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A calorimetric study of the influence of calcium on the stability of bovine α-lactalbumin

Tonya Hendrix, Yuri V. Griko*, Peter L. Privalov

Department of Biology, The Johns Hopkins University, Baltimore, MD 21218, USA

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Abstract

Bovine α-lactalbumin has been studied by differential scanning calorimetry with various concentrations of calcium to elucidate the effect of this ligand on its thermal properties. In the presence of excess calcium, α-lactalbumin unfolds upon heating with a single heat-absorption peak and a significant increase of heat capacity. Analysis of the observed heat effect shows that this temperature-induced process closely approximates a two-state transition. The transition temperature increases in proportion with the logarithm of the calcium concentration, which results in an increase in the transition enthalpy as expected from the observed heat capacity increment of denaturation. As the total concentration of free calcium in solution is decreased below that of the proteins, there are two temperature-induced heat absorption peaks whose relative area depends on the calcium concentration, such that further decrease of calcium concentration results in a increase of the low-temperature peak and a decrease of the high-temperature one. The high-temperature peak occurs at the same temperature as the unfolding of the holo-protein, while the low-temperature peak is within the temperature range associated with the unfolding of the apo-protein. Statistical thermodynamic modeling of this process shows that the bimodal character of the thermal denaturation of bovine α-lactalbumin at non-saturated calcium concentrations is due to a high affinity of Ca^{2+} for α-lactalbumin and a low rate of calcium exchange between the holo- and apo-forms of this protein. Using calorimetric data, the calcium-binding constant for α-lactalbumin has been determined to be 2.9×10^8 M^{-1}.

Keywords: α-Lactalbumin; Calcium binding; Binding constant; Calorimetry

* Corresponding author. Tel.: +1-410-516-6037; fax: +1-410-516-5213.
E-mail address: griko@jhunix.hcf.jhu.edu (Y.V. Griko)

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1. Introduction

α-Lactalbumin (LA) is an acidic, low molecular weight globular protein produced in the lactating mammary gland as a regulatory component of the lactose biosynthesis [1,2]. Its physical characteristics and folding properties are significantly affected by specific interactions with Ca^{2+}. The removal of bound calcium greatly decreases the thermal stability of LA but the protein retains essentially the same folded conformation [3,4]. On the other hand, according to a number of authors [5–7], the removal of Ca^{2+} at neutral pH and low ionic strength converts LA into a molten globule state. Yutani et al. [8] have studied calorimetrically the calcium-free apo-LA, which is supposed to be in the molten globule state in low ionic strength solutions: they did not observed any excess heat effect upon heating. It was therefore assumed that this state of LA is close to the unfolded state (see also Okazaki et al. [7]). However, Relkin et al. [9] studied LA at low concentrations of calcium using a Perkin-Elmer scanning calorimeter: they observed two heat absorption peaks upon heating the solution. The first peak was attributed to the thermal denaturation of the apo-form and the second one to the holo-form. This observation did not attract much attention because to overcome the insensitivity of the instrument used, the calorimetric experiments were conducted using high concentrations of protein (3 mM) and very fast heating rates (≥ 10 K min^{-1}), which are inappropriate for the quantitative thermodynamic analysis. However, later calorimetric studies showed that at room temperature and neutral pH the apo-LA is in the partly unfolded state if the ionic strength of the solution is low [10]. This partly unfolded state occurs because removal of Ca^{2+} results in the appearance of strong repulsive forces between uncompensated negative charges at the calcium-binding site. In the presence of monovalent salts apo-LA has a native-like structure that unfolds cooperatively upon heating with significant heat absorption, although at much lower temperatures than the holo-LA [3,10].

In this paper we demonstrate, using highly sensitive scanning microcalorimetry techniques, that a solution of LA at pH 8.0 and low concentrations of calcium indeed represents a mixture of apo- and holo-forms that do not readily interconvert and therefore unfold at different temperatures. These calorimetric data permit a reliable determination of the binding constant for calcium, a quantity difficult to obtain by other methods because of the low stability of the apo-form of LA.

