Thermal Denaturation of Human Lactoferrin and Its Effect on the Ability To Bind Iron

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Thermal stability of human milk lactoferrin was studied by differential scanning calorimetry and compared with that of recombinant human lactoferrin produced in *Aspergillus awamori.* Maximum peak temperature, transition enthalpy, and activation energy of lactoferrin as isolated from human milk (67.0 °C, 2276 kJ/mol, and 275.5 kJ/mol, respectively) increased significantly when lactoferrin was fully saturated with iron (90.6 °C, 3209 kJ/mol, and 387.6 kJ/mol, respectively) which indicates that the binding of iron to lactoferrin is an important factor in the stabilization of its structure. Similar results were obtained for recombinant human lactoferrin, indicating a high degree of resemblance between both proteins. The ability of human lactoferrin to bind iron after heat treatment was also studied, remaining practically intact after treatments of 72 °C for 20 s or 135 °C for 8 s, while more severe treatments reduced markedly this ability.

Keywords: *Human lactoferrin; thermal denaturation; iron-binding ability*

INTRODUCTION

Lactoferrin is a member of the transferrin family of iron-binding proteins and it consists of a single polypeptide chain with a molecular weight of 80 000. Crystallographic analysis has revealed that lactoferrin is composed of two globular lobes of similar size which are connected by a single short bridging peptide. Each lobe consists of two domains that form a cleft enclosing the binding site for a metal ion, the structure of which confers to this protein the ability to bind reversibly two ferric ions per molecule (Anderson et al., 1987). Although, the affinity of lactoferrin for iron is much greater than that of transferrin, it occurs in milk practically devoid of iron. This unsaturated state could be responsible for many of the biological functions proposed for lactoferrin which include a bacteriostatic effect by reducing the availability of iron to microorganisms, the inhibition of iron-catalyzed oxidation reactions, or the regulation of the intestinal absorption of iron (Sánchez et al., 1992a). However, other functions suggested for lactoferrin appear to be unrelated to the level of iron bound, such as a bactericidal effect due to the interaction of a structural domain of lactoferrin with the bacterial surface (Bellamy et al., 1992), a growth-promoting activity on certain cellular lines, or its participation in the inflammatory response by modifiying the release of cytokines (Sánchez et al., 1992a).

Lactoferrin is a major protein in human milk (Hennart et al., 1991), while in bovine milk is markedly lower, as 0.1 mg/mL (Sánchez et al., 1988). The great concentration of lactoferrin in human milk supports the idea that it must play an important role in the development of the newborn. Therefore, as infant formulas usually manufactured from cow's milk are practically devoid of lactoferrin, the supplementation of artificial milks with this protein seems to have a considerable interest. Moreover, there is also an increasing demand for natural compounds acting as antibiotics to supplement specialty foods and pharmaceutical products, in which lactoferrin could exert such role (Smithers et al., 1996).

Two sources can be potentially used to obtain lactoferrin. First, lactoferrin from bovine milk, which is usually obtained by large-scale methods from the whey obtained in the cheese-making process (Calvo, 1994). The other system to obtain lactoferrin developed in the last years is the production of recombinant lactoferrin in microorganisms (Ward et al., 1992; Liang and Richardson, 1993) or in transgenic animals (Krimpenfort, 1993). Recently, a system to obtain commercial quantities of human recombinant lactoferrin has been developed in *Aspergillus awamori* (Ward et al., 1995).

As the interest in producing lactoferrin to be used in infant formula and other products is growing, the thermal stability of this protein should be evaluated to design treatments which ensure that its biological activity is maintained. To date, few studies have been carried out on the thermal stability of human lactoferrin and those carried out have shown contradictory results. Ford et al. (1977) found that the treatment at 70 °C for 15 s caused 95% of lactoferrin denaturation, while Goldblum et al. (1984) reported that 100% of lactoferrin remained intact in similar conditions. On the other hand, the effect of heat treatment on bovine lactoferrin has been widely studied, including the effect on some aspects related to its biological activity (Abe et al., 1991; Kawakami et al., 1992; Sánchez et al., 1992b; Oria et al., 1993; Paulsson et al., 1993). In the present work the thermal stability of human milk lactoferrin and recombinant human lactoferrin has been studied by

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differential scanning calorimetry (DSC) and the thermodynamic parameters found for thermal denaturation of both proteins have been compared. The effect of heat treatment on the ability of human lactoferrin to bind iron has been also studied.

