Structural Basis for Difference in Heat Capacity Increments for Ca^{2+} Binding to Two α-Lactalbumins

Ann Vanhooren,* Kristien Vanhee,* Katrien Noyelle,* Zsuzsa Majer,† Marcel Joniau,* and Ignace Hanssens*
*Interdisciplinary Research Center, Katholieke Universiteit Leuven Campus Kortrijk, B-8500 Kortrijk, Belgium; and †Department of Organic Chemistry, Eötvös Loránd University, Budapest 112, Hungary

ABSTRACT Thermodynamic parameters for the unfolding of as well as for the binding of Ca^{2+} to goat α-lactalbumin (GLA) and bovine α-lactalbumin (BLA) are deduced from isothermal titration calorimetry in a buffer containing 10 mM Tris-HCl, pH 7.5 near 25°C. Among the different parameters available, the heat capacity increments (ΔC_p) offer the most direct information for the associated conformational changes of the protein variants. The ΔC_p values for the transition from the native to the molten globule state are rather similar for both proteins, indicating that the extent of the corresponding conformational change is nearly identical. However, the respective ΔC_p values for the binding of Ca^{2+} are clearly different. The data suggest that a distinct protein region is more sensitive to a Ca^{2+}-dependent conformational change in BLA than is the case in GLA. By analysis of the tertiary structure we observed an extensive accumulation of negatively charged amino acids near the Ca^{2+}-binding site of BLA. In GLA, the cluster of negative charges is reduced by the substitution of Glu-11 by Lys. The observed difference in ΔC_p values for the binding of Ca^{2+} is presumably in part related to this difference in charge distribution.

INTRODUCTION

α-Lactalbumin (LA) is a small globular protein. Its shape resembles that of a prolate ellipsoid. A deep cleft divides the molecule roughly into two lobes. One lobe, the α-domain, contains four α-helices, the other is characterized by the presence of a β-sheet (Acharya et al., 1989, 1991; Pike et al., 1996). A loop of 10 amino acids that spans the cleft is strongly Ca^{2+} binding (Stuart et al., 1986; Acharya et al., 1989, 1991) and is assumed to play a role in the mutual positioning of the two domains. In the absence of Ca^{2+} and under mild denaturing conditions, a number of specific tertiary interactions dissolves cooperatively during a process that behaves as a two-state transition (Hiraoka et al., 1980; Kuwajima et al., 1986). As a result, the protein adopts a slightly expanded molten globule state. In this state of LA, the β-domain is significantly unfolded whereas the α-domain retains its native helices as well as a native-like tertiary fold (Baum et al., 1989; Alexandrescu et al., 1993; Peng and Kim, 1994; Wu et al., 1995). The further unfolding of these secondary structures under stronger denaturing conditions occurs noncooperatively (Vanderheeren and Hanssens, 1994, 1999; Griko et al., 1994).

Molten globule states are universal stable intermediates in folding processes of globular proteins (Arai and Kuwajima, 2000; Haynie and Freire, 1993; Ptitsyn, 1994), and much attention is being devoted to elucidate the characteristics that determine their structure and stability. The careful study of mutant proteins with a directed substitution offers an excellent approach to this problem. However, comparative studies of the unfolding behavior of closely related natural proteins can offer additional solutions.

Bovine α-lactalbumin (BLA) is known to be less stable than goat α-lactalbumin (GLA) (Segawa and Sugai, 1983). The observation that the amino acid sequences of GLA and BLA differ by only seven amino acids invited us to analyze the different thermodynamic parameters for the unfolding of both proteins under identical conditions of pH and low ionic strength. In a neutral to slightly basic medium and near room temperature the apo-forms of both LAs convert from the native to the molten globule state while the Ca^{2+}-bound proteins are in their native conformation. Therefore, the analysis of the heat exchange (ΔH_{exp} values) from isothermal titration calorimetry (ITC) measurements with Ca^{2+} at different temperatures yields the thermodynamic parameters (ΔH, ΔS, ΔG, ΔC_p) for the binding of the Ca^{2+} ion in addition to those for the conformational transition of the apo-proteins. Several authors have shown that even monovalent cations bind in a specific way to LAs (Permyakov et al., 1985; Hiraoka and Sugai, 1985; Desmet et al., 1987), the ability of cations to interact with LA being optimal when the ionic radius equals that of Ca^{2+} (Desmet, 1992). To avoid small monovalent cations we performed our measurements in a Tris-HCl buffer. However, Tris-H^+ possesses a large deprotonation enthalpy. Upon eventual protonation or deprotonation of the protein that might occur during conformational changes and Ca^{2+} binding, the measured heat exchange (ΔH_{exp} values) also includes a contribution for the transfer of those protons from or to the components of the Tris-HCl buffer. Therefore, the impact of the deprotonation or protonation of the buffer components is measured to eventually correct the observed heat exchange (ΔH_{exp}). Of the resulting thermodynamic parameters (ΔH, ΔS, ΔG, ΔC_p), derived for the conformational transition and for the Ca^{2+} binding of the LAs, especially the heat capacity

Received for publication 14 March 2001 and in final form 10 October 2001.

Address reprint requests to Dr. Ignace Hanssens, Interdisciplinary Research Center, Katholieke Universiteit Leuven Campus Kortrijk, B-8500 Kortrijk, Belgium; Tel.: 32-56-246413; Fax: 32-56-246997; E-mail: ignace.hanssens@kulak.ac.be.

0006-3495/02/01/407/11 $2.00

increments (ΔC_p values) are closely related to changes in the exposure of hydrophobic surface (Livingstone et al., 1991; Privalov and Makhatadze, 1992; Oobataka and Ooi, 1993; Freire, 1995). Therefore, the ΔC_p values offer direct information on the extent of the conformational changes of LAs (Griko 1999, 2000).

Our analysis indicates that the heat capacity increments for the transition from the native-like state of apo-GLA and -BLA to their respective molten globule-like state (ΔC_{pNE}) have approximately one-half the value of those for the complete unfolding of an LA and are rather similar for both protein species. The ΔC_{pNE} values agree with the observation that the molten globule state of an LA is characterized by a highly unfolded β-domain and a well-conserved native-like fold of the α-domain (Baum et al., 1989; Alexandrescu et al., 1993; Peng and Kim, 1994; Wu et al., 1995). In contrast, the heat capacity increments for the binding of Ca^{2+} (ΔC_{pCa2+}) are larger for BLA than for GLA. These differences in ΔC_{pCa2+} occur as well for Ca^{2+} binding to the apo-LAs in their molten globule states as for binding to the native states. Therefore, a distinct region within BLA is clearly more sensitive to a Ca^{2+}-dependent exposure to solvent than is the corresponding region in GLA. By careful analysis of the respective tertiary structures of the native proteins we observed an increased accumulation of negatively charged amino acids near the Ca^{2+}-binding site of BLA. In GLA the cluster of negative charges is less pronounced as Glu-11, which participates in the negative cluster in BLA, is mutated to a positively charged Lys. Obviously, this different distribution of charged amino acids near the Ca^{2+}-binding site contributes to the observed difference in ΔC_{pCa2+} values between apo-GLA and -BLA.

MATERIALS AND METHODS

Materials

BLA was purchased from Sigma (St. Louis, MO). The protein was decalcified by applying a sample in 10 mM EDTA and 10 mM NH_4HCO_3, pH 8.5, to a sephacryl-HR-100 column. Elution was done with 5 mM NH_4HCO_3, pH 8.5. The protein fraction was checked for its Ca^{2+} and Na^+ content by atomic absorption spectroscopy; typically it contained less than 0.05 and 0.08 mol of the respective cation/mol of protein. Preparations meeting these requirements were lyophilized and stored at -20°C until use. As NH_4HCO_3 disintegrates upon lyophylization, salt-free protein samples were obtained.

GLA was prepared from fresh milk whey. After addition of Tris and EDTA to final concentrations of 50 and 1 mM, respectively, and adjustment of the pH to 7.5 with HCl, the whey was applied to a Phenyl-Sepharose column (Pharmacia, Uppsala, Sweden). Apo-GLA was bound hydrophobically to the column (Lindahl and Vogel, 1984; Noppe et al., 1998), whereas the other whey proteins were eluted with the Tris-EDTA buffer, pH 7.5. α-Lactalbumin was eluted by changing the eluting buffer to 50 mM Tris, 1 mM Ca^{2+}, pH 7.5. The Ca^{2+}-GLA was then demetallized as described above.

Lyophilized GLA and BLA samples were also checked for EDTA content, using a method based on the formation of a fluorescent terbium-EDTA-salicylic acid complex (Lee, 2001). Samples containing 500 μl of a mixture of terbium nitrate and sodium salicylate (1 mM each), 1 ml of protein solution (15–80 μM), and 500 μl of Capso buffer (0.1 M, pH 11) were shaken for 15 min and transferred into a 10 × 10-mm cuvette. The resultant fluorescence was measured with an Aminco-Bowman Series 2 spectrofluorimeter (excitation 340 nm, emission 545 nm) and compared with a calibration curve of samples containing EDTA (0–50 μM). The residual EDTA-content for both BLA and GLA was below the detection limit, indicating that the molar ratio EDTA/protein was less than 0.02.

