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Effect of Heat Treatment on Denaturation of Bovine α-Lactalbumin: Determination of Kinetic and Thermodynamic Parameters

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The effect of heat treatment on the denaturation of α-lactalbumin was studied, under different conditions, over a temperature range of 78−94 °C. The concentration of the residual immunoreactive protein after different treatments was determined by kinetic analysis, obtaining D and Z values. Thermodynamic parameters were also calculated. Denaturation of α-lactalbumin, measured by the loss of immunoreactivity, could be described as an order of reaction of $n = 1.5$. Results obtained indicated that α-lactalbumin was more heat-sensitive when treated in milk than in phosphate buffer. The protein was also denatured more rapidly in the apo form than in the calcium-saturated form. Besides, the thermal stability of apo-α-lactalbumin decreased with the binding of oleic acid.

KEYWORDS: Heat denaturation; bovine milk; native lactalbumin; apo-α-lactalbumin; kinetics; thermodynamics

INTRODUCTION

α-Lactalbumin is the second most abundant protein in bovine whey, after β-lactoglobulin, its concentration in mature milk being ~1.2 mg/mL (*1*). It is an acidic, low molecular weight globular protein (14.2 kDa) that is produced in the mammary gland during lactogenesis (*2*).

One of the most interesting features of α-lactalbumin is its ability to bind metal cations. The protein displays a high affinity binding site for Ca^{2+} and several Zn^{2+} binding sites. When isolated from milk, α-lactalbumin contains about one ion of calcium per mole of protein. The binding site for calcium also binds, although more weakly, other ions such as Mg^{2+}, Mn^{2+}, Na^{+}, and K^{+} (*3*, *4*). The removal of calcium from α-lactalbumin causes an increase in the fluorescence intensity and shifts the maximum wavelength to a higher value, reflecting a conformational change of the protein (*5*). Furthermore, it has been observed by circular dichroism that the structure of the apo form is similar to the molten globule state in which the secondary structure remains unchanged, whereas the tertiary structure is altered (*6*). The binding of calcium to α-lactalbumin increases significantly protein stability against denaturation by heat and other denaturing agents such as urea and guanidine hydrochloride (*4*).

Another interesting property of α-lactalbumin is its ability to interact with lipid membranes and fatty acids (*7*−*10*). It has been shown that α-lactalbumin binds 5-doxylstearic, stearic, palmitic, and oleic acids (*7*, *9*, *10*), the binding parameters depending upon the protein state. Studies performed by fluorescence spectroscopy and partition equilibrium indicated that the bovine and human α-lactalbumin apo forms display one binding site for oleic acid, the association constant being $4.6 \times 10^{6}\ M^{-1}$ (*7*). However, native α-lactalbumin was reported to be unable to bind fatty acids (*7*, *10*).

The main function of α-lactalbumin is to participate in lactose biosynthesis as the regulatory component of the lactose synthase complex (*2*). Recent studies have indicated that a multimeric form of α-lactalbumin, isolated from human casein by anion-exchange chromatography, can induce apoptosis in tumor and immature cells but not in healthy differentiated cells (*11*, *12*). Likewise, α-lactalbumin derived from human or bovine whey could be converted to the apoptotic-inducing form when removing calcium bound, by treatment with EDTA, and passing it through an anion-exchange column previously conditioned with oleic acid (*10*). This active form of the protein, called "human or bovine α-lactalbumin made lethal to tumor cells" (HAMLET or BAMLET, respectively), was described as a complex formed by apo-α-lactalbumin and oleic acid (*10*, *13*). Furthermore, it has been shown that this complex induces apoptosis in vivo, it slows tumor development in the brain of rats, and it reduces skin papillomas in humans (*11*).

On the other hand, whey is a subproduct produced in large scale in the cheesemaking process. One of the main concerns of whey processors is to produce whey protein products, such as concentrates, isolates, or fractions enriched in β-lactoglobulin or α-lactalbumin, with specific functionalities (*14*). In this sense, the potential use of α-lactalbumin as a supplement for special food or pharmaceutical products has encouraged the study of

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its physicochemical and biological properties. Heat treatments are extensively used for preservation and, thus, the thermal stability of α-lactalbumin should be evaluated to design treatments that ensure the maintenance of its biological activity.

