

Comparative study on heat stability of camel and bovine apo and holo α -lactalbumin

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The stability of camel α -lactalbumin (α -la) against heat denaturation was measured, using circular dichroism (CD) and fluorescence spectroscopy, as well as differential scanning calorimetry (DSC). The experiments were performed in the presence of saturating concentrations of calcium as well as in the presence of EDTA, yielding to the apo form of α -la. The change in heat capacity (ΔC_p) suggests a greater contribution of hydrophobic interactions to the stability of holo camel α -la than in its bovine counterpart. Overall the results obtained in this study suggest a greater stability of camel α -la than the bovine protein in both holo and apo states. Also CD experiments showed similar secondary structure for camel and bovine α -la and secondary structure of camel α -la was better preserved than that of bovine α -la during heat denaturation. The differences in thermal stability between the proteins from two species can be primarily ascribed to the difference in the quantity of hydrophobic interactions involved in their folding.

Keywords: α -lactalbumin; differential scanning calorimetry; circular dichroism; fluorescence; camel.

α -Lactalbumin (α -la) is a small, acidic and hydrophilic metallo-protein present in milk of all mammals (Hill & Brew, 1975). Several functions have been ascribed to α -la during the neonatal period. One of the most important biological functions of this protein is to participate as the non-catalytic regulatory subunit (specifier component) in lactose synthase complex (Calderone et al. 1996). Recently, a folding variant of α -la was shown to induce apoptosis in tumor and immature cells (Casbarra et al. 2004). It has been suggested that active form of α -la may reduce the pool of potentially malignant cells in gut, inducing their apoptosis (Svensson et al. 2000). Native α -la consists of a large α -helical domain (composed of three major α -helices and two short 3_{10} -helices) and a small β -sheet domain (composed of a series of loops and of a small three-stranded anti-parallel β -pleated sheet), which are connected by a calcium-binding loop. A deep cleft between them divides two domains (Permyakov & Berliner, 2000). Two hydrophobic regions (called aromatic clusters I and II) are located in the interior of the globular structure

(Chrysin et al. 2000) and four disulphide bridges also stabilize the structure of this protein (Kuhlman et al. 1997).

One of the most interesting features of α -la is its ability to bind metal cations. The protein has a high affinity calcium-binding site in a loop between two helices and has multiple binding sites for other physiologically significant cations (Vanhooren et al. 2002). Calcium binding strongly influences the α -la tertiary structure, molecular stability and is required for the formation of disulphide bond in the reduced and denatured α -la (Rao & Brew, 1989).

Since α -la is known to adapt different folds as a response to the medium condition, it has been extensively used in protein folding studies (Lala & Keul, 1992). α -La is known to be a very suitable protein object for elucidation of protein folding mechanism and one of the most popular model systems for studies of molten globule (MG) state, which generally contains native-like secondary structures but lacks stable tertiary structures (Paci et al. 2001). The calcium-depleted apo-form of α -la at neutral pH can be induced to adopt a partly unfolded state or MG upon moderate heating (Griko & Remeta, 1999). On the other hand, the acid state (A-state) of this protein at very low pH values, so called the classical MG state, produced calcium release during protonation of its binding sites (Hill & Brew,

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LALBA_CAMDR      KQFTKCKLSDELKDMNGHGGITLAEWICII FHMSGYDTETVVSNNNGNREY 50
Q28049_BOVIN     EQLTKEVFRLEKDLKGYGGVSLPEWVCTAFHTSGYDTQAI VQNNSTEY 50
                  *:***:  ***:***:*.**:* ** *****:*.**.. **

LALBA_CAMDR      GLFQINNKIWCRDNENLQSRNICDISCDKFLDDDLTDDKMKCAKKILDKEG 100
Q28049_BOVIN     GLFQINNKIWCKDDQNP HSSNICNISCDKFLDDDLTDDIMCVKKILDKVG 100
                  *****:***: * ***:***** **.****** *

LALBA_CAMDR      IDYWLAHKPLCSEKLEQWQCEKW 123
Q28049_BOVIN     INYWLAHKALCSEKLDQWLCEKL 123
                  *:*****.******:** **

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Fig. 1. Sequence alignment of bovine and camel α -La. Protein sequence alignment was done with CLUSTALW (v. 2.0.11; <http://www.ebi.ac.uk>). PubMed accession numbers are P00710 (camel) and Q28049 (bovine). Identical (*), conserved (:), and semi-conserved (.) amino acids in two sequences are marked.