2. Results

Fig. 1 gives the temperature dependence of the partial heat capacity of LA in 10 mM Tris–HCl (pH 8.0) in the presence of different concentration of $CaCl_2$. The concentration of LA, in all cases, was 0.1 mM and the heating rate was 1 K min^{-1}. The protein used in this experiment initially contained bound calcium which had not been specially removed. The addition of calcium to the buffer increased the peak area and peak maximum and correlated with an increase in temperature and enthalpy of LA unfolding (Table 1). This stabilizing effect of calcium on the holo-LA is rather small and appears to be proportional to the logarithm of calcium concentration (see inset in Fig. 1).

Fig. 2 shows the temperature dependencies of the partial heat capacities of LA in 10 mM Tris (pH 8.0) with different relative concentrations of EDTA that was used to decrease the concentration of free Ca^{2+} and holo-LA. As the concentration of EDTA increases, the major heat absorption peak decreased in area giving rise to a sec-

Table 1
Thermodynamic characteristics of LA unfolding in 10 mM Tris (pH 8.0) in the presence of varying amounts of $CaCl_2$

[Salt] (mM)	T_m (°C)	ΔH (T_m) (kJ mol^{-1})	K_a (25°C) (mol^{-1})
0.0 $CaCl_2$	64.1	271.0	–
0.1 $CaCl_2$	65.2	297.6	1.9×10^8
1.0 $CaCl_2$	67.9	316.3	1.8×10^7
2.0 $CaCl_2$	68.9	318.0	9.5×10^6
10.0 $CaCl_2$	70.0	313.8	–

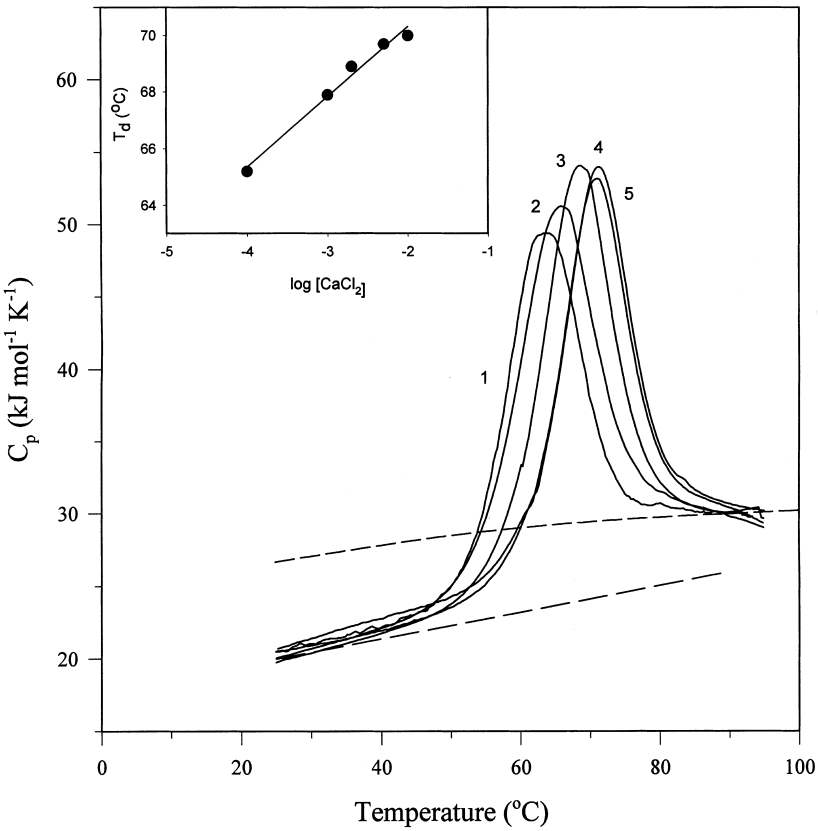

Fig. 1. The calorimetrically measured heat capacity profiles of LA in 10 mM Tris (pH 8) and various amounts of $CaCl_2$: (1) 0 mM; (2) 0.1 mM; (3) 1 mM; (4) 10 mM; and (5) 100 mM. The inset shows the dependence of the denaturation temperature of LA on the log of the $CaCl_2$ concentration in the range from 0.1 to 10 mM. The dashed line shows the extrapolation of the heat capacity of the native LA with a slope typical of other stable globular proteins [12].