Studies of the thermal denaturation of bovine α-lactalbumin have been performed using different techniques such as circular dichroism (*15*), sodium dodecyl sulfate (SDS) electrophoresis (*16*), capillary electrophoresis (*17*), and immunochemical techniques (*18*, *19*) and differential scanning calorimetry (*20*, *21*). However, there are discrepancies in the results obtained due probably to the use of different media to treat the protein and different methods to measure its denaturation.

The aim of this work was to study the effect of heat treatment on bovine α-lactalbumin in different conditions in order to facilitate the design of heat treatments that preserve its possible biological function. The present work includes the study of bovine α-lactalbumin in the native form and in the apo form with and without oleic acid bound. Likewise, the denaturation of α-lactalbumin was also determined in bovine milk samples.

MATERIALS AND METHODS

Materials. Fresh raw bovine milk samples were kindly supplied by CLESA (Utebo, Zaragoza, Spain). Sodium barbital was provided by Panreac (Barcelona, Spain). Sephadex G-100, G-50, and G-25, agarose, complete and incomplete Freund's adjuvants, and oleic acid were supplied by Sigma (Poole, U.K.). The polyacrylamide gels, gel buffers, and molecular weight marker were purchased from Pharmacia (Uppsala, Sweden). The rest of the reagents and chemicals not specifically referred to were of analytical grade.

Isolation of Bovine α-Lactalbumin. Bovine milk samples were skimmed by centrifugation at 2000*g* for 30 min at 4 °C. Whey was obtained by ultrafiltration in an Amicon DC 2A system fitted with a hollow fiber cartridge, Diaflo H1P100. This system supplies whey proteins of molecular mass below 100 kDa. The whey obtained was concentrated in ultrafiltration cells with YM-3 Diaflo Amicon membranes. α-Lactalbumin was isolated by gel filtration chromatography on Sephadex G-100 (90 × 5 cm) equilibrated with 25 mmol/L sodium acetate and 50 mmol/L NaCl buffer, pH 6.5, at 4 °C. Fractions enriched in α-lactalbumin were pooled, concentrated, and chromatographed under the same conditions on a Sephadex G-50 column (75 × 3 cm). The protein obtained was dialyzed against distilled water and lyophilized. The purity of α-lactalbumin was checked by SDS-PAGE on Phastsystem equipment (Pharmacia, Uppsala, Sweden). Electrophoresis was performed on 8−25% acrylamide gradient gels. Protein samples were dissolved in 10 mmol/L Tris-HCl buffer, pH 8.0, containing 1 mmol/L EDTA, 2.5% SDS, and 0.01% bromophenol blue and boiled for 5 min. A molecular weight marker was included in the gels. The gels were Coomasie Blue-stained and destained until a colorless background was achieved. α-Lactalbumin obtained was 98% pure as determined by optical density.

Preparation of Bovine Apo-α-lactalbumin and Incubation with Oleic Acid. Calcium bound to α-lactalbumin was removed to obtain the apo form according to the method of Murakami et al. (*22*). An amount of 12 mg of α-lactalbumin was dissolved in 1.2 mL of 10 mmol/L Tris-HCl buffer, pH 8.5, containing 100 mmol/L EDTA. The mixture was incubated at room temperature for 1 h and, afterward, it was applied on a Sephadex G-25 column (25 × 1 cm) equilibrated with the same buffer. Fractions eluted in the void volume of the column were pooled, dialyzed against distilled water, and lyophilized. Apo-α-lactalbumin was characterized by fluorescence spectroscopy as described by Barbana et al. (*7*). The spectrum reached the maximum of fluorescence intensity at 330 and 350 nm for native lactalbumin and apo-α-lactalbumin, respectively.

Apo-α-lactalbumin (125 μg/mL) was incubated with oleic acid dissolved in ethanol at 37 °C for 1 h. The concentration of oleic acid added was twice the molar concentration of the protein, and the final concentration of ethanol in the protein solution was 3%.

