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Energetics of structural domains in α-lactalbumin

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Abstract

α-Lactalbumin is a small, globular protein that is stabilized by four disulfide bonds and contains two structural domains. One of these domains is rich in α-helix (the α-domain) and has Cys 6-Cys 120 and Cys 28-Cys 111 disulfide bonds. The other domain is rich in β-sheet (the β-domain), has Cys 61-Cys 77 and Cys 73-Cys 91 disulfide bonds, and includes one calcium binding site. To investigate the interaction between domains, we studied derivatives of bovine α-lactalbumin differing in the number of disulfide bonds, using calorimetry and CD at different temperatures and solvent conditions. The three-disulfide form, having a reduced Cys 6-Cys 120 disulfide bond with carboxymethylated cysteines, is similar to intact α-lactalbumin in secondary and tertiary structure as judged by its ellipticity in the near and far UV. The two-disulfide form of α-lactalbumin, having reduced Cys 6-Cys 120 and Cys 28-Cys 111 disulfide bonds with carboxymethylated cysteines, retains about half the secondary and tertiary structure of the intact α-lactalbumin. The remaining structure is able to bind calcium and unfolds cooperatively upon heating, although at lower temperature and with significantly lower enthalpy and entropy. We conclude that, in the two disulfide form, α-lactalbumin retains its calcium-binding β-domain, whereas the α-domain is unfolded. It appears that the β-domain does not require α-domain to fold, but its structure is stabilized significantly by the presence of the adjacent folded α-domain.

Keywords: α-lactalbumin; calorimetry; disulfide bonds; domains; ellipticity

α-lactalbumin (LA) is a small (14.26 kDa), globular protein which, from crystallographic studies of baboon and human LA (Acharya et al., 1989, 1991), consists of two domains divided by a deep cleft (Fig. 1). One of these domains is discontinuous, being formed by the N- and C-terminal parts of the polypeptide chain, and is held together by two disulfide bonds, Cys 6-Cys 120 and Cys 28-Cys 111. This domain is rich in α-helix and is usually designated the α-domain. The β-domain includes two short stretches of β-sheet, a short α-helix, and the calcium-binding site, which is formed by three Asp side chains and two main-chain carbonyls. This continuous domain includes two disulfide bonds: Cys 61-Cys 77 and Cys 73-Cys 91.

At neutral pH and in the presence of calcium, LA unfolds cooperatively at high temperatures, with significant increase of enthalpy and heat capacity (Griko et al., 1994). At lower pH, LA unfolds in two stages. The first stage is highly cooperative and proceeds with significant and sharp heat absorption, but at this stage the heat capacity does not reach the value expected for the fully unfolded polypeptide chain. This value is reached at the second stage, which proceeds without a significant heat absorption peak. It is therefore unclear whether this second stage represents a cooperative process with a small enthalpy, or is a gradual process. Analysis of the change of optical properties associated with these two stages, of their dependence on the presence of calcium, and comparison with the unfolding of the structurally related equine lysozyme, permits the assignment of the first cooperative stage to the unfolding of the β-domain of LA and the second stage to the unfolding of the α-domain (Griko et al., 1994, 1995). The presence of domains that have temperature-dependent unfolding was also verified by fluorescence studies (Vanderheeren & Hanssens, 1994). However, it was unclear as to how independent are these domains, i.e., whether one can fold and be stable without the other. This point needs to be clarified in order to understand the nature of the intermediate state of LA, which is usually regarded as a liquid-like "molten globule" state (Kuwajima, 1977; Ptitsyn, 1992), rather than a partly unfolded state of a two-domain protein with one retained domain.

Peng and Kim (1994) have investigated the state of the isolated α-domain of LA using a single-chain, recombinant model of this domain in which residues 39 and 81 are connected by a short linker of three glycines. They therefore attributed the α-helical segment 81-104 to this domain, in contrast to our dissection of LA's two domains as shown in Figure 1. They found that this α-domain has the same tertiary fold and the same secondary structure as that in intact LA, but lacks extensive, specific side-chain packing.

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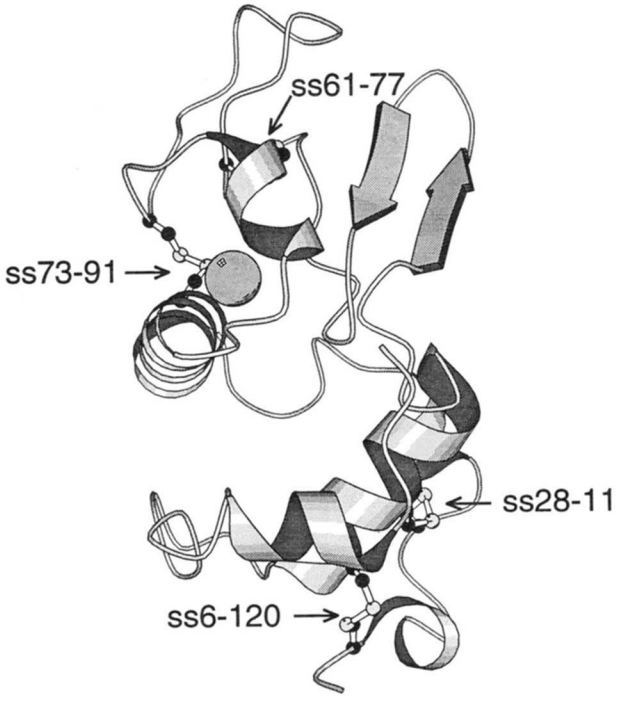

Fig. 1. Representation of the crystal structure of baboon α-lactalbumin (Acharya et al., 1989) generated from coordinates deposited in the Brookhaven Protein Data Bank (1ALC) using the program MOLSCRIPT (Kraulis, 1991). Calcium is represented as a solid sphere and the four disulfide bonds are highlighted.

