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Stability of equine lysozyme

I. Thermal unfolding behaviour

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Abstract

The thermal denaturation of Ca^{2+}- and apo-forms of equine lysozyme was followed by using far and near UV circular dichroism and intrinsic fluorescence methods. The difference found between the temperature dependence of the ellipticity at 222 nm and 287 nm, which show two stages in the thermal transition, and those at 228 nm and 294 nm, which indicate only one stage over a wide range of temperatures reflects that different subdivisions of the protein molecule are characterized by a different stability, cooperativity and pathway of denaturation. The first transition, reflected in the increase of the ellipticity at 222 nm and 287 nm, coincides with the transition detected by fluorescence and occurs at 30–50°C for the apo-form and at 50–60°C for the Ca^{2+}-form of lysozyme. It seems to correlate with the transfer of some tryptophan residues to a more hydrophobic environment and with a local rearrangement of the tertiary and secondary structures. The unfolding transition detected by the decrease of the ellipticity at all wavelengths occurs nearly in the same temperature region for the apo- and Ca^{2+}-forms, i.e. 50–80°C and 55–80°C, respectively. The presence of a Ca^{2+}-binding loop in equine lysozyme may be partly responsible for the drastic destabilization of its structure as a whole both in the presence but especially in the absence of Ca^{2+} in comparison with hen and human lysozymes.

Keywords: Equine lysozyme; Thermal denaturation; Circular dichroism; Fluorescence

Introduction

Equine lysozyme belongs to the family of c-type lysozyme-α-lactalbumin proteins which are characterized by remarkable homologies in the sequences and in the three-dimensional structures [1–5]. In contrast to most c-type lysozymes equine lysozyme is a Ca^{2+}-binding protein like all α-lactalbumins [6–8]. The binding site includes three aspartate residues (Asp 86, 91 and 92) contributing their side chain carboxylate groups to ligand Ca^{2+} and Lys 83 and Asn 88 contributing two backbone carbonyl groups (the residue numbering is based on the equine lysozyme numbers of Fig. 1 in Ref. [1]). As a consequence of the binding property the following question can be put forward: how does the presence of the Ca^{2+}-loop influences the structure and stability of the

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equine lysozyme molecules at whole in comparison with the corresponding properties of non-Ca^{2+}-binding lysozymes and Ca^{2+}-binding α-lactalbumins. The latter proteins demonstrate during GuHCl denaturation an equilibrium intermediate named "molten globular" with unfolded tertiary structure and native-like secondary structure [9–11].

The present paper is devoted to the investigation of the thermal unfolding transition of equine lysozyme in the presence and absence of Ca^{2+}. The thermal denaturation of hen and human lysozymes and bovine α-lactalbumin can be described by a two-state transition model as it has been shown both by microcalorimetry and spectroscopic methods [12–16]. Hen and human lysozymes are very stable proteins, at pH 4–4.5 their thermal unfolding occur at temperatures higher than 70°C [12–14]. However, the thermostability of bovine α-lactalbumin drastically depends on the binding of Ca^{2+} by the protein [15,16]. Kuroki et al. [17] have shown that the creation of the Ca^{2+}-binding site in human lysozyme by replacing Gln 86 and Ala 92 with aspartate residues, stabilizes this protein against protease digestion and thermal denaturation in comparison with the wild type lysozyme: if the latter one reaches a maximum activity at 70°C then for the Asp 86/92 apo- and the Ca^{2+}-loaded mutants 65°C and 80°C are found, respectively.

In the present work we used far UV and near UV circular dichroism and intrinsic fluorescence, to follow the changes of the protein tertiary and secondary structures upon heating.

2. Materials and methods

Equine lysozyme was isolated from horse milk, purified and decalcified as described before [6,18]: the Ca^{2+} content was 0.03 mole Ca^{2+} per mole of protein. For the measurements of protein concentration the extinction coefficient at 280 nm for equine lysozyme was $E_{1\%} = 23.5$ [7].

The circular dichroism measurements were performed on a Jasco J-600 A spectropolarimeter [19]. The fluorescence spectra were registered with an Aminco SPF-500 spectrofluorimeter. The ratio of fluorescence intensities at a fixed wavelength on the wings of the spectrum (I_{370}/I_{330}) was used to evaluate the spectrum position changes. The effect of trivial thermal quenching of the emission upon heating can be eliminated by the method of analysis of Bushueva et al. [20].