ond lower-temperature peak located at 27°C while the transition temperature of both peaks remained constant. This observation suggests that the influence of calcium on the thermodynamic properties of LA depends on the concentration range of Ca^{2+}. At concentrations of Ca^{2+} where one can expect that all calcium-binding sites are saturated, we observe a single heat-absorption peak that increases slowly in proportion to the logarithm of calcium concentration. At concentrations where the free calcium in the solution is less than the protein's concentration and not all molecules of LA are bound calcium, two heat-absorption peaks are observed. The low-temperature peak can be assigned to the apo-form of LA and the area of this peak has a sigmoidal dependence on the relative EDTA concentration, R (see inset to Fig. 2).

Fig. 3 presents the denaturation enthalpies of holo- and apo-LA measured under various solvent conditions (see Table 2) plotted against the transition temperature. It also includes previously reported enthalpies of LA unfolding [10,11]. The figure shows that all the calorimetrically measured enthalpies of LA denaturation are in reasonable correspondence. Regression analysis of this data set, which includes enthalpy values for apo-LA, gives an enthalpy dependence on temperature equal to 4.3 kJ K^{-1} mol^{-1}. This value is lower than previously reported [10], but is in good agreement with the experimentally measured denaturation heat capacity increment if the heat

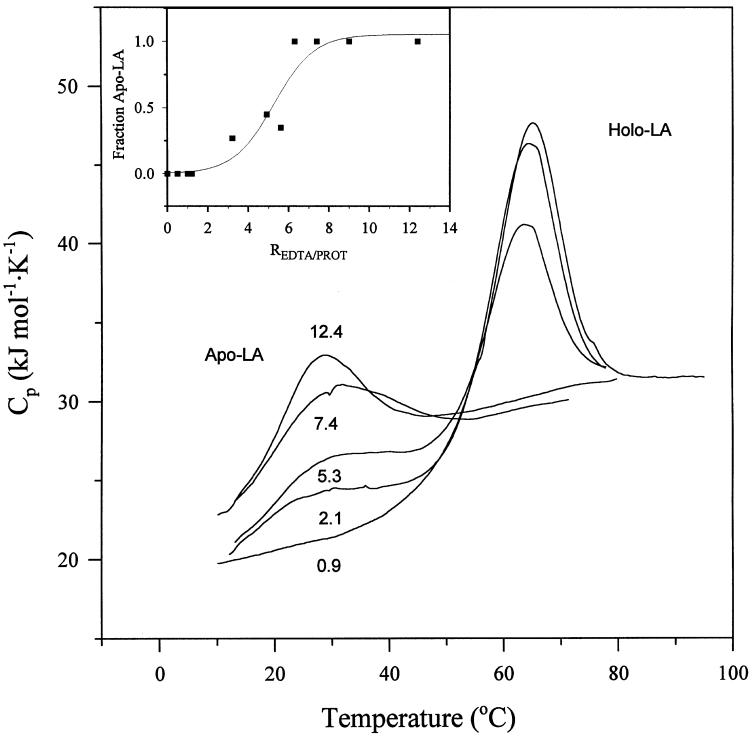

Fig. 2. The calorimetrically measured heat capacity profiles of LA in 10 mM Tris (pH 8) and various amounts of EDTA expressed as $R = [\text{EDTA}]/[\text{protein}]$, and indicated by the curves. The inset shows the calculated fraction of apo-LA vs. R, the fractional amount of EDTA.

capacity function of the native state is extrapolated with a slope equal to that found for other stable globular proteins [12], which is indicated in Fig. 1 by the dashed line.