APPARATUS

Calorimetry of proteins was performed in a DuPont thermal analyzer (model DSC 10, Nemours, Germany), using a thermal analyst 2000 system.

PROCEDURE

Preparation of Proteins. Human lactoferrin was isolated from milk, kindly supplied by healthy donors, following a method described by Bezwoda and Mansoor (1986), with minor modifications. Briefly, samples of human milk were skimmed, acidified with HCl, and centrifuged to obtain whey. The whey was dialyzed against 5 mM sodium barbital, 50 mM NaCl, pH 7.4, and applied to a column of Cibacron Blue Sepharose equilibrated with the same buffer. The column was eluted in two steps, first with the same buffer containing 1 M NaCl and second, with 2 M NaCl. Lactoferrin fractions were pooled, dialyzed against 15 mM potassium phosphate, 150 mM NaCl, pH 7.4 (PBS) or deionized water, and concentrated by ultrafiltration or liophylized. Lactoferrin was stored at −20 °C until it was used.

The purity of isolated lactoferrin was assessed by SDS−PAGE using gels with a gradient of acrylamide 8−25% (Phast System, Pharmacia, Uppsala, Sweden) and densitometric analysis, and it was found to be higher than 96%. The iron saturation of lactoferrin was less than 15%, estimated from the ratio of absorbances at 280 and 465 nm.

Human recombinant lactoferrin from *A. awamori* was kindly donated by Agennix Inc. (Houston, TX), and it was used in the calorimetric experiments without further purification. The iron saturation of native recombinant lactoferrin was less than 18%. To obtain lactoferrin (both from human milk and recombinant origin) with 100% of iron saturation, an excess of iron was added as FeNTA (ferrinitrilotriacetate) and held for 3 h at room temperature before analysis.

Differential Scanning Calorimetry (DSC). Solutions of lactoferrin from human milk or of recombinant origin were prepared in PBS or human skimmed milk, at a protein concentration of approximately 100 mg/mL which was determined by spectrometry using the absorptivity at 280 nm as 1.2 and 1.4 g^{-1} L cm^{-1} for lactoferrin as isolated and in the iron-saturated form, respectively. Samples and references (10 μL) were sealed in DuPont aluminum pans, the references consisted of pans containing PBS or human skimmed milk which had been previously heated.

DSC scans were programmed at heating rates of 2, 3, 4, 5, 7, 10, and 20 °C/min in the temperature range of 35−110 °C. Samples were analyzed at least in triplicate. To check that there was no renaturation of protein after treatment, denaturated samples were cooled to 30 °C and rescanned in the same conditions.

Several thermodynamic parameters can be afforded from the DSC transition peak. The enthalphy change was estimated from the peak area using a straight baseline drawn from the onset to the end of thermal transition. Temperatures of maximum heat absorption (T_{max}), onset temperatures (T_s), and enthalpy change (ΔH_{cal}) of denaturation were plotted as a function of heating rate, and then, the denaturation temperature, onset temperature, and enthalphy change of denaturation were estimated by extrapolation to a rate of 0 °C.

To assess the complexity of the denaturation process, a parameter measured directly from the transition peak, the width at half-peak height ($\Delta T_{1/2}$), may be used. This parameter allows the van't Hoff enthalpy for a two-state process to be calculated (Wright, 1982):

$$\Delta H_{VH} = 4RT_{max}^{\ 2}/\Delta T_{1/2} \qquad (1)$$

where T_{max} is the maximum peak temperature, and R is the gas constant. Therefore, the comparison of the value obtained for ΔH_{VH} with the calorimetric enthalpy change (ΔH_{cal}) calculated from the thermogram obtained, may indicate how closely the denaturation reaction approximates to a two-state process. Lack of correspondence between the two values indicates a decreased cooperativity due to the presence of intermediates or to intermolecular associations (Privalov, 1982).

The kinetic parameters for denaturation of a protein may be calculated from the information contained within the DSC thermogram. To calculate the kinetic parameters for denaturation of human milk or recombinant lactoferrin we have used Kissinger's method (1957), which is based on the relationship between the heating rate and the maximum peak temperature. For any order of reaction, E_a/R is determined from the slope of $\ln(\beta/T_{max}^2)$ vs $1/T_{max}$:

$$(\beta/T_{max}^{\ 2}) = (AE_a/R)e^{-E_a/RT_{max}} \qquad (2)$$

where β is the scanning rate, T_{max} is the maximum peak temperature, E_a is the activation energy, A is the preexponential factor, and R is the gas constant.