In all measurements hen egg-white lysozyme was taken as a reference protein to test the validity of our approaches and to compare its properties with those of equine lysozyme. For hen lysozyme transitions detected by CD and fluorescence methods occur in the same temperature region. Van 't Hoff thermodynamic parameters, calculated from thermal dependencies of CD ellipticities at different wavelengths coincide with each other and with the data in the literature [12–14].

Lysozymes tend to aggregate at neutral pH upon heating. In the case of equine lysozyme preliminary experiments of changes of scattered light showed that the apo- or Ca^{2+}-forms of this protein did not aggregate at pH 4.5 while the fluorescence intensities at 350 nm and 370 nm did not show any pH dependence between pH 3 and 8. Therefore, the study of the thermal denaturation was carried out at pH 4.5, a condition for which we can be sure that the equine lysozyme exists in the Ca^{2+}-form in the presence of 10 mM $CaCl_2$.

3. Results

Figure 1 shows the plot of the reciprocal fluorescence intensity at 360 nm versus T/η (temperature, in K/viscosity, in cP) pH 4.5 for the thermal denaturation of the apo- and Ca^{2+}-forms of equine lysozyme and hen egg-white lysozyme: thermal transitions occur at 33–40°C, 50–57°C and 72–80°C respectively. The fluorescence intensity ratio I_{370}/I_{330} decreases (blue shift of spectrum) in the low temperature parts of the plots up to 50°C and 60°C for the apo- and Ca^{2+}-forms of equine lysozyme respectively and increases (red shift of spectrum) at higher temperatures (data not shown).

The near and far UV CD spectra of the Ca^{2+}-

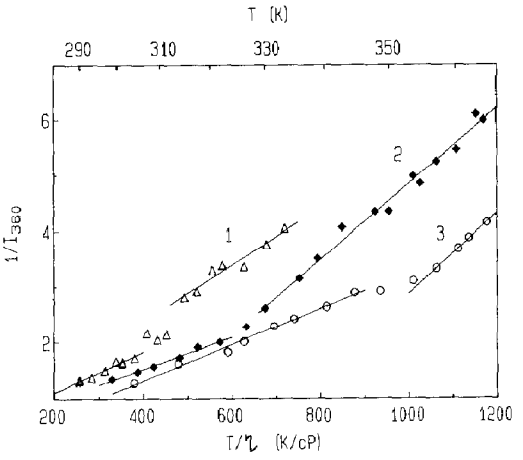

Fig. 1. The dependence of reciprocal fluorescence intensity at 360 nm vs. T/η (T–temperature, η–solvent viscosity). Conditions: 10 mM Na-acetate, 70 mM NaCl, pH 4.5, protein concentrations: 0.2 mg ml^{-1}. (1) Equine lysozyme apo-form; (2) equine lysozyme Ca^{2+}-form, 10 mM $CaCl_2$; and (3) hen egg-white lysozyme.

loaded and apo-equine lysozyme at pH 4.5 are presented in Figs. 2 and 3. They correlate very well with the spectra of equine lysozyme at pH 7.5, reported previously [7], but in the region of 275–285 nm we additionally detected the fine

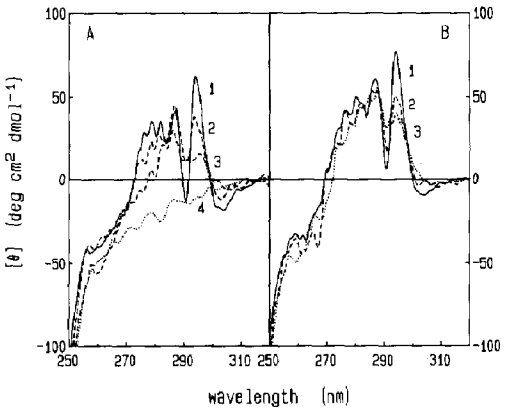

Fig. 2. The near UV CD spectra of equine lysozyme as a function of temperature. Conditions: 10 mM Na-acetate, 70 mM NaCl, pH 4.5, Protein concentration: 0.6 mg ml^{-1}. (A) Ca^{2+}-form, 10 mM $CaCl_2$ at (1) 18.8°C, (2) 19°C after the cooling of the unfolded protein, (3) 62°C, and (4) 87°C. (B) Apo-form at (1) 18°C, (2) 18°C after the cooling of the denatured protein, and (3) 48°C.