In this paper, we present the results of optical and thermodynamic studies of derivatives of bovine LA having no, one, two, or four reduced disulfide bonds. We assumed that this bovine protein does not differ significantly from baboon and human LA, which are highly homologous in sequence and have been studied crystallographically. Using CD and scanning microcalorimetry, we show that α-domain is unstable without its two disulfide bonds. However, unfolding of the α-domain does not lead to the unfolding of the β-domain, which still folds and unfolds in a cooperative manner and binds calcium specifically, although it is destabilized significantly in the absence of the α-domain.

Results

The three-disulfide derivative of bovine LA (3SS-LA) has the Cys 6 and Cys 120 residues reduced and blocked by carboxymethylation to prevent disulfide bond formation. In the two-disulfide derivative (2SS-LA), the Cys 6, Cys 120, Cys 28, and Cys 111 disulfide bonds are reduced and blocked. All four cysteines are reduced and blocked in R-LA (see the Materials and methods). The intact native protein is designated N-LA.

CD spectroscopy was used for structural characterization of N-LA and its derivatives and Figure 2 shows near- and far-UV CD spectra at low temperature (2 °C). It should be noted that these CD spectra of N-LA and its disulfide derivatives are very similar to those reported by Ewbank and Creighton (1993b).

Asymmetry in the environment of aromatic groups in a protein is reflected in the magnitude of the ellipticity at 270 nm, permitting assessment of the preservation of tertiary structure. At this wavelength, 3SS-LA has the same ellipticity as N-LA, and 2SS-LA has less than half of the ellipticity of N-LA, whereas R-LA shows no ellipticity. It appears that removal of the Cys6-Cys120 disulfide bond has marginal effects on LA tertiary structure (Kuwajima et al., 1990; Ikeguchi et al., 1992), but additional removal of the Cys 28-Cys 111 disulfide bond leads to significant disruption of tertiary structure. Removal of all four disulfide bonds results in the complete absence of tertiary structure in R-LA at 2 °C.

The ellipticity in the far UV, and particularly at 222 nm, is usually regarded as an index of the α-helical content, i.e., secondary structure. At low temperature, the ellipticity of 3SS-LA at 222 nm is even larger in magnitude than that of N-LA, which might mean some increase of helicity upon removal of one disulfide bond (Fig. 2B). The ellipticity of 2SS-LA at 222 nm is significantly smaller than that of N-LA, but is larger than that of R-LA. The spectra of R-LA is quantitatively similar to that of N-LA at 90 °C (data not shown), a spectrum typical for all heat-denatured proteins at that temperature (Privalov et al., 1989). We therefore conclude that 2SS-LA has less helicity than N-LA, but still contains some secondary structure. It should be remembered that the majority of α-helical structure in LA, but not all, is contained in the α-domain (Fig. 1).

The CD spectra of N-LA and its derivatives do not change significantly in the presence of excess calcium, indicating that, in 10 mM Tris, pH 8, they retain bound calcium (Fig. 2). This observation is in agreement with the high association constant for calcium binding to N-LA and its several derivatives (Ewbank & Creighton, 1993b). However, the removal of calcium by EDTA significantly affects the structure of all the disulfide derivatives of LA. In the presence of EDTA, 2SS-LA retains very little of its secondary structure and has no tertiary structure. Under the same conditions, 3SS-LA loses only part of its secondary and tertiary structure. As expected, N-LA loses very little secondary structure and not much tertiary structure.

Figure 3 shows the change in ellipticity of N-LA and its derivatives in the near (Fig. 3A) and far (Fig. 3B) UV upon heating in 10 mM Tris, pH 8.0. In every case, the ellipticity at 270 nm decreases in a sigmoidal manner over a rather narrow temperature range and does not change upon further heating. The final magnitude of the ellipticity for all the derivatives is similar, regardless of the number of disulfide crosslinks. This suggests that they are all unfolded to the same extent at temperatures above 80 °C. Although 2SS-LA has less than half of the ellipticity of N-LA at low temperature, the transition still appears to be sigmoidal, indicating that disruption of the remaining structure is a cooperative process.

Although the ellipticity at 222 nm changes in a sigmoidal manner with increasing temperature, it is more complicated than the temperature-induced changes at 270 nm (Fig. 3B). With increasing temperature, the ellipticity of 2SS-LA at 270, which is significantly lower than that of N-LA and 3SS-LA at low temperature, decreases in a sigmoidal manner to the value expected for the completely unfolded protein (Privalov et al., 1989), and does not change on further heating. The ellipticity of N-LA and 3SS-LA also decreases in a sigmoidal manner, but not to the level expected for the fully unfolded protein, and continues to decrease monotonically on further heating. The extrapolation of its temperature dependence shows that the fully unfolded state of N-LA and 3SS-LA is reached above 100 °C.