DE-MALDI-TOF mass spectroscopy revealed that each purified protein consists of a single component with a molecular mass of 14,186 and 14,178 dalton for GLA and of BLA, respectively. The analysis was performed by M-Scan Ltd (Ascot, UK) using a Voyager STR Biospectrometry Research Station laser-desorption mass spectrometer coupled with delayed extraction.

Tris was a product of Merck (Darmstadt, Germany). HEPES and MOPS were purchased from Boehringer (Mannheim, Germany). Tris-HCl buffer was obtained by direct titration of Tris with HCl. Any back titration is avoided to exclude Na^+ and other small cations from the buffer solution. These cations are suspected to bind competitively to the specific Ca^{2+} site (Hiraoka and Sugai, 1985; Desmet et al., 1987). Buffer solutions of HEPES and MOPS were obtained by titration of the above sulfonic acids with either KOH or NaOH to distinguish the impact of K^+ and/or Na^+ cations.

BLA and GLA concentrations were determined by ultraviolet (UV)-spectrophotometry using the value $\epsilon_{280} = 28{,}500\ (\text{mol/l})^{-1}\ \text{cm}^{-1}$ for both proteins. This value has been determined for BLA (Kronman and Andreotti, 1964). An identical molar extinction coëfficient is applicable to both proteins as GLA and BLA contain Trp, Tyr, and Cys groups at identical locations (Gill and von Hippel, 1989).

Circular dichroism

Circular dichroism (CD) measurements were made on a Jasco J-600 spectropolarimeter (Jasco, Tokyo, Japan), using 5-mm cuvettes and a protein concentration of ~25 μM. At each temperature of the transition curve, measurements were started 5 min after temperature equilibration of the sample. Evidence that an equilibrium state had been obtained was provided by the fact that the ellipticity values were identical in heating and cooling runs, provided that the samples had not been exposed to high temperatures (>345 K) for more than 15 min.

Isothermal titration calorimetry

Microcalorimetric titration measurements were made in a MicroCal MCS isothermal titration calorimeter (MicroCal Inc., Northampton, MA). In a typical experiment, 1.3 ml of ~35 μM α-lactalbumin in 10 mM Tris-HCl, pH 7.5, near 25°C, was titrated in 20 steps with 40 μl of 3.5 mM $CaCl_2$. The Ca^{2+} concentration of the titrant was determined using a Perkin-Elmer 3300 absorption spectrometer. During titration, the injection syringe was rotated at 200 rpm. The values of the molar enthalpy changes were determined from the titration curves after subtraction of a baseline measured in the absence of protein. To allow for a valid comparison of the results, special attention is given to titrate all the samples with Ca^{2+} from the same stock solution. In this way possible errors resulting from inaccurate ion content are neutralized when comparing the resulting thermodynamic parameters.

RESULTS

Near-UV ellipticity changes of GLA and BLA as a function of temperature

Several authors have shown that even monovalent cations bind in a specific way to LAs (Permyakov et al., 1985; Hiraoka and Sugai, 1985; Desmet et al., 1987), the ability of

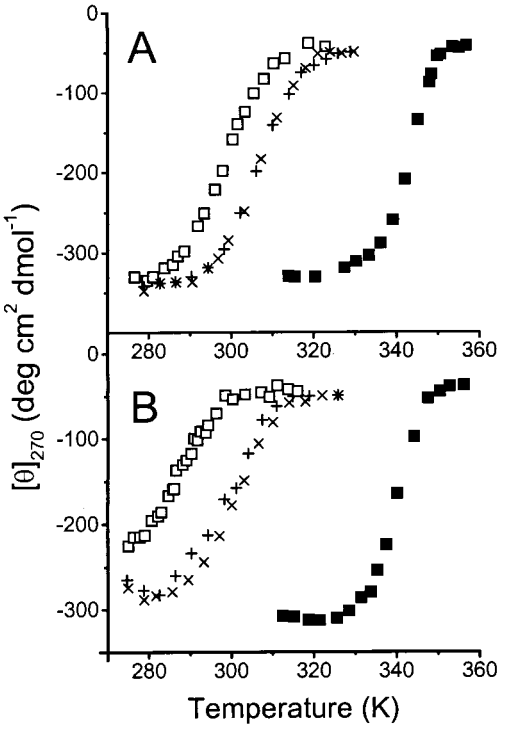

FIGURE 1 Ellipticity at 270 nm as a function of temperature for GLA (*A*) and BLA (*B*). The solvent conditions are 25 μM GLA in 10 mM Tris-HCl, pH 7.5, and 2 mM Ca^{2+} (■), in 10 mM Tris-HCl, 2 mM EGTA, pH 7.5 without further additions (□) or with 10 mM NaCl (×) and in 10 mM MOPS-NaOH, 2 mM EGTA, pH 7.5 (+).

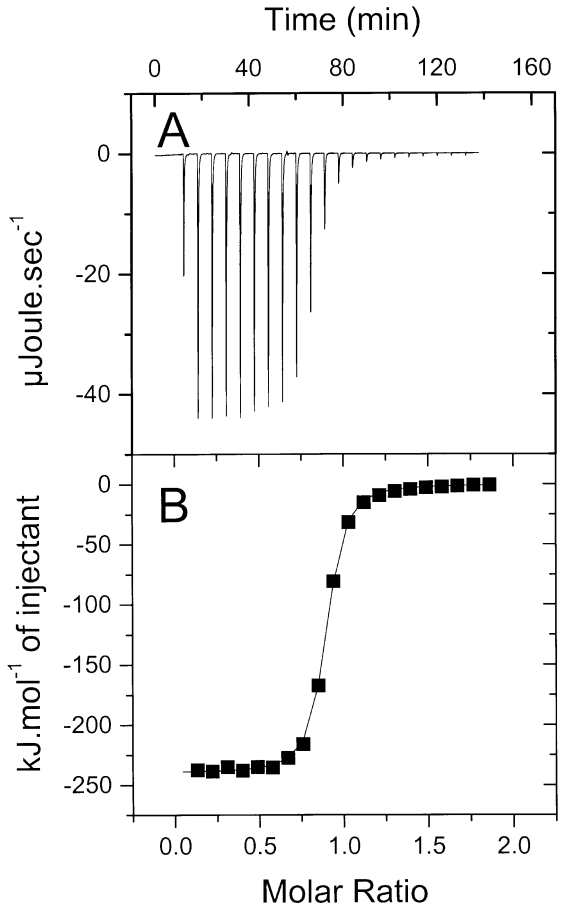

FIGURE 2 (*A*) Profile of the heat exchange measurement for a calorimetric titration of apo-BLA in 10 mM Tris-HCl with Ca^{2+}. The sample solution (1.3 ml of 35 μM BLA at 313.7 K) was titrated with 2 μL injections of 3.5 mM Ca^{2+}. (*B*) Profile of the heat exchange per mole of injected Ca^{2+} as a function of the molar ratio of the injectant to BLA for the above titration. The line through the experimental points represents the optimal fit for a monoligand binding. From the curve fitting $\Delta H_{Ca2+,exp}$ and $K_{Ca2+,app}$ are obtained.

the cations to interact with LA being optimal when the ionic radius equals that of Ca^{2+} (Desmet, 1992). To avoid small monovalent cations we preferred a buffer of Tris/Tris-H^+ in this comparative study of Ca^{2+}-binding to apo-GLA and -BLA.

Before starting the calorimetric titrations we first verified the unfolding of the apo- and Ca^{2+}-bound proteins under the solvent conditions of the subsequent calorimetric titrations. The unfolding of tertiary structure of LAs is monitored by the change of their near-UV ellipticity. Because of the immobilization of aromatic side chains by specific contacts, native LA shows a pronounced negative ellipticity near 270 nm. In the molten globule state, the interactions of the aromatic residues are randomized and the near-UV ellipticity approaches zero. The open and filled squares of Fig. 1, *A* and *B*, represent the mean residue ellipticity at 270 nm of apo- and Ca^{2+}-bound GLA and BLA, respectively, in 10 mM Tris-HCl as a function of temperature.

At 276 K (3°C) the ellipticity values of apo-GLA and Ca^{2+}-GLA in 10 mM Tris-HCl are nearly the same (Fig. 1 *A*, open and filled squares), indicating that in both conditions GLA reaches a quasiidentical native state. In contrast, at 276 K and in the mentioned buffer conditions, the ellipticity value of apo-BLA is less negative than that of Ca^{2+}-BLA (Fig. 1 *B*, open and filled squares). This observation suggests that in the buffer of 10 mM Tris-HCl only a fraction of apo-BLA is folded into the native state at 276 K.