Preparation of the Antisera. Antisera against bovine α-lactalbumin were developed in rabbits. A volume of 0.5 mL of α-lactalbumin (1 mg/mL) was homogenized with 0.5 mL of complete Freund's adjuvant and administered in several subcutaneous injections in the back. After 4 weeks, the animals were boosted following the same protocol as in the first immunization, although using incomplete Freund's adjuvant. Ten days later, the animals were bled from the ear vein. Immunoelectrophoresis was used to check the antisera (*23*), which showed to be specific for α-lactalbumin.

Measurement of α-Lactalbumin Concentration. The concentration of α-lactalbumin was determined by radial immunodiffusion (*24*). Agarose at 1% in 25 mmol/L barbital buffer and 0.3 mol/L NaCl, pH 8.2, were prepared containing 0.5% specific antiserum and spread onto 9 × 12 cm glass plates, previously coated with 0.8% agarose in distilled water, and air-dried. In the solidified gel, 2 mm thick, 20 wells of 3.5 mm diameter were cut out. Samples and appropriate standards of α-lactalbumin (7 μL) were applied to the wells, and the plates were incubated in moist chambers at room temperature for 48 h. After immunodiffusion, the plates were washed in 10 mmol/L potassium phosphate and 0.25 mol/L NaCl buffer, pH 7.4, for 24 h with frequent changes. Finally, the plates were washed once with distilled water and allowed to air-dry. The gel was stained with Coomasie Blue [250 mg/L in methanol/distilled water/acetic acid (45:49:6, v/v/v)] and then destained in methanol/acetic acid/glycerol/distilled water (25:8:2:65, v/v/v/v). Standard curves were made by plotting the square of the diameter values of the precipitating rings versus the concentration of the protein standards.

Heat Treatment. Twenty-five microliter samples of skimmed milk or α-lactalbumin samples in 10 mmol/L potassium phosphate and 0.15 mol/L NaCl buffer, pH 7.4, were introduced into glass capillary tubes (1.5−1.6 mm outer diameter, 1.1−1.2 mm inner diameter) and then sealed with a microflame; their hermeticity was checked by immersion in tepid water. The capillaries were immersed in a temperature-controlled water bath (±0.1 °C) at five different temperatures: 78, 82, 86, 90, and 94 °C. Heated samples, in duplicate, were removed from the bath at different intervals for each temperature and immediately cooled by immersion in an ice−water bath. The concentration of undenatured α-lactalbumin was measured by radial immunodiffusion as described above.

Calculation of *D* and *Z* Values. *D* values (time required for 90% denaturation at constant temperature) were calculated for each treatment from the graphical representation of the logarithm of concentration as a function of time. *D* values were calculated as the reciprocal of the slope obtained by regression analysis. The effect of temperature on the *D* value was also studied, and the *Z* value (degrees necessary to reduce *D* value in one logarithmic cycle) was calculated by regression analysis. *Z* was obtained from the slope of the line drawn by regression analysis when representing the logarithm of *D* values versus the corresponding temperatures.

Kinetic Analysis. Reaction Order. The denaturation process for bovine α-lactalbumin can be described by the general rate equation

$$-\mathrm{d}C/\mathrm{d}t = kC^n \quad (1)$$

where $-\mathrm{d}C/\mathrm{d}t$ represents the rate of protein denaturation, k the rate constant, C the protein concentration at each time, and n the reaction order.

For $n = 1$

$$-\mathrm{d}C/\mathrm{d}t = kC \qquad -\mathrm{d}C/C = k\,\mathrm{d}t$$

and integrating

$$\ln(C_0/C_t) = kt \quad (2)$$

is obtained where C_0 is the initial protein concentration (for time $t = 0$) and C_t the concentration of undenatured protein at each holding time. When the denaturation process follows first-order kinetics, the graphical representation of eq 2 gives a straight line and the value b of the ordinate intercept (time $t = 0$) is close to 0. By regression analysis, the value of the constant k is obtained from the slope of the lines.

For $n \neq 1$, when the general eq 1 is integrated

$$(C_t/C_0)^{1-n} = 1 + (n-1)kt \quad (3)$$

is obtained. For non-first-order reactions the representation of the results according to eq 3 yields straight lines, and from its slope the rate constant k is obtained.

