Thermodynamic Characterization of the Partially Unfolded State of Ca^{2+}-Loaded Bovine α-Lactalbumin: Evidence That Partial Unfolding Can Precede Ca^{2+} Release†

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ABSTRACT: The thermal denaturation of bovine α-lactalbumin (BLA) was studied at pH 7.5 and at various Ca^{2+} concentrations using near-UV circular dichroism and differential scanning calorimetry. The Ca^{2+} dependence of the denaturation equilibria proves that, in the transition region, partially unfolded α-lactalbumin consists of a mixture of Ca^{2+}-loaded and Ca^{2+}-free protein. The thermodynamic parameters of the unfolding of these two species were determined at 68 °C and were then compared with one other, with the thermodynamic parameters deduced from calorimetric titration of α-lactalbumin with Ca^{2+}, and with those derived from Ca^{2+} titration of a mutant human lysozyme having an engineered Ca^{2+}-binding site. This comparison indicated that (a) the unfolding curves for Ca^{2+}-BLA deduced from the near-UV ellipticity change are more able to distinguish between unfolding with and without Ca^{2+} release than those deduced from differential scanning calorimetry, (b) the Ca^{2+}-loaded denaturated state of BLA is more folded than the Ca^{2+}-free protein at 68 °C, and (c) a heat-induced unfolding process, consisting of an initial Ca^{2+} release, followed by a conformational relaxation, is unlikely to occur at the experimental pH and in the millimolar region of Ca^{2+} concentrations, due to the large free energy requirement of the initial step. A more probable mechanism would be unfolding via a Ca^{2+}-loaded intermediately unfolded state, with subsequent Ca^{2+} release.

Thermodynamic parameters related to protein folding−unfolding equilibria have received considerable interest with regard to the prediction of the stability of engineered proteins. Much attention has been given to conformational changes in α-lactalbumins (Hayne & Freire, 1993; Murphy & Freire, 1992; Kuwajima, 1989) since the equilibrium conditions between the compact native state, the intermediate folded states, and the unfolded random state are easily obtained. At neutral pH and moderate temperatures, the protein exhibits a multiple-step thermal denaturation: in the first stage, the native conformation is converted into the so-called "molten globule" state, which is still compact but sufficiently loose to allow access to its hydrophobic core and to confer high mobility on the aromatic residues of the core (Dolgikh et al., 1985; Segawa & Sugai, 1983). When the temperature is increased further, this intermediate state, with its poorly defined tertiary but native-like secondary structure, progressively unfolds through a series of conformations each with less residual structure (Griko et al., 1994; Vanderheeren & Hanssens, 1994).

α-Lactalbumins have also received attention because of their high-affinity Ca^{2+}-binding site (Hiraoka et al., 1980), which has been identified as a short loop structure, consisting of ten residues, five of which contribute liganding atoms (Stuart et al., 1986; Acharya et al., 1991). The physicochemical aspects of the metal binding to the protein have been thoroughly investigated. Ca^{2+} binding stabilizes the protein in its compact native state, and current dogma is that partial unfolding is always accompanied by loss of Ca^{2+} (Mc Kenzie & White, 1991). Recently, we reported that, at neutral pH and moderate Ca^{2+} concentrations (2 mM), some metal ion remains associated with bovine α-lactalbumin (BLA) after thermal denaturation of its native structure (Vanderheeren & Hanssens, 1994). At 80 °C, the thermally denaturated Ca^{2+}-loaded BLA has more secondary structure than does apo-BLA. Moreover, at this temperature, the molten globule character of Ca^{2+}-loaded BLA is conserved in such a way that the protein retains an accessible hydrophobic core, while the hydrophobic core of the apoprotein is largely lost.

To obtain a clearer understanding of protein denaturation in the presence of Ca^{2+}, the thermodynamic parameters of these changes were analyzed near the transition region. The results indicate that, at 68 °C and neutral pH, the unfolding of native Ca^{2+}-BLA occurs via an intermediately unfolded Ca^{2+}-loaded state, which then releases its Ca^{2+}, rather than by direct release of Ca^{2+} from the native protein, followed by its subsequent unfolding.

EXPERIMENTAL PROCEDURES

Materials. BLA was purchased from Sigma and decalcified by applying a sample in 10 mM EDTA and 10 mM

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$(NH_4)_2CO_3$, pH 8.5, to a Sephacryl HR-100 column. It was eluted with 5 mM $(NH_4)_2CO_3$, pH 8.5. The protein fraction was checked for its Ca^{2+} and Na^+ contents by atomic absorption spectrometry; 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. The protein concentrations of BLA solutions were determined from their absorbance at 280 nm, as well as from the equivalence point on Ca^{2+} titration (Desmet & Van Cauwelaert, 1988). The mutant human lysozyme, M4, in which Ala 83, Glu 86, Asn 88, and Ala 92 are replaced by Lys, Asp, Asp, and Asp, respectively, contains the entire Ca^{2+}-binding site of BLA; its preparation and characteristics have been described previously (Haezebrouck et al., 1993). All experiments were performed in 10 mM Tris-HCl buffer (pH 7.5), containing an appropriate amount of the required metal ions, or 2 mM EDTA when working in Ca^{2+}-free conditions. Na^+ ions were excluded from the buffer solutions, since they bind competitively to the specific Ca^{2+} site with a binding constant of 200 M^{-1} (Desmet et al., 1987). Ca^{2+} and Mn^{2+} concentrations in the stock and working solutions were determined using a Perkin-Elmer 3300 atomic absorption spectrometer.