Furthermore, the near-UV CD curves indicate that the transition from the unfolded to the native conformation for apo-GLA as well as that for apo-BLA (Fig. 1, *A* and *B*, open squares) is mainly completed at 320 K (47°C). The Ca^{2+}-bound proteins stay native up to the latter temperature (Fig. 1, *A* and *B*, filled squares). This increased thermal stability resulting from Ca^{2+} binding, which is a general property of all α-lactalbumins (Stuart et al., 1986), is useful for the analysis of the ITC measurements.

Isothermal titration calorimetry of apo-GLA and -BLA with Ca^{2+}

Fig. 2, *A* and *B*, represents a typical example of an isothermal titration experiment. In Fig. 2 *A*, the heat exchange is

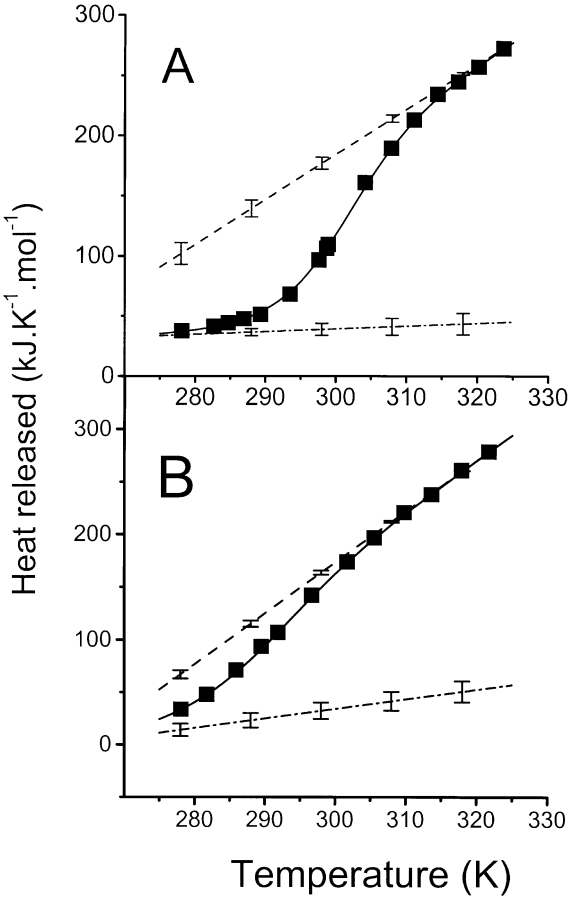

FIGURE 3 Molar heat release ($-\Delta H_{Ca2+,exp}$) for Ca^{2+} binding to GLA (*A*) and BLA (*B*). Black squares (■) represent experimental values. The full lines represent the curves fitted according to the combined Eqs. 1 through 7. Dashed lines represent the temperature dependence of $-\Delta H_{Ca2+,EN}$. Dash-dot lines represent the temperature dependence of $-\Delta H_{Ca2+,N}$. The error bars represent standard deviation.

shown at subsequent injections of Ca^{2+} into an apo-BLA solution at 313.6 K (40.5°C). By deconvolution of the subsequent titration curve (Fig. 2 *B*), the molar heat exchange for Ca^{2+} binding ($\Delta H_{Ca2+,exp(T)}$) is calculated. In case sufficient data points are gathered to reproduce accurately the drop of the heat release near the equivalence point, also a reliable value for the Ca^{2+}-binding constant ($K_{Ca2+,exp(T)}$) can be deduced. The further treatment of both series of data are described in the following sections.

Influence of eventual proton exchange on the $\Delta H_{Ca2+,exp(T)}$ values

The experimental values for molar release of heat upon Ca^{2+} binding ($-\Delta H_{Ca2+,exp(T)}$) to GLA and BLA in a buffer of 10 mM Tris-HCl, pH 7.5, near 298 K, are plotted as a function of temperature in Fig. 3, *A* and *B*, respectively. A number of distinct differences between both sigmoid curves are observed: 1) the transition domain, characterized by the steepest part of the curve, is situated at a higher temperature for GLA than for BLA; 2) in the center of the transition domain, the slope is larger for GLA than for BLA; 3) at the extremities of the respective transition domains, the slope is smaller for GLA than for BLA.

Before starting the analysis of the temperature dependence of the $\Delta H_{Ca2+,exp(T)}$ values we looked for the eventual contribution resulting from protonation of Tris or deprotonation of Tris-H^+. Indeed, if LA would loose or acquire a proton upon Ca^{2+} binding and/or changing conformation these protons should be transferred to or withdrawn from the buffer components. In this case the $\Delta H_{Ca2+,exp(T)}$ values would have to be corrected for the eventual deprotonation enthalpy of the buffer ($\Delta H_{deprot(T)}$). The evaluation of this contribution is realized by measuring the binding of Ca^{2+} to the LAs in buffers with different deprotonation enthalpies. The real Ca^{2+}-binding enthalpy can then be calculated according to:

$$\Delta H_{Ca2+,real(T)} = \Delta H_{Ca2+,exp(T)} - n_{H+} \times \Delta H_{deprot(T)} \quad (1)$$

in which n_{H+} designates the number of protons that are released ($n_{H+} > 0$) or taken up ($n_{H+} < 0$) by the buffer.

We measured the $\Delta H_{Ca2+,exp(T)}$ values for the binding of Ca^{2+} to GLA and BLA in 10 mM MOPS, HEPES, and Tris-HCl, respectively, at the low and high temperature extremes of the transition curve. As the equilibrium Tris + $H^+ \leftrightarrow$ Tris-H^+ is sensitive to temperature, a Tris-HCl buffer of pH 7.5 near 25°C (298.1 K) shifts from pH 7.95 near 9.5°C (282.6 K) to pH 6.95 near 45°C (318.1 K). We accounted for this pH shift by adjusting the pH of the MOPS and HEPES buffer to pH 7.95 near 9.5°C and to pH 6.95 near 45°C, respectively. This large difference in pH by no means invalidates our comparative study.

The $\Delta H_{Ca2+,exp(T)}$ values determined at pH 7.95 near 9.5°C are summarized in Table 1. Interestingly, the values vary as to whether the sulfonic acids (MOPS and HEPES) have been titrated with KOH or NaOH to attain pH 7.95 near 9.5°C. However, the respective Ca^{2+} binding enthalpies in MOPS and HEPES buffer are, within the experimental error, identical with the $\Delta H_{Ca2+,exp(T)}$ values that are obtained in Tris-HCl buffer to which 10 mM KCl or NaCl is added. In the presence of 10 mM K^+ and Na^+, respectively, the $\Delta H_{Ca2+,exp(T)}$ values of GLA and BLA are identical in the different buffer systems and are not influenced by the deprotonation enthalpy of the buffer. This observation allows us to conclude that neither GLA nor BLA exchange an appreciable amount of protons upon Ca^{2+} binding at the lower temperature limit of the transition curves.

Concerning the observation that the $\Delta H_{Ca2+,exp(T)}$ values of GLA and BLA are dependent on the nature of the dissolved monovalent cation, the near UV ellipticity curves presented by the right (+) and diagonal (×) crosses in Fig. 1, *A* and *B*, show that the transition of both apoproteins is

TABLE 1 Experimental enthalpy values for the binding of Ca^{2+} to GLA and BLA in different buffer conditions

Buffers	ΔH_{deprot}* (kJ mol^{-1})	GLA T_{exp} (°C)	GLA $\Delta H_{Ca2+,exp}$† (kJ mol^{-1})	BLA T_{exp} (°C)	BLA $\Delta H_{Ca2+,exp}$† (kJ mol^{-1})
pH 7.95 at 9.5°C					
Tris-HCl 10 mM	48.1	9.7	−41.3	9.3	−50.3
Tris-HCl 20 mM	48.1	9.1	−38.9	9.9	−42.7
Tris-HCl 10 mM, KCl 10 mM	48.1	9.3	−30.4	9.5	−28.9
HEPES-KOH 10 mM	20.3	9.0	−29.4	9.0	−32.3
MOPS-KOH 10 mM	21.2	10.3	−31.3	9.0	−29.9
Tris-HCl 10 mM, NaCl 10 mM	48.1	9.2	−18.5	9.2	−9.5
HEPES-NaOH 10 mM	20.3	9.2	−17.1	8.6	−9.5
MOPS-NaOH 10 mM	21.2	9.3	−18.0	9.1	−10.4
pH 6.95 at 45°C					
Tris-HCl 10 mM	46.5	45.1	−256.0	45.1	−260.9
Tris-HCl 10 mM, NaCl 10 mM	46.5	45.1	−249.7	45.1	−257.8
MOPS-NaOH 10 mM	22.6	45.1	−246.8	45.1	−256.1

*Values for ΔH_{deprot} for MOPS and HEPES were obtained from Fukada and Takahashi (1998); the ΔH_{deprot}-values for Tris were obtained from Samland et al. (2001).