The ordinate intercept b (time $t = 0$) for a non-first-order reaction should be $b = 1$, if the process follows the estimated reaction order.

Kinetic Parameters. The temperature and the denaturation constant are related according to the Arrhenius equation

$$k = A\, e^{-E_A/RT}$$

where k represents the rate constant for the denaturation process, A the Arrhenius constant, E_A the apparent energy of activation, R the universal gas constant, and T the absolute temperature. Taking natural logarithms

$$\ln k = \ln A - (E_A/R)(1/T) \quad (4)$$

is obtained. When the natural logarithm of the denaturation constant versus the inverse of the absolute temperature is plotted according to expression 4, the E_A value from the slope, and the ln A value from the ordinate intercept are obtained. Thus, the thermodynamic parameter changes in enthalpy ($\Delta H^{\ddagger}$), entropy ($\Delta S^{\ddagger}$), and free energy of activation (ΔG) are obtained using the equations

$$\Delta H^{\ddagger} = E_A - RT$$

$$\Delta S^{\ddagger} = R[\ln A - \ln(K_B/h_p) - \ln T]$$

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$$

where K_B is the Boltzmann constant and h_p the Planck constant.

RESULTS AND DISCUSSION

In this work, the denaturation of bovine α-lactalbumin was studied by measuring the loss of reactivity with specific antibodies using radial immunodiffusion. High correlations were obtained ($r^2 \geq 0.98$) in the standard curves when representing the square diameter of precipitating rings versus the corresponding concentrations of the protein standards of α-lactalbumin. Thus, a linear response can be ensured when samples with concentrations in the range of the standards are measured.

As a first step, preliminary experiments were performed to determine an appropriate range of temperatures to study α-lactalbumin denaturation by the capillary method. It was observed that native α-lactalbumin in phosphate buffer at a concentration of 125 μg/mL denatured very slowly at 74 °C. Treatment at that temperature for 6 h caused only 20% denaturation of native α-lactalbumin and, thus, a range from a higher temperature was chosen, between 78 and 94 °C.

The degree of loss of α-lactalbumin immunoreactivity increased with temperature and time of treatment (**Figure 1**). It was also observed that native α-lactalbumin was more heat labile when it was treated in milk than in phosphate buffer at a similar concentration. Furthermore, the immunoreactivity of native α-lactalbumin decreased more rapidly at a high concentration than at a low concentration. The time of denaturation for a given temperature was longer for native α-lactalbumin than for apo-α-lactalbumin, and the binding of oleic acid to apo-α-lactalbumin caused a decrease in the thermal stability of the protein. The graphs show results of individual experiments, whereas mean values of data from at least two experiments were used to calculate all of the kinetic parameters. D and Z values were calculated as described under Materials and Methods (**Table 1**). The variation coefficients of D values calculated from the different experiments for all samples and all temperatures were <6%. D values changed as a function of temperature (**Figure 2**).

Table 1. D Values and Z Values for Native α-Lactalbumin in Phosphate Buffer and in Milk and for Apo-α-lactalbumin in Phosphate Buffer with and without Oleic Acid Bound at Different Temperatures.

	phosphate buffer				
	native[a]	native[b]	apo[b]	apo + oleic acid[b]	milk[a]
D_{78} (s)	10213	54054	7874	5714	3254
D_{82} (s)	7399	19616	6097	5263	2032
D_{86} (s)	3668	13155	4237	2673	1745
D_{90} (s)	2339	6970	3389	2217	1164
D_{94} (s)	1408	5753	2525	1483	1139
Z (°C)	18.0	16.7	32.1	20.5	34.7

[a] Protein concentration = 1.25 mg/mL. [b] Protein concentration = 125 μg/mL.