Measurement of Iron-Binding Capacity. The ability of lactoferrin to bind iron after heat treatment was determined by a procedure adapted from Klausner et al. (1983). Human lactoferrin as isolated at a concentration of 1 mg/mL was dissolved in 25 mM Tris, 100 mM NaCl, 10 mM sodium bicarbonate, pH 7.8, and subjected to different heat treatments. After heat treatment, 500 μL of lactoferrin solution was mixed with a 2-fold molar excess of iron (150 μL of a radiolabeled iron solution) and incubated at room temperature for 1 h. The iron-radiolabeled solution was prepared by mixing unlabeled $FeCl_3$ and $^{59}FeCl_3$ (specific activity of 37.5 mCi/mg, Dupont, Nemours, Belgium) in a molar ratio of 400:1 and this solution was mixed with disodium nitrilotriacetate in a molar ratio of 1:8.

To eliminate the aggregates of protein the solution of lactoferrin was centrifuged at 10000*g* for 15 min, the supernatant was removed and the pellet, if any, was washed twice with 500 μL of 25 mM Tris, 100 mM NaCl, 10 mM sodium bicarbonate, pH 7.8. The amount of iron in the supernatant, pellet, and the two washings was estimated by radioactivity counting in a gamma counter. The free iron in the supernatant (500 μL) was removed by gel filtration on prepacked NAP-10 columns (Pharmacia, Uppsala, Sweden) equilibrated with PBS and fractions of 500 μL were collected. The concentration of lactoferrin in each fraction was determined by the Bradford method (1976) using human lactoferrin as standard. The radioactivity associated to each fraction was also measured in a gamma counter, and the molar ratio of iron/lactoferrin was calculated considering the specific activity of the iron solution.

Statistical Evaluation. Statistical significance of differences between values obtained for denaturation of lactoferrin in human milk and PBS was assessed by ANOVA using Scheffe T-test. Estimates of uncertainty for the values of peak maximum temperature, denaturation enthalpy, and onset temperature of transition when extrapolated to 0 °C/min and for the estimate of activation energy were determined by linear regression.

RESULTS

Thermal analysis of lactoferrin from human milk and from *A. awamori* was performed by DSC. Denaturation thermograms of lactoferrin as isolated and in the iron-saturated form in PBS at a heating rate of 10 °C, are shown in Figure 1 and in both cases lactoferrin exhibits only one denaturation peak. The position and the shape of the endothermic peaks are affected by the iron saturation of lactoferrin. Thus, as it is shown in Table 1, temperatures of maximum heat absorption and

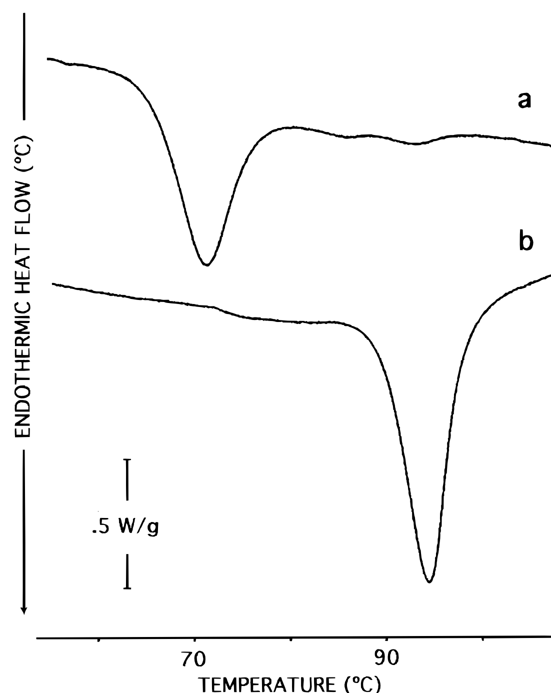

Figure 1. Differential scanning calorimetry thermograms of human milk lactoferrin as isolated (a) and as 100% iron-saturated form (b). The amount of analyzed protein was 1 mg per pan in PBS and the scanning was performed at a heating rate of 10 °C/min. Heat flow was recorded as watts per gram.

