Innovative Romanian Food Biotechnology *Vol. 9, Issue of September, 2011*

Received June 10, 2011 / Accepted July 10, 2011

RESEARCH ARTICLE

EFFECT OF CALCIUM ADDITION ON THE THERMAL DENATURATION OF BOVINE APO-α-LACTALBUMIN – A PRELIMINARY STUDY

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Abstract

Bovine α-lactalbumin is known to be present in molten globule form in its apo-state (i.e., Ca^{2+} depleted state). In this preliminary study, heat-induced conformational changes were analyzed using the fluorescence spectroscopy at very low pH in the absence and the presence of calcium ions. The heat treatment caused the decrease of intrinsic fluorescence, evidencing the increase of intramolecular quenching of the tryptophans.

The presence of two classes of tryptophan residues (exposed and buried) was reflected also in the Stern–Volmer plot, and the K_{SV} values were used for estimating the exposed tryptophan residues in the protein.

Introduction

α-lactalbumin (α-LA) is a small globular protein secreted in mammalian milks. It is a calcium-binding metalloprotein, which becomes a stable molten globule (MG or A-state) under low-pH, Ca-free and low-salt conditions, under thermal stress or in the presence of mild denaturants (Nitta, 2002). A number of studies have shown that the overall architecture of the α-LA molten globule has extensive native-like character, particularly in the α-domain where native-like helices are arranged in a manner that bears a close relationship to that found in the native structure (Bai *et al*., 2001). However, partly folded states or molten globules of α-LA are also formed at neutral pH by removing the single protein-bound calcium; by adding a denaturant or by cleaving the 28–111 disulfides bridge (Kuwajima, 1996). In particular, it is an important system through which to probe the nature of interactions that define the fold of a polypeptide main-chain prior to the close packing of the side-chains that result in the fully native structure (Arai and Kuwajima, 2000).

The biological function of α-LA is to participate in lactose biosynthesis, as the non-catalytic regulatory subunit of the lactose synthase complex (Brew, 2003). Recently, a folding variant of human α-LA was shown to induce apoptosis in tumor cells and immature cells (Svanborg *et al*., 2003). Besides these functions, α-LA has the ability to bind histones (Permyakov *et al.,* 2004) and also some of its chymotryptic peptides present bactericidal activity (Pellegrini *et al.,* 1999).

One of the most interesting features of α-LA is its ability to bind metal cations. It has a strong Ca^{2+}

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binding site, which also weakly binds Mg^{2+}, Mn^{2+}, Na^{+} and K^{+}, and also has several distinct Zn^{2+} binding sites (Noyelle and Dael, 2002). The binding of Ca^{2+} to α-LA causes pronounced structural changes, mostly in tertiary structure, but not in secondary structure (Kronman, 1989). Furthermore, the binding of cations to the Ca^{2+} binding site increases protein stability against denaturation by heat and by various other denaturing agents such as urea and guanidine hydrochloride (Permyakov and Berliner, 2000).

This study represents a preliminary step in assessing the influence of metal cations on the mechanism of thermal denaturation of α-LA at low pH. Therefore, fluorescence spectroscopy technique was used to evaluate the conformational and structural changes of apo-α-LA induced by thermal treatments in the absence and respectively presence of calcium chloride. We consider that this information will contribute substantially to the elucidation of structure-function relationship.

Materials and methods

Materials

Ca^{2+}-depleted bovine α-LA and acrylamide were obtained from Sigma (Sigma-Aldrich Co, St. Louis, MO). The protein was used without further purification. All other chemicals used were of analytical grade.

Heat-treatment

1 mg ml^{-1} g of protein was dispersed in 10 mM Tris-HCL buffer with and respectively without 5 mM $CaCl_2$ and pH of the mixture was adjusted to 2.0 with HCl 1 N. The mixture was stirred at room temperature for 15 min. 0.5 ml of protein solutions were filled in plastic tubes (1 cm diameter). The thermal treatment experiments were conducted in a thermostatic water bath at temperatures ranging from 60°C to 80°C for 15 min. After thermal treatment, the tubes were immediately cooled in ice water to prevent further denaturation. All experiments were performed within 3 minutes after the heat treatment.