D values obtained for the denaturation of α-lactalbumin in milk were comparable with those found by Lyster (*18*) and Levieux (*25*) using radial immunodiffusion. However, D values obtained were lower than those determined by Jeanson et al. (*19*) using an ELISA method. These differences could be attributed to the different technique used to measure α-lactalbumin concentration as well as to the different thermal treatment. When milk is heated, the heating and cooling times may vary greatly depending on the sample volume and the type of container used to heat the samples. In the present work, 25 μL aliquot portions of samples were heated into glass capillary tubes, whereas samples of 1 mL were employed by Jeanson et al (*19*). The Z value of 34.7 °C obtained here for α-lactalbumin treated in milk confirmed the high heat resistance of α-lactalbumin compared with other milk proteins (*26*, *27*). This value was higher than the value of 20.5 °C calculated previously (*19*).

The thermoresistance of α-lactalbumin at a similar protein concentration was greater when the protein was treated in phosphate buffer than in milk, differences among D values being higher for the lower temperatures studied. The lower thermoresistance of α-lactalbumin in milk has been attributed in part to the reaction of the sulfhydryl groups of β-lactoglobulin with disulfide bonds present in α-lactalbumin forming intermolecular complexes, probably via disulfide interchange (*18*, *25*). In fact, it has been reported that the addition of specific reagents for sulfhydryl groups to skim milk reduced ∼25-fold the rate of denaturation of α-lactalbumin at 85 °C (*18*). In addition, the presence of α-casein decreases the temperature of denaturation of α-lactalbumin and of other whey proteins such as β-lactoglobulin, lactoferrin, and serum albumin when measured by differential scanning calorimetry (*28*) and electrophoresis (*29*). This would explain the higher thermoresistance observed for α-lactalbumin and β-lactoglobulin when treated in whey than when treated in milk (*25*, *29*). Moreover, the greater decrease of milk pH in comparison with that of phosphate buffer could also contribute to the lower thermoresistance of α-lactalbumin when treated in milk, as has been also reported for lactoferrin (*27*).

From the results obtained in this work, the heat denaturation of α-lactalbumin has been shown to be dependent on protein concentration. α-Lactalbumin was less resistant at a concentration of 1.25 mg/mL than at 125 μg/mL. This fact could be due to the trend of the protein to form aggregates at the high concentration, which would lead to a decrease of accessible epitopes in the protein and, thus, to a decrease of immunoreactivity (*17*).

On the other hand, D values were much higher for α-lactalbumin in the native form than in the apo form at a similar protein

Figure 1. Effect of heat treatment on the denaturation of native α-lactalbumin in phosphate buffer (■, 125 μg/mL; ●, 1.25 mg/mL) and in milk (○) and of apo-α-lactalbumin in phosphate buffer (125 μg/mL) with and without oleic acid bound (△, ▲) at different temperatures. C_t represents the concentration of α-lactalbumin measured by radial immunodiffusion at each holding time.

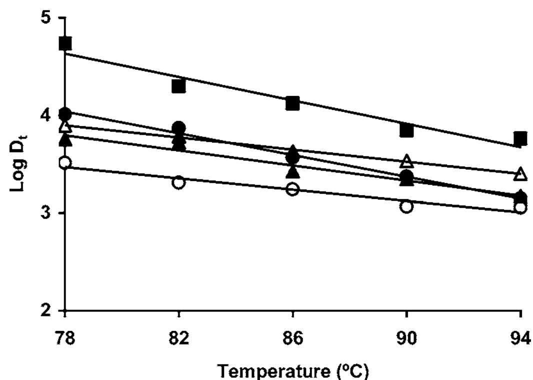

Figure 2. Effect of temperature on D values for the denaturation of native α-lactalbumin in phosphate buffer (■, 125 μg/mL; ●, 1.25 mg/mL) and in milk (○) and of apo-α-lactalbumin in phosphate buffer (125 μg/mL) with and without oleic acid bound (△, ▲).