1975). Since α -la has many important biological functions, which depend on the conformational states, the interest in the molecular and biological properties of this protein continues. Most of the studies on α -la were carried out using bovine or human proteins. Camel milk contains α -la with a concentration around 2.2 mg/ml. Camel milk also lacks β -lactoglobulin (Haddadin et al. 2008; El-Hatmi et al. 2006, 2007). To our knowledge, most of the studies on the stability of camel milk proteins were done on total milk (Farah, 1992; Elagamy, 2000) or total whey proteins (Farah, 1986; Leveux, 2006; Laleye, 2008). Study of pure camel α -la in a controlled buffering condition may give more particular information about its structure and heat stability.

Conti et al. (1985) reported there are two variants of α -la in camel milk. Generally camel α -la is composed of 123 amino acids (Beg et al. 1985) and has a molecular mass of 14.43 kDa whereas its bovine counterpart with the same number (but somewhat different composition) of amino acid residues has a molecular mass of 14.18 kDa. According to the sequence alignment data (Fig. 1), the similarity and identity between these proteins are 82.9 and 69.1%, respectively. These similarities and differences and also potential ability of α -la to adopt cytotoxic MG-like state folding have stimulated the present study. It was aiming to investigate the differences in the structures and stabilities between camel and bovine α -la in apo and calcium saturated (holo) states in comparative biophysical studies such as differential scanning calorimetry (DSC), fluorescence and circular dichroism (CD).

Material and Methods

Milk from camel (*Camelus dromedarius*) was provided by the Department of Clinical Sciences and Faculty of Veterinary Medicine of the University of Tehran. Bovine α -la was obtained from Sigma chemical Co. Other chemicals were of analytical grade (Sigma) and used without further purification. All solutions prepared with double distilled water were kept at 4 °C until use.

Purification of camel α -la

After defatting, major fractions of caseins and whey proteins of camel milk were precipitated using ammonium sulphate (26.4%). The supernatant was dialyzed against double distilled water for 96 h at 4 °C and applied to an UF membrane (cut off 30 kDa). The filtrate collected was applied to another UF membrane (cut off 10 kDa) to increase the protein concentration. The purity (>97%) of α -la sample prepared by this method, was estimated by SDS-PAGE (15% acrylamide gel; Schägger & von Jagow, 1987) by Coomassie blue staining. Purified camel α -la, which was retained on 10 kDa UF membrane, was collected and lyophilized for further usages.

Preparation of apo-form of camel α -la

Camel apo α -la was generated from the native protein by dissolving it in 20 mM-Tris-HCl buffer, pH 7.5, containing 3.5 mM-EDTA removing bound calcium (Svensson et al. 2000). The induced conformational change and the increase of exposure of hydrophobic sites on the surface of apo-form of α -la were confirmed by CD spectroscopy and the increase of 8-anilino-1-naphthalenesulfonic acid (ANS)-fluorescence, respectively.

Protein assay

The protein concentration was determined using the Bradford reagent (Bio-Rad) with bovine serum albumin (BSA) as the standard protein (Bradford, 1976).

Calorimetric measurements

DSC measurements of α -la samples (70 μ M) were carried out in an N-DSC II differential scanning calorimeter having 0.324 ml cell at a heating rate of 2 K/min. All buffers and samples were thoroughly degassed by stirring in an evacuated chamber at room temperature. Prior to the loading, the instruments and the cells were pressurized to about 2 atm to prevent the formation of gas bubbles during the heating. Subtraction of the buffer baseline was performed

Table 1. The thermodynamic parameters of thermal unfolding of bovine and camel proteins in calcium loaded (holo) and calcium depleted (apo) states