3. Discussion

It is clear from the above that LA solutions having concentrations of calcium lower than that of protein contain a mixture of the apo- and holo-forms that do not interconvert readily. This slow interconversion suggests that the binding constant of Ca^{2+} to LA is very high, and correspondingly the rate of calcium release by the holo-form (as well as by EDTA) is much lower than the rate of calcium binding by the apo-form [13]. If we take into account that these two forms differ drastically in stability and the denatured protein does not specifically bind calcium, we can describe the observed process by the following scheme:

$$\begin{array}{ccc} \text{P} + \text{L} & \leftrightarrow & \text{PL} \\ \downarrow & & \uparrow \\ \text{D} & & \end{array} \qquad (1)$$

where P is the apo-LA; PL is the holo-LA with bound ligand; D is the denatured LA; and L is the ligand, i.e. calcium ion. Such a process was analyzed in detail by Brandts and Lin [14] and Shrake and Ross [15]. Using the formalism suggested by these authors, we simulated the calorimetrically determined heat capacity functions of LA in the presence of various concentration of calcium. In this simulation we assumed, in accordance with our previous finding [10], that the enthalpy and heat capacity effect of calcium binding by LA are zero.

The results of the simulations are shown in Fig. 4a and the parameters for the simulations are given in the legend to Fig. 4. We found that the binding constant decreases with the increase of the calcium concentration and calculated an aver-

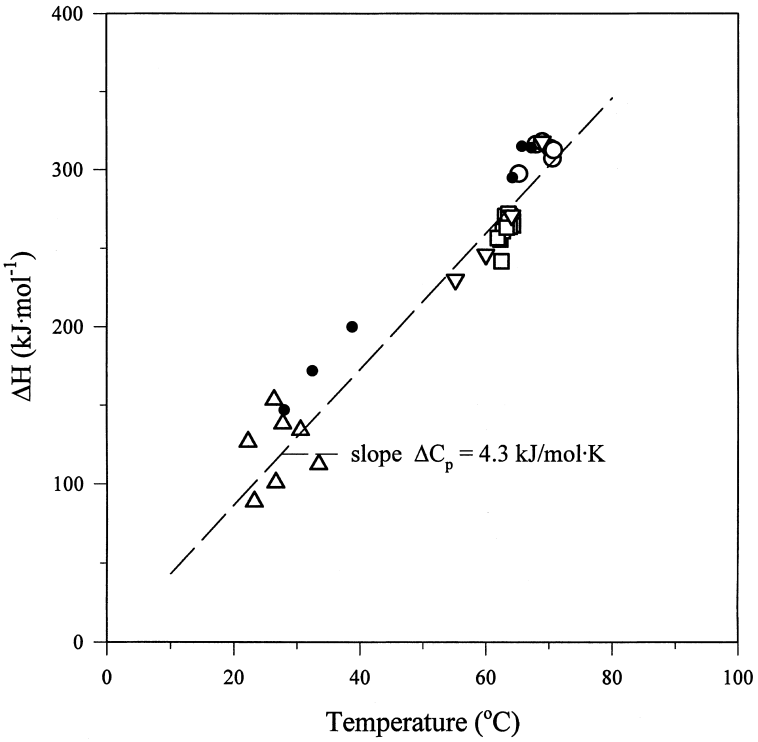

Fig. 3. The enthalpy of LA denaturation plotted vs. the transition temperature: (-△-) apo-LA; (-□-) holo-LA; (-○-) LA in presence of calcium as shown in Table 1; (-●-) from [10]; and (-▽-) from [11].

age calcium binding constant K_a of 2.9×10^8 M^{-1}.

The binding constant of Ca^{2+} to LA has been determined by many authors using various modifications of the Hummel–Dryer procedure and the reported values between 10^6 and 10^9 M^{-1} spread across three orders of magnitude (for review see Kronman [2]). According to Kronman [2], the values which are considered to be the most reliable values were obtained by his group and lie between 1.5×10^6 M^{-1} [16] and 2.5×10^6 M^{-1} [17]; however, all their measurements were carried out at room temperature (20–25°C) at neutral pH. As shown in Fig. 2, the denaturation of apo-LA in solutions with pH 8.0 begins below 10°C and at 20°C a significant portion of apo-LA are denatured. Therefore, the values of the binding constant offered by Kronman have all been underestimated. This situation can explain the lower values of the apparent binding constant for calcium. The value of binding constant that we obtained, 2.9×10^8 M^{-1}, is close to the value $K_a = 5 \times 10^7$ M^{-1} obtained from a direct titration isotherm at 5°C [18].