concentration. The influence of calcium on the stability of bovine α-lactalbumin has been widely studied by differential scanning calorimetry. In the presence of an excess of calcium, α-lactalbumin unfolds upon heating with a single heat absorption peak of ~64.1 °C. When calcium was removed from the protein by treatment with EDTA, the maximum temperature of denaturation decreased to a value of ~35 °C (*20*). The difference in thermoresistance of both forms of α-lactalbumin is probably due to the conformational change that occurs when calcium is removed from the protein as has been observed by fluorescence spectroscopy and circular dichroism (*5*, *6*). A similar behavior has been observed with other metalloproteins such as lactoferrin or transferrin, which are more resistant to heat denaturation when iron-saturated (*27*). However, the binding of oleic acid to apo-α-lactalbumin produced a decrease in thermal stability of the protein as can be deduced from the lower D values obtained for the protein with the fatty acid bound. This effect is not due to the presence of ethanol in the protein solution because similar results were obtained for the apo form heat-treated in the presence and in the absence of 3% ethanol at three different temperatures studied (results not shown). These results are in agreement with those obtained by Polverino de Laureto et al. (*30*), who found a reduction of the circular dichroism signal at 270 nm for apo-α-lactalbumin in the presence of oleic acid, indicating a considerable change in the tertiary structure of the protein. The effect of binding of oleic acid to apo-α-lactalbumin is contrary to that observed for the binding of fatty acids to other fatty acid-binding proteins such as β-lactoglobulin (*31*).