Table 1. Thermal Parameters for Denaturation of Human Milk Lactoferrin in PBS at a Heating Rate of 10 °C/min[a]

	T_{max}[b] (°C)	ΔH_{cal}[c] (kJ/mol)	$\Delta T_{1/2}$[d] (°C)	ΔH_{VH}[e] (kJ/mol)	ΔH_{cal}:ΔH_{VH}
native hLF[f]	71.85	2623	6.25	632	3.81
iron-saturated hLF[f]	94.52	3677	4.58	980	4.15

[a] Results are shown as mean of at least three independent samples. [b] Peak maximum temperature. [c] Denaturation enthalpy change. [d] Half-width denaturation peak. [e] Van't Hoff enthalphy of denaturation; it was calculated from $\Delta H_{VH} = 4RT_{max}^2/\Delta T_{1/2}$. [f] hLF, lactoferrin from human milk.

enthalpy change shift to higher values when lactoferrin is fully saturated with iron. Moreover, the half-peak height of the thermogram of lactoferrin as isolated is wider than that of the iron-saturated lactoferrin curve, indicating that the former presents a lower cooperativity in the denaturation process. The relationship between the calorimetric enthalpy change obtained from the thermogram and the van't Hoff enthalpy (ΔH_{cal}:ΔH_{VH}) has also been calculated for lactoferrin as isolated and iron-saturated protein. This ratio in both cases approaches 4, indicating the presence of intermediates in the denaturation process. Normally, for small single-domain globular proteins the denaturation implies a cooperative transition and the calorimetric enthalpy change is equal to the van't Hoff enthalpy being the ratio ΔH_{cal}:ΔH_{VH} around 1 (Privalov, 1982). However, in proteins in which the conformation is stabilized by interactions among several domains the ratio ΔH_{cal}:ΔH_{VH} is greater than 1, such is the case of lactoferrin.

The maximum peak temperature and calorimetric enthalpy change of denaturation are affected by the heating rate as is shown in Figures 2 and 3, which suggests that the denaturation process is kinetically determined. The lines correspond to the best fit of straight lines for which correlation coefficients ranged from 0.87 to 0.98. The enthalpy change at a heating rate of 2 °C/min has not been considered due to the difficulty in determining the baseline. Both thermodynamic parameters increase with the heating rate, and the values obtained by extrapolation to 0 °C/min are shown in Table 2. The binding of iron to either lactoferrin obtained from human milk or to the recombinant lactoferrin from *A. awamori* increases the denaturation temperature and enthalpy change about 23 °C and 1000 kJ/mol, respectively, indicating that the binding of iron is an important factor on the thermal stability of this protein. Denaturation temperature and enthalpy change of human milk lactoferrin as isolated (67.0 °C and 2276 kJ/mol, respectively) and in the iron-saturated form (90.6 °C and 3209 kJ/mol, respectively) are very close to those found for recombinant lactoferrin (67.3 °C and 2165 kJ/mol for lactoferrin as isolated; and 89.6 °C and 3248 kJ/mol for iron-saturated recombinant lactoferrin). Renaturation was not observed for any of the samples analyzed by DSC.