	T_m (°C)	ΔH_{cal} (kcal/mol)	ΔH_{vH} (kcal/mol)	$\Delta H_{vH}/\Delta H_{cal}$	ΔC_p (kcal/mol K)
Camel α -la (Holo)	71.7±0.1	53.3±9.3	62.4±5.4	1.17	1.69±0.38
Bovine α -la (Holo)	67.4±2.4	58.2±10	66.3±4.1	1.11	0.80±0.15
Camel α -la (Apo)	39.6±3.5	17.3±1.6	24.9±4.4	1.43	0.27±0.09
Bovine α -la (Apo)	32.4±0.1	32.1±2.2	36.7±2.7	1.14	0.89±0.3

and the values of T_m , ΔS , ΔH_{cal} , ΔH_{vH} , and ΔC_p were calculated from thermograms using CpCalc software (version 2.1). The reversibility of the unfolding was checked routinely by re-heating of the cooled sample immediately following the first scan. The thermodynamic parameters of camel and bovine α -la unfolding are presented in Table 1 and are the average of three replications.

Fluorescence spectroscopy

Fluorescence intensity was measured with a Cary Eclipse spectrofluorimeter (Varian, Australia). Intrinsic tryptophan fluorescence was measured in protein solutions (10 μ M in 20 mM-Tris-HCl buffer, pH 7.5). All experiments were performed using a 1-cm path length fluorescence cuvette, 5 nm slit size and excitation wavelength of 280 nm.

The assessment of ANS binding by apo- and holo-forms of bovine and camel α -la was carried out after 15 min incubation with 20 μ M-ANS. During fluorescence measurements of ANS- α -la complex (excitation 385 nm), the emission spectra were recorded between 400 and 600 nm on Cary-Eclipse spectrofluorimeter. The bandwidths for both excitation and emission were 10 nm.

CD-measurements

The CD spectra of camel and bovine α -la (holo- and apo-forms) were recorded on a Jasco J-810 instrument equipped with a thermoelectric sample holder. A cuvette of 1-mm path length was used. The protein concentration in the CD experiments was 15 μ M. The results were expressed in molar ellipticity $[\theta]$ (deg cm² dmol⁻¹) based on a mean amino acid residue weight (MRW) of 115.3 for bovine α -la (Yang et al. 2006) and 117.3 for camel α -la. The molar ellipticity was determined as $[\theta]_{\lambda} = (100 \text{ MRW } \theta_{\text{obs}}/cl)$, where θ_{obs} is the observed ellipticity in degrees at a given wavelength, c is the protein concentration in mg/ml and l is the length of the light path (cm). Secondary structure estimation from the far-UV CD spectra was calculated using circular dichroism spectra by neural networks (CDNN) (Böhm et al. 1992). Changes of the secondary structures of camel and bovine α -la have been followed by monitoring the ellipticity at 222 nm in the far-UV region. Temperature scans were performed stepwise, allowing the sample to equilibrate at each temperature for at least 3 min. The average of three measurements was plotted, and error bars indicate the standard deviation from the mean. Data were fitted with sigmoidal curve using SigmaPlot 10.0 software.

Results and Discussion

Camel and bovine α -la heat denaturation measurements

DSC thermogram shows the excess heat capacity (C_p , sample minus reference buffer) as a function of temperature. In this study, camel and bovine α -la in 2 mM-calcium as well as in 3.5 mM-EDTA was heated and their unfolding was followed by DSC. The thermograms obtained for all proteins showed classic single endothermic peak typical of protein denaturation (Fig. 2). The heat capacity change upon unfolding (ΔC_p) is the thermodynamic property that can be connected most directly to the exposure of hydrophobic surface areas and disorganization of water shell around the protein (Gómez et al. 1995). The change in heat capacity between the native and unfolded states (ΔC_p) of camel and bovine α -la in the presence of calcium was found to be 1.7 and 0.8 kcal mol⁻¹ K⁻¹, respectively. Higher ΔC_p of camel α -la than that of bovine counterpart under the same experimental conditions suggests a greater contribution of hydrophobic interactions to the stability of camel α -la than in its bovine counterpart. Sequence comparison of bovine and camel α -la shows greater hydrophobicity of the amino acid sequence 25–35 in the hydrophobic core (see Fig. 1). This is in good agreement with the higher ΔC_p and higher intensity of ANS fluorescence (Fig. 3B) observed for holo camel α -la.