†Standard deviations of multiple experiments were typically ± 1.5 kJ/mol.

shifted to higher temperatures by the addition of 10 mM Na^+. Therefore the $\Delta H_{Ca2+,exp(T)}$ values at 9.5°C in the absence and presence of Na^+ (and presumably also of K^+) are influenced by small differences in protein conformation depending on whether Na^+ (or K^+) is added to the solution or not. Nevertheless, the fact that the $\Delta H_{Ca2+,exp(T)}$ values in the presence of equal concentrations of Tris-H^+, K^+, or Na^+ ions are different (Table 1) indicates that the ion effect is not a pure question of ionic strength. It affirms the idea that Na^+ and even that K^+ bind in a specific way to LAs (Permyakov et al., 1985; Hiraoka and Sugai, 1985; Desmet et al., 1987).

The results of Table 1 show that also near 45°C (318.1 K) at pH 6.95 and in the presence of 10 mM Na^+ (10 mM MOPS-NaOH and 10 mM Tris-HCl with 10 mM NaCl), the $\Delta H_{Ca2+,exp(T)}$ values of GLA and of BLA are identical within experimental error. The values are not dependent on the deprotonation enthalpy of the buffer. As a consequence, also under the experimental conditions of the high temperature extreme of the transition curves, no noticeable amount of protons is exchanged.

The fact that the Ca^{2+} binding enthalpies of GLA and BLA are not influenced by the buffer deprotonation enthalpy, neither before nor after the transition domain, strongly suggests that the $\Delta H_{Ca2+,exp(T)}$ values do not require corrections for protonation or deprotonation of the buffer within the whole temperature region investigated.

Analysis of the temperature dependence of the $\Delta H_{Ca2+,exp(T)}$ values

From the above results we have learned that the experimental enthalpy values for Ca^{2+} binding to apo-GLA and -BLA (Fig. 3, *A* and *B*) do not contain noticeable contributions from proton exchange with the buffer substances. For the further analysis of the $\Delta H_{Ca2+,exp(T)}$ values we assumed that, in the considered temperature range (276–320 K), native apo-LA equilibrates with the expanded molten globule state and that all Ca^{2+}-bound LA is native. The latter assumption is in agreement with the constant near-UV CD signal of Ca^{2+}-LA below 320 K (Fig. 1). Under the conditions of the above assumptions $\Delta H_{Ca2+,exp(T)}$ consists of only two contributions, one for Ca^{2+} binding to the fraction of native apo-LA ($\alpha_{N(T)}$) and another for Ca^{2+} binding to the fraction of molten globule ($1 - \alpha_{N(T)}$). The respective molar enthalpies are represented by $\Delta H_{Ca2+,N(T)}$ and $\Delta H_{Ca2+,EN(T)}$. The subscript N refers to Ca^{2+} binding to native apo-LA, and the subscript EN emphasizes that Ca^{2+} binding to expanded LA also includes a transition to the native state of the protein. Hence,

$$\Delta H_{Ca2+,exp(T)} = \alpha_{N(T)} \times \Delta H_{Ca2+,N(T)} + (1 - \alpha_{N(T)}) \times \Delta H_{Ca2+,EN(T)} \quad (2)$$

The values of the enthalpy change in the above equation are temperature dependent. They can be related to the respective values at the standard temperature (298 K) by the heat capacity increments $\Delta C_{pCa2+,N}$ and $\Delta C_{pCa2+,EN}$. Assuming that the respective heat capacity increments do not depend significantly on temperature, we write

$$\Delta H_{Ca2+,N(T)} = \Delta H_{Ca2+,N(298)} + \Delta C_{pCa2+,N} \times (T - 298) \quad (3)$$

and

$$\Delta H_{Ca2+,EN(T)} = \Delta H_{Ca2+,EN(298)} + \Delta C_{pCa2+,EN} \times (T - 298) \quad (4)$$

TABLE 2 Summary of the thermodynamic parameters obtained from isothermal titration of GLA and BLA with Ca^{2+} at different temperatures

Parameter		GLA	BLA
$\Delta H_{Ca2+,EN(298)}$	(kJ mol^{-1})	−177.4 ± 5	−164.3 ± 2
$\Delta H_{Ca2+,N(298)}$	(kJ mol^{-1})	−38.9 ± 5	−32.4 ± 8
$\Delta H_{NE(298)}$	(kJ mol^{-1})	138.5 ± 10	131.9 ± 10
$\Delta S_{NE(298)}$	(J K^{-1} mol^{-1})	463 ± 32	460 ± 35
$T_{(298)} \times \Delta S_{NE(298)}$	(kJ mol^{-1})	138.0 ± 10	137.1 ± 10
$\Delta G_{NE(298)}$	(kJ mol^{-1})	+0.5 ± 0.2	−5.2 ± 0.5
$\Delta C_{pCa2+,EN}$	(kJ K^{-1} mol^{-1})	−3.74 ± 0.2	−4.83 ± 0.1
$\Delta C_{pCa2+,N}$	(kJ K^{-1} mol^{-1})	−0.23 ± 0.2	−0.92 ± 0.1
ΔC_{pNE}	(kJ K^{-1} mol^{-1})	3.51 ± 0.3	3.91 ± 0.2

The parameters $\Delta H_{Ca2+,EN(298)}$, $\Delta H_{Ca2+,N(298)}$, $\Delta S_{NE(298)}$, $\Delta C_{pCa2+,EN}$, and $\Delta C_{pCa2+,N}$ are obtained directly from the fittings in Fig. 3, *A* and *B*. The other parameters are deduced as complementary from classical thermodynamic equations.

$\alpha_{N(T)}$ in Eq. 2 is further expressed as a function of $K_{NE(T)}$, the equilibrium constant for the unfolding from the native to the expanded state of the apoprotein

$$\alpha_{N(T)} = 1/(1 + K_{NE(T)}) \quad (5)$$

The equilibrium constant in its turn is expressed as a function of the characteristic thermodynamic parameters

$$K_{NE(T)} = \exp-(\Delta H_{NE(298)} - T\Delta S_{NE(298)} + \Delta C_{pNE}(T - 298 - T \ln T/298))/RT \quad (6)$$

$\Delta H_{NE(298)}$ and $\Delta S_{NE(298)}$ are the transition enthalpy and entropy at 298 K, respectively.

Finally, as $\Delta H_{Ca2+,N(T)}$ is the enthalpy exchange for Ca^{2+} binding to native apo-LA whereas $\Delta H_{Ca2+,EN(T)}$ combines the value for Ca^{2+} binding with that for a conformational change from an expanded to a native Ca^{2+} protein, the enthalpy change for the unfolding of apo-LA ($\Delta H_{NE(298)}$) is substituted in Eq. 6 by ($\Delta H_{Ca2+,N(298)} - \Delta H_{Ca2+,EN(298)}$). The related heat capacity change (ΔC_{pNE}) is also substituted by ($\Delta C_{pCa2+,N} - \Delta C_{pCa2+,EN}$).

$$\Delta H_{NE(298)} = \Delta H_{Ca2+,N(298)} - \Delta H_{Ca2+,EN(298)} \quad (7)$$

$$\Delta C_{pNE} = \Delta C_{pCa2+,N} - \Delta C_{pCa2+,EN} \quad (8)$$

The results of the curve fittings according to the combined Eqs. 2 through 8 are shown in Fig. 3, *A* and *B*. The full line in each of these figures, represents the best fit of the $-\Delta H_{exp(T)}$ values as a function of temperature. The fitting offers the numeric values of five independent parameters: $\Delta H_{Ca2+,EN(298)}$, $\Delta C_{pCa2+,EN}$, $\Delta H_{Ca2+,N(298)}$, $\Delta C_{pCa2+,N}$, and $\Delta S_{NE(298)}$. These values are collected in Table 2. To enable a direct comparison of the complementary thermodynamic parameters, the resulting values of $\Delta H_{NE(298)}$, of ΔC_{pNE}, of the product $T_{(298)} \times \Delta S_{NE(298)}$, and of $\Delta G_{NE(298)}$ are also added to Table 2. In Fig. 3, *A* and *B*, the temperature dependence of the respective $\Delta H_{Ca2+,EN(T)}$ (Eq. 4) and $\Delta H_{Ca2+,N(T)}$ values (Eq. 3) is also represented. The $\Delta H_{Ca2+,EN(T)}$ values are represented by the dashed lines. These lines are tangent to the experimental enthalpy values in the high temperature region, and their slopes equal the respective $\Delta C_{pCa2+,EN}$ values. The $\Delta H_{Ca2+,N(T)}$ values are presented by the dash-and-dot lines. These lines are supposed to touch the experimental curve at sufficiently low temperatures. Their slopes equal $\Delta C_{pCa2+,N}$.