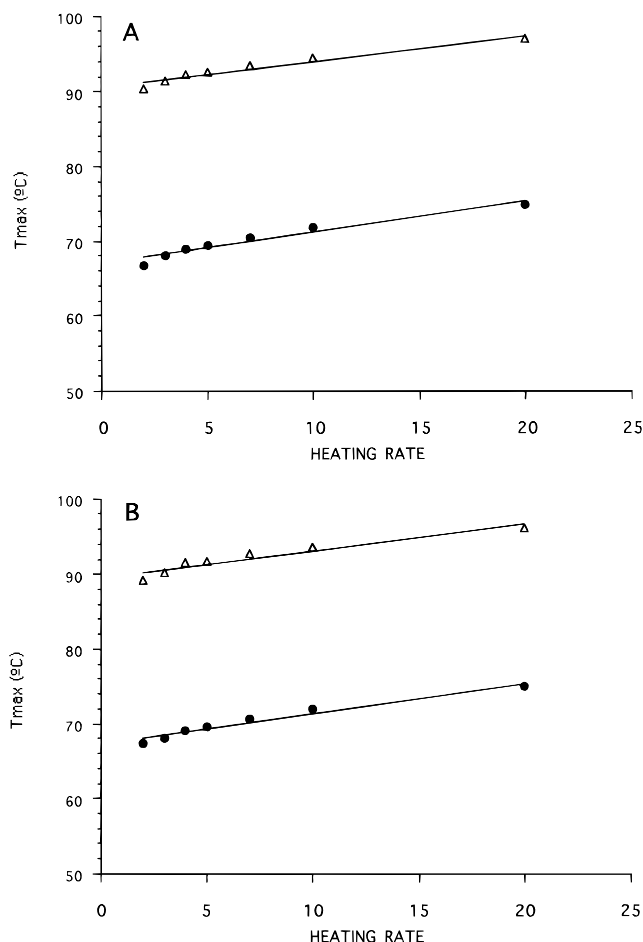

Figure 2. Maximum peak temperature (T_{max}) at different heating rates for denaturation of lactoferrin as isolated (●) and in the iron-saturated form (△) from human milk (A) or from recombinant origin (B).

We have also studied the denaturation of lactoferrin in human skimmed milk and the results obtained were compared with those of lactoferrin in PBS (Table 3). The enthalpy change of denaturation was significantly lower for lactoferrin from human milk and from recombinant origin heated in human skimmed milk than for those heated in PBS ($p < 0.01$). Significant differences were also found in the values of the maximum peak temperature corresponding to denaturation of lactoferrin from

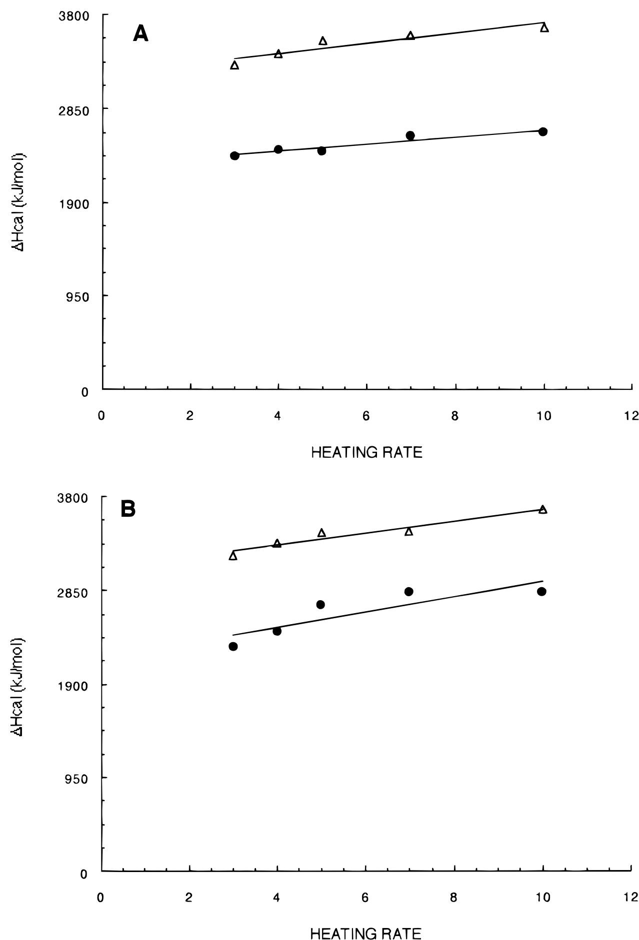

Figure 3. Enthalpy change (ΔH_{cal}) at different heating rates for denaturation of lactoferrin as isolated (●) and in the iron-saturated form (△) from human milk (A) or from recombinant origin (B).

Table 2. Thermal Parameters for Denaturation of Human Lactoferrin in PBS, Extrapolated to 0 °C/min

	T_{max}[a] (°C)	ΔH_{cal}[b] (kJ/mol)	T_s[c] (°C)
native hLF[d]	66.97	2276	62.25
iron-saturated hLF[d]	90.57	3209	87.44
native rLF[e]	67.29	2165	62.22
iron-saturated rLF[e]	89.58	3248	85.50

[a] Peak maximum temperature. [b] Denaturation enthalpy. [c] Onset temperature. [d] hLF, lactoferrin from human milk. [e] rLF, lactoferrin from recombinant *A. awamori.*