Intrinsic fluorescence spectroscopy

After heat-treatment, the protein solutions were diluted in buffer of appropriate pH. Intrinsic fluorescence measurements were made on a LS-55 luminescence spectrometer (PerkinElmer Life Sciences, Shelton, CT) with a quartz cell of 10 mm path length, using an excitation wavelength of 292 nm. The emission was collected between 300 and 420 nm. The excitation and emission slits were both 10 nm, and the scan speed was 200 $nm \cdot min^{-1}$. Measurements were made at 25 °C.

Fluorescence quenching experiments

Fluorescent quenching experiments were performed with acrylamide. Apo-α-LA was incubated in buffers for 1 h at 25 °C. 8 M acrylamide were prepared freshly in 10 mM Tris-HCl buffer at pH 7.5. Quenching titrations with acrylamide were performed by sequentially adding aliquots of the quencher stock solutions to the samples denatured at different pH and gently stirred. The excitation wavelength was set at 292 nm, and the fluorescence emission spectra were scanned from 300 to 400 nm. Fluorescence intensity was corrected for background, adjusted for dilution by the titrant, and corrected for inner filter effects. The inner filter effect due to acrylamide absorption was corrected according to the Eq. (1) (Eftink and Ghiron, 1981):

$$F_{corr} = F \cdot 10^{A/2} \qquad (1)$$

where F_{corr} and F are the corrected and observed fluorescence intensities, respectively, and A is the absorbance at 292 nm. The fluorescence quenching data in the presence of acrylamide were analyzed by fitting to Stern–Volmer equation (Eq. 2) (Lehrer, 1971):

$$\frac{F_0}{F} = 1 + K_{SV}[Q] \qquad (2)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, $[Q]$ is the concentration of the quenchers, and K_{SV} is Stern-Volmer quenching constant.

Statistical analysis of data

Each experiment was carried out at least in triplicate. All standard deviation errors and linear regression were calculated using Microsoft Excel.

Results and discussions

Intrinsic fluorescence intensity

Fluorescence has been extensively used to obtain information on the structural and dynamic properties of proteins, since the spectral parameters of fluorescence emission spectra such as position, shape, and intensity are dependent on the electronic and dynamic properties of the chromophore environments, (Prajapati *et al.*, 1998).

Tryptophan fluorescence is very sensitive to the environment, so that it can be used to prove modifications of the tertiary structure. α-LA has four tryptophan residues at positions 26, 60, 104, and 118 (France and Grossman, 2000). Out of these four tryptophans, only Trp^{60} is exposed to the solvent in the native state and contributes only 7% to the total fluorescence of α-LA.

Figure 1 shows the fluorescence spectra of apo-α-LA after incubation in the presence and absence of calcium chloride. It can be seen an increase in fluorescence intensity in the absence of Ca^{2+} at pH 2.0, evidencing the reduction of intramolecular quenching of the tryptophans. The substantial changes in the tryptophan fluorescence spectrum that occur on the absence of Ca^{2+} from α-LA suggest that a major change in molecular conformation has occurred.

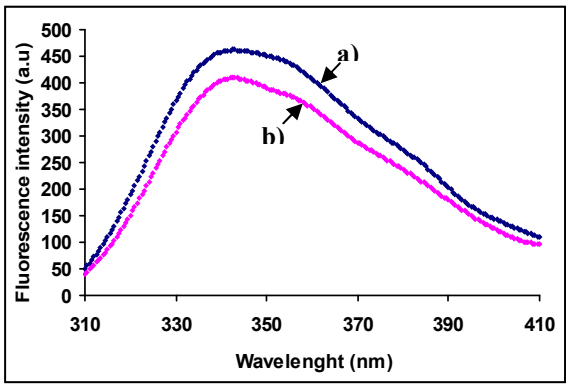

Figure 1. *The fluorescence spectra of α-LA*

The molten globule form (in the absence of calcium) exhibited an increase (~13%) in fluorescence intensity relative to the form in the presence of calcium ions.