The decrease of the binding constant of calcium with increasing calcium concentration (see Table 1) might be caused by an increasing nonspecific influence of the ionic strength resulting in decreased electrostatic interactions. The presence of excess free calcium is also responsible for the stabilization of holo-LA at high concentration of calcium. This effect seems to be entropic resulting from the entropy of mixing of the calcium released upon LA denaturation with the calcium of the bulk solution. The positive entropy of mixing decreases with increasing concentrations of Ca^{2+} in the bulk solution, thus increasing the transition temperature. This situation resembles that of DNA, the stability of which depends on the ionic strength and this dependence is entropic in nature caused by entropy of mixing of the released counter ions [19,20]. The suggested ef-

Table 2
Thermodynamic characteristics of LA unfolding in 10 mM Tris (pH 8.0) in the presence of various amount of EDTA[a]

R_{EDTA}	T_m^{apo} (°C)	Θ^{apo} (kJ mol^{-1})	T_m^{holo} (°C)	Θ^{holo} (kJ mol^{-1})	ΔH_{cal} (kJ mol^{-1})	ΔH_{tot} (kJ mol^{-1})	K_a (M^{-1})
			63.1	270.5	264.0	270.5	
0.5			64.3	264.5	254.0	264.5	3.3×10^7
1.0			63.8	263.7	260.0	263.7	
1.2			62.3	255.5	253.0	255.5	8.0×10^8
3.2	26.4	153.4	62.7	260.9	222.0	231.9	2.9×10^8
4.9	27.8	138.2	61.9	256.5	202.0	203.3	3.4×10^8
5.6	23.3	88.8	63.3	263.1	192	202.1	2.3×10^8
6.3	26.7	100.8			81.0	100.8	
7.4	33.5	112.4			95.0	112.4	
9.0	30.6	134.0			117.0	134.0	
12.4	22.3	126.6			118.0	126.6	

[a] R_{EDTA} is the ratio of the concentration of EDTA to protein; T_m^{apo} and T_m^{holo} are temperatures of the heat absorption maximum for the holo- and apo- forms; Θ^{apo} and Θ^{holo} are the corresponding heat effects; and K_a is the association constant for Ca^{2+}.

fect of excess free calcium on polyelectrolyte properties and the stability of LA, does not exclude the possibility of its direct interaction with the protein at the additional low-affinity sites demonstrated earlier [21,22].

In order to demonstrate the influence of the calcium-binding constant on the character of LA denaturation, Fig. 4b presents the results of the LA heat capacity function simulation for different calcium binding constants. It shows that the denaturation of LA occurs in two different temperature ranges only if the binding constant is above 10^8 M^{-1}.

4. Materials and methods

Bovine α-lactalbumin was obtained from Sigma Chemical Co. Purity of the protein was monitored by PAGE under native and denatured conditions. The concentration of protein solutions was measured spectrophotometrically using an extinction coefficient of $E^{1\%}_{280\ nm} = 20.9$ [23] with correction for light-scattering effects. Ethylenediaminetetraacetic acid (EDTA) and calcium chloride were obtained from Sigma.

Calorimetric experiments were performed with a Nano-DSC differential scanning calorimeter by Calorimetric Sciences Corporation [24] at scanning rate 1 K min^{-1} and excess pressure 1.5 atm. The molar heat capacity was calculated for bovine α-lactalbumin according to [25] using a mol wt. 14 300 Da and a partial specific volume $V = 0.709$ cm^3 g^{-1}. The concentration of protein used in the calorimetric experiments was 1.5–2.5 mg ml^{-1}. The temperature dependencies of the heat capacity of LA in the unfolded state was calculated at different temperatures according to [12].

The concentration of free Ca^{2+} in solution, $[Ca^{2+}]$, was determined by the general equation:

$$[Ca^{2+}] = \frac{[Ca]}{[EDTA] - [Ca]} K^{-1}$$

where $K = 1.98 \times 10^8$ M^{-1} is the Ca^{2+} binding constant to EDTA [26], [Ca] is the total concentration of calcium in solution and [EDTA] is the concentration of EDTA in solution. Since the used concentrations of EDTA were two orders of magnitude higher than that of LA, the presence of the protein does not affect the concentration of the free calcium much.