Isothermal Titration Calorimetry. Microcalorimetric titration measurements were carried out in a MicroCal MCS isothermal titration calorimeter. In a typical experiment, either 1.3 mL of 20 μM α-lactalbumin was titrated in 20 steps with 100 μL of 0.35 mM $CaCl_2$ or a 2.67 mM solution of $MnCl_2$ was used for titration of a 108 μM solution of BLA. For a typical experiment with Ca^{2+} in the presence of Mn^{2+}, a 102 μM solution of BLA in 7.59 mM $MnCl_2$ was titrated with 2.5 mM $CaCl_2$ solution. The values of the binding constants and molar enthalpy changes were determined from the titration curves after subtraction of a baseline measured in the absence of protein. The reported values were obtained by averaging the results of at least five measurements. The software for automatic data collection and analysis was supplied by the manufacturer.

Circular Dichroism. The thermal transitions for BLA in the absence or presence of various Ca^{2+} concentrations were determined from the mean residue ellipticity (deg cm^2 $dmol^{-1}$) at 270 nm. All circular dichroism measurements were carried out in a Jasco J-600 spectropolarimeter, using 10 mm path-length cuvettes. The BLA concentration was 22.8 μM. According to the desired composition, a concentrated BLA solution was diluted with buffer and with a Ca^{2+} stock solution. Each measurement was started at least 10 min after temperature equilibration of the sample, controlled by means of an immersed, calibrated thermocouple. Evidence that equilibrium states were obtained is given by the fact that the ellipticity values were identical on heating and cooling the samples, provided they had not been exposed to more than 80 °C for several minutes.

Differential Scanning Calorimetry. Calorimetric scans were carried out on a MicroCal MCS differential scanning calorimeter, using software supplied by the manufacturer for automatic data collection and analysis. The samples were degassed for 15 min at room temperature prior to scanning at rates of 60 °C/h. The protein concentration used was 215 μM in a buffer solution containing an excess of Ca^{2+} over protein, ranging from 260 μM to 1.4 mM. Excess heat capacity functions were determined after subtraction of a baseline, calculated from the progress of the reaction. At scanning rates of 12 °C/h, the maximal heat absorptions were at identical temperatures as at 60 °C/h, but, due to the low rate of heat supply, the values for the excess heat capacities were inaccurate at the slower scanning rate.

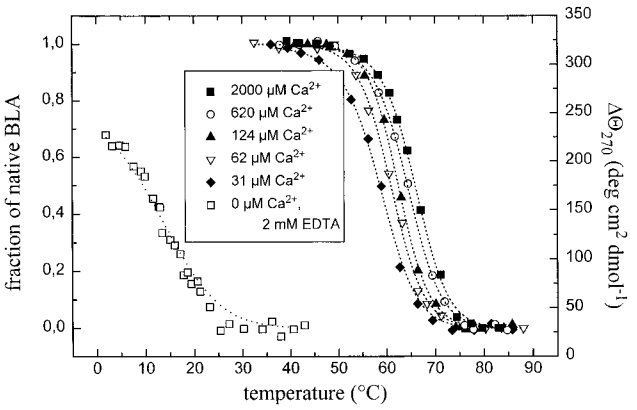
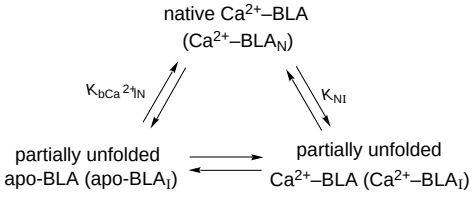


FIGURE 1: Thermal transition curves for BLA measured by the ellipticity change at 270 nm (right ordinate) and transformed to the fractions of BLA in the native form (left ordinate). Conditions: 22.8 μM BLA in 10 mM Tris-HCl, pH 7.5, at the Ca^{2+} concentrations indicated. The dashed lines through the points represent the curves from which the equilibrium constants were obtained.

RESULTS

Recently we reported that, in 10 mM Tris-HCl at pH 7.5 and 2 mM Ca^{2+}, some strongly bound Ca^{2+} ions remain associated with BLA after thermal unfolding of the protein's native structure. At 80 °C, an intermediately unfolded state of Ca^{2+}-loaded BLA is obtained. Although this intermediate shows no near-UV ellipticity, it still retains more far-UV ellipticity than is seen in apo-BLA at this temperature. Furthermore, it interacts better with hydrophobic probes than do either the native protein or the protein in a more unfolded state (Vanderheeren & Hanssens, 1994). These properties are characteristics of a molten globule state. Further characterization of this partially unfolded Ca^{2+}-BLA requires determination of the thermodynamic relations (enthalpy, entropy, and heat capacity changes) in the three steps in the following cycle:



These relations were compared at 68 °C, the temperature at which, at neutral pH and with a large excess of Ca^{2+}, the equilibrium between native Ca^{2+}-BLA and partially unfolded Ca^{2+}-BLA is close to unity.

Thermodynamic Relationhips between Partially Unfolded apo-BLA and Native Ca^{2+}-BLA. Figure 1 shows the temperature dependence of the mean residue ellipticity at 270 nm of BLA in 10 mM Tris-HCl (pH 7.5) in the presence of varying amounts of Ca^{2+}. The decrease in ellipticity value in the various curves is related to the thermal unfolding of the native tertiary structure (Dolgikh et al., 1981; Segawa & Sugai, 1983; Kuwajima et al., 1985). The fraction of BLA in the native form (left ordinate) was calculated from the ellipticity change (right ordinate). Almost all the apo-BLA (in 2 mM EDTA) had lost its native conformation by 30