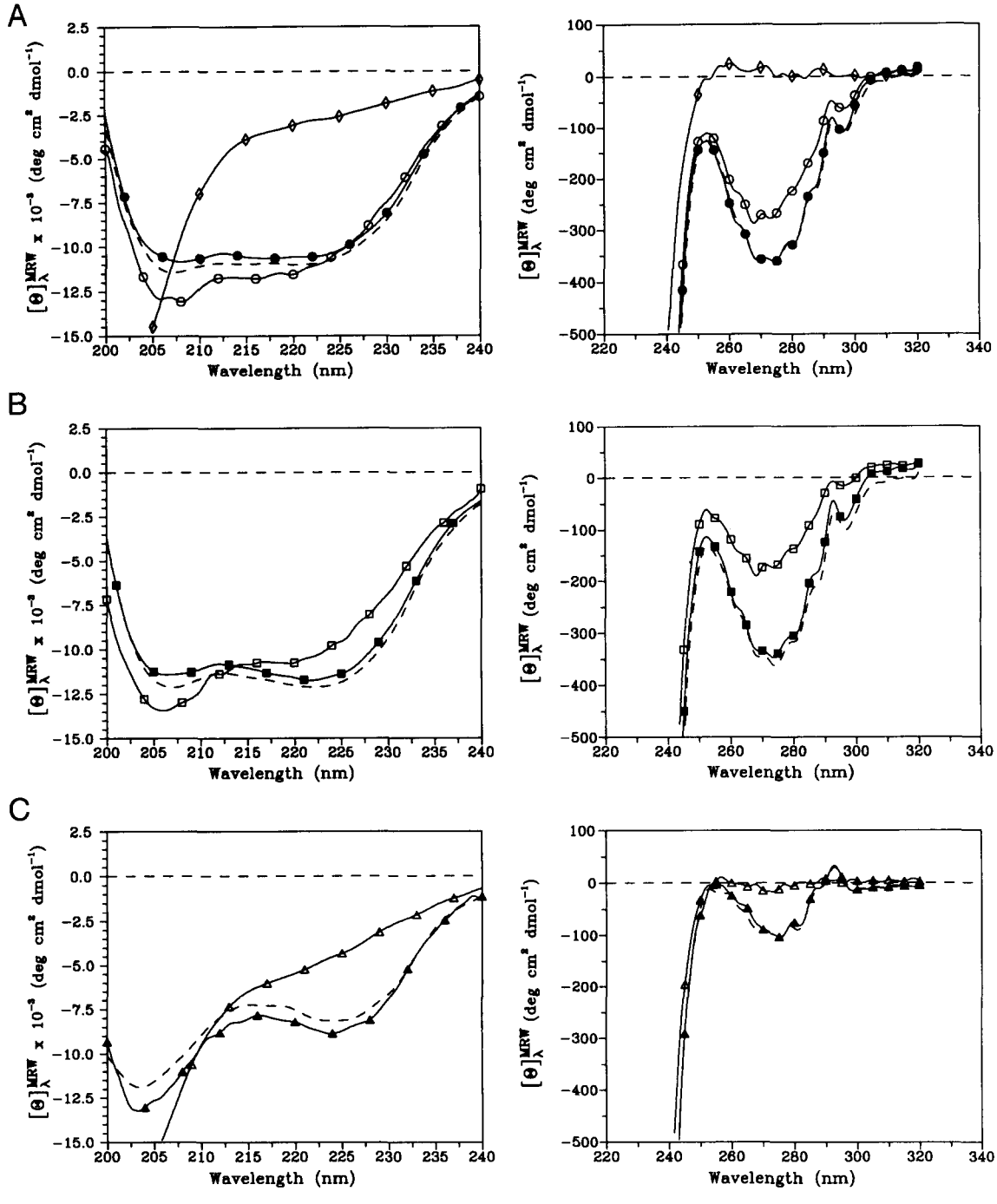

Fig. 2. Near-UV and far-UV CD spectra of (**A**) N-LA (○); (**B**) 3SS-LA (□); and (**C**) 2SS-LA (△) at 2 °C. Dashed lines represent spectra in 10 mM Tris, pH 8.0. Open and closed symbols represent spectra acquired in the same buffer with 1 mM EDTA or 2 mM $CaCl_2$, respectively (10 mM Tris, pH 8.0, plus 2 mM $CaCl_2$; and 10 mM Tris, pH 8.0 and 1 mM EDTA). Spectra of R-LA (◇) in 10 mM Tris, pH 8.9, is indicated in A. Symbols are used to distinguish the curves, which were measured continuously.

The calorimetric scans of all derivatives of LA are highly reversible and produce the reverse heat effect upon cooling, with a small shift in the transition temperature caused by slow kinetics of folding (Fig. 4). Reversibility of the observed heat effect permits treatment using equilibrium thermodynamics. Figure 5 shows the partial molar heat capacity as a function of temperature for N-LA, 3SS-LA, and 2SS-LA in the presence of excess calcium. At room temperature, the partial heat capacity of all three samples is similar and has a low magnitude. With temperature increase, they all show heat absorption peaks and a significant rise in the heat capacity, almost to the level expected for the fully unfolded polypeptide chain. Unfortunately, 2SS-LA cannot be heated above 80 °C due to its increased tendency to aggregate relative to N-LA (Ewbank & Creighton, 1993a). De-