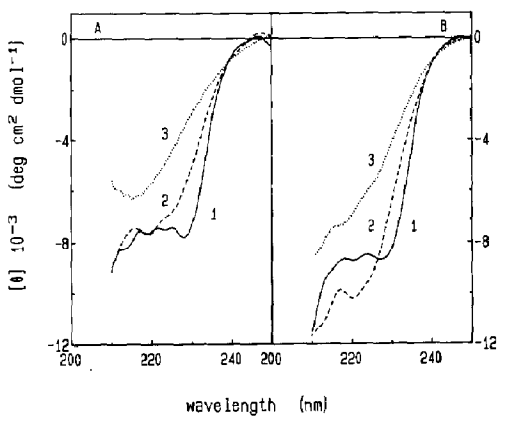

Fig. 3. The far UV CD-spectra of equine lysozyme at different temperatures. Conditions: 10 mM Na-acetate, 70 mM NaCl, pH 4.5, protein concentration: 0.6 mg ml^{-1}. (A) Ca^{2+}-form, 10 mM $CaCl_2$ at (1) 22.4°C, (2) 62°C, and (3) 85.3°C. (B) Apo-form at (1) 18.4°C, (2) 48°C, and (3) 78.5°C.

structure of the spectrum, consisting of three little but reproducible maxima which are more distinct in the presence of Ca^{2+}.

The Ca^{2+}- and apo-lysozyme spectra at 18°C are very similar. The minima and maxima are found at the same wavelengths. However, in the region between 270 and 310 nm, the apo-spectrum is clearly shifted towards more positive ellipticity values. In the far UV a reduction of the negative ellipticity in the region of 210–230 nm is observed in the presence of Ca^{2+}. These observations are in close agreement with those published before [7].

Figures 4 and 5 show the temperature dependencies at pH 4.5 of the ellipticities at 287 (289) nm, 294 nm, 222 nm and 228 nm for the apo- and Ca^{2+}-forms of equine lysozyme respectively. The plots of the ellipticities at 287 or 289 nm and 222 nm demonstrate two transitions occurring during the thermal denaturation in the absence and presence of Ca^{2+}. In contrast the ellipticities at 228 nm and 294 nm show only one stage.

All plots of optical activity demonstrate the pre-denaturational quenching of the CD signal as it was observed for hen lysozyme (data not shown). For apo-lysozyme the slope of the diminution of the ellipticity at 294 nm at temperatures below 50°C is pronounced and perhaps reflects the gradual unfolding of the molecule conformation.

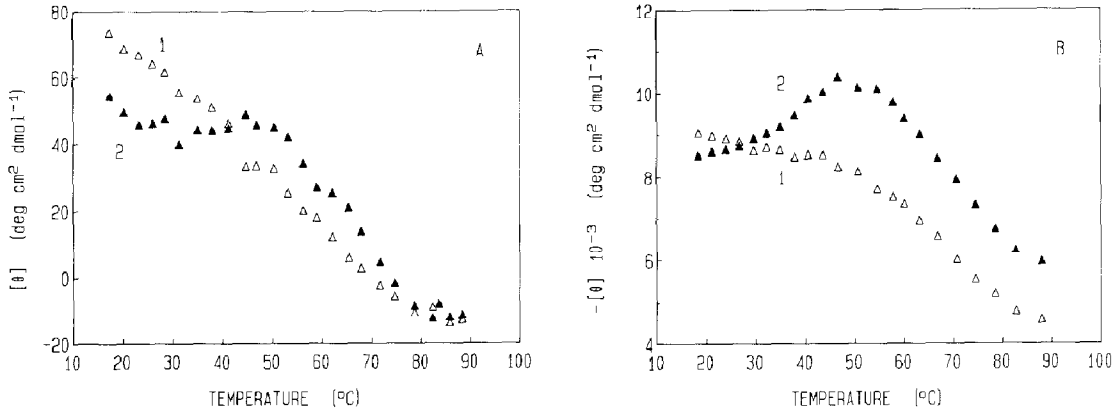

Fig. 4. Temperature dependence of the ellipticity of the apo-form of equine lysozyme. Conditions: 10 m*M* Na-acetate, 70 m*M* NaCl, pH 4.5, protein concentration: 0.6 mg ml^{-1}. (A) In near UV at 294 nm (1), and at 289 nm (2). (B) In far UV at 228 nm (1), and at 222 nm (2).