Table 3. Comparison of the Thermal Parameters for Denaturation of Human Lactoferrin in PBS and in Human Skimmed Milk[a]

	human milk lactoferrin		recombinant lactoferrin	
	T_{max} (°C)	ΔH_{cal} (kJ/mol)	T_{max} (°C)	ΔH_{cal} (kJ/mol)
PBS	71.85 ± 0.21	2623 ± 19	72.04 ± 0.17	2844 ± 38
human milk	71.27 ± 0.10	1811 ± 97	72.03 ± 0.18	1899 ± 18

[a] Peak maximum temperature (T_{max}) and denaturation enthalpy change (ΔH_{cal}) of human milk lactoferrin and of recombinant lactoferrin from *A. awamori* were measured at a heating rate of 10 °C/min. The amount of protein in each pan was approximately 1 mg. Results are shown as mean ± SD of at least three independent samples.

human milk heated in PBS when compared with that heated in human milk ($p < 0.01$).

The activation energy for thermal denaturation of lactoferrin was calculated by the Kissinger method (1957). As it is shown in Figure 4, the Kissinger plots for recombinant and milk lactoferrin are very close to straight lines, with correlation coefficients of 0.99 in all cases. The activation energy values were calculated from the slopes of those straight lines and the values obtained for recombinant and milk lactoferrin were compared. The activation energy for iron-saturated lactoferrin from human milk was of 387.6 kJ/mol (433.8−341.5, confidence limits at 95%), higher than that of the protein as isolated which was of 275.5 kJ/mol (304.0−247.0, confidence limits at 95%). The values obtained for recombinant human lactoferrin were for the iron-saturated protein of 367.8 kJ/mol (406.5−328.8, confidence limits at 95%) and for the protein as isolated 284.4 kJ/mol (326.9−242.0, confidence limits at 95%).

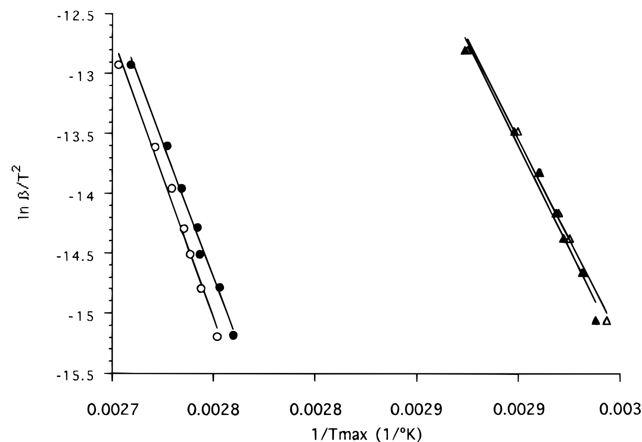

Figure 4. Kissinger plot for heat denaturation of native (△, ▲) and iron-saturated lactoferrin (○, ●) from human milk (△, ○) or from recombinant origin (▲, ●). T_{max} is the peak temperature (degrees Kelvin) and β is the scanning rate.

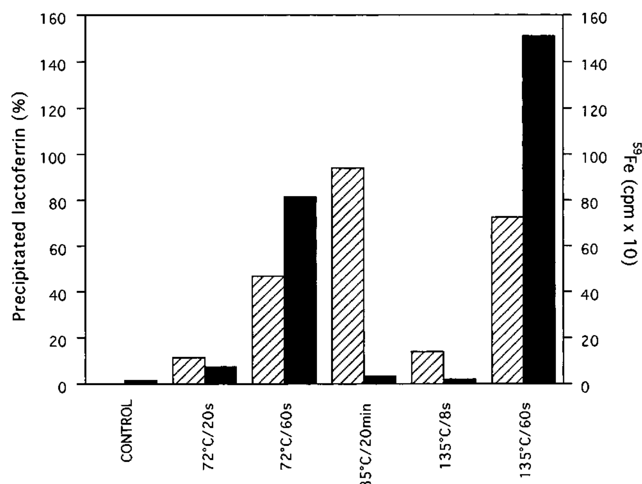

Figure 5. Effect of heat treatment on the binding of iron to human lactoferrin. Hatched bars represent the percentage of precipitated lactoferrin separated by centrifugation after the heat treatment, and the solid bars represent the radiolabeled iron associated to the aggregates of lactoferrin. Results are the mean of three independent experiments.