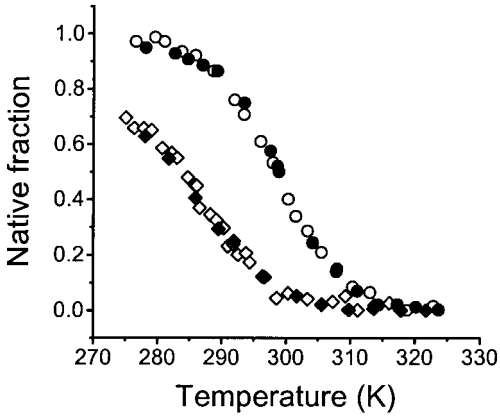

FIGURE 4 Thermal transition curves for apo-GLA (*circles*) and apo-BLA (*diamonds*) calculated from the ellipticity change at 270 nm (*open symbols*) and from the molar enthalpy change upon Ca^{2+} binding (*black symbols*), respectively. For the calculation of the fractions of native BLA from the ellipticity change it has been assumed that the compact native state of apo-BLA has the same ellipticity value as native Ca^{2+}-loaded BLA.

It is a prerequisite of a two-state equilibrium that different physico-chemical characteristics register the same transition. As a verification of this exigency we compared the thermal transitions resulting from the ellipticity changes (Fig. 1, *A* and *B*) with those obtained from the titration calorimetry (Fig. 3, *A* and *B*). The fractions of native apo-GLA, deduced from ellipticity changes, are easily calculated as the limit values at both extremes of the transition curve are clearly obtained (Fig. 1 *A*). For the calculation of the fractions of native apo-BLA from the ellipticity changes, we supposed that the near-UV CD signal of native apo-BLA equals the value of native Ca^{2+}-BLA (Fig. 1 *B*). The native fractions, deduced from titration calorimetry, are calculated for both apo-LAs according to Eq. 2. The different results are plotted in Fig. 4. The agreement of both series of fractional changes for GLA as well as for BLA, is a confirmation that near-UV CD changes and heat exchanges register the same unfolding equilibrium and that the assumption of a two-state transition, made to deduce the fitting procedure, is reliable.

Ca^{2+}-binding constants

At each temperature an apparent constant for the binding of Ca^{2+} to LA is obtained by simulation of the ITC titration curve (Fig. 2 *B*). However in the lower temperature region,

TABLE 3 Summary of $K_{Ca2+,app}$, $K_{NE,apoLA}$, and $K_{Ca2+,N}$ values for GLA and BLA

Temperature (K)	$K_{Ca2+,app}$ (mol^{-1} L)	$K_{NE,apoLA}$	$K_{Ca2+,N}$ (mol^{-1} L)
GLA			
314.7	$(15.0 \pm 0.3) \times 10^6$	28 ± 1.1	$(4.4 \pm 0.4) \times 10^8$
317.3	$(3.4 \pm 0.2) \times 10^6$	54 ± 2.9	$(1.8 \pm 0.2) \times 10^8$
320.1	$(1.4 \pm 0.1) \times 10^6$	108 ± 5.9	$(1.5 \pm 0.2) \times 10^8$
BLA			
313.7	$(8.3 \pm 0.4) \times 10^6$	210 ± 7.6	$(17.6 \pm 1.2) \times 10^8$
317.8	$(3.1 \pm 0.1) \times 10^6$	570 ± 14	$(17.7 \pm 0.9) \times 10^8$
321.7	$(0.9 \pm 0.1) \times 10^6$	1542 ± 43	$(14.0 \pm 1.5) \times 10^8$

Below 310 K apparent binding constants of 2.8×10^7 and larger are obtained. No account is taken of these values as the product of $K_{Ca2+,app}$ times the concentration of the dissolved macromolecule (35 μM) exceeds 1000 in these conditions, which prohibits the definition of valuable data (ITC Users Manual of MicroCal Inc.).

Ca^{2+} binding to LA is very strong resulting in very steep ITC titration curves near the equivalence points. In this case, the simulation procedure offers an inaccurate, mostly underestimated value of the binding constant. This is the case whenever the product of the binding constant times the concentration of the dissolved macromolecule exceeds a value of 1000 (Users Manual of MicroCal Inc.). In our titration experiments, using a protein concentration of 35 μM, apparent binding constants of 2.8×10^7 and larger are obtained below 310 K. Therefore, no account is taken of these values. Above 310 K, the experimental decrease of the heat exchange near the equivalence point becomes spread over several injections of the Ca^{2+} solution (Fig. 2 *B*) and the apparent Ca^{2+}-binding constants ($K_{Ca2+,app(T)}$) are smaller than 2.8×10^7. These values are gathered in Table 3.

The term apparent binding constant ($K_{Ca2+,app(T)}$) refers to the fact that in our ITC measurements Ca^{2+} binds to an equilibrium mixture of native and expanded apo-LA. However, in the conditions of our experiments and below 320 K, Ca^{2+}-bound LA is compactly folded (Fig. 1). $K_{Ca2+,app(T)}$ defined in this way can be related with $K_{Ca2+,N(T)}$, the constant for Ca^{2+} binding to native apo-LA, and with $K_{NE(T)}$, the constant for the equilibrium between native and expanded apo-LA, by the following equation

$$K_{Ca2+,app(T)} = \frac{[Ca^{2+} - LA_N]}{([LA_N] + [LA_E]) \times [Ca^{2+}]} = \frac{K_{Ca2+,N(T)}}{(1 + K_{NE(T)})} \quad (9)$$

The thermodynamic parameters in Table 2 allow us to calculate $K_{NE(T)}$ values at different temperatures. From the available $K_{Ca2+,app(T)}$ and calculated $K_{NE(T)}$ values, constants for Ca^{2+} binding to native LA ($K_{Ca2+,N(T)}$) are obtained. These values are added in Table 3. They are also introduced, together with $\Delta H_{Ca2+,N(298)}$ and $\Delta C_{pCa2+,N}$ values (from Table 2), in a van't Hoff-equation equivalent to Eq. 6 to calculate $\Delta S_{Ca2+,N(298)}$ and subsequently $K_{Ca2+,N(298)}$. The latter values equal 1.1 ($\pm$ 0.2) $\times 10^9$ and 3.9 ($\pm$0.8) $\times 10^9$ for GLA and BLA, respectively. These binding constants and the corresponding thermodynamic parameters are collected in Table 4. Taking into account the partial unfolding of both apo-LAs at 298 K, the apparent constants for the binding of Ca^{2+} to the mixed population of native and expanded apo-GLA or -BLA (estimated $K_{Ca2+,app(298)}$) are 5.7 ($\pm$1) $\times 10^8$ and 4.3 ($\pm$0.8) $\times 10^8$, respectively.

TABLE 4 Thermodynamic parameters for the binding of Ca^{2+} to native apo-LA at 298 K

Parameter		GLA	BLA
$\Delta H_{Ca2+,N(298)}$	(kJ mol^{-1})	-38.9 ± 5	-32.4 ± 8
$\Delta S_{Ca2+,N(298)}$	(J K^{-1} mol^{-1})	$+42 \pm 15$	$+75 \pm 25$
$T_{(298)} \times \Delta S_{Ca2+,N(298)}$	(kJ mol^{-1})	$+12.6 \pm 5$	$+22.3 \pm 8$
$\Delta G_{Ca2+,N(298)}$	(kJ mol^{-1})	-51.5 ± 0.4	-54.7 ± 0.5
$K_{Ca2+,N(298)}$	(mol^{-1} L)	1.1 ($\pm$0.2) $\times 10^9$	3.9 ($\pm$0.4) $\times 10^9$
$\Delta C_{pCa2+,N}$	(kJ K^{-1} mol^{-1})	-0.23 ± 0.2	-0.92 ± 0.1

DISCUSSION

The final goal of this ITC study is the mutual comparison of the thermodynamic properties for the thermal unfolding and for the Ca^{2+} binding of apo-GLA and -BLA. To avoid small monovalent cations we performed our measurements in a Tris-HCl buffer (10 mM Tris-HCl, pH 7.5 near 25°C).

The thermodynamic parameters for the thermal unfolding of an LA are frequently deduced from differential scanning calorimetry (DSC) data (Dolgikh et al., 1985; Pfeil and Sadowski, 1985; Xie et al., 1993; Ptitsyn and Uversky, 1994). A plot of a DSC scan represents the heat capacity as a function of temperature. The excess heat absorption upon unfolding of the protein offers a bell-shaped heat capacity peak; under the peak an "S-shaped" baseline shift occurs as a consequence of the different specific heat capacities of the protein states before and after the transition. In the case of an apo-LA this offers a specific inconvenience. Indeed, at neutral pH and low ionic strength (10 mM Tris-HCl, pH 7.5 near 25°C) apo-LA already starts to denature at temperatures below 0°C. This results in an incomplete DSC transition peak (Yutani et al., 1992; Griko et al., 1994; Veprintsev et al., 1997) and in a lack of reference values needed to draw the "S-shaped" baseline. As a consequence the calculation of fractional changes of the protein states as a function of temperature, as well as the computation of the transition enthalpy is compromised. Nevertheless, to enable the analysis of the data, it has been postulated that any native Ca^{2+}-LA has the same heat capacity as the native apo-LA variant (Griko et al., 1994; Griko, 1999, 2000). Our results,

however, indicate that this hypothesis does not hold for every type of LA.