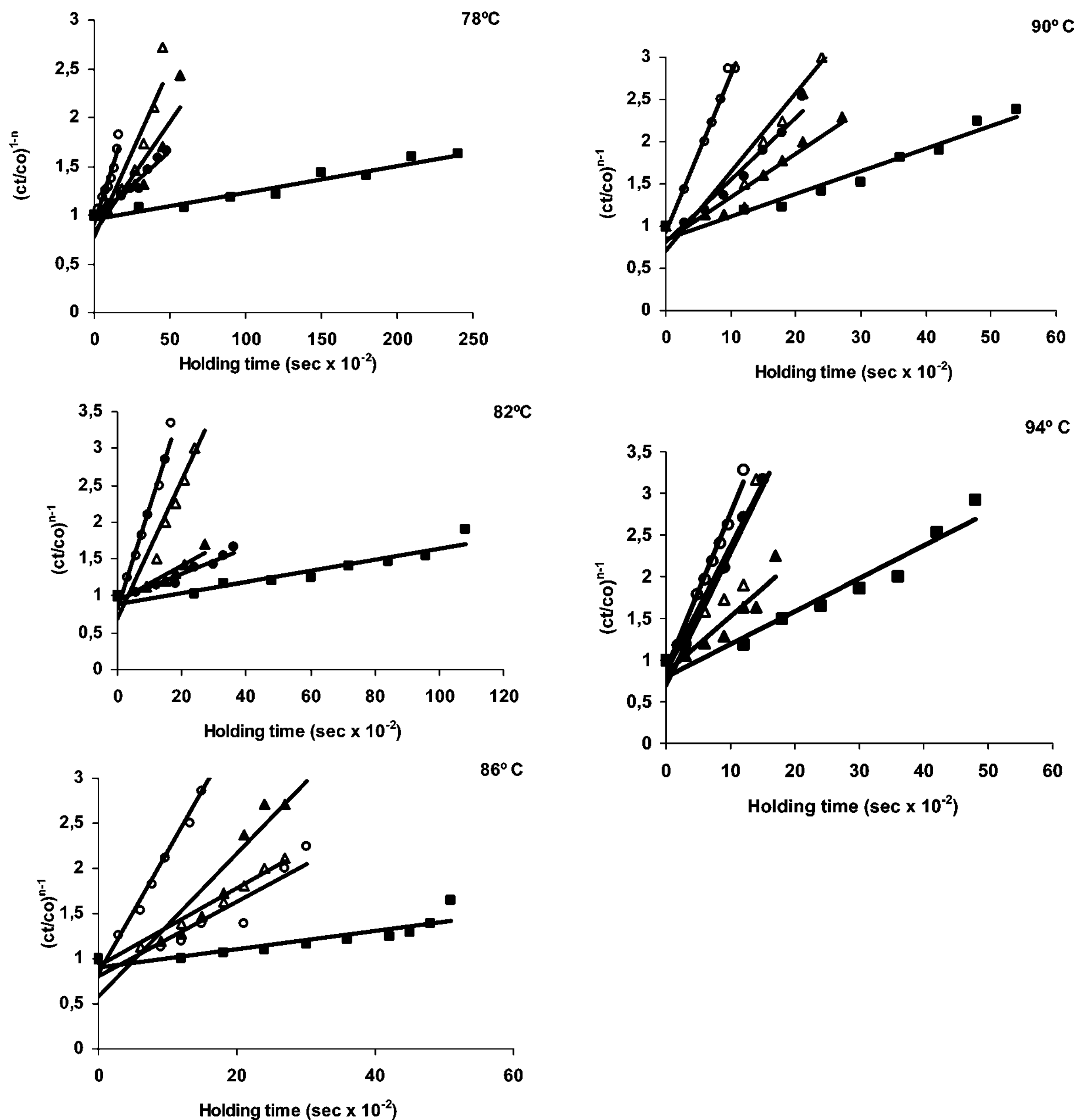

Figure 3. Denaturation of native α-lactalbumin in phosphate buffer (■, 125 μg/mL; ●, 1.25 mg/mL) and in milk (○) and of apo-α-lactalbumin in phosphate buffer (125 μg/mL) with and without oleic acid bound (△, ▲) assuming a reaction order of $n = 1.5$. C_t is the undenatured protein concentration at each holding time, and C_0 is the initial protein concentration.

Kinetic Analysis. The process of the decrease of immunoreactive α-lactalbumin with heat treatment at different temperatures with time was subjected to reaction kinetic analysis. The graphical representation of the denaturation process of α-lactalbumin considering a reaction order of $n = 1.5$ is shown in **Figure 3**, and values of K were determined (**Table 2**). For $n = 1.5$ the correlation coefficients r^2 were >0.85 and b values were close to 1. These results indicate that the value of $n = 1.5$ is suitable to describe the process in the range of temperatures studied. Other authors, using immunodiffusion techniques (*18*), electrophoresis (*29*), and high-performance liquid chromatography (*32*), have indicated that heat denaturation of α-lactalbumin follows a first-order reaction. However, considering that the kinetic parameters of denaturation of α-lactalbumin vary with protein concentration (**Figure 1**), an order of reaction >1 should be assumed.

When the natural logarithm of the rate constant was plotted versus the reciprocal of the absolute temperature, a linear relationship was observed in the temperature range studied, which allowed the calculation of activation energy values (**Table 3**). This relationship has also been found to be linear for the denaturation of α-lactalbumin in skim milk in the range of temperatures used in this work. However, a change in the slope

Table 2. Rate Constants for Denaturation (k, Seconds × 10^4) of Native α-Lactalbumin in Phosphate Buffer and in Milk and of Apo-α-lactalbumin in Phosphate Buffer with and without Oleic Acid Bound, Assuming a Reaction Order of $n = 1.5$

	phosphate buffer				
	native[a]	native[b]	apo[b]	apo + oleic acid[b]	milk[a]
78 °C	1.40	0.27	2.26	3.55	4.73
82 °C	1.79	0.75	2.45	5.20	10.88
86 °C	4.30	1.03	4.13	7.95	13.56
90 °C	7.35	2.67	5.21	9.43	18.42
94 °C	15.01	3.94	6.72	15.87	18.72

[a] Protein concentration = 1.25 mg/mL. [b] Protein concentration = 125 μg/mL.

Table 3. Thermodynamic Parameters[a] for Denaturation of Native α-Lactalbumin in Phosphate Buffer and in Milk and for Apo-α-lactalbumin in Phosphate Buffer with and without Oleic Acid Bound, Assuming a Reaction Order of 1.5