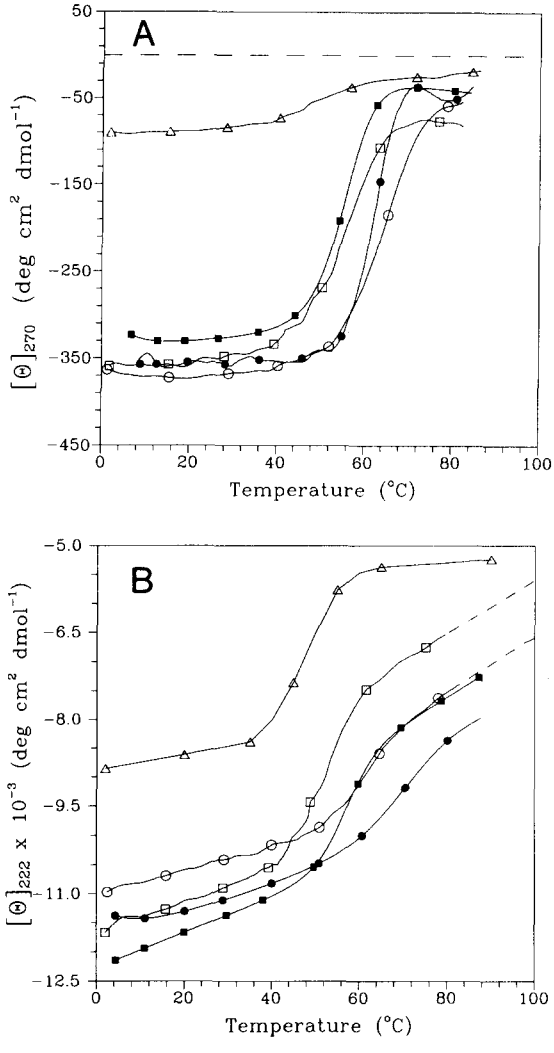

Fig. 3. Temperature dependence of the ellipticity of N-LA (○), 3SS-LA (□), and 2SS-LA (△) at **(A)** 270 nm and **(B)** 222 nm in 10 mM Tris, pH 8.0 (open symbols), and in the same buffer and plus 2 mM $CaCl_2$ (filled symbols). Symbols are used to distinguish the curves, which were measured continuously at a constant heating rate of 0.5 K/min.

creasing the number of disulfide bonds reduces the transition temperature as well as the area of the heat absorption peak (i.e., the calorimetric enthalpy of transition) and its sharpness, which depends on the enthalpy and cooperativity of the considered process.

The heat capacity functions of N-LA and its derivatives in 10 mM Tris, pH 8.0, with varying calcium concentrations are presented in Figure 6. Without excess calcium, the temperature-induced transition in N-LA and 3SS-LA takes place at significantly lower temperature and with smaller enthalpy than in the presence of 2 mM $CaCl_2$. The van't Hoff enthalpies of the considered process, determined from the profile of observed excess heat absorption given the assumption that this process represents a two-state transition, are also smaller (Table 1). In the presence of 1 mM EDTA, the heat absorption peak is preserved only for N-LA. It starts below room temperature. Nevertheless, the full excess calorimetric enthalpy in this peak is in a good correspondence with the van't Hoff enthalpy. For 2SS-LA in 10 mM Tris,

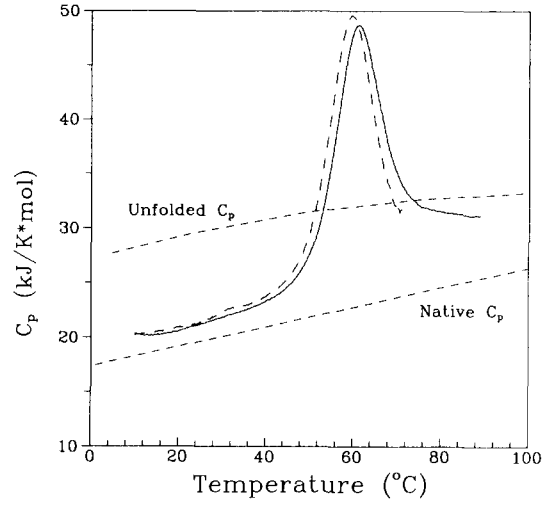

Fig. 4. Calorimetrically measured partial heat capacity function of 3SS-LA upon heating (solid line) and subsequent cooling (dashed line) with the same rate (1 K/min) in 10 mM Tris, pH 8.0, 2 mM $CaCl_2$. The temperature dependence of the heat capacity of the unfolded and native LA are included for reference (see the Materials and methods).

one can see only a trace of the heat absorption peak observed in the presence of 2 mM $CaCl_2$, and the transition appears sharper than expected from the observed heat effect, i.e., the calorimetric enthalpy is much lower than the apparent van't Hoff enthalpy (see also Fig. 3).

Discussion

All thermodynamic parameters of the observed temperature-induced transitions of N-LA and its derivatives are listed in Table 1. It shows that the calorimetric enthalpy determined from the area of the heat absorption peak is in good correspondence

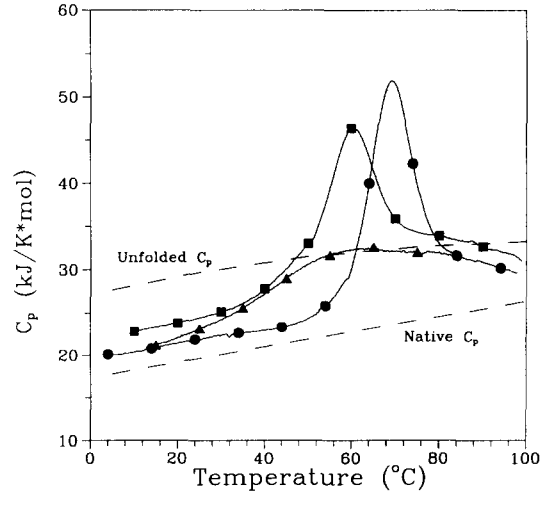

Fig. 5. Calorimetrically measured partial molar heat capacity functions of N-LA (●), 3SS-LA (■), and 2SS-LA (▲) acquired in 10 mM Tris, pH 8.0, plus 2 mM $CaCl_2$. Symbols are used to distinguish the curves, which were measured continuously at a constant heating rate of 1 K/min.