In our study we derived the thermodynamic parameters for the unfolding of apo-GLA and -BLA in 10 mM Tris-HCl from ITC measurements with Ca^{2+}. The analysis of ITC data is enabled by the fact that the temperature intervals for the thermal unfolding of the apo- and the Ca^{2+}-LAs are clearly separated from each other (open and filled squares in Fig. 1, *A* and *B*). The ITC-data offer at each temperature an absolute value of the heat exchange on Ca^{2+} binding (Fig. 3, *A* and *B*). Under the conditions of our experiments (pH 7–8) the $\Delta H_{bCa2+,exp}$ values are free from contributions due to protonation or deprotonation of buffer substances. The data are analyzed assuming that the transition between the native and the molten globule state of LA is in a two-state equilibrium and that, within the considered temperature range, constant heat capacity increments can be assigned to the transition. To verify the reliability of the above assumptions we compared the thermal transitions resulting from our ITC analysis with those obtained from the near-UV ellipticity changes. The good overlap of the fractional changes derived by both methods (Fig. 4) points out that after all the combined assumption of a global two-state equilibrium and a constant heat capacity increment for the transition, holds within the conditions of our study. Moreover, our thermal analyses are based on the agreement of the calorimetric enthalpy values (Fig. 3, *A* and *B*) and of the van't Hoff enthalpies for two-state transition (Eqs. 2–8). The ability to fit a curve of experimental enthalpy values as a function of temperature to the combined equations is in a complementary way confirmatory for the applicability of the assumptions.

In agreement with observations of Segawa and Sugai (1983), our near-UV CD transition curves (Fig. 1, *A* and *B*) indicate that native apo-GLA is more thermostable than apo-BLA. This statement is further evidenced by the transition curves resulting from the calorimetric titrations (Fig. 3, *A* and *B*). The free energy values for the transition (ΔG_{NE}) of both LAs result from positive ΔH_{NE} and positive ΔS_{NE} values (Table 2), therefore the thermal unfolding of both apo-LAs is entropy driven. However, the larger free energy change for the transition of apo-GLA than for apo-BLA (+0.5 kJ/mol and −5.2 kJ/mol, respectively, at 298 K) results from a larger melting enthalpy for the former than for the latter apo-protein (138.5 and 131.9 kJ mol^{-1}, respectively, at 298 K). In the transition region the ΔS_{NE} values are very similar for both proteins (463 J K^{-1} mol^{-1} and 460 J K^{-1} mol^{-1}, respectively, at 298 K). Comparable results are obtained by Desmet (1992).

The most important contribution of the present study comes from the observed differences in heat capacity increment. The heat capacity change between two protein states (ΔC_p) is the thermodynamic property that can be connected most directly to the exposure of surface area (Ladbury and Chowdry, 1996). Several research groups have developed algorithms relating the ΔC_p values to the area of hydrophobic and hydrophilic surfaces that become exposed and hydrated during the unfolding of the protein (Livingstone et al., 1991; Privalov and Makhatadze, 1992; Oobataka and Ooi, 1993; Freire, 1995). In those equations, the contributions from the exposure of hydrophobic surface are dominant. In BLA a clear correlation has been found between the $\Delta C_{p\ NE}$ value and the amount of residual secondary structure of the denatured protein (Griko, 1999, 2000). At room temperature the ΔC_p value between completely unfolded and native LA is estimated at 7.5 to 8.0 kJ K^{-1} mol^{-1} (Haynie and Freire, 1993; Griko 1999), whereas that between the molten globule and the native state of LAs is 3.5 to 4.0 kJ K^{-1} mol^{-1} (Freire, 1995; Griko, 1999). Our ITC study offers $\Delta C_{p\ NE}$ values of 3.51 and 3.91 kJ K^{-1} mol^{-1} for the thermal transition of apo-GLA and -BLA, respectively. It has been pointed out that, in the molten globule state of LA, the β-sheet domain is significantly unfolded, whereas the α-helix domain retains its native helices as well as its tertiary fold (Baum et al., 1989; Alexandrescu et al., 1993; Peng and Kim, 1994; Wu et al., 1995). The fact that the ΔC_p values for the expansion of native apo-GLA and -BLA to the molten globule state are nearly one-half of those for complete unfolding, is in good correspondence with the above statement. Furthermore, the mutual resemblance of both ΔC_{pNE} values suggests that the extent of the temperature-induced conformational change is nearly identical for both apo-LAs.

In contrast to the good resemblance of the ΔC_{pNE} values, clear differences are revealed between the respective $\Delta C_{pCa2+,N}$ and $\Delta C_{pCa2+,EN}$ values (Table 2). The values of $\Delta C_{pCa2+,N}$ amount to −0.23 for GLA and −0.92 kJ K^{-1} mol^{-1} for BLA. By several authors (Kuroki et al., 1992; Griko et al., 1994; Vanderheeren et al., 1996; Hendrix et al., 2000) Ca^{2+} binding to native apo-LA is supposed to not affect the conformation of the protein, and the heat capacity change for Ca^{2+} binding to this state is estimated to approach zero. The small value of $\Delta C_{pCa2+,N}$ for GLA (−0.23 kJ K^{-1} mol^{-1}) is in fair agreement with this point of view. However, this statement does not apply to BLA. The relatively important $\Delta C_{pCa2+,N}$ value for BLA (−0.92 kJ K^{-1} mol^{-1}) indicates that Ca^{2+}-BLA is more compactly folded than native apo-BLA. A recent x-ray diffraction study reveals that on removal of Ca^{2+} from crystallized BLA, a minor expansion of the metal binding site triggers a more important separation of the helical and the β-sheet domain at the opposite face of the protein molecule (Chrysina et al., 2000). Such conformational differences between native apo- and Ca^{2+}-conformers undoubtedly contribute to the observed $\Delta C_{pCa2+,N}$ value for BLA. However, the poor $\Delta C_{pCa2+,N}$ value for GLA suggests that the latter protein variant, in its native state, is less susceptible for the above as well as for eventual other events due to Ca^{2+}-induced access of solvent into its hydrophobic regions. This different influence of Ca^{2+} is unexpected as

```
                    10          20          30          40          50          60
GLA     EQLTKCEVFQ  KLKDLKDYGG  VSLPEWVCTA  FHTSGYDTQA  IVQNNDSTEY  GLFQINNKIW
BLA               R  E        G           T
          --α helix A--         --------α helix B------       -----β-sheet----

                    70          80          90          100         110         120
GLA     CKDDQNPHSR  NICNISCDKF  LDDDLTDDIV  CAKKILDKVG  INYWLAHKAL  CSEKLDQWLC  EKL
BLA               S                       M   V
                                -----Ca2+ site-/---/--α helix C---------    --helix--
```

FIGURE 5 Amino acid sequences of GLA and BLA. The complete sequence of GLA is given; the sequence of BLA only highlights the positions that differ from GLA. The amino acids that approach each other in the tertiary structure creating a negatively charged cluster around the Ca^{2+}-binding site are printed boldly. The location of the principal secondary structural elements is also marked.

the Ca^{2+}-binding site is very much conserved among the different LAs (Acharya et al., 1989). Indeed, all 10 amino acids of the Ca^{2+}-binding loop are identical in GLA and BLA (Fig. 5, Lys-79–Asp-88). To investigate possible origins for different $\Delta C_{pCa2+,N}$ values in GLA and BLA we have inspected the location of the differing amino acids within the spatial structure of those proteins. In BLA, seven amino acids differ from GLA (Fig. 5). Three neutral residues are conservatively replaced by other neutral ones (Ala-30-Thr, Val-90-Met, and Ala-92-Val). One neutral amino acid in GLA is replaced by a positively charged one in BLA (Gln-10-Arg). Two charged amino acids become neutral (Asp-17-Gly and Arg-70-Ser), and one positively charged residue in GLA is replaced by a negatively charged one in BLA (Lys-11-Glu). Strikingly, Glu-11 in BLA contributes to a large cluster of negative charges (see Fig. 6). The cluster is composed of Asp-78, -82, -83, -84, -87, and -88, which are located on the Ca^{2+}-binding loop, of Asp-37, which is located in a loop between helix B and the β-sheet, of Glu-7 and -11 on helix A near the NH_2-terminus, and of the terminal Glu-1. As a consequence, quite different subparts of the molecule encounter each other in the negatively charged cluster. Undoubtedly, in a medium of low ionic strength and in the absence of a metal ion, the realization of a close approach between the Ca^{2+}-binding loop and the NH_2-terminal region within this protein is hindered by the strong repulsion of charges from both substructures. Upon binding, Ca^{2+} accommodates the carboxyl groups of Asp-82, -87, and -88 (Stuart et al., 1986) thereby considerably reducing the repulsion between the above substructures. In contrast, the repulsion between the above substructures in apo-GLA is already counteracted by the presence of a