Curves a and b represent the spectra in the presence and in absence of calcium chloride, respectively. The excitation wavelengths were 292 and the emission data were collected between 300 and 420 nm.

Increasing the temperature affects the fluorescence properties of apo-α-LA. Figure 2 shows the fluorescence spectra of apo-α-LA before and after incubation at different temperatures for 15 min. The thermal treatment induces a slight decrease in the fluorescence intensity, evidencing the increase of intramolecular quenching of the tryptophans, and potentially reversible structural changes.

α-LA exists as a partially folded conformer (U form) at acid pH. A second partially folded conformer (H form) is formed above 60°. Sommers and Kronman (1980) compared the changes in tryptophan fluorescence which occur on forming U and H. These authors analyzed the fluorescence properties of the bovine protein and an N-bromo-succinimide derivative, and determined which tryptophan residues give rise to such changes in fluorescence. Trp^{28} and Trp^{109} in the native state transfer their excitation energy to Trp^{63} whose fluorescence is quenched by a pair of vicinal disulfide bridges.

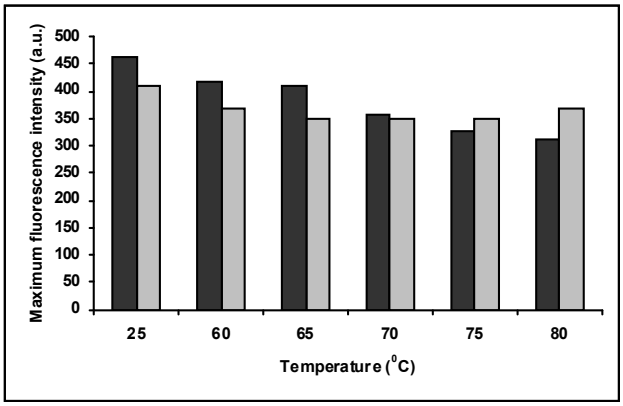

Figure 2. *Heat-induced structural changes of apo-α-LA in the absence (dark) and presence (grey) of calcium chloride as monitored by maximum fluorescence intensity*

This process persists in the U form of the protein, but is absent in the H conformer. Most of the

change in fluorescence seen in the N ⇌U conversion is due to increase in yield of Trp^{28}, while the changes in fluorescence occurring on formation of the H form are due to exposure of Trp^{63} and elimination of its quenching and/or excited state transfer from 28 to 109.

The maximum emission wavelength of the intrinsic fluorescence spectrum of the protein (λ_{max}) was used to follow the structural changes of the protein induced by temperature. Partially folded states are expected to have λ_{max} lying between that of the native and fully denatured state (Demarest *et al.*, 1999). The maximum emission of tryptophan in the absence of calcium was red-shifted from 343 nm to 346 nm with increasing temperature up to 75°C, and then blue-shifted to 342.5 nm at higher temperature (80°C). These results indicated that tryptophan residues are more solvent exposed in the apo- form and, therefore, reflect a conformational change in the protein caused by the removal of the calcium bound to the protein.

This indicates the possible release of intramolecular quenching of tryptophan fluorescence and is associated with a displacement of the emission maximum towards higher wavelengths. A shift in the wavelength of maximum fluorescence emission toward higher wavelengths, caused by a shift in the excitation wavelength toward to the red edge of the absorption band arise due to slow rates of solvent reorientation around an excited state fluorophore. This depends on the motional restriction imposed on the solvent molecules in the immediate vicinity of the fluorophore (Chaudhuri *et al.*, 2010).