The enthalpy of denaturation (ΔH) depends on the temperature at which denaturation occurs as judged by the area of heat absorption peak (Sanchez-Ruiz, 1995). In Fig. 2, the dashed line represents the polynomial baseline fit with the area under the curve representing the calorimetric enthalpy (ΔH) and the peak of the curves representing the midpoint of the unfolding transition (T_m). Values of calorimetric enthalpy (ΔH_{cal}), van't Hoff enthalpy (ΔH_{vH}), ΔC_p and T_m deduced from the thermograms are given in Table 1. Larger enthalpy change for bovine α -la in similar solvent conditions indicates that larger amount of energy is necessary for its unfolding as compared with the camel protein counterpart (Hendrix et al. 2000). The enthalpy of denaturation we found for camel α -la is more than what Levieux et al. (2006) have reported before. This might be because of differences in methods or solvent conditions; they obtained thermodynamic parameters indirectly by immunochemical method for camel α -la in milk.

As reported in Table 1, the $\Delta H_{vH}/\Delta H_{cal}$ ratio of the camel protein is close to one, indicating that the heat unfolding follows two state process (Sturtevant, 1987), similar

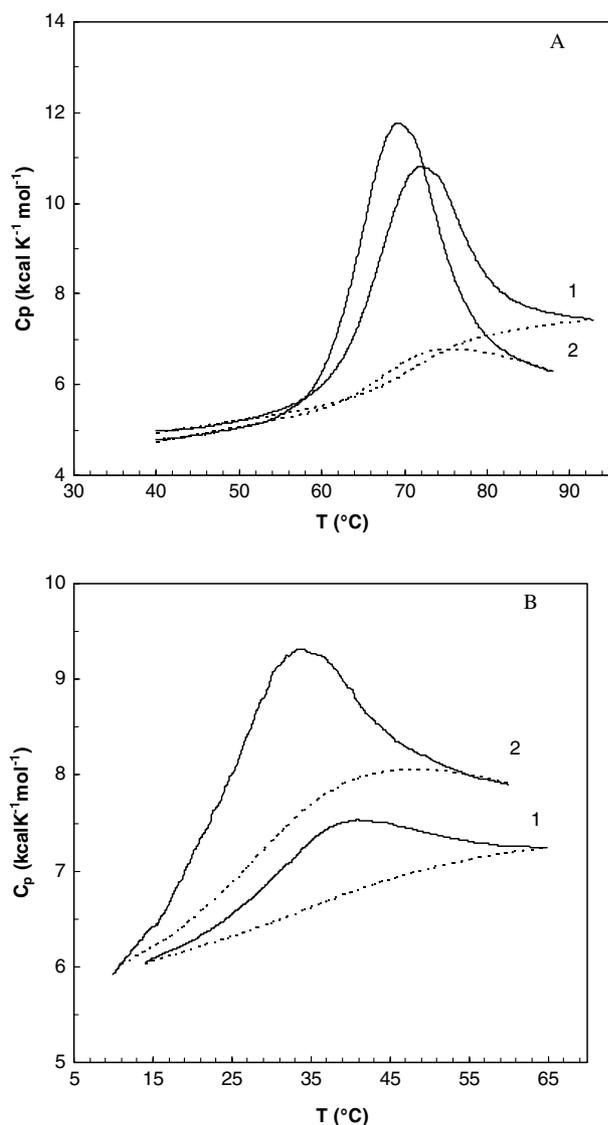


Fig. 2. Plot of measured heat capacity against temperature for camel (1) and bovine (2) α -La in 20 mM-Tris-HCl, pH 7.5, in the presence of (A) 2 mM- CaCl_2 (holo form) or (B) 3.5 mM-EDTA (apo form). Protein concentration was 70 μM . Dashed lines represent the polynomial baseline fit.

to what was reported previously for bovine α -la in similar solvent conditions (Griko et al. 1994). Superimposition of the DSC curves for all α -la upon re-heating of the cooled sample shows that the unfolding transition is reversible for all of them. As reflected by its T_m , bovine α -la is less stable than camel α -la. The stability of α -la is dependent on binding of calcium to the primary α -la calcium-binding site (Greene et al. 1999). As shown in Fig. 2, for apo α -la in the presence of EDTA very broad transitions were obtained starting at low temperatures.