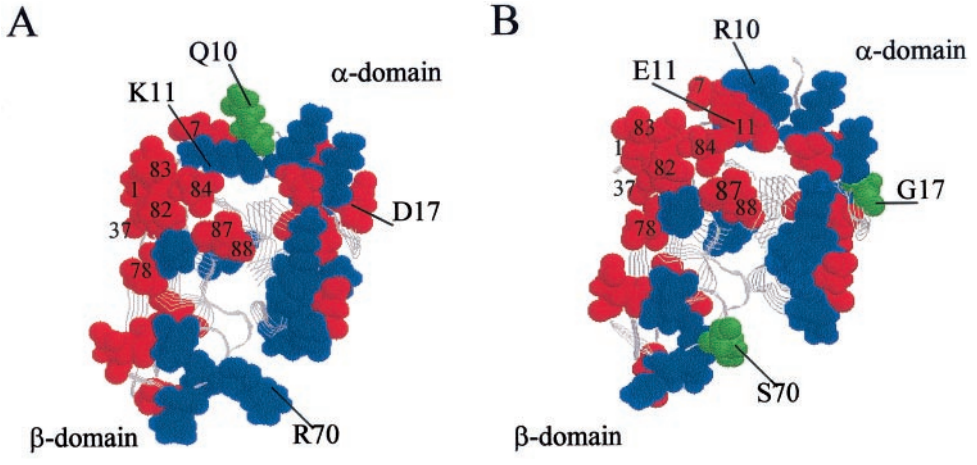

FIGURE 6 Space distribution of charges in LA at pH 7.5. The crystal structures of GLA (*A*) and BLA (*B*) are generated from the coordinates deposited under the codes 1HFYa and 1HFZa, respectively, in the Brookhaven Protein Data Bank (Pike et al., 1996). The negatively charged Asp and Glu residues are presented in red. The positively charged Lys, Arg, and His residues are in blue. The amino acids that are differently charged in GLA and BLA are also explicitly marked. Only those neutral amino acids that meet the above requirement are drawn space filled in green. The negatively charged amino acids within the Ca^{2+}-binding loop as well as those immediately surrounding this loop (<10 Å) are numbered according to their sequence in the peptide chain. The positions of the COOH-terminal residues 121 to 123 are not clearly defined from the electron density maps. Therefore none of those amino acids are drawn.

positive Lys in position 11 (Fig. 6 *A*) and the binding of Ca^{2+} is in this case hardly able to effect a closer mutual approach of the mentioned substructures. Additional evidence for the suggestion that in 10 mM Tris-HCl the compact state of native apo-BLA suffers more from repulsive charges than the equivalent apo-GLA comes from the larger temperature shift when 10 mM NaCl is added (compare the curves marked by open squares and diagonal crosses in Fig. 1, *A* and *B*). Indeed, the larger temperature shift indicates that apo-BLA is stabilized more than apo-GLA by a reduction of the repulsive forces.

Finally, in our study we determined Ca^{2+} binding constants for GLA and BLA. Because it has been found that α-LAs bind Ca^{2+} with high specificity (Hiraoka et al., 1980), the binding constant for Ca^{2+} at a neutral pH and near 25°C has been determined by different authors. Most published values concern apparent constants for the binding of Ca^{2+} to apo-BLA that is partially in a molten globule state. Although there is no full agreement, most authors found evidence for a binding constant between 10^8 and 10^9 (Permyakov et al., 1981; Bryant and Andrews, 1984; Kuwajima et al., 1986; Desmet and Van Cauwelaert, 1988; Berliner and Johnson, 1988; Vanderheeren et al., 1996). Our estimated $K_{Ca2+,app}$ values at 298 K (5.7×10^8 and 4.3×10^8 for apo-GLA and -BLA, respectively) are in good agreement with these data. Furthermore, our study makes clear that larger values must be considered for the binding constant of Ca^{2+} to native apo-LA ($K_{Ca2+,N(T)}$); at 298 K these values are 1.05×10^9 and 3.95×10^9 for GLA and BLA, respectively. As well the negative $\Delta H_{Ca2+,NE}$ as the positive $\Delta S_{Ca2+,NE}$ values contribute favorably to the large binding constants. Interestingly, the larger $K_{Ca2+,N(298)}$ value for BLA results from a more favorable entropy contribution for Ca^{2+} binding to native apo-BLA than to -GLA (Table 4). This can be interpreted by the fact that the large cluster of exclusively negative groups near the Ca^{2+}-binding site of BLA offers a better probability that Ca^{2+} will be trapped than is offered by the corresponding surroundings in GLA.

By application of standard thermodynamic equations we calculated $K_{Ca2+,N(T)}$ at 338 K (65°C), a temperature at which native Ca^{2+}-LA is destabilized (results not tabulated). The respective values for the binding to GLA and BLA are 1.3×10^8 and 3.9×10^8. The latter value is close to $K_{Ca2+,N(T)} = 2.9 \times 10^8$, obtained from Ca^{2+} exchange between BLA and EDTA at 338 K (Hendrix et al., 2000). This indicates that even at that high temperature, native Ca^{2+}-LA does not tend to dissociate in a direct way. In an earlier paper we deduced that the Ca^{2+} release at that temperature is mediated by a partial unfolding of Ca^{2+}-bound LA (Vanderheeren et al., 1996). In agreement with this finding it has been shown that derivatives of LA with a reduced number of disulfide bonds are able to bind Ca^{2+} and to keep the β-domain folded, even in the absence of the native structure of the α-domain (Hendrix et al., 1996; Wu et al., 1996). Furthermore, the lack of specific side-chain packing within the α-domain results in a significant thermal destabilization of the Ca^{2+}-bound β-domain (Hendrix et al., 1996; Wu et al., 1996). This pattern of destabilization, with a preliminary disturbance of the α-domain and subsequent Ca^{2+} release and unfolding of the β-domain, presumably also accounts for the pattern of the temperature-induced destabilization of Ca^{2+}-LA with four intact disulfide bridges (Vanderheeren et al., 1996). In contrast to this, by forced decalcification of LA the β-domain region destabilizes and unfolds at low temperatures. In a complementary way of what has been described above, the significant interdomain interaction effects that the unfolding of the β-domain results in a reduction of the side-chain packing in the α-domain (Baum et al., 1989; Alexandrescu et al., 1993; Peng and Kim, 1994; Wu et al., 1995). The contribution of the extended accumulation of negative charges near the Ca^{2+}-binding site to this ability for interdomain interaction has to be investigated more thoroughly.

We thank Wim Noppe for the purification and decalcification of the α-lactalbumins. This research was supported by grant G-0253-97 from the Flemish Fund for Scientific Research. Kristien Vanhee is a bursary of the Onderzoeksraad K. U. Leuven.

REFERENCES

Acharya, K. R., J. Ren, D. I. Stuart, D. C. Phillips, and R. E. Fenna. 1991. Crystal structure of human α-lactalbumin at 1.7 Å resolution. *J. Mol. Biol.* 221:571–581.

Acharya, K. R., D. I. Stuart, N. P. C. Walker, M. Lewis, and D. C. Phillips. 1989. Refined structure of baboon α-lactalbumin at 1.7 Å resolution. *J. Mol. Biol.* 208:99–127.

Alexandrescu, A. T., P. A. Evans, M. Pitkeathly, J. Baum, and C. M. Dobson. 1993. Structure and dynamics of the acid-denatured molten globule state of α-lactalbumin. *Biochemistry*. 32:1707–1718.

Arai, M., and K. Kuwajima. 2000. Role of the molten globule state in protein folding. *Adv. Prot. Chem.* 53:209–282.

Baum, J., C. M. Dobson, P. A. Evans, and C. Hanley. 1989. Characterization of a partly folded protein by NMR methods: studies on the molten globule state of guinea pig α-lactalbumin. *Biochemistry*. 28:7–13.

Berliner, L. C., and J. D. Johnson. 1988. α-Lactalbumin and calmodulin. *In* Calcium-Binding Proteins, Volume II: Biological Functions. M.V. Thompson, editor. CRC Press, Inc., Boca Raton, FL. 79–116.

Bryant, D. T. W., and D. Andrews. 1984. High affinity binding of Ca(II) to bovine α-lactalbumin in the absence and presence of EGTA. *Biochem. J.* 220:617–620.

Chrysina, E. A., K. Brew, and K. R. Acharya. 2000. Crystal structures of apo- and holo-bovine α-lactalbumin at 2.2 Å resolution reveal an effect of calcium on inter-lobe interactions. *J. Biol. Chem.* 275:37021–37029.

Desmet, J. 1992. The effect of metal ion binding on protein stability: a thermodynamic analysis of the binding to α-lactalbumin. Ph.D. thesis. Katholieke Universiteit Leuven, Belgium.