In the presence of calcium, e very small red shift was observed with increasing temperature (1-1.5 nm). Since λ_{max} of 354 nm corresponds to a fully denatured protein, the protein is close to complete denaturation when heating at 75°C in the absence of calcium ions and at 60^0C in the presence of 5 mM $CaCl_2$ at very low pH.

However, the Trp fluorescence emission maximum of the protein in the presence of Ca^{2+} was 342.5 nm, identical to it value measured in the absence of cations. These results suggest that α-LA also forms a molten-globule like state at very low pH in the presence of calcium.

It seems that in the presence of 5 mM $CaCl_2$ lowers the transition temperature of protein unfolding. It has been shown that, when α-LA binds less than 1 mol of calcium (or other divalent cation) per mole of protein, the thermal transition temperature of this apo-α-LA decrease to about 35^0C (Relkin, 1996). Addition of calcium ions to the apo-α-LA increases the transition temperature to about 66^0C (Relkin et al., 1993).

There is a distinct correlation between α-LA stability and the Ca^{2+} association constant (Bratcher and Kronman, 1984), namely, that the increased sensitivity to pH and ionic strength below pH 5.2 is due to a decrease in the Ca^{2+} binding affinity. Such effects appear more pronounced in the case of apo-α-LA as a consequence of changes in the degree of ionization of the carboxyl groups that are ordinarily involved in coordination of the Ca^{2+} cation at the metal binding site in holo-α-LA.

Acrylamide quenching experiments

To estimate the degree of exposure of tryptophan residues involved in the different states of apo-α-LA, the tryptophan fluorescence intensity at different condition and temperatures was measured in the presence of acrylamide. Acrylamide quenches the fluorescence of surface exposed and partially buried tryptophan residues but not those buried in the hydrophobic core of a protein. Therefore, acrylamide quenching can be successfully used to provide insights into conformational changes of proteins by probing the solvent accessibility of fluorescent moieties (Eftink and Selvidge, 1982).

Quenching titrations were performed with sequentially added aliquots of acrylamide solutions to protein samples. The peak wavelength of the fluorescence spectra was found not to be shifted significantly by the presence of acrylamide, suggesting that the protein conformation is not affected by acrylamide (data not shown).

The extent of quenching and hence the value of K_{SV} depends on the degree to which the quencher achieves the encounter distance of the fluorophore. Acrylamide can penetrate both hydrophobic as well as hydrophilic regions of the protein and this is indicated by the linear plot of F_0/F, against [Q]

suggesting that all four tryptophan residues of apo-α-LA are equally accessible to the quencher.

Figure 3 shows Stern-Volmer plots for the tryptophan fluorescence quenching of apo-α-LA by acrylamide in the absence and respectively in the presence of 5 mM $CaCl_2$. Stern–Volmer constants (K_{SV}) were obtained from the slope of each linear relationship.

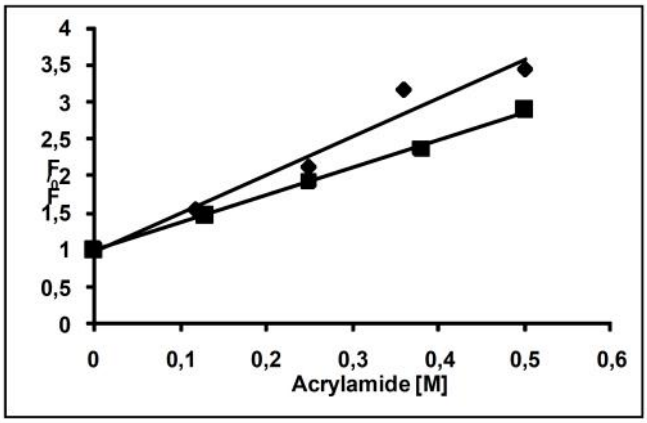

Figure 3. *Acrylamide quenching of tryptophan fluorescence of apo-α-LA in the absence (square) and in the presence of $CaCl_2$ (diamonds)*