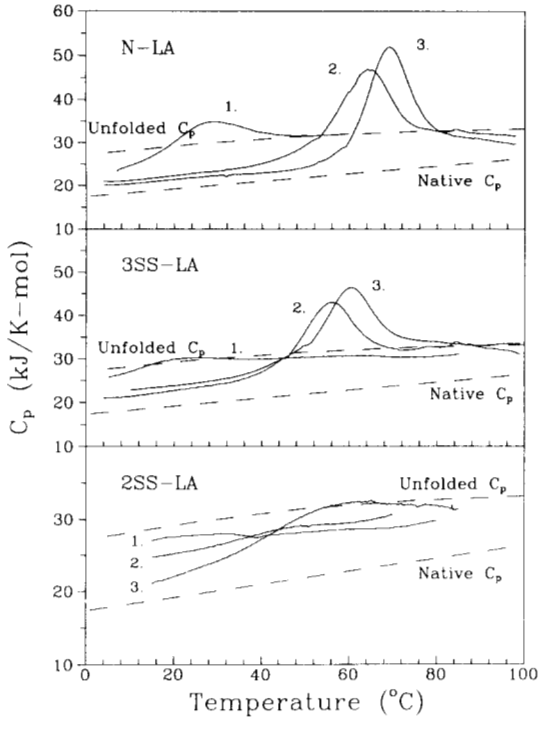

Fig. 6. Calorimetrically measured partial molar heat capacity of N-LA, 3SS-LA, and 2SS-LA acquired in (1) 10 mM Tris, pH 8.0, 1 mM EDTA; (2) 10 mM Tris, pH 8.0; and (3) 10 mM Tris, pH 8.0, 2 mM $CaCl_2$.

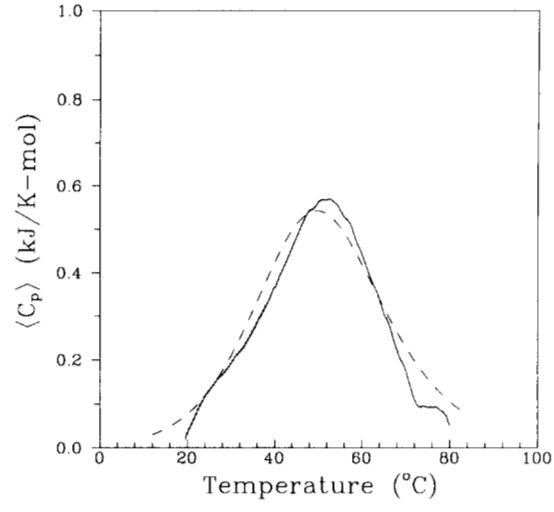

Fig. 7. Temperature dependence of the calorimetrically determined excess heat capacity of 2SS-LA in 10 mM Tris, pH 8.0, 2 mM $CaCl_2$ (solid line) and its simulation (dashed line) obtained by assuming that the temperature-induced process represents a two-state transition that proceeds with an enthalpy equal to 118 kJ/mol and a heat capacity increment of 4.3 kJ/K·mol.

with the van't Hoff enthalpy, obtained from the sharpness of the heat absorption curve, assuming that it represents a two-state transition. The close correspondence of these two enthalpies is a strong argument that the observed process is a highly cooperative two-state transition for all considered cases. This conclusion is supported by simulation of the observed heat absorption curve using parameters listed in Table 1 and assuming that a temperature-induced process represents a two-state transition. As shown in Figure 7, the correspondence between the measured and simulated functions is very good, even in the case of 2SS-LA in the presence of 2 mM $CaCl_2$, its broad transition notwithstanding. In cases when the calorimetric enthalpy is larger than the van't Hoff enthalpy, the heat capacity function of temperature cannot be simulated by a single two-state transition (Privalov & Potekhin, 1986). When the van't Hoff enthalpy is larger than the calorimetric one, it is an indication that not all protein molecules in the studied solution participate in the temperature-induced process, i.e., the solution is heterogeneous. This we observe in the case of 3SS-LA in 10 mM Tris the excess heat absorption in this case is very small but sharp, i.e., calorimetric enthalpy is much smaller than the van't Hoff enthalpy. In the case of 2SS-LA, the initial heat capacity is much higher than expected for a fully folded protein, and CD in the

Table 1. *Calorimetric parameters for the unfolding of N-LA, 3SS-LA, and 2SS-LA at pH 8.0*[a]

Solvent condition	Sample	T_t (°C)	$\Delta H^{cal}(T_t)$ (kJ/mol)	$\Delta H^{vH}(T_t)$ (kJ/mol)	$\Delta S(T_t)$ (kJ/K·mol)	$\Delta G(25\,°C)$ (kJ/mol)
10 mM Tris,	N-LA	68.9	318	330	0.93	28
2 mM $CaCl_2$	3SS-LA	60.5	246	233	0.74	18
	2SS-LA	52.6	81	89	0.25	2
10 mM Tris	N-LA	64.1	271	251	0.81	21
	3SS-LA	55.2	230	221	0.70	15
	2SS-LA	38.8	73	80	0.23	–
10 mM Tris	N-LA	28.5	180	182	0.60	2
1 mM EDTA	3SS-LA	17.4	41	65	0.14	–
	2SS-LA	–	–	–	–	–

[a] Experimental error in estimation of ΔH^{cal} is of the order of 25 kJ/mol and for ΔS^{cal}, 0.10 kJ/K·mol. ΔG (25°) was determined by extrapolation of ΔH and ΔS values to 25 °C, assuming that the heat capacity increment does not depend on temperature and equals 4.3 kJ/K·mol in all cases. Only the transition temperature was determined for 3SS-LA in the presence of EDTA because the heat absorption upon folding is too small to accurately measure.

near UV is close to that of the unfolded protein (Fig. 3). It therefore appears that, under these conditions, part of the sample is initially unfolded and does not participate in the heat-induced unfolding process, but a small part of molecules does unfold cooperatively in rather narrow temperature range. This seems to be a very general situation with calcium-binding proteins, resulting from slow exchange of calcium (slower than the rate of unfolding) between the apo and holo forms of these molecules (see Griko et al., 1995). A deficit of calcium causes the protein solution to become a heterogeneous mixture of two forms, the apo and holo, which have different stabilities. At temperatures in which the apo form is unstable, we observe unfolding only of the holo form, the amount of which depends on the concentration of calcium. This circumstance prevents detailed study of the stability of 2SS-LA; it cannot be studied in solutions with low concentrations of calcium, but high concentrations of calcium also increase the tendency of these molecules to aggregate upon unfolding.