It is amply documented that removal of metal ions or prosthetic groups from holo protein leads to local or overall conformational changes, reduction of stability, and

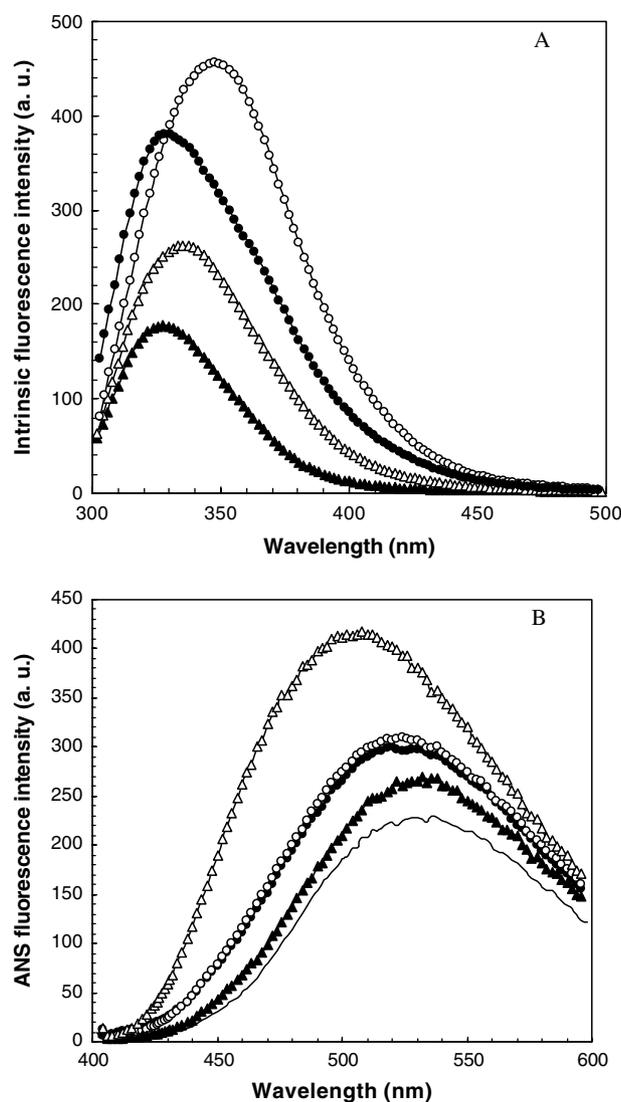


Fig. 3. The intrinsic (A) and ANS fluorescence emission spectra (B) of 10 μM -camel apo α -la (○), camel holo α -la (●), bovine apo α -la (△), bovine holo α -la (▲), and ANS alone (-) in 20 mM-Tris-HCl, pH 7.5, in the presence of 2 mM- CaCl_2 (holo form) or 3.5 mM-EDTA (apo form) at 20 $^{\circ}\text{C}$. The excitation wavelengths were 280 nm (A) and 350 nm (B) with slits of 5 and 10 nm, respectively.

enhanced chain flexibility (Polverino de Laureto et al. 2002). When calcium was depleted the maximal temperatures of denaturation of bovine and camel proteins were decreased.

