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Two steps in the transition between the native and acid states of bovine α-lactalbumin detected by circular polarization of luminescence: Evidence for a premolten globule state?

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Abstract

A few studies indirectly support the existence of an intermediate in the transition of Ca^{2+}-saturated bovine α-lactalbumin (α-LA) from the native (N) to the acidic (A) state, known as the molten globule state. However, direct experimental evidence for the appearance of this intermediate has not been obtained. The signal of circular polarization of luminescence (CPL) is sensitive to fine conformational transitions because of its susceptibility to changes in the environmental asymmetry of fluorescent chromophores in their excited electronic states. In the present study, CPL measurements were applied using the intrinsic tryptophan fluorescence of α-LA as well as the fluorescence of 8-anilino-1-naphthalenesulfonic acid (ANS) bound to α-LA. CPL of tryptophan and ANS was measured in the pH range of 2.5–6 in order to find direct experimental evidence for the proposed intermediate. CPL (characterized by the emission anisotropy factor, g_{em}) depends on the asymmetry of the protein molecular structure in the environment of the tryptophan and the ANS chromophores in the excited electronic state. The pH dependence of both the g_{ab}, absorption anisotropy factor determined by CD, and the ANS steady state fluorescence, showed a single transition at pH 3–3.7 as already reported elsewhere. This transition was interpreted as being a result of a change of the α-LA tertiary structure, which resulted in a loss of asymmetry of the environment of both the tryptophan residues and the ANS hydrophobic binding sites. The pH dependence of the tryptophan and ANS g_{em} showed an additional conformational transition at pH 4–5, which coincided with the pK_a of Ca^{2+} dissociation (pK_a 5), as predicted by Permyakov et al. (1981, *Biochem Biophys Res Commun 100*:191–197). The titration curve showed that there is a pH range between 3.7 and 4.1 in which α-LA exists in an intermediate state between the N- and A-state. We suggest that the intermediate is the premolten globule state characterized by a reduced Ca^{2+} binding to the α-LA, native-like tertiary structure, and reduced asymmetric fluctuation of the tertiary structure on the nanosecond time scale. This intermediate resembles the "critical activated state" theoretically deduced by Kuwajima et al. (1989, *J Mol Biol 206*:547–561). The present study demonstrates the power of CPL measurements for the investigation of folding/unfolding transitions in proteins.

Keywords: absorption anisotropy factor; ANS fluorescence; circular dichroism; emission anisotropy factor; molten globule state; protein folding

Ca^{2+}-saturated bovine α-lactalbumin has a pH-dependent transition from the native (N) to acidic (A) form. The A-state of α-LA is well characterized as a molten globule state (Kuwajima, 1989; Ewbank & Creighton, 1991; Chyan et al., 1993; Creighton & Ewbank, 1994; Peng & Kim, 1994; Peng et al., 1995). The MG-state of proteins is characterized by ordered native secondary structure, similar to the N-state, and disordered tertiary structure. In this conformation, the protein molecule has a compact structure, but lacks the dense packing of side chains (Ptitsyn, 1987; Kuwajima, 1989). The loss of packing of the tertiary structure was detected in particular by the reduced molar ellipticity in the near-UV range (CD of aromatic residues). Hence, the decrease of the molar ellipticity at 270–275 nm was used as a test of the N → MG transition.

Ptitsyn, Kuwajima, and coworkers employed a series of conformationally sensitive methods in order to show that the N → A transition in α-LA is an N → MG transition (Ptitsyn, 1987;

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Abbreviations: ANS, 8-anilino-1-naphthalenesulfonic acid; ANS-CPL, CPL related to ANS fluorescence; ANS-g_{em}, emission anisotropy factor related to ANS binding; α-LA, bovine α-lactalbumin; CPL, circular polarization of luminescence; MG, molten globule; preMG, premolten globule; Trp-CPL, CPL related to tryptophan residues; Trp-g_{em}, emission anisotropy factor related to tryptophan residues.