Finally, the effect of several heat treatments on the ability of human lactoferrin to bind iron was also studied. Samples containing lactoferrin after heat treatment were centrifuged to separate the denatured proteins and the radioactivity associated to it shown in Figure 5. When lactoferrin was subjected to the most severe heat treatments (72 or 135 °C for 60 s or 85 °C for 20 min) partial precipitation of the protein was found. However, the aggregates of lactoferrin heated at 72 °C or at 135 °C for 60 s were still able to bind

Table 4. Effect of the Heat Treatment on the Ability To Bind Iron of Human Lactoferrin (hLF)[a]

	mol of Fe/mol of hLF
control	2.00 ± 0.32
72 °C/20 s	1.52 ± 0.31
72 °C/60 s	1.56 ± 0.24
85 °C/20 min	0.95 ± 0.20
135 °C/8 s	1.65 ± 0.42
135 °C/60 s	1.55 ± 0.29

[a] Lactoferrin as isolated from human milk was subjected to the heat treatment and after cooling was mixed with an excess of radiolabeled iron (^{59}Fe) to obtain the full saturation of the protein. The protein aggregates were removed by centrifugation and the free iron by gel filtration. Results are shown as mean ± SD of three independent samples.

iron, in contrast to those of lactoferrin heated at 85 °C for 20 min. Furthermore, the ability to bind iron of the lactoferrin that remained soluble after the different heat treatments was examined and compared with that of the unheated protein (Table 4). The molar ratio of iron/lactoferrin was found to be 2 for the unheated lactoferrin, which agrees with the well-known capacity of lactoferrin to bind two atoms of iron per molecule. Most of the heat treatments applied to human lactoferrin caused a decrease in the ability to bind iron of about 25%, but when lactoferrin was subjected to the most severe treatment (85 °C for 20 min) the ability to bind iron decreased to 50%.

DISCUSSION

DSC thermograms for denaturation of human lactoferrin from breast milk or recombinant human lactoferrin expressed in *A. awamori* show single endotherm curves in which maximum peak temperature and enthalpy change values increase when lactoferrin is saturated with iron as it had been previously found for human lactoferrin (Hadden et al., 1994). These results contrast with those reported for iron-saturated lactoferrin from bovine milk which show a double-peak endotherm (Rüegg et al., 1977; Sánchez et al., 1992b; Paulsson et al., 1993). This double peak has been attributed to a difference in the heat sensitivity of the two lobes of lactoferrin (Evans and Williams, 1978). However, it could also be explained by the presence of monoferric species in bovine lactoferrin samples, as it has been shown that human serum transferrin and ovotransferrin partially saturated with iron display DSC curves with several peaks and only when the protein is completely saturated appears a single peak (Lin et al., 1994).

Denaturation temperature (temperature of maximum heat absorption extrapolated to 0 °C/min) and enthalpy change are two thermodynanic parameters that indicate the thermal stability of a protein. Thus, enthalpy change reflects a net absorption of energy due to disruption of internal hydrogen bonds in protein and in water, the energy released when new bonds are formed between the protein and water and between the molecules of water around the apolar groups, and the endothermic contribution of the disruption of van der Waals bonds between apolar groups, which is negligible (Paulsson and Dejmek, 1990). Our results show that the denaturation temperature and enthalpy change are similar either in lactoferrin from human milk or in recombinant lactoferrin, increasing about 23 °C and 1000 kJ/mol, respectively, when the protein is iron-saturated with regard to that as isolated, with low iron content. The increase in the value of those thermodynamic parameters with the iron saturation was also found for bovine lactoferrin (Rüegg et al., 1977; Sánchez et al., 1992b; Paulsson et al., 1993), indicating that the binding of iron to lactoferrin is an important factor in the thermal resistance of the protein structure. Similar stabilizing effects have been found for other ligands bound to proteins, such as fatty acids bound to β-lactoglobulin and albumin or calcium bound to α-lactalbumin (Bernal and Jelen, 1985; Puyol et al., 1994). However, the values of denaturation temperature and enthalphy change for bovine lactoferrin were lower than those obtained for human lactoferrin in the same conditions (about 10 °C and 2000 kJ/mol lower, respectively) (Sánchez et al., 1992b), indicating that human lactoferrin shows a higher thermal stability than bovine lactoferrin.