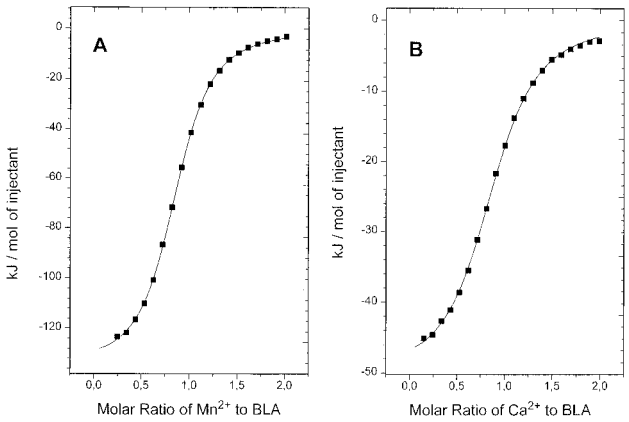


FIGURE 2: Enthalpy exchange on titration of (A) 1.3 mL of 108 μM apo-BLA with 5 μL injections of 2.67 mM Mn^{2+} and (B) 1.3 mL of 102 μM BLA in 7.59 mM Mn^{2+} with 5 μL injections of 2.51 mM Ca^{2+}. The other solvent conditions are 10 mM Tris-HCl, pH 7.5, and 30.65 °C.

°C, while Ca^{2+}-loaded BLA only unfolded above 45 °C. Thus, the thermodynamic properties for Ca^{2+} binding between 30 and 45 °C include a change from the intermediately unfolded state of apo-BLA (apo-BLA_I) to the native state of Ca^{2+}-BLA (Ca^{2+}-BLA_N). In addition, an extrapolation of the thermodynamic properties from this temperature region to higher temperatures has to include the effect of this conformational change on Ca^{2+} binding. In order to emphasize the fact that the thermodynamic values for the binding of Ca^{2+} to apo-BLA_I include the transition to Ca^{2+}-BLA_N, the subscript IN (intermediate−native state) will be used; it will also be added to the related Ca^{2+}-binding constant:

$$K_{bCa^{2+}IN} = [Ca^{2+}\text{-}BLA_N]/[\text{apo-}BLA_I][Ca^{2+}] \quad (1)$$

In order to determine the enthalpy and heat capacity change of the binding of Ca^{2+} to apo-BLA ($\Delta H_{bCa^{2+}IN}$ and $\Delta C_{p\ bCa^{2+}IN}$, respectively), isothermal titration calorimetry measurements were performed at different temperatures. Values of −181.9 and −226.3 kJ/mol were obtained at 30.65 and 41.79 °C, respectively, from which a molar heat capacity change of −3.99 kJ/(mol·K) was calculated. (It is taken into account that 4% of apo-BLA is in the native state at 30.65 °C.)

At 30 °C, the affinity of Ca^{2+} for BLA is too strong to permit the determination of its binding constant and the Gibbs free energy in a direct titration experiment. Other metal ions, such as Mn^{2+}, can bind competitively to the same high-affinity Ca^{2+} site (Desmet & Van Cauwelaert, 1988; Desmet et al., 1991a), and the Ca^{2+}-binding constant can thus be obtained from competitive binding experiments between Ca^{2+} and Mn^{2+} (Bryant & Andrews, 1984; Desmet & Van Cauwelaert, 1988). From calorimetric titration of apo-BLA with Mn^{2+} at 30.65 °C (Figure 2A), a binding constant ($K_{bMn^{2+}IN}$) of 2.0×10^5 M^{-1} was calculated. In 7.59 mM Mn^{2+} the apparent binding constant for Ca^{2+} ($K_{bCa^{2+}app}$) was found to be 1.32×10^5 (Figure 2B). From the above data, the direct binding constant for Ca^{2+} is calculated with the equation for competitive binding:

$$(K_{bCa^{2+}app})^{-1} = (K_{bCa^{2+}IN})^{-1} + (K_{bMn^{2+}IN}/K_{bCa^{2+}IN})[Mn^{2+}] \quad (2)$$

This gave a value of 2.07×10^8 M^{-1} for $K_{bCa^{2+}IN}$, in agreement with the value obtained by others (Permyakov et al., 1981, 1987; Segawa & Sugai, 1983). The corresponding $\Delta G_{bCa^{2+}IN}$ at 30.65 °C was −42.6 kJ/mol, and subtraction of $\Delta H_{bCa^{2+}IN}$ from $\Delta G_{bCa^{2+}IN}$ gave a value of −139.3 kJ/mol for $T\Delta S_{bCa^{2+}IN}$. The thermodynamic parameters at 30.65 °C, for Ca^{2+} binding to intermediately unfolded apo-BLA, were converted to 68 °C using the $\Delta C_{p\ bCa^{2+}IN}$ value and are presented in Table 1 (row 2).

Unfolding of Ca^{2+}-BLA at 68 °C. The unfolding of BLA in the presence of different Ca^{2+} concentrations was studied by circular dichroism and by differential scanning calorimetry.

(A) Circular Dichroism. From the mean residue ellipticity change at 270 nm (Figure 1), the fraction of protein in the partially unfolded state (α_I) or in the native tertiary structure (α_N) was calculated. The ratio of these fractions also represents the ratio of the total concentration of BLA in the unfolded state to the total concentration of native protein. At a given temperature, it might be expected that this ratio would represent the unfolding constant of BLA. It is obvious that the relationship α_I/α_N is Ca^{2+} dependent, and thus, in the presence of Ca^{2+}, this ratio only represents an apparent unfolding constant (K_{NIapp}):

$$K_{NIapp} = \alpha_I/\alpha_N = ([\text{apo-}BLA_I] + [Ca^{2+}\text{-}BLA_I])/([\text{apo-}BLA_N] + [Ca^{2+}\text{-}BLA_N]) \quad (3)$$