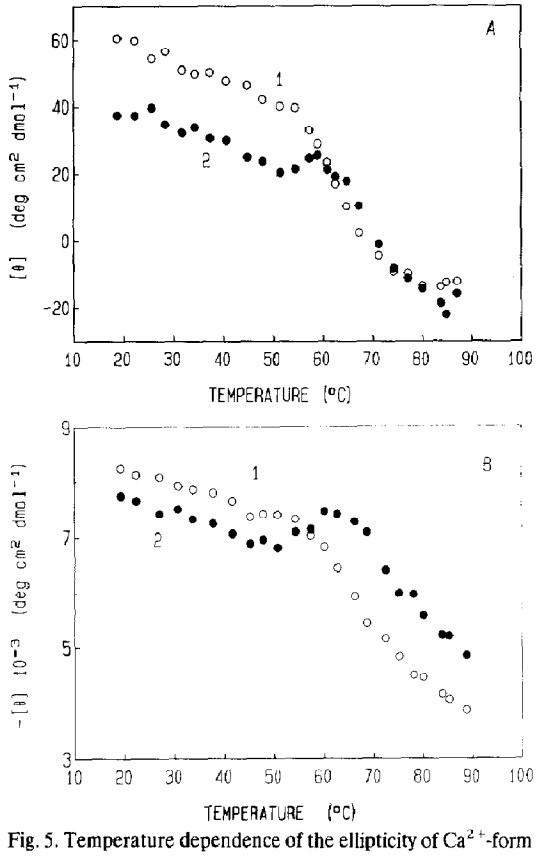

Fig. 5. Temperature dependence of the ellipticity of Ca^{2+}-form of equine lysozyme. (A) In near UV at 294 nm (1), and at 287 nm (2). (B) In far UV at 228 nm (1), and at 222 nm (2). Conditions as in Fig. 4.

For the apo-protein, a careful analysis of the 289 nm and 222 nm data shows a first transition which coincides with an increase of the optical activity between 30 and 50°C and an estimated mid-point of transition, $T_m = \pm 45$°C, while the second transition correlates with a diminution of the optical activity above 50°C. In the presence of Ca^{2+}, the first transition starts at 50 °C with T_m being ± 57°C and a second transition occurs above 60°C. For the 294 nm and the 228 nm data, either in the absence or presence of Ca^{2+}, only one transition of unfolding is observed starting at 50 ° and 55°C, respectively. This transition represents the unfolding of the protein as a whole: we therefore use it to compare the unfolding of Ca^{2+}- and apo-equine lysozyme with both hen and human lysozymes. In Fig. 6 we compare the thermal denaturation of hen, human, apo- and Ca^{2+}-equine lysozyme as measured by the ellipticity at 228 nm: the transitions for equine lysozyme are clearly less cooperative and occur at a T_m of 70°C.

It is important to note that the end of the first transition and the beginning of the second one are superimposed at about 47–50°C and 60–62°C for the apo- and Ca^{2+}-forms, respectively. We consider that these temperature regions coincide with the existence of some kind of intermediate

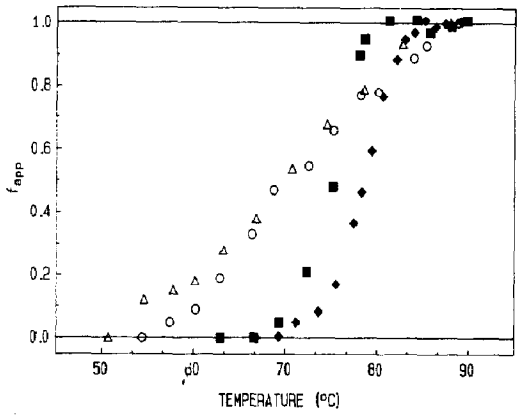

Fig. 6. The thermal denaturation of hen (■), human (◇), apo (△) and Ca^{2+}- (○) equine lysozyme. The degree of unfolding of the protein (f_{app}) is measured by the ellipticity at 228 nm as a function of temperature. Conditions as in Fig. 4. (The data of the unfolding for equine lysozyme followed at 294 nm and for hen and human lysozymes followed at 289 nm, coincide with those at 228 nm but have not been plotted here).

state in the general process of the equine lysozyme unfolding.