Fluorescence study of camel and bovine α -la

Intrinsic fluorescence emission measurements of camel and bovine α -la in the presence of 2 mM-calcium and 3.5 mM-EDTA were performed using excitation wavelength of 280 nm. As indicated in Fig. 3A, the intrinsic fluorescence maximum emission wavelength of camel and bovine holo α -la is 328 nm, which implies that the Trp

Table 2. The secondary structure content of camel and bovine α -la

	Alpha-helix	Beta-sheet	Beta-turn	Random coil
Camel α -la (Holo)	42.2 \pm 1.6	13.6 \pm 0.4	15.8 \pm 2.0	28.3 \pm 2.1
Bovine α -la (Holo)	43.1 \pm 2.7	13.3 \pm 1.9	15.6 \pm 0.6	27.9 \pm 3.0
Camel α -la (Apo)	34.7 \pm 2.7	16.5 \pm 1.8	16.7 \pm 0.4	32.2 \pm 2.5
Bovine α -la (Apo)	33.4 \pm 2.5	16.8 \pm 1.6	16.7 \pm 0.4	33.0 \pm 2.4

The relative proportion of secondary structures before and after calcium removal at 25 °C was calculated by CDNN (Böhm et al. 1992)

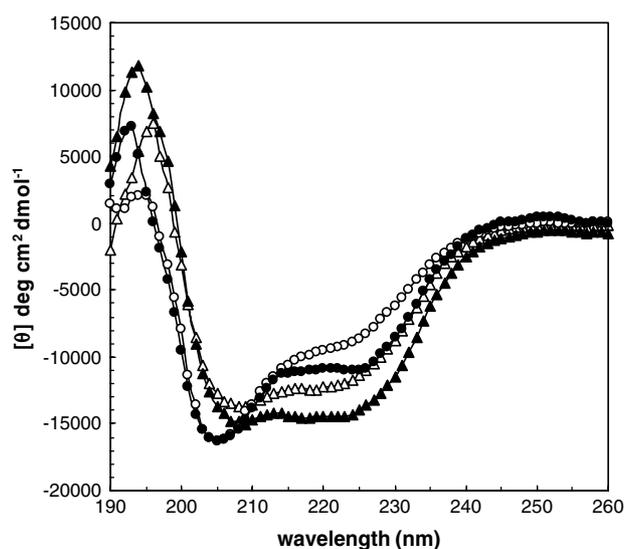


Fig. 4. Far-UV circular dichroism spectra of 15 μ M-camel apo α -la (\circ), camel holo α -la (\bullet), bovine apo α -la (\triangle), and bovine holo α -la (\blacktriangle) in 20 mM-Tris-HCl (pH 7.5) at 25 °C.

environment is very similar in the native structure of both α -la. The maximum of emission intensity of camel α -la is greater than the maximum of emission intensity of bovine α -la. Primary structure of camel α -la contains 5 Trp, 4 Phe and 3 Tyr while its bovine counterpart contains 4 Trp, 4 Phe and 4 Tyr. The large difference between the emissions of two proteins is mainly due to the extra Trp in camel α -la. The removal of calcium bound to both α -la provokes an increase in their fluorescence intensities and a red shift of their maxima. The λ_{\max} after calcium removal, were 347 nm and 335 nm for camel and bovine α -la, respectively. These changes suggest that Trp residues are more exposed to solvent in the apo form of both α -la reflecting the conformational changes caused by removal of the bound calcium. The shift of λ_{\max} after calcium removal is greater in the case of camel α -la than in the case of bovine α -la.

ANS binding studies indicate the quantity of hydrophobic sites available on the surface of protein able to bind the ANS probe. Hence, to some extent, ANS binding depicts the overall three-dimensional (3D) structure in solution (Cardamone & Puri, 1992; Hawe et al. 2008) and the status of hydrophobic surface clusters. It has been shown that ANS has a strong affinity for a solvated