The Ca^{2+} dependence of K_{NIapp} provides information on the unfolding of Ca^{2+}-BLA. Since the native tertiary structure of apo-BLA is lost above 30 °C, its concentration ([apo-BLA_N]) at 68 °C is negligible. If, at the same temperature, the concentration of Ca^{2+}-BLA in the intermediately unfolded state ([Ca^{2+}-BLA_I]) can be considered negligible as compared with that of [apo-BLA_I], then the apparent unfolding constant is related to the Ca^{2+}-binding constant ($K_{bCa^{2+}IN}$) as follows:

$$K_{NIapp} = (K_{bCa^{2+}IN}[Ca^{2+}])^{-1} \quad (4)$$

Figure 3A shows the values for K_{NIapp}, obtained as described above, as a function of the reciprocal of the free Ca^{2+} concentration at various temperatures. If eq 4 is valid, the intercept on the ordinate of these graphs should be zero, but this is clearly not the case. Consequently, an equilibrium between native and intermediately unfolded Ca^{2+}-BLA must also be taken into account:

$$K_{NIapp} = ([Ca^{2+}\text{-}BLA_I] + [\text{apo-}BLA_I])/[Ca^{2+}\text{-}BLA_N] = K_{NI} + (1/K_{bCa^{2+}IN}[Ca^{2+}]) \quad (5)$$

Values for K_{NI}, the unfolding constant for Ca^{2+}-loaded BLA, and $K_{bCa^{2+}IN}$, were obtained at several temperatures from the ordinate intercepts and the slope of the lines in Figure 3A; these are presented in Table 2. The natural logarithms of the equilibrium constants as a function of temperature were fitted to the equation:

$$\ln K = -(\Delta H_{Tr}/RT) + (\Delta S_{Tr}/R) - (\Delta C_p/RT)(T - T_r - T\ln(T/T_r)) \quad (6)$$

where the reference temperature, T_r, was fixed at 68 °C (341 K). Because floating of three adjustable parameters, ΔH_{T_r}, ΔS_{T_r}, and ΔC_p gives various combinations that fit to the experimental results and since there is an inherent assumption

Table 1: Summary of the Various Thermodynamic Parameters Obtained from Direct Analyses of the Experiments[a]

method	transition	T (°C)	ΔH (kJ mol^{-1})	$T\Delta S$ (kJ mol^{-1})	ΔG (kJ mol^{-1})	K (M^{-1})
titration calorimetry	apo-BLA$_I$ + Ca^{2+} → Ca^{2+}-BLA$_N$	30.65	−181.9 ± 6	−139.3 ± 6	−42.6 ± 1	(2.1 ± 1)10^8
		68	−330.9 ± 11	−314.3 ± 8	−16.6 ± 5	(0.4 ± 2)10^3
CD	apo-BLA$_I$ + Ca^{2+} → Ca^{2+}-BLA$_N$	68	−332.5 ± 4	−310.0 ± 4	−22.5 ± 0.1	(2.8 ± 0.1)10^3
	Ca^{2+}-BLA$_N$ → Ca^{2+}-BLA$_I$	68	281.6 ± 3	283.8 ± 3	−2.2 ± 0.3	2.2 ± 0.2
DSC	apo-BLA$_I$ + Ca^{2+} → Ca^{2+}-BLA$_N$	68	−355.1 ± 2	−333.9 ± 2	−21.2 ± 0.1	(1.8 ± 0.1)10^3
	Ca^{2+}-BLA$_N$ → Ca^{2+}-BLA$_I$	68	339.7 ± 4	338.1 ± 4	1.6 ± 0.3	0.57 ± 0.05
titration calorimetry	apo-M4$_N$ + Ca^{2+} → Ca^{2+}-M4$_N$	25	−28.7 ± 1	11.0 ± 1	−39.7 ± 0.3	(9.0 ± 1)10^{6a}
		68	−30.1 ± 1	11.1 ± 0.3	−41.2 ± 0.3	(2.1 ± 0.2)10^6

[a]Data from Haezebrouck et al. (1993).

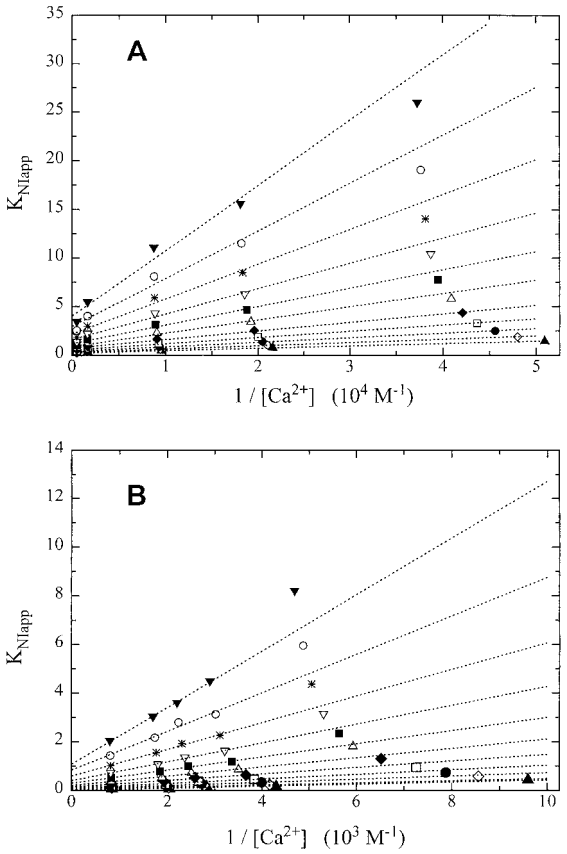


FIGURE 3: Apparent unfolding constants of Ca^{2+}-loaded BLA as a function of the reciprocal of the Ca^{2+} concentration, calculated from (A) the ellipticity change at 270 nm and (B) the excess heat capacity. Solvent conditions: 10 mM Tris-HCl, pH 7.5, and different Ca^{2+} concentrations. Lines through the data present the optimal fits according to eq 5. Temperatures vary from 60 °C (data of lowest lines) to 70 °C (data of highest lines).

in eq 6 that ΔC_p is independent of temperature, the latter value was fixed at an experimental value.