K_{SV} values for the protein was found to be 3.56±0.9 mol^{-1} L for the experiments performed in the absence of calcium ions and 5.23±0.85 mol^{-1} L for the experiments performed in the presence of 5 mM $CaCl_2$, respectively. Sharma and Kishore (2008) suggested K_{SV} value of 1.97 mol^{-1} L for the native protein at neutral pH, whereas a value of 7.16 mol^{-1} L was reported for denatured protein in 8.5 mol^{-1} L urea.

Figure 4 shows the K_{SV} values calculated after heat treatment. Analyzing the results, one can see significant increase in the solvent accessibility of tryptophan residues in the presence of cations up to 60^0C (6.69±0.95 mol^{-1} L), whereas in the absence of $CaCl_2$, the K_{SV} values are significant higher in the higher temperature range.

The K_{SV} values were obtained from the slope of the linear dependence of F_0/F versus the free concentration of acrylamide. The data with error bars were expressed as mean ± S.D. (n= 3).

When heating at 80°C in the absence of calcium, a significant increase in K_{SV} from 3.56±0.9 mol^{-1} L to 6.76±0.4 mol^{-1} L was observed, which is almost 1.9 times higher compared to the un-treated protein. The observed K_{SV} in these conditions suggest the protein has an intermediate conformation in between native and denatured state.

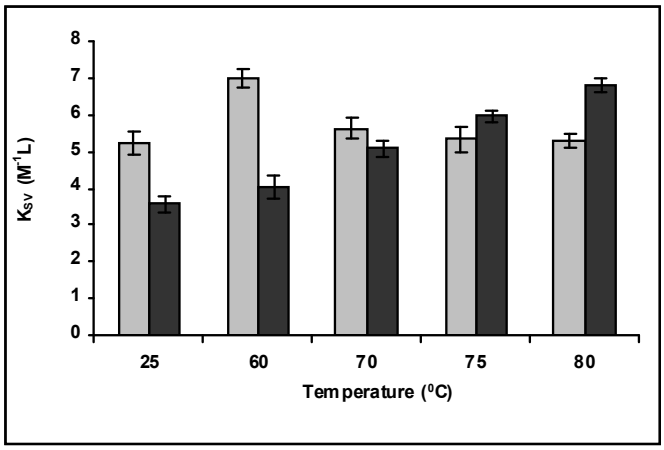

Figure 4. *The Stern–Volmer quenching constants (K_{SV}) of apo-α-LA fluorescence in the absence (dark) and presence (grey) of calcium chloride as a function of temperature*

These findings suggest that in the un-treated state the tryptophan residues are shielded from the quencher to the highest degree compared with the denatured state.

Conclusions

In this study, some fluorescence spectroscopy techniques were used to monitor the heat induced changes in terms of conformation of bovine α-LA in the absence and presence of calcium chloride. The heat treatment caused the decrease of intrinsic fluorescence of the protein in the absence of Ca^{2+}, whereas a slightly increase in fluorescence was observed when heating at 80^0C in buffer with 5 mM $CaCl_2$. Our results therefore constitute one of the first reports of the K_{SV} values of α-LA in different state.

Further studies are needed in order to elucidate the influence of cations addition on the protein behavior during heat treatment. These specific and complex issues are currently developed in our laboratories (www.bioaliment.ugal.ro) within the SPD-BIOTECH Project (www.spd.biotech.ugal.ro).

Acknowledgement

This work has benefited from financial support through the 2010 POSDRU/89/1.5/S/52432 project, *Organizing the national interest postdoctoral school of applied biotechnologies with impact on Romanian bioeconomy*, project co-financed by the European Social Fund through the Sectoral Operational Programme Human Resources Development 2007-2013.

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