On the other hand, the thermal denaturation of lactoferrin in human skimmed milk has also been studied by DSC, and the results compared with those found when lactoferrin was treated in PBS, as the protein thermal behavior may be influenced by the composition of the medium. DSC study of lactoferrin in skimmed milk was feasible because only lactoferrin shows an endothermic peak during heating, as it was assessed by subjecting skimmed milk to DSC in the same conditions. This fact is due to the concentration of proteins in human whey which is too low to produce any endothermic peak. Furthermore, even if there were some caseins (the major proteins of milk) left they would not give any endothermic peak (Paulsson and Dejmek, 1990). We have found that the enthalpy change for denaturation of lactoferrin from both origins is considerably lower when they are heated in human skimmed milk than in PBS. This finding could be due to a decrease of pH associated with changes in calcium equilibria when milk is heated, as it has been also found for denaturation of bovine lactoferrin in milk (Sánchez et al., 1992b).

Another parameter may be calculated directly from the width at half-peak height of the transition peak, the theorical van't Hoff enthalpy. When this value approaches the actual value of enthalpy it means that the protein denaturation follows a two-state kinetic model (Privalov, 1982). The denaturation enthalpy change measured from the transition peak for denaturation of human lactoferrin is about 4-fold higher than the van't Hoff enthalpy, indicating that partially unfolded intermediates exist in equilibrium with the native state during the thermal denaturation process. This finding is in agreement with the three-dimensional structure of human lactoferrin which consists of two similar lobes, each of them further organized into two domains with a site for iron in the cleft between them (Anderson et al., 1987).

The activation energy for denaturation of lactoferrin from human milk or from recombinant origin has been determined by the method of Kissinger (1957). The activation energy values for iron saturated lactoferrin are higher than those obtained for both proteins in the unsaturated form, which confirm the previous results on the higher thermal stability of lactoferrin saturated with iron. Moreover, the values obtained for human recombinant lactoferrin and lactoferrin from milk are very close which indicates the high similarity between the two proteins. Although this method is applied to single denaturation reactions and as it has been shown

above, lactoferrin undergoes a multistate thermal denaturation, we have used it to obtain a kinetic approximation to thermal denaturation of human milk lactoferrin and compare with that for recombinant lactoferrin. The Kissinger plot we have obtained for human lactoferrin gives a straight line which indicates a good correspondence with single reactions (Taylor and Fryer, 1993).

The iron-binding ability of human lactoferrin after heat treatment was also studied; it remains over 75% after most of applied treatments, except for lactoferrin heated at 85 °C for 20 min when it decreased to 50%. Despite the small decrease in the ability of lactoferrin to bind iron, the most severe treatments cause partial precipitation of lactoferrin. Thus, the most suitable treatments appear to be 72 °C for 20 s or 135 °C for 8 s which correspond to those used for mild pasteurization and UHT treatments, respectively, because there was no precipitation of protein.

The ability of bovine lactoferrin to bind iron following heat treatment was studied by Kawakami et al. (1992), who found that lactoferrin retained over 85% of its iron-binding ability when treated in a medium with an ionic strength of 0.01 at a range of different heat treatments, but decreased markedly at an ionic strength of 0.1. These results are not comparable to those found in the present study since the experimental conditions are different. Furthermore, the spectrometric analysis to estimate iron-binding ability of lactoferrin used by those authors is not very appropriate as the heat treatment may cause turbidity in the samples. The methodology used in the present study permits the differentiation of the iron bound to the intact lactoferrin from that bound to lactoferrin aggregates, therefore, it is more suitable to study the effect of heat on the ability of lactoferrin to bind iron.

The results found in this work show that the binding of iron to human lactoferrin is an important factor in the resistance of the protein structure to thermal treatment, as was previously found for bovine lactoferrin. Furthermore, our results show that the thermal behavior of recombinant lactoferrin is very similar to that of lactoferrin obtained from human breast milk. The thermal stability of human lactoferrin is higher than that reported for bovine lactoferrin, therefore it would be more suitable to be used in products which have to be subjected to heat treatment. The ability of human lactoferrin to bind iron which is responsible for many of its biological functions is maintained in a high proportion after some treatments, which correspond to mild pasteurization or to UHT treatments; however, more severe treatments could markedly decrease this ability. Finally, the effect of heat treatment on lactoferrin is influenced at least in part by the composition of medium, thus, this aspect should be considered by determining the thermostability of lactoferrin in each product or by treating it separately and adding it aseptically to the product.

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