In this way, the mean value for $\Delta C_{p\ bCa^{2+}IN}$ [−3.99 kJ/(mol·K)], determined by titration calorimetry at 30.65 and 41.79 °C, was used to calculate the fit between ln $K_{bCa^{2+}IN}$ and temperature. The related values for $\Delta H_{bCa^{2+}IN}$ and $\Delta S_{bCa^{2+}IN}$ at 68 °C are shown in row 3 of Table 1.

To estimate the heat capacity change for the unfolding of Ca^{2+}-BLA without loss of the metal ion ($\Delta C_{p\ NI}$), we rely on the observation that, in α-lactalbumin as well as in Ca^{2+}-binding lysozymes, the global process of Ca^{2+} binding plus conformational change can be subdivided. The binding contribution is equal for both proteins, but the contribution of the conformational change is dependent on the nature of the protein (Desmet et al., 1989, 1991a,b). Similar conclusions were made by Kuroki et al. (1989), who created a mutant human lysozyme with an engineered Ca^{2+}-binding

Table 2: Equilibrium Constants Obtained from the Thermal Transition Curves for Ca^{2+}-BLA, Measured by Near-UV CD

T (°C)	K_{NI}	$K_{bCa^{2+}IN}$ (M^{-1})	T (°C)	K_{NI}	$K_{bCa^{2+}IN}$ (M^{-1})
60	0.226	41 391	66	1.191	5 277
61	0.301	29 886	67	1.604	3 827
62	0.402	21 640	68	2.170	2 784
63	0.541	15 713	69	2.949	2 035
64	0.729	11 432	70	4.017	1 489
65	0.889	7 353			

site by replacing Glu 86 and Ala 92 with Asp residues. Using X-ray crystallography, they demonstrated that the coordination pattern around the Ca^{2+} ion was quite similar to that seen in baboon α-lactalbumin (Inaka et al., 1991). The X-ray patterns also showed that the overall tertiary structure of the mutant did not change when Ca^{2+} was bound. The thermodynamic parameters obtained for Ca^{2+} binding, therefore, do not contain a contribution for conformational changes (Kuroki et al., 1992a). By replacing four residues of the Ca^{2+}-binding loop, rather than two, the whole Ca^{2+} site of α-lactalbumin has been inserted into the M4 mutant of human lysozyme. The near- and far-UV CD spectra indicate that this mutant likewise binds Ca^{2+} without any conformational change (Haezebrouck et al., 1993). We have therefore determined the enthalpy change for the binding of Ca^{2+} to the M4 mutant of human lysozyme at different temperatures. The binding enthalpy varied from −28.69 kJ/mol at 25 °C to −28.82 kJ/mol at 35 °C. The derived molar heat capacity change of −0.032 kJ/(mol·K) represents the contribution of Ca^{2+} binding to $\Delta C_{p\ bCa^{2+}IN}$. Since the total heat capacity change for Ca^{2+} binding and conformational change from I to N is −3.99 kJ/(mol·K), the heat capacity change for the unfolding of Ca^{2+}-BLA without loss of Ca^{2+} ($\Delta C_{p\ NI}$) is 3.96 kJ/(mol·K). Upon introduction of this $\Delta C_{p\ NI}$ value, the best fit (according to eq 6) for ln K_{NI} as a function of temperature gave the values of ΔH_{NI} and ΔS_{NI} at 68 °C shown in Table 1 (row 4).

In a further step, the temperature dependence of the different populations of BLA molecules was calculated from the obtained thermodynamic parameters for 215 μM BLA in the presence of either 260 μM or 1.4 mM Ca^{2+}. These results, plotted in Figure 4A, show that, at the higher Ca^{2+} concentration, the metal ion remained mainly protein bound on unfolding. Above 75 °C, the fraction of intermediately unfolded Ca^{2+}-BLA decreased, with an increase in the proportion of the unfolded apo-BLA. From the calculated fractions of partially unfolded apo- and Ca^{2+}-BLA at the temperature at which the native state no longer exists and from the appropriate enthalpy changes at 68 °C (Table 1, rows 3 and 4), apparent enthalpy changes of +315 and +292 kJ/mol were estimated for the unfolding of native Ca^{2+}-BLA in 260 μM and 1.4 mM Ca^{2+}, respectively.

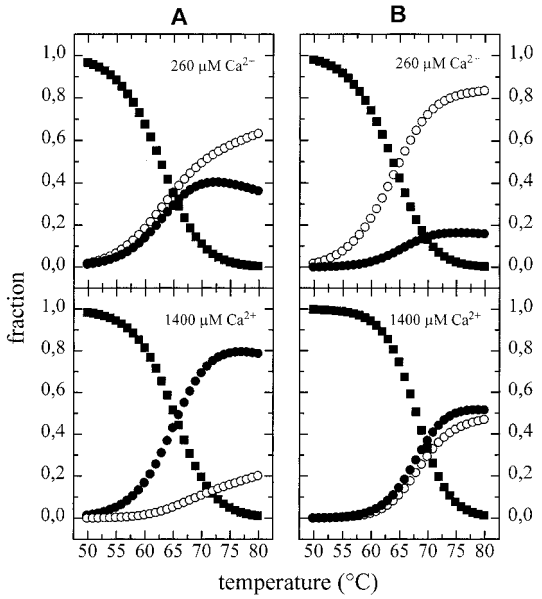


FIGURE 4: Fractions of intermediately unfolded apo-BLA (○), native Ca^{2+}-BLA (■), and intermediately unfolded Ca^{2+}-BLA (●) as a function of temperature, calculated from (A) the ellipticity change at 270 nm and (B) the excess heat capacity, respectively. Conditions: 215 μM BLA in 10 mM Tris-HCl, pH 7.5, and 260 μM or 1.4 mM Ca^{2+}.