	phosphate buffer									phosphate buffer					
	native[b] E_A = 165 kJ mol^{-1}			native[c] E_A = 178 kJ mol^{-1}			milk[b] E_A = 88 kJ mol^{-1}			apo[c] E_A = 79 kJ mol^{-1}			apo + oleic acid[c] E_A = 96 kJ mol^{-1}		
	$\Delta H^‡$	$\Delta S^‡$	$\Delta G^‡$	$\Delta H^‡$	$\Delta S^‡$	$\Delta G^‡$	$\Delta H^‡$	$\Delta S^‡$	$\Delta G^‡$	$\Delta H^‡$	$\Delta S^‡$	$\Delta G^‡$	$\Delta H^‡$	$\Delta S^‡$	$\Delta G^‡$
78 °C	161.88	0.173	100.84	174.94	0.148	123.01	85.37	–0.067	109.05	75.67	–0.092	108.27	93.27	–0.038	106.72
82 °C	161.84	0.173	100.15	174.91	0.148	122.42	85.34	–0.068	109.32	75.64	–0.092	108.65	93.23	–0.038	106.88
86 °C	161.81	0.173	99.45	174.88	0.148	121.83	85.31	–0.068	109.59	75.61	–0.093	109.02	93.20	–0.038	107.03
90 °C	161.78	0.173	98.76	174.85	0.148	121.24	85.27	–0.068	109.86	75.57	–0.093	109.39	93.17	–0.038	107.19
94 °C	161.74	0.173	98.06	174.82	0.148	120.65	85.24	–0.068	110.13	78.54	–0.093	109.76	93.13	–0.038	107.34

[a] Parameters shown are apparent energy of activation (E_A) and change of the enthalpy ($\Delta H^‡$), entropy ($\Delta S^‡$), and free energy of activation ($\Delta G^‡$). [b] Protein concentration = 1.25 mg/mL. [c] Protein concentration = 125 μg/mL.

was found for temperatures >95 °C as has also been observed for other milk proteins (*18*, *29*).

Values of the activation energy estimated in this work are similar to those reported for native α-lactalbumin in skim milk (*29*, *32*) and in phosphate buffer (*33*) but lower than those reported by others (*20*). Differences between these values may be due to different experimental conditions and techniques used in different works. We found that the values of activation energy and the change in enthalpy of activation were nearly two times higher for native lactalbumin than for apo-α-lactalbumin. These results are in agreement with those obtained by Hendrix et al. (*34*) using differential scanning calorimetry. Differences obtained in the activation energy indicate that a larger amount of energy is necessary to activate the native molecule to start denaturation compared with the apo form of the protein.

From the value of the activation energy, thermodynamic parameters, such as the change of enthalpy of activation ($\Delta H^‡$), entropy of activation ($\Delta S^‡$), and the free energy of activation ($\Delta G^‡$) were calculated as described under Materials and Methods (**Table 3**).

For each sample of protein studied, similar values for the thermodynamic parameters were obtained at the different temperatures studied. The high values of the change in enthalpy of activation obtained for native α-lactalbumin in phosphate buffer indicated that it undergoes a considerable change in conformation during denaturation. The positive values found for the variation in entropy of activation indicated that there is not a significant process of aggregation. Therefore, these values are typical of a reaction mechanism in which the denaturation of the protein is the rate-determining process that predominates over an aggregation process. Similar results have been reported for other milk proteins in the range of temperatures studied in this work (*26*, *27*). In contrast, the lower values for the apparent energy of activation and the change in enthalpy of activation along with a negative change in entropy of activation found for apo-α-lactalbumin and α-lactalbumin in milk suggest the existence of a small aggregation process in which intermolecular bonds are formed and, therefore, the state of the order of the system increases. It is known that apo-α-lactalbumin, partially unfolded, is much more prone to protein aggregation than the native form. This property derives from the fact that in the apo form, hydrophobic patches can be exposed, which lead to protein aggregation via hydrophobic interactions (*5*). Besides, the free energy value for the process of denaturation of α-lactalbumin in the different forms studied obtained for all temperatures is in the same range of values obtained for most of the globular proteins (*26*, *27*).

The results found in this work show that the binding of calcium to bovine α-lactalbumin is an important factor in the resistance of the protein structure to thermal treatment. Also, the effect of heating on α-lactalbumin is greatly influenced by the composition of the medium and the concentration of protein. Thus, these aspects should be considered in the design of heat treatments of α-lactalbumin in order to preserve its putative biological function when it is added to special food or pharmaceutical products.

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NOTE ADDED AFTER ASAP PUBLICATION

The original posting of November 11, 2005, contained two equations numbered 3. The second of these has been changed to number 4 with the posting of November 22, 2005.

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