Kuwajima, 1989). It was also shown that the MG-state in α-LA can be produced by removal of the bound Ca^{2+} ion at low ionic strength and above room temperature. It was proposed (Kuwajima, 1977; Kuwajima et al., 1981, 1989; Kronman & Bratcher, 1984), that the N → A transition of Ca^{2+}-α-LA and apo-α-LA is not a simple two-state transition, but that another intermediate state is stabilized at some point in the pH titration. Moreover, Lala and Kaul (1992) suggested that the intermediate of apo-α-LA is a premolten globule. The preMG was thought to be similar to the MG-state, but with partial local packing. A preMG was also postulated for the cytochrome *c* transitions (Jeng & Englander, 1991; Nötling & Sligar, 1993). It is still not known whether the N → A transition is a simple two-state transition. In order to answer this question, one has to apply a multiple probe test. Hence, new methods that are sensitive enough to detect changes in packing of side chains in proteins are required. For this purpose, we used measurements of circular polarization of luminescence. The effectiveness of the CPL measurements in studies of the conformational changes in proteins has been demonstrated by Steinberg and his coworkers (Steinberg, 1978a) and Gafni and coworkers (Schauerte et al., 1992, 1995).

Spontaneous emission (luminescence) of a chiral molecule can be partially circularly polarized: a molecule that exhibits different absorption of left- and right-hand polarized light (CD), is expected to emit circularly polarized light (Steinberg, 1978a). CPL is expressed by the emission anisotropy factor g_{em}, which is the difference between the left- and right-handed circularly polarized light components of the total fluorescence emission. This definition is analogous to the definition of the CD, $\Delta\epsilon = \epsilon_l - \epsilon_r$, which can be expressed by the absorption anisotropy factor g_{ab} (Kuhn, 1958; Steinberg et al., 1974; Steinberg, 1975):

$$g_{ab} = \frac{\mathrm{Im}(A|p|B)(B|m|A)}{|(A|p|B)|^2} = \frac{P_{la} - P_{ra}}{(P_{la} + P_{ra})/2} = \frac{\epsilon_l - \epsilon_r}{\epsilon} \quad (1)$$

$$g_{em} = \frac{\mathrm{Im}(A'|p|B')(B'|m|A')}{|(A'|p|B')|^2} = \frac{P_{le} - P_{re}}{(P_{le} + P_{re})/2} = \frac{\Delta f}{f/2}, \quad (2)$$

where p and m are the electric and magnetic dipole moment operators, respectively; A and B are the quantum states involved in the absorption transitions; A' and B' are the quantum states involved in the emission processes; P_a and P_e are probabilities of left-handed (l) or right-handed (r) circularly polarized absorption (a) and emission (e), respectively; ϵ is the molar extinction coefficient; f is the total luminescence intensity; and Δf is its circularly polarized part. From the definition mentioned above, a negative value for the g_{ab}- or g_{em}-factor shows that right-handed circularly polarized absorption or emission is dominant. When the left-handed portion of the total absorption or emission is larger, the *g*-factor is positive. The CPL method and its multiple applications have been reviewed in detail elsewhere (Richardson & Riehl, 1977; Steinberg, 1978a, 1978b; Riehl & Richardson, 1986).

Thus, CPL reflects a chirality of the chromophore in the excited electronic state in the same way as CD reflects a chirality in the ground electronic state. g_{ab} and g_{em} are characteristic of the electronic wave functions at the equilibrium position of the nuclei in the ground and excited electronic states, respectively, but not to the nuclei wave functions themselves (Steinberg et al., 1974; Steinberg, 1975). The susceptibility of electron density (represented by the electron wave function) to an environmental electric field leads to the susceptibility of the g_{ab}- and g_{em}-factor to changes in the environment of the chromophore in the protein molecule. Obviously, the electron density of the excited electronic state is more expanded and more sensitive to the external electric fields than that of the ground electronic state (Calvert & Pitts, 1966; Barltrop & Coyle, 1975). Because of this, the g_{em}-factor is expected to be more sensitive to small changes in the chirality of the chromophore. Part of the chirality of the chromophore can be induced by the environmental asymmetry. Therefore, changes in the *g*-factor can reflect changes in the environmental asymmetry. g_{ab}, obtained from the CD measurements, depends on the changes of the environmental asymmetry of the chromophore and its changes in its ground electronic state. The CPL measurements (g_{em}) give the corresponding changes for the chromophore in its excited electronic state. Environmental asymmetry of the chromophore is an essential characteristic of ordered protein structures. Therefore, the CPL signal is expected to reflect conformational transitions in a structural subdomain close to a fluorescent probe. The CPL spectroscopy is thus expected to be a sensitive technique for the detection of the folding-unfolding transitions in proteins.