Figure 8 is a plot of the transition enthalpy versus transition temperature for all of the LA derivatives and for data obtained previously for N-LA (Griko et al., 1994). The enthalpy of the cooperative transition for N-LA and 3SS-LA are very similar if compared at the same temperature, and have a similar temperature dependence. The slope of this dependence, 4.3 kJ/K·mol, is very close to the calorimetrically measured heat capacity increment of the observed cooperative transition. This shows that the temperature dependence of the enthalpy of cooperative transition in N-LA and 3SS-LA results mainly from the heat capacity increment associated with this transition, and this increment does not depend significantly on temperature and solvent conditions. It also confirms our previous conclusion that the effect of calcium binding on the enthalpy and the heat capacity of N-LA are very small, and the influence of calcium on the thermostability of LA is mainly entropic (Griko et al., 1994, 1995). Because the heat capacity increment associated with conformational changes in proteins results mainly from the hydration of exposed nonpolar groups (Privalov & Makhatadze, 1990; Makhatadze & Privalov, 1995), one can conclude that the cooperative transition that we observe in N-LA and its disulfide derivatives is associated with unfolding of a rather extended hydrophobic core.

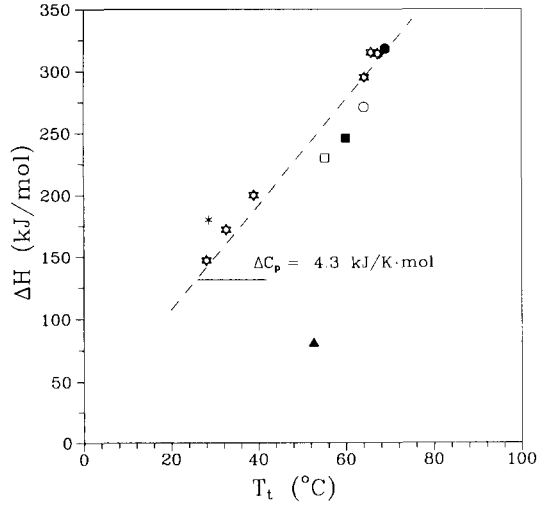

Fig. 8. Calorimetrically measured enthalpy of temperature-induced unfolding as a function of transition temperature of N-LA (○), 3SS-LA (□), and 2SS-LA (△) acquired in 10 mM Tris, pH 8.0. Filled symbols represent data acquired in the same buffer plus 2 mM $CaCl_2$. The * represents N-LA acquired in the same buffer plus 1 mM EDTA. Data of Griko et al. (1994) for N-LA (✡) in various concentrations of Tris buffer are also included.

As discussed in our previous papers (Griko et al., 1994, 1995), the temperature-induced cooperative transition in N-LA, which depends on the presence of calcium, is likely to be associated with the unfolding of its calcium-binding β-domain. As a result of this transition, the protein is in a partially unfolded state with a retained but highly fluctuating α-domain that unfolds with further increase of temperature up to 100 °C. Unfolding of this domain is associated with an increase of heat capacity to the level expected for the completely unfolded polypeptide chain and with a rather small increase in enthalpy. This second stage of unfolding is therefore monotonic and it is difficult to judge whether it represents a cooperative transition or a gradual unfolding of a highly fluctuating domain.

The removal of one disulfide crosslink, Cys 6-Cys 120, and carboxymethylation of the resulting cysteines does not significantly change the structure (Ikeguchi et al., 1992; Ewbank & Creighton, 1993b) or thermodynamic properties of LA. It leads to a slight increase in its helicity and some decrease in the temperature of cooperative transition. As in the case of N-LA, the cooperative transition of 3SS-LA is followed by a stage of monotonic decrease of residual helicity, perhaps associated with the gradual unfolding of the α-domain. The removal of a second disulfide bond, Cys 28-Cys 111, results in a very significant change in the CD signal in the far and near UV which decreases to less than half of the original magnitude at 270 and 222 nm (Ewbank & Creighton, 1993b). It is also notable that, for 2SS-LA, the second stage of the temperature-induced ellipticity changes at 222 nm disappears (Fig. 3). This confirms that the second stage of ellipticity change at 222 nm is associated with the α-domain and that this domain unfolds upon removal of its two disulfide bonds and carboxymethylation of the corresponding cysteines. This might result both from the increase of unfolding entropy of α-domain at the removal of covalent crosslinks, and from the introduction of bulk blocking groups in its structure (Ikeguchi et al., 1992).