Desmet, J., I. Hanssens, and F. Van Cauwelaert. 1987. Comparison of the binding of Na^+ and Ca^{2+} to bovine α-lactalbumin. *Biochim. Biophys. Acta.* 912:211–219.

Desmet, J., and F. Van Cauwelaert. 1988. Calorimetric experiments of Mn^{2+}-binding to α-lactalbumin. *Biochim. Biophys. Acta.* 957:411–419.

Dolgikh, D. A., L. V. Abaturov, I. A. Bolotina, E. V. Brazhnikov, V. E. Bychkova, R. I. Gilmanshin, Y. O. Lebedev, G. V. Semisotnov, E. I. Tiktopulo, and O. B. Ptitsyn. 1985. Compact state of a protein molecule

with pronounced small-scale mobility: bovine α-lactalbumin. *Eur. Biophys. J.* 13:109–121.

Freire, E. 1995. Thermodynamics of partly folded intermediates in proteins. *Annu. Rev. Biomol. Struct.* 24:141–165.

Fukuda, H., and K. Takahashi. 1998. Enthalpy and heat capacity changes for the proton dissociation of various buffer components in 0.1 M potassium chloride. *Proteins Struct. Funct. Genet.* 33:159–166.

Gill, S. C., and P. H. von Hippel. 1989. Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* 182: 319–326.

Griko, Y. V. 1999. Denaturation versus unfolding: energetic aspects of residual structure in denaturated α-lactalbumin. *J. Prot. Chem.* 18: 361–369.

Griko, Y. V. 2000. Energetic basis of structural stability in the molten globule state: α-lactalbumin. *J. Mol. Biol.* 297:1259–1268.

Griko, Y. V., E. Freire, and P. L. Privalov. 1994. Energetics of the α-lactalbumin states: a calorimetric and statistical thermodynamic study. *Biochemistry.* 33:1889–1899.

Haynie, D. T., and E. Freire. 1993. Structural energetics of the molten globule state. *Proteins Struct. Funct. Genet.* 16:115–140.

Hendrix, T. M., Y. Griko, and P. Privalov. 1996. Energetics of structural domains in α-lactalbumin. *Protein Sci.* 5:923–931.

Hendrix, T. M., Y. Griko, and P. Privalov. 2000. A calorimetric study of the influence of calcium on the stability of bovine α-lactalbumin. *Biophys. Chem.* 84:27–34.

Hiraoka, Y., T. Segawa, K. Kuwajima, S. Sugai, and N. Moroi. 1980. α-Lactalbumin: a calcium metalloprotein. *Biochem. Biophys. Res. Commun.* 95:1098–1104.

Hiraoka, Y., and S. Sugai. 1985. Equilibrium and kinetic study of sodium- and potassium-induced conformational changes of apo-α-lactalbumin. *Int. J. Peptide Protein Res.* 26:252–261.

Kronman, M. J., and R. E. Andreotti. 1964. Inter- and intramolecular interactions of α-lactalbumin: I. The apparent heterogeneity at acid pH. *Biochemistry.* 3:1145–1151.

Kuroki, R., K. Nitta, and K. Yutani. 1992. Thermodynamic changes in the binding of Ca^{2+} to a mutant human lysozyme (D86/92): enthalpy-entropy compensation observed upon Ca^{2+} binding to proteins. *J. Biol. Chem.* 267:24297–24301.

Kuwajima, K., Y. Harushima, and S. Sugai. 1986. Influence of Ca(II) binding on the structure and stability of bovine α-lactalbumin studied by circular dichroism and magnetic resonance spectra. *Int. J. Peptide Protein Res.* 27:18–27.

Ladbury, J. E., and B. Z. Chowdry. 1996. Sensing the heat: the application of isothermal titration calorimetry to thermodynamic studies of biomolecular interactions. *Chem. Biol.* 3:791–801.

Lee, Y. C. 2001. Fluorimetric determination of EDTA and EGTA using terbium-salicylate complex. *Anal. Biochem.* 293:394–402.

Lindahl, L., and H. J. Vogel. 1984. Metal-ion dependent hydrophobic interaction chromatography of α-lactalbumins. *Anal. Biochem.* 140: 394–402.

Livingstone, J. R., R. S. Spolar, and M. T. Record. 1991. The contribution to the thermodynamics of protein folding from reduction in water-accessible nonpolar surface area. *Biochemistry.* 30:4237–4244.

Noppe, W., P. Haezebrouck, I. Hanssens, and M. De Cuyper. 1998. A simplified purification procedure of alpha-lactalbumin from milk using Ca^{2+}-dependent adsorption in hydrophobic expanded bed chromatography. *Bioseparation.* 8:153–158.

Oobataka, M., and T. Ooi. 1993. Hydration and heat-stability effects on protein unfolding. *Prog. Biophys. Mol. Biol.* 59:237–284.

Peng, Z., and P. S. Kim. 1994. A protein dissection study of a molten globule. *Biochemistry.* 33:2136–2141.

Permyakov, E. A., L. A. Morozova, and E. A. Burstein. 1985. Cation binding effects on the pH, thermal and urea denaturation transitions in α-lactalbumin. *Biophys. Chem.* 21:21–31.

Permyakov, E. A., U. V. Yarmolenko, L. P. Kalinichenko, L. A. Morozova, and E. A. Burstein. 1981. Calcium binding of α-lactalbumin: structural rearrangement and association constant evaluation by means of intrinsic protein fluorescence changes. *J. Biol. Chem.* 256:8582–8586.

Pfeil, W., and M. L. Sadowski. 1985. A scanning calorimetric study of bovine and human apo-α-lactalbumin. *Stud. Biophys.* 109:163–170.

Pike, A. C. W., K. Brew, and K. R. Acharya. 1996. Crystal structures of guinea-pig, goat and bovine α-lactalbumin highlight enhanced conformational flexibility of regions that are significant for its action in lactose synthase. *Structure.* 4:691–703.

Privalov, P. L., and G. I. Makhatadze. 1992. Contribution of hydration and noncovalent interactions to the heat-capacity effect on protein unfolding. *J. Mol. Biol.* 224:715–723.

Ptitsyn, O. B. 1994. Kinetic and equilibrium intermediates in protein folding. *Protein Eng.* 7:593–596.

Ptitsyn, O. B., and V. N. Uversky. 1994. The molten globule is a third thermodynamic state of protein molecules. *FEBS Lett.* 341:15–18.

Samland, A. K., I. Jelesarov, R. Kuhn, N. Amrhein, and P. Macheroux. 2001. Thermodynamic characterization of ligand-induced conformational changes in UDP-*N*-acetylglucosamine enolpyruvyl transferase. *Biochemistry.* 40:9950–9956.

Segawa, T., and S. Sugai. 1983. Interactions of divalent metal ions with bovine, human and goat α-lactalbumin. *J. Biochem. (Tokyo).* 93: 1321–1328.

Stuart, D. I., K. R. Acharya, N. P. C. Walker, S. G. Smith, M. Lewis, and D. C. Phillips. 1986. α-Lactalbumin possesses a novel calcium binding loop. *Nature.* 324:84–87.

Vanderheeren, G., and I. Hanssens. 1994. Thermal unfolding of bovine α-lactalbumin. *J. Biol. Chem.* 269:7090–7094.

Vanderheeren, G., and I. Hanssens. 1999. Spectroscopic study of the thermal unfolding of α-lactalbumin. *Recent Res. Dev. Biochem.* 1:45–61.

Vanderheeren, G., I. Hanssens, W. Meijberg, and A. Van Aerschot. 1996. Thermodynamic characterization of the partially unfolded state of Ca^{2+}-loaded α-lactalbumin: evidence that partial unfolding can precede Ca^{2+} release. *Biochemistry.* 35:16753–16759.

Veprintsev, D. B., S. E. Permyakov, E. A. Permyakov, V. V. Rogov, K. M. Cawthern, and L. J. Berliner. 1997. Cooperative thermal transitions of bovine and human apo-α-lactalbumins: evidence for a new intermediate state. *FEBS Lett.* 412:625–628.

Wu, L. C., Z. Peng, and P. S. Kim. 1995. Bipartite structure of the α-lactalbumin molten globule. *Nature Struct. Biol.* 2:281–286.

Wu, L. C., B. Schulman, Z. Peng, and P. S. Kim. 1996. Disulfide determinants of calcium-induced packing in α-lactalbumin. *Biochemistry.* 35:859–863.

Xie, D., V. Bhakuni, and E. Freire. 1993. Are the molten globule and the unfolded states of apo-α-lactalbumin enthalpically equivalent? *J. Mol. Biol.* 232:5–8.

Yutani, K., K. Ogasahara, and K. Kuwajima. 1992. Absence of the thermal transition in apo-α-lactalbumin in the molten globule state. *J. Mol. Biol.* 228:347–350.