(B) Differential Scanning Calorimetry. As with the CD unfolding curves, the curves in Figure 5, showing the excess heat capacity as a function of temperature, are shifted toward higher temperatures in the presence of a larger excess of Ca^{2+} over protein. The molar enthalpy changes at the transition temperature were determined by integrating the area under the peak for each Ca^{2+} concentration and are shown in Table 4. These were used to calculate the enthalpies at 68 °C, using $\Delta C_{p\ \mathrm{NI}}$ equal to a value of +3.96 kJ/(mol·K). From the analysis of the CD unfolding curves, it was expected that the enthalpy values would decrease from +315 kJ/mol at the lower Ca^{2+} concentration to +292 kJ/mol at the higher. The actual values obtained from direct calorimetric measurements (Table 4) were not sufficiently accurate to clearly see the expected trend, but their mean magnitude was within the expected range.

As with the CD results, we tried to determine the ratio α_I/α_N from the heat capacity scans at different temperatures and different Ca^{2+} concentrations. To do so, the peak surface integrated below a given temperature was divided by the one above that temperature and defined as an apparent unfolding constant (K_{NIapp}). Figure 3B represents the plots of those K_{NIapp} values as a function of the reciprocal of the free Ca^{2+} concentration. Again, the intercepts on the ordinate were not zero, due to an equilibrium between native and intermediately unfolded Ca^{2+}-BLA. The values for K_{NI}, derived from the intercepts on the ordinate, and the values for $K_{\mathrm{bCa^{2+}IN}}$, derived from the slopes, are shown in Table 3 and the related thermodynamic values in Table 1 (rows 5 and 6).

To test the internal coherence of the thermodynamic parameters, these values were used to calculate the temperature dependence of various populations of BLA molecules under the conditions of 215 μM BLA in the presence of 260 μM or 1.4 mM Ca^{2+}; these populations are shown in Figure 4B. From the fractions of intermediately unfolded apo- and Ca^{2+}-BLA in the absence of the native state and from the appropriate enthalpy changes at 68 °C (Table 1, rows 5 and 6), the apparent molar transition enthalpy was calculated as 352 kJ/mol at 260 μM Ca^{2+} and 347 kJ/mol at 1.4 mM Ca^{2+}. These deduced enthalpy changes were greater than the calorimetric values obtained by integrating the excess heat capacity as a function of temperature (Table 4). This discrepancy will be discussed in the next section.

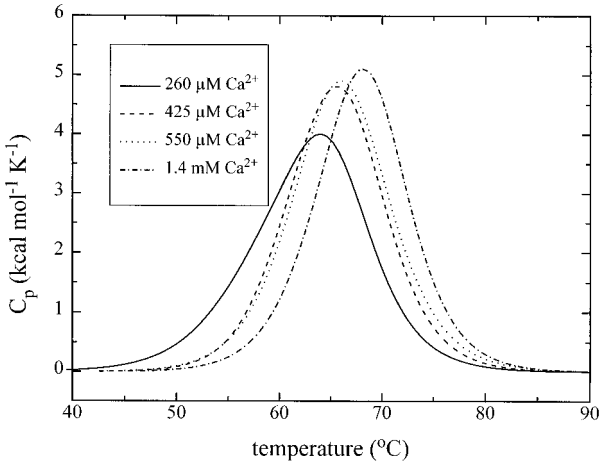


FIGURE 5: Excess heat capacity curves for 215 μM BLA in 10 mM Tris-HCl, pH 7.5, and excess Ca^{2+}.

Table 3: Equilibrium Constants Obtained from the Excess Heat Capacity Curves on Thermal Denaturation of Ca^{2+}-BLA

T (°C)	K_{NI}	$K_{\mathrm{bCa^{2+}IN}}$ (M^{-1})	T (°C)	K_{NI}	$K_{\mathrm{bCa^{2+}IN}}$ (M^{-1})
60	0.035	33 014	66	0.299	3 705
61	0.053	22 889	67	0.403	2 585
62	0.071	15 228	68	0.590	1 824
63	0.103	10 684	69	0.837	1 261
64	0.147	7 502	70	1.077	860
65	0.210	5 266			

Table 4: Calorimetric Enthalpy Values Derived from the Excess Heat Capacity Curves on Thermal Unfolding of BLA at Different Ca^{2+} Concentrations

$[Ca^{2+}]_t$ (μM)	T_m (°C)	ΔH_{T_m} (kJ mol^{-1})	$\Delta H_{68°C}$ (kJ mol^{-1})
260	64.2	301.7	316.8
425	65.5	314.7	324.6
550	66.0	328.9	336.9
700	66.8	290.2	295.0
1400	68.2	316.6	315.8