Unfolding of the α-domain leads to significant destabilization of the remaining part of LA. This destabilization, however, is caused not by increase of the entropy of unfolding, as one might expect for a structure with less disulfide crosslinks (Table 1). To the contrary, the entropy of cooperative transition in 2SS-LA is lower than in N-LA. Thus, destabilization results mainly from a decrease of the enthalpy of unfolding, which appears to be significantly lower than that expected from the functional dependencies found for N-LA and 3SS-LA (Fig. 8). This decrease in the enthalpy and entropy of cooperative transition cannot be explained by significantly lower calcium-binding ability of 2SS-LA, which results in heterogeneity of sample; i.e., not all 2SS-LA molecules are in holo form in the presence of 2 mM $CaCl_2$. Indeed, in that case, the van't Hoff enthalpy of the observed process would be larger than the calorimetric one, but these two enthalpies are in good correspondence. Therefore, this decrease of the enthalpy and entropy can be associated only with some structural changes in the cooperative unit, either in its initial or final state in the observed temperature-induced process. How-

ever, this decrease in enthalpy and entropy of the cooperative transition, which can be assigned to unfolding of the β-domain, is caused by the removal of disulfide bonds in the α-domain and its consequent unfolding. It is unreasonable to assume that, in 2SS-LA, the β-domain unfolds less upon heating than in N-LA. It appears, therefore, that unfolding of the α-domain has a direct effect on the folded state of the β-domain and either reduces the size of this domain or makes it looser. The Gibbs energy of stabilization of this domain at room temperature is only about 2 kJ/mol (Table 1). This gives, for the fraction of unfolded molecules,

$$F = \frac{\exp(-\Delta G/RT)}{1 + \exp(-\Delta G/RT)}, \quad (1)$$

the value 0.3 at 25 °C, i.e., the β-domain without support of the α-domain spends 30% of the time in the unfolded state. Therefore, in an NMR experiment, one will observe it as almost "molten" at 25 °C. However, the structure of this domain is certainly not liquid-like. It strongly fluctuates, but it should have specific long-range interactions given its native-like fold, which is required to form the calcium-binding site. Ewbank and Creighton (1993b) came to the same conclusion when observing electrophoretically the unfolding of 2SS-LA by urea, which proceeded with a sigmoidal change of mobility. The main advantage of calorimetric study of the temperature-induced unfolding of 2SS-LA is that it permits us to show unequivocally that this is a highly cooperative process that is approximated well by a two-state transition.

Using calorimetric data, we can determine the calcium-binding parameters of N-LA and its derivatives. If K is the calcium-binding constant and **a** is a free calcium activity (concentration), the stability of the holo and apo forms of N-LA should then differ on:

$$\delta\Delta G(T,a) = RT \ln(1 + K\mathbf{x}\mathbf{a}). \quad (2)$$

According to Table 1, $\delta\Delta G$(25 °C, 2.0 mM $CaCl_2$) = 26 kJ/mol. This gives, for the binding constant of calcium, the value 1.6×10^7, which is very close to that found before by Ewbank and Creighton (1993b) and other authors (see for review Kronman, 1989). A much larger value, 10^9, was reported by Desmet et al. (1989), who used titration calorimetry to study calcium binding by LA. They also found that binding of calcium by LA proceeds with the negative enthalpy, which disagrees with our results. The negative enthalpy in those titration experiments was observed because they performed it at 25 °C, at which apo LA is partly unfolded (Fig. 6). Therefore, what had been measured in the titration calorimetric experiment was, in fact, the enthalpy of folding of LA induced by calcium binding and not the enthalpy of calcium binding by the native apo LA (see also Grikio et al., 1995). It should be noted that the fact that apo N-LA is 80% native at 25 °C was noticed also by Ewbank and Creighton (1993b). Unfortunately, we cannot determine the calcium-binding constant of 2SS-LA from the obtained calorimetric data because its apo form is absolutely unstable at any temperature. Assuming that the fraction of folded molecules in the absence of calcium is less than 0.1 at 25 °C, we can only state that the Gibbs energy of 2SS-LA stabilization by 2 mM $CaCl_2$ should be more than 7 kJ/mol and, therefore, the binding constant of calcium should be higher than 10^4. Thus, it is still high as noticed also by Ewbank and Creighton (1993b).

If the β-domain of LA is not independent of the α-domain, but is more stable in its presence, this stabilizing effect should be mutual. In other words, the unfolding of β-domain should also destabilize the α-domain. This does not, however, necessarily mean that the α-domain is completely unstable without the β-domain. It might be less stable, more flexible, and extensively fluctuating while maintaining a native-like fold. This is just what has been observed for the isolated α-domain by Peng and Kim (1994).

The main conclusion that follows from our results is that the β-domain of LA is able to fold and to keep its structure and calcium-binding ability in the absence of the native structure of the α-domain. Disruption of the α-domain results in significant destabilization of the β-domain. However, even in this destabilized state, with a disrupted α-domain, unfolding of the β-domain is a cooperative process that proceeds with significant enthalpy and entropy increase.

Materials and methods

Bovine LA was obtained from milk according to Armstrong et al. (1967). 3SS-LA was obtained by partial reduction by dithiothreitol and subsequential modification by iodoacetic acid of the Cys 6-Cys 120 disulfide bond as described by Kuwajima et al. (1990). 2SS-LA and R-LA were obtained by reduction with dithiothreitol and modification by iodoacetic acid as described by Ewbank and Creighton (1993a, 1993b). Figure 9 shows the population of disulfide derivatives as a function of time at 15 °C. To accumulate the maximum amount of 2SS-LA, the reaction with dithiothreitol was allowed to proceed for 30 min at 15 °C.

