appeared quite similar for both pH values, and an increase in the unordered regions at expense of β-sheet ones has been observed in the two cases. The results obtained for S_0 confirm the opening of the κ-CN structure by heating at both pH values.

However, analyzing the ANS–κ-CN interaction, several differences appeared according to the pH of the medium. The ANS binding at pH 7.2 showed an important contribution of hydrophobic interactions. Then, the structural transformation produced by strong heating with the opening of β-sheet regions to give disordered structures can be plausibly associated with a higher exposure of apolar side groups of the protein. The fact that the κ-CN Trp residue was located in a region which would be structured as a β-sheet can account for the increase in the number of bound ANS molecules which appeared in the Trp neighborhood by strong heating, possibly as a consequence of the opening of such structure with exposition of apolar binding sites.

On the contrary, since the ANS binding at pH 5.2 presented electrostatic characteristics, it is possible to assume that charged side chains of the κ-CN take part in the binding sites. The structural transformation which accompanied the aggregation of the protein by heating did not produce any increment of such kind of sites; that is, there was no further exposure of positively charged residues such as Lys located in or near apolar environments. However, the number of bound ANS molecules near the Trp residue experimented an increase, which suggests that the opening of the β-sheet region harboring the Trp residue contributed by approaching it to more occupied ANS-binding sites. Accordingly, a red shift was observed in the absorbance spectra of the protein heated at 95 °C at this pH, indicating an increase in the polarity of the Trp neighborhood.

The decrease in number of ANS molecules bound in the Trp neighborhood at the lower pH would indicate that this region would participate in the self-association of κ-CN particles to form micelles or submicelles.

Therefore, κ-CN undergoes—due to a strong heating—an aggregation process whose mechanism and aggregates structure depend on the medium pH. The aggregates obtained are less compact, especially at pH 7.2, and richer in disordered regions, with changes in the exposition of apolar side chains to the medium. These changes could indubitably play an important role in the expression of their functional properties, a possibility which deserves further consideration because of its potential importance in the food industry.

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