Figures 2 and 3 show the CD spectra of these intermediate states in the absence (48°C) and presence of Ca^{2+} (62°C). In the absence of Ca^{2+} the near UV spectrum (Fig. 2B) is only slightly different from the spectrum at 18°C, except for the Cotton effects at 294 and 305 nm which change markedly and the minimum at 290 nm which practically disappears. The far UV spectrum demonstrates a pronounced enhancement of the band in the 210–227 nm region and a reduction of the CD signal in the 227–237 nm range compared to the spectrum at 18°C. In the presence of Ca^{2+} the near UV spectrum at 62°C is drastically reduced compared to 18°C, although the fine structure in the spectral region 270–280 nm is still present. In the far UV spectrum the value of the ellipticities in the 223–240 nm region is increased but remains nearly constant in the 218–222 nm region.

Figures 2 and 3 also show the CD spectra of the denatured protein at 78–87°C: the tertiary structure is unfolded but the secondary structure is only partially unfolded because the negative CD band in the far UV is still pronounced. The thermal denaturation of equine lysozyme is nearly but not fully reversible. The fact, however, that the end of the first transition and beginning of the second one superimpose, prevents us from calculating the effective parameters of the denaturation as can be done for hen egg white lysozyme.

4. Discussion

The thermal stability of hen egg white and human lysozymes is high: the midpoints of transitions, T_m, are 76°C and 78°C respectively, while the unfolding itself is a two-state highly cooperative transition of both tertiary and secondary structure (see Fig. 6). Both lysozymes behave very similar although they differ by 41% in primary structure. Equine lysozyme also differs by 40% in sequence from both former lysozymes [1]: however, it has one distinct property, namely the presence of a Ca^{2+}-binding loop, nearly identical to the "elbow" described in α-lactalbumin [8].

Very few data are available on equine lysozyme: the binding constant for Ca^{2+} was determined [6] and from thermodynamic results [7] we concluded that apo-equine lysozyme is in a partially unfolded state (U) at 25°C and ionic strength 0.01 of the buffer. The argument for this was that binding of Ca^{2+} to the apo-state was accompanied by a large exothermic enthalpy change, suggesting a conformational change. In this respect, apo-equine lysozyme resembles apo-bovine α-lactalbumin. The CD spectra under different experimental conditions were also published [7]. However, no interpretation of the different maxima present in the near UV-spectrum of equine lysozyme ([7] and Fig. 2 this paper) is available yet which makes an unambiguous explanation of the thermal phenomena observed in this paper impossible. However, a tentative explanation of the spectrum can be proposed on the basis of the CD-spectrum of hen lysozyme, extensively studied by Cowburn et al. [21]. The spectrum in the region 270–300 nm has been attributed to tryptophan residues. In hen lysozyme, Ikeda et al. [22] assigned the negative CD band at 305 nm to Trp 109. If the same is true in equine lysozyme then its environment depends on the Ca^{2+}-binding of the protein, because the negative

Cotton effect at 305 nm increases upon Ca^{2+}-binding. In contrast, the ellipticity in the 270–300 nm region decreases upon binding. Therefore Trp 109 cannot be the main contributor to this band at the same time, which agrees with the conclusion of Tanaka et al. [23] concerning the hen lysozyme spectrum. Trp 28 and Trp 63 too cannot be responsible since the first one is located to far from the Ca^{2+}-binding loop while oxidation of Trp 63 does not change the spectrum [23]. This leaves only Trp 64 and Trp 112 as possible contributors to the 287 nm and the 294 nm CD peaks.