Separation of the different disulfide derivatives of LA was performed on a Pharmacia FPLC using a Mono Q anion exchange column with a linear gradient of NaCl in 10 mM Tris-HCl buffer, pH 8.2, containing 1 mM $CaCl_2$. Identity of the chromatographic peaks was deduced from their elution sequence and confirmed using mass spectrometry and PAGE under na-

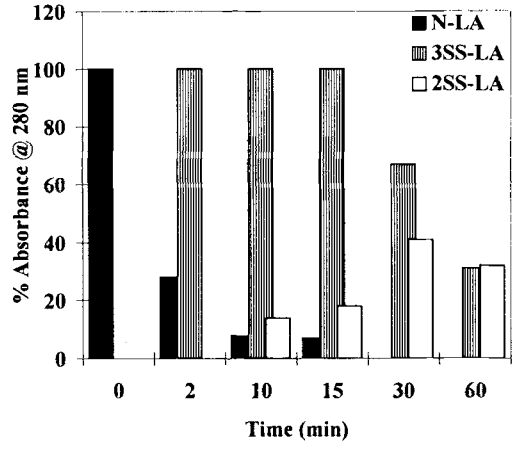

Fig. 9. Relative absorbance of N-LA, 3SS-LA, and 2SS-LA as a function of time of reaction with dithiothreitol at 15 °C. Data was taken from FPLC profiles of the separation of reaction products after reaction with iodoacetic acid.

tive conditions. The FPLC peaks were homogeneous according to molecular weight and charge.

The efficacy of reduction and modification was provided by high-resolution mass spectrometry. Mass spectra of N-LA, 3SS-LA, and 2SS-LA were acquired using a Kompact MALDI III time of flight mass spectrometer (Kratos Analytical, Manchester, England) at the Mid-Atlantic Mass Spectrometer Center. Protein solution (0.3 mL) was placed on a sample slide and mixed with 0.3 mL of matrix (saturated sinapinic acid in 1:1 ethanol:water) (Hillenkamp & Karas, 1990). The experimentally observed masses for N-LA (14,291 Da), 3SS-LA (14,397 Da), and 2SS-LA (14,492 Da) were very close to the calculated values, 14,260 for N-LA, 14,376 for 3SS-LA, and 14505 for 2SS-LA, assuming 58 Da for a carboxymethyl group. The existence of one major peak at the correct position demonstrated the purity of the samples.

The concentration of protein was measured spectrophotometrically using an extinction coefficient of $E_{280} = 20.9$ (Wetlaufer, 1961) for all derivatives. All experiments were performed in 10 mM Tris HCl buffer, pH 8.0, except where indicated.

Calorimetric measurements were performed with the new scanning calorimeter described by Privalov et al. (1995), which is produced now by the Calorimetry Science Corporation (CSC) under the trademark Nano-DSC. Measurements were conducted at a heating rate of 1 K/min using rather low concentrations of proteins (about 1 mg/mL) and low ionic strength solutions to avoid aggregation because the derivatives of LA have an increased tendency to aggregate. The absence of aggregates was checked by the light-scattering effect. The partial specific heat capacity of the proteins was determined according to the procedure described by Privalov and Potekhin (1986) using a molecular mass of 14,260 kDa and a partial specific volume of 0.709 cm^3/g. Due to the low protein concentrations used for calorimetry, the error in the determination of the absolute values of partial heat capacities of LA derivatives is rather large, about 3 kJ/K·mol.

The calorimetric transition enthalpy, $\Delta H(T_t)^{cal}$, was determined from the area of the heat absorption peak by extrapolating the heat capacity of the initial and final states to the mid-transition temperature, T_t, in accordance with the previously determined temperature dependencies of these functions: linear for the native state, and for the unfolded one, calculated by summing the heat capacity values of individual amino acid residues (Privalov & Makhatadze, 1990). The van't Hoff enthalpy, ΔH^{vH}, was determined by a best fit approximation of the excess heat capacity, assuming that the temperature-induced process is a two-state transition that proceeds with the calorimetrically measured heat capacity increment, ΔC_p:

$$C_p(T) = C_p(T)^N + \Delta H^{VH}(dF/dT) + F(T)\Delta C_p, \qquad (3)$$

where $F(T)$ is the fraction (population) of the unfolded molecules (for details see Privalov & Potekhin, 1986; Privalov et al., 1995). The entropy of transition was determined at the transition temperature, T_t, as:

$$\Delta S(T_t)^{cal} = \frac{\Delta H(T_t)}{T_t}. \qquad (4)$$

All these estimates were done using the program included with the Nano-DSC.

The enthalpy and entropy of transition were reduced to standard temperature T_0 (25 °C = 298.15 K) by well-known equations, assuming that the heat capacity increment does not depend significantly on temperature and, in the all cases, equals 4.3 kJ/K·mol:

$$\Delta H(T_0) = \Delta H(T_t) + \int_{T_0}^{T_t} \Delta C_p\, dT \approx \Delta H(T_t) - \Delta C_p(T_t - T_0) \qquad (5)$$

$$\Delta S(T_0) = \Delta S(T_t) + \int_{T_0}^{T_t} \Delta C_p\, d\ln T \approx \Delta S(T_t) - \Delta C_p \ln(T_t/T_0). \qquad (6)$$

CD measurements were obtained with a Jasco J710 spectropolarimeter equipped with a thermostatted cell-holder. Temperature was controlled using a NESLAB RTE 210 programmable circulating water bath. The results were calculated in terms of molar ellipticity using a mean residue weight of 115 Da for N-LA, 3SS-LA, and 2SS-LA, and 118 Da for R-LA.

All studies of temperature-induced changes of LA and its derivatives, both CD and DSC, were done with continuous heating using constant heating rates (1 K/min) and were continuously processed by computer over the whole temperature range studied. The symbols on the curves presented in the figures are therefore not points measured individually, but simply designate the derivative being measured.

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