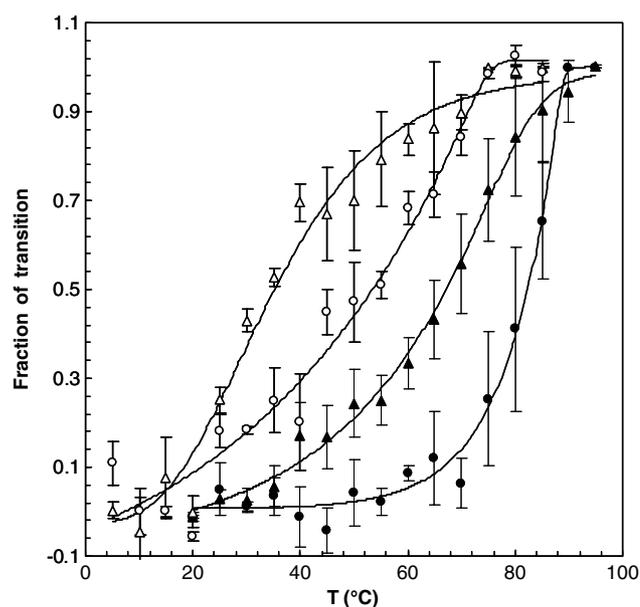


Fig. 5. Thermal unfolding profile of α -La monitored by ellipticity at 222 nm; Total α -la concentration was 15 μ M in 20 mM-Tris-HCl buffer, pH 7.5, containing 2 mM- CaCl_2 (holo form) or 3.5 mM-EDTA (apo form). The lines represent the sigmoidal curve fit to the unfolding data and error bars denote standard deviation from the mean values. Symbols are identical to those of Fig. 4.

hydrophobic core of the MG state. However, it binds weakly both with unfolded chains and with native proteins (Semisotnov et al. 1987). As mentioned before, two hydrophobic clusters have been detected in the native structure of α -la (Acharya et al. 1989). ANS fluorescence measurements were made to probe the accessibility of the hydrophobic areas after calcium removal in the studied α -la. As shown in Fig. 3B a greater surface hydrophobicity of camel α -la than that of bovine protein at equivalent protein concentration can be explained by a greater hydrophobicity of N-terminal part of the α -helical domain of this α -la than its bovine counterpart. Apo bovine α -la shows ANS binding resembling MG state. For camel α -la little ANS fluorescence intensity increase after calcium removal was observed (Fig. 3B). This might be due to partial unfolding of the molecule. This remains in agreement with less value of ΔC_p of camel α -la in the apo state and bigger red shift of λ_{\max} of fluorescence emission after calcium removal

(Fig. 3A) in camel α -la reflecting bigger conformational changes.

The results of intrinsic and extrinsic fluorescence studies suggest that conformational integrities of both camel and bovine α -la are sensitive to calcium removal. However, camel α -la shows greater change in exposure of buried hydrophobic areas upon calcium depletion.

Far-UV ellipticity changes of bovine and camel α -la

The far-UV CD spectra characterize the secondary structures of proteins due to the peptide bond absorption, and changes in this spectrum usually reflect the major backbone changes in proteins (Kelly & Price, 2000). The far-UV CD spectra of bovine and camel α -la were recorded at room temperature, in the presence of 2 mM-calcium or 3.5 mM-EDTA (Fig. 4). The relative proportions of secondary structures are presented in Table 2. Table 2 shows that the secondary structure of camel α -la is very similar to that of bovine α -la. Calcium loaded holo forms of these proteins are slightly more structured than their apo forms (see Fig. 4).

As shown in Fig. 4 and Table 2, and based on the results obtained from the fluorescence studies, it is clear that the removal of calcium from these proteins causes pronounced structural changes mostly in tertiary structure but also slight changes in secondary structures. As seen in Table 2, after calcium removal, a reduction in α -helix content and a slight elevation in β -structures were observed. Formation of non-native β -sheets in destabilizing condition was reported previously to increase propensity of some proteins to aggregation (Norma & Greenfield, 1999). Non native β -structure formation and exposure of hydrophobic interior of the protein in the apo state (Fig. 2B) possibly gives to this protein capability to penetrate biological membranes what could explain its subsequent cytotoxic activity. As shown in Fig. 5 thermal denaturation of camel α -la was observed at higher temperatures in both apo and holo states. Similar changes happen in β -sheet structure since similar results were obtained at 217 nm. Consequently, these results indicate that the secondary structure of camel α -la is better preserved than that of bovine α -la during heat denaturation.

In 2 mM-calcium, even at 90 °C, the value of ellipticity at 222 nm is higher than that observed in the presence of 3.5 mM-EDTA (data not shown). This indicates that, even at 90 °C, calcium remains bound to camel α -la and influences its conformation. This result remains in agreement with the results reported previously for bovine α -la (Vanderheeren & Hanssen, 1994).

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