With these data in mind, the following conclusions concerning equine lysozyme can be derived from the results presented in this paper. First, it is clear from the general appearance of the unfolding curves measured by tryptophan fluorescence (Fig. 1) and CD in the far and near UV (Figs. 4–6) that apo- and Ca^{2+}-equine lysozyme have a lower thermal stability than both hen and human lysozymes, with the apo-form being the least stable. Moreover, the cooperativity of the unfolding is much lower. Secondly, the CD data demonstrate that the thermal unfolding of equine lysozyme is not a simple two state highly cooperative process as observed for hen and human lysozymes. The thermal denaturation for the apo- and Ca^{2+}-form is characterized by two consecutive transitions when measured at 222 nm and 287 nm and only one, when measured at 228 and 294 nm. The first transition is characterized by an increase in optical activity in the near (287 nm) and far UV (222 nm), a Ca^{2+} dependent T_m, a shift to shorter wavelength of the tryptophan fluorescence (data not shown) and a change in tryptophan fluorescence intensity (Fig. 1). While the latter points confirm our conclusion that tryptophan residues are responsible for the Cotton effect at 287 nm, the short wavelength shift accompanying this first transition suggest a transfer of some tryptophan residues to a more hydrophobic environment. If it is accepted that an optical activity arises most commonly from the internal residues which have no free rotation [24,25], then an enhancement of the CD ellipticity in the near UV, as observed during the first transition, suggests that some tryptophan residues change their environment to make more hydrophobic contacts. The fact that this transition is detected at 287 nm and not at 294 nm is indicative of the fact that the transition occurs in one part of the equine lysozyme molecule while the tryptophan residues responsible for the ellipticity at 294 nm do not sense any change in their environment.

The increase of the ellipticity in the 222 nm zone (and decrease around 228 nm) during the first unfolding transition cannot be interpreted unambiguously. Indeed, the α-helix and β-sheet give a pronounced contribution to the CD spectrum at 222 nm but the magnitude of their contributions are less important at 228 nm [26]. Moreover, to quantify the changes in the multiple classes of secondary structure accurate experimental data are needed to as low as 180 nm [26], which is not the case for our present results.

All these observations suggest that we are not dealing with a three-state transition with an intermediate involving the protein in globo such as in the case of α-lactalbumin GuHCl denaturation. We propose that at the end of the first transition (Figs. 4 and 5) an intermediate state (U′) is present which is not denaturated since it is characterized by a pronounced spectrum in the near and far UV (Figs. 2 and 3). The intermediate is a consequence of a temperature induced and Ca^{2+}-dependent change in both secondary and tertiary structure in one part of the lysozyme molecule which is more vulnerable to a temperature increase. The lysozyme molecule is divided in two halves by a crevice [27]: in one halve the β-structured fragments are situated whereas in the second halve the N-terminal and C-terminal fragments are located. The first transition detected at 287 nm is Ca^{2+}- dependent and since the Ca^{2+}-binding loop (83–93) is situated in the first halve, it is most likely that the first transition occurs in this part of the molecule and that Trp 64 is the most important contributor at 287 nm and not Trp 112.

The second thermal transition detected by the decrease of the ellipticity at 287 nm and 222 nm correlates with the decrease of the ellipticity at 294 nm and 228 nm (Figs. 4 and 5). These diminutions of the CD signal at all wavelengths reflect the unfolding of the tertiary and secondary

structure of the equine lysozyme molecule as a whole, similar to the thermal denaturation observed for hen and human lysozymes (see also Fig. 6). The red shift of the fluorescence spectrum or the increase of the ratio I_{370}/I_{330} respectively (data not shown), for both the Ca^{2+}- and apo-equine lysozyme undoubtedly are caused by a thermal denaturation of the protein structure as a whole. The results of Fig. 6 demonstrate quite conclusively that equine lysozyme is destabilized drastically compared to hen and human lysozymes. The cooperativity is much lower: although a calculation of a transition enthalpy for equine lysozyme from the data in Fig. 6 is not permitted since the denaturation does not occur according to a pure two-state model, the comparison of the slopes of the unfolding transitions is a confirmation of the decreased cooperativity.

In conclusion, we believe that the presence of the Ca^{2+}-binding loop in equine lysozyme is mainly responsible for the lower stability of the protein as a whole compared with hen and human lysozymes and the presence of two consecutive transition steps in the thermal denaturation. The independent unfolding behaviour of different parts of equine lysozyme in the presence and absence of Ca^{2+} strongly supports the idea that the protein molecule consists of structural units folded independently [28]. For equine lysozyme we propose a equilibrium structural intermediate which is the consequence of a rearrangement of a local conformation or a Ca^{2+}-dependent part of the protein globule, in contrast with the model of a "molten globule" intermediate state of α-lactalbumin [9–11].

Acknowledgments

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