Tryptophan fluorescence is a signal commonly used in protein structural studies (Burstein, 1977; Lakowicz, 1983; Permyakov, 1993), and has been widely used in the study of α-LA (Bell et al., 1975; Sommers & Kronman, 1980; Kronman et al., 1981; Permyakov et al., 1981; Murakami et al., 1982; Murakami & Berliner, 1983; Kronman & Bratcher, 1984).

Another spectroscopic probe that was used in the search for the MG-state in proteins is the hydrophobic molecule 8-anilino-1-naphthalenesulfonic acid. By steady-state fluorescence spectroscopy it was shown that ANS is bound to the α-LA molecule in the A-state. The binding can be monitored by the two orders of magnitude increase in its blue fluorescence (Mulqueen & Kronman, 1982; Fitzgerald & Swaisgood, 1989). The fluorescence intensity of the ANS-α-LA mixture is significantly enhanced in the N → A transition. This effect is probably the result of a tight ANS-α-LA interaction in the less-ordered conformational state that makes the hydrophobic core accessible to penetration and binding of nonpolar molecules (Mulqueen & Kronman, 1982; Kuwajima, 1989; Semisotnov et al., 1991).

In the present study, the circular polarization of tryptophan fluorescence of Ca^{2+}-α-LA was measured in order to test the hypothesis that the N → A transition is not a simple two-state transition. CPL of bound ANS was also introduced as an additional probe for investigating N → A transition. An additional transition in the pH range of 4–5 was found. This is thought to be a transition from the N to an intermediate that can be considered as a preMG-state.

Results

Tryptophan CPL and CD spectra

The tryptophan CPL spectrum of an α-LA solution in the native Ca^{2+}-saturated state (pH 5.3, 2.7 mM $CaCl_2$) showed values of $g_{em} = 3 \times 10^{-4}$. This value was slightly wavelength-dependent in the range of 320–360 nm (Fig. 1). At 310 nm, g_{em} seemed to be increased, but with high uncertainty because of the low fluorescence intensity. A higher value of g_{em} at 310 nm (relative

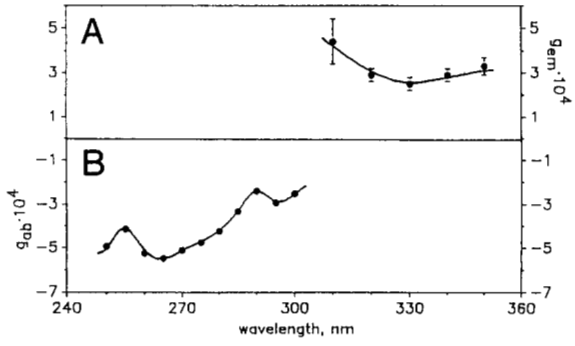

Fig. 1. Spectra of the absorption anisotropy factor g_{ab} **(B)** and the emission anisotropy factor g_{em} **(A**; excitation at 280 nm) of bovine α-lactalbumin in 10 mM phosphate buffer, pH 5.3, and 2.7 mM $CaCl_2$.

to the values in the 320–360 nm range) may be caused by a higher contribution of tyrosine fluorescence to the total CPL signal. The absorption anisotropy factor, g_{ab}, of α-LA under the same conditions showed negative values. The wavelength dependence of the g_{ab}-factor was in general similar to that of the CD spectrum in the near-UV range (Ewbank & Creighton, 1991; Chyan et al., 1993; Peng & Kim, 1994; Peng et al., 1995).