The simulated heat capacity function was generated as suggested by Brandts and Lin [14] and was fitted to the calorimetrically determined function by a non-linear regression analysis program from Philip Sherrod.

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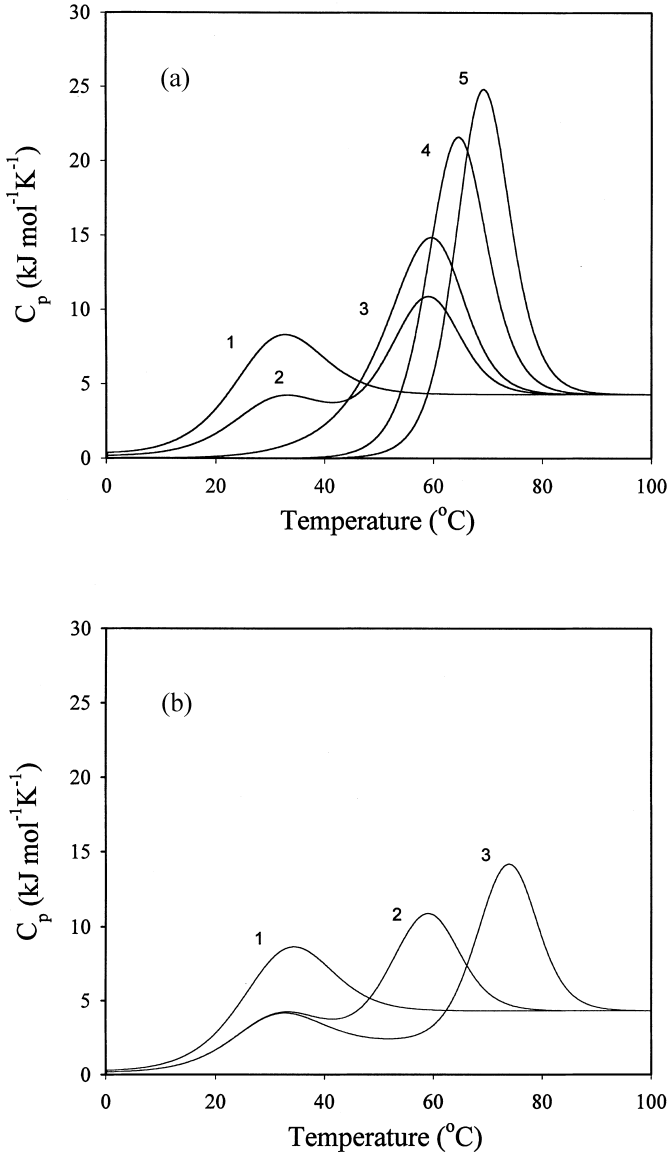

Fig. 4. Simulation of the heat capacity profiles of LA in the presence of different concentrations of ligand (panel a) and at different values of the Ca^{2+} binding constant (panel b). Parameters used for simulation are: concentration of protein = 1×10^{-4} M; $K_a = 1 \times 10^7$ M^{-1}; the temperature of denaturation of apo-LA = 300 K; the enthalpy of denaturation of apo-LA (ΔH_d) = 118 kJ mol^{-1}; the heat capacity increment of denaturation ($\Delta C_{p,d}$) = 4.3 kJ mol^{-1} K^{-1}; the enthalpy of calcium binding (ΔH_a) = 0 kJ mol^{-1}; the heat capacity effect of calcium binding ($\Delta C_{p,a}$) ~ 0 kJ $K^{-1}mol^{-1}$; and the number of binding sites $N = 1$. In panel a, the total concentration of ligand is: (1) 1×10^{-8} M; (2) 5×10^{-5} M; (3) 1×10^{-4} M; (4) 5×10^{-4} M; and (5) 2×10^{-3} M. In panel b, the values of association constant are: (1) 1×10^4 M^{-1}; (2) 1×10^7 M^{-1}; (3) 1×10^8 M^{-1}; and the total concentration of ligand is 5×10^{-5} M.

lactalbumin for calorimetric measurements. This study was supported by National Institute of Health Grant GM 48036.

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