Relationship between Intermediately Unfolded and Native apo-BLA. The van't Hoff analysis of the equilibrium constants, derived from the thermal transition curves for Ca^{2+}-BLA and measured by the ellipticity change at 270 nm, provides thermodynamic values for the unfolding processes which are at least as reliable as those derived from the excess heat capacity. This encouraged us to analyze the temperature-dependent ellipticity changes of apo-BLA (Figure 1, open squares), in this way. Since, in the absence of Ca^{2+}, only Ca^{2+}-free native and intermediately unfolded BLA need to be considered, the temperature dependence only results from the temperature-dependent unfolding equilibrium constant between native and intermediately unfolded apo-BLA (K_{NIapoBLA}). Thus the temperature-dependent fractions of native apo-BLA, $\alpha_{N(T)}$, can be fitted to the combined expressions (6) and (7).

$$\alpha_{N(T)} = 1/(1 + K_{\mathrm{NIapoBLA}}) \qquad (7)$$

As previously described, the number of variables can be reduced by estimating $\Delta C_{p\ \mathrm{NI}}$ as 3.96 kJ/(mol·K). The values

obtained for $\Delta H_{\mathrm{NIapoBLA}}$ and $T\Delta S_{\mathrm{NIapoBLA}}$ at 30.65 °C are then +159.5 and +168.0 kJ/mol, respectively.

The difference between the enthalpy change for the reaction "apo-BLA_I + Ca^{2+} ⇔ $CaBLA_N$" (Table 1, row 1) and the enthalpy change for "apo-BLA_I ⇔ apo-BLA_N" (reverse of $\Delta H_{\mathrm{NIapoBLA}}$) is −22.3 kJ/mol. This value is consistent with the above mentioned enthalpy change observed for Ca^{2+} binding to the M4 mutant of human lysozyme (−28.69 kJ/mol at 25 °C and −28.82 kJ/mol at 35 °C). It also agrees well with earlier observations that Ca^{2+} binding to the mutant protein is accounted by Ca^{2+} binding to the binding loop of BLA, without any contribution from protein conformational changes (Haezebrouck et al., 1993). It was of interest to determine the other thermodynamic parameters for Ca^{2+} binding to the M4 mutant of human lysozyme at 25 °C and to convert them to 68 °C; these are shown in Table 1 (rows 7 and 8).

DISCUSSION

The thermal transition curves derived from the ellipticity change at 270 nm (Figure 1) and from differential scanning calorimetry (Figure 5) show that, at a moderate Ca^{2+} concentration (2 mM), a large proportion of BLA remains Ca^{2+} loaded after the thermally induced destabilization of its tertiary structure. In thermal unfolding studies at Ca^{2+} concentrations of 20−50 μM (Kuwajima et al.,1985; Van Dael et al., 1992) and in a study of guanidine hydrochloride-induced unfolding at millimolar Ca^{2+} concentrations (Ikeguchi et al., 1986), no Ca^{2+}-loaded intermediately unfolded state of BLA was detected, and therefore it was generally believed that, in all circumstances, the partial unfolding of α-lactalbumins is accompanied by loss of Ca^{2+}. However, analysis of the present thermal transition curves confirmed our earlier conclusions, based on differences in hydrophobic behavior and differences in the far-UV ellipticity of Ca^{2+}- and apo-BLA at 80 °C, that some Ca^{2+} remains bound to the protein after heat-induced unfolding to the intermediate state (Vanderheeren & Hanssens, 1994). The present analysis also agrees with the observations of Kuroki et al. (1992b), who reported that their Ca^{2+}-binding mutant of human lysozyme retained bound Ca^{2+} in its unfolded state. Furthermore, from Figure 4, it is clear that the fraction of the thermally unfolded intermediate of Ca^{2+}-bound BLA will be small at a Ca^{2+} concentration of 20−50 μM, which is in good agreement with the findings of Kuwajima et al. (1985) and those of Van Dael et al. (1992). The reason why, even at millimolar Ca^{2+}, no Ca^{2+}-loaded, denaturant-induced intermediate of BLA was detected (Ikeguchi et al., 1986) is not investigated, but it is possible that Ca^{2+} binding may be blocked by the binding of denaturant.

The aim of this work was to characterize the thermodynamic parameters for the transition from native Ca^{2+}-loaded BLA to intermediately unfolded Ca^{2+}-loaded BLA (Ca^{2+}-BLA_N → Ca^{2+}-BLA_I) and to compare these with those obtained when unfolding is accompanied by Ca^{2+} release (Ca^{2+}-BLA_N → apo-BLA_I + Ca^{2+}). The thermal transition curves derived from CD and DSC measurements offered the required thermodynamic values at 68 °C (Table 1, rows 3−6). The resultant populations of the various BLA molecules are presented in Figure 4. Their mutual differences indicate that the partial change in ellipticity at 270 nm and in the partial heat absorption does not give an equivalent estimate of the progress of the unfolding transition.

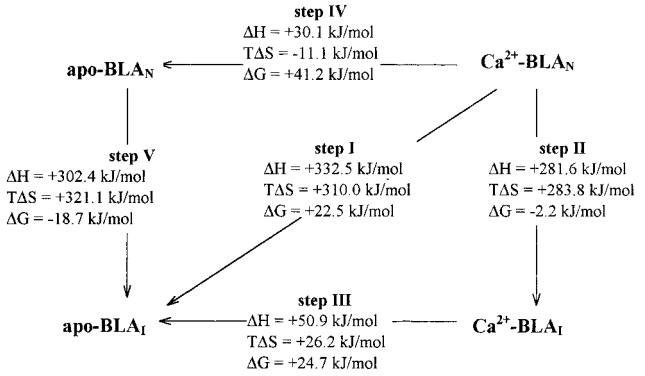


FIGURE 6: Cycle showing the thermodynamic values (ΔH, $T\Delta S$, ΔG) for different transitions of apo-BLA and Ca^{2+}-BLA, determined at 68 °C.

Therefore, the relationship of these physical properties to the unfolding transition will be considered in more detail.

The fraction of intermediately unfolded BLA, determined from the vanishingly small ellipticity at 270 nm, represents the fraction of aromatic groups that become mobile. In the absence of Ca^{2+}, the van't Hoff enthalpy value derived from this ellipticity (Figure 1, open squares) is consistent with the calorimetric value, indicating that the ellipticity change adequately represents the global event of partial unfolding. Similar results have been reported for the partial unfolding of goat apo-α-lactalbumin (Desmet et al., 1991b). In addition, for a correct estimation of the apparent unfolding constants in the presence of Ca^{2+} (eq 5), the Ca^{2+}-free and Ca^{2+}-bound intermediates of BLA must contribute equally to the ellipticity change. As the same minimum value of the near-UV ellipticity is obtained in the presence and in the absence of Ca^{2+}, this is the case.

When deriving the apparent unfolding constants from the excess heat capacity curves, one has to assume that the enthalpy changes for unfolding with and without release of Ca^{2+} are the same. However, the release of Ca^{2+} itself already accounts for a heat absorption of 30 kJ/mol, and unfolding with release of Ca^{2+} results in a more unfolded state than unfolding with retention of Ca^{2+}; the enthalpy value for unfolding with loss of Ca^{2+} is therefore larger than that for unfolding without Ca^{2+} release. In the apparent unfolding constant values, the contribution of unfolding with loss of Ca^{2+} will be overestimated. This effect can be clearly seen upon comparison of Ca^{2+}-BLA_I and apo-BLA_I fractions in Figure 4A,B (CD and DSC experiments, respectively).

Further evidence for the good reliability of the thermodynamic values obtained from the CD transition curves comes from their agreement to the values derived from titration calorimetry (Table 1, rows 2 and 3) and from the excess heat capacity of Ca^{2+}-BLA near 68 °C (Table 4), respectively. For the reasons mentioned, the thermodynamic data obtained from the CD measurements will be used in the further discussion.

Using the collected thermodynamic data at 68 °C (Table 1), the cycle presented in Figure 6 can be constructed. In this scheme, the data for steps I and II, representing the partial unfolding of native Ca^{2+}-BLA with release or retention of Ca^{2+}, respectively, were determined from the thermal unfolding values measured by near-UV CD (Table 1, rows 3 and 4). Step III represents the Ca^{2+} release in the partially unfolded state, its thermodynamic parameters being calculated from the difference between steps I and II. Step IV represents the release of Ca^{2+} from the native Ca^{2+}-BLA,

resulting in the native state of apo-BLA (unstable at 68 °C). As the conformation of BLA does not change during this transition, the magnitude of the thermodynamic parameters was determined by the binding of Ca^{2+} to the mutant M4 of human lysozyme that does not change its conformation (Table 1, row 8). The data for step V, the transition of native apo-BLA to its unfolded state at 68 °C, were obtained from the differences between steps I and IV.

In this cycle, the ΔH and $T\Delta S$ values for step II (+281.6 and + 283.8 kJ/mol), the partial unfolding of Ca^{2+}-BLA, are smaller than the respective values for step V (+302.4 and 321.1 kJ/mol), the partial unfolding of apo-BLA. As the native states have the same conformation, the smaller transition values stem from the fact that intermediately unfolded Ca^{2+}-BLA is less unfolded than is intermediately unfolded apo-BLA. This agrees with our earlier findings (Vanderheeren & Hanssens, 1994) that, at 70 °C, 2 mM Ca^{2+}, and neutral pH, Ca^{2+}-BLA has a resulting hydrophobic pocket while the apoprotein does not.

The thermodynamic parameters, presented in Figure 6, help to understand the unfolding behavior of Ca^{2+}-BLA. Decalcification of α-lactalbumin at room temperature results in a partially denaturated apoprotein, supporting the hypothesis that the unfolding of Ca^{2+}-BLA at higher temperatures results from a weakened Ca^{2+} affinity, such that the first step of the heat-induced unfolding would be Ca^{2+} release. However, the values for the thermodynamic parameters for Ca^{2+} binding to the M4 mutant of human lysozyme make it clear that the free energy and the binding constant of the specific Ca^{2+}-binding loop barely change with temperature (Table 1, row 8). Therefore, at 68 °C and millimolar Ca^{2+} concentrations, unfolding via steps IV and V is unlikely. From the thermodynamic data, it follows that unfolding via steps II and III is more probable. The thermodynamic parameter responsible for the destabilization of Ca^{2+}-BLA in step II is the entropy increase, which is related to expansion of the protein structure without loss of Ca^{2+}. Also the entropy increase in step III is related to further unfolding of the protein structure, as the entropy of the Ca^{2+} release from the specific Ca^{2+} loop (cf. step IV) is negative.

In conclusion, we would like to give some interpretation on the molecular basis of our results. The present observations indicate that Ca^{2+} stabilizes not only the native state but also a partially unfolded one. This latter effect results from the stabilization of secondary structure elements (Vanderheeren & Hanssens, 1994). Since the Ca^{2+}-binding loop of α-lactalbumins (with ligand contributions by Lys 79, Asp 82, Asp 84, Asp 87, and Asp 88) contributes, at the same time, to the amino-terminal side of a 3_{10}-helix (residues 76−82) and to the carboxyl terminus of the α-helix C (residues 86−99) (Acharya et al., 1989), these helical structures are the first structural elements to be stabilized by Ca^{2+}. The ability of Ca^{2+} to stabilize the more compact native state may result from the fact that these secondary structural elements are immobilized through the Ca^{2+}-occupied loop in such a way that optimal interactions with neighboring structures are possible. At high temperatures when several bonds are weakened, these neighboring structures would then dissociate before the release of Ca^{2+}.

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