The difference in the orientational distribution of the molecules in the ground and excited electronic states, known as photoselection, is zero when the absorption and emission dipole moments are parallel (Steinberg, 1978a). Photoselection can generate an artifactual CPL signal in a sample taken from a nonrandomly oriented population of chromophores. Such a sample can preferentially absorb one of the two circularly polarized components of the incident light, even when the excitation beam is nonpolarized. With reference to the optical alignment of our instrument, the angle between the excitation beam and the direction of fluorescence collection was 180°. Under this setting, no photoselection was possible (Steinberg, 1978b). Other evidence for this is presented below.

pH dependence of tryptophan CPL and CD

The tryptophan emission anisotropy factor g_{em} contained additional information on the N → A transition that was not detected by the CD measurement. Figure 2 shows the results of the pH dependence of the anisotropy factors in both absorption (g_{ab}) and emission (g_{em}) throughout the N → A transition. The main observation is the detection of a conformational transition with a $pK_{a1}(g_{em})$ value of 4.9. This transition is in addition to the transition with a $pK_{a2}(g_{em})$ value of 2.9, which was also detected in the CD measurement with a $pK_a(g_{ab})$ of 3.1 at 272 nm and 3.0 at 292 nm.

The significance of the differences in the g_{em} values in the three pH ranges is demonstrated in Table 1. The two pH-transitions detected by the emission anisotropy factor g_{em}, could not be accounted for by a change of photoselection for the simple reason that there were no changes in $g_{ab}(272)$ or $g_{ab}(292)$ when $g_{em}(340)$ was reduced from $(3.0 \pm 0.4) \times 10^{-4}$ to $(1.5 \pm 0.3) \times 10^{-4}$ during the titration from pH 5.5 to pH 4.

At a pH < 3, the absorption anisotropy factor g_{ab} at 272 nm was small, but still negative, whereas $g_{ab}(292)$ nm was also small, but of a positive value. There were probably both tryptophan and tyrosine contributions in the g_{ab} at 272 nm, whereas mainly tryptophan contributed the g_{ab}-factor at 292 nm.

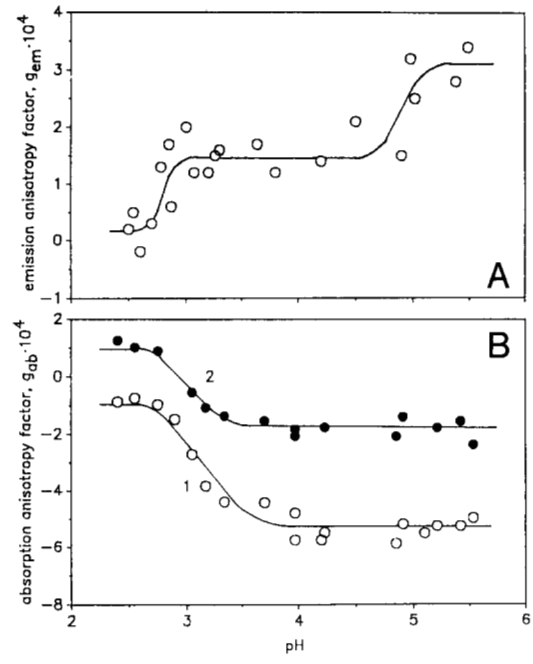

Fig. 2. pH dependence of absorption anisotropy factor g_{ab} at 272 **(B1)** and 292 nm **(B2)** and emission anisotropy factor g_{em} at 340 nm **(A)** of bovine α-lactalbumin in 2.7 mM $CaCl_2$.

pH dependence of ANS-CPL

Figure 3 shows the pH dependence of the ANS emission anisotropy factor ANS-g_{em} and ANS fluorescence intensity at 490 nm (excitation at 365 nm). The fluorescence intensity showed a sharp increase between pH 3.7 and pH 3. This appeared to be a single transition with an apparent pK_a of 3.3–3.4, which is close to the pK_a of the near-UV CD-detected acidic transition. The blue ANS fluorescence enhancement was interpreted earlier by Semisotnov et al. (1991) as an indication of binding of ANS to α-LA in the A-state. The pH dependence of the ANS-g_{em} also showed a single transition, but with a very different pK_a that

Table 1. *Emission anisotropy factor for tryptophan and ANS fluorescence of bovine α-lactalbumin in different pH ranges*[a]

	Emission anisotropy factor, $g_{em} \cdot 10^4$	
pH range	Tryptophan (N)	ANS (N)
5.5–5.0	3.02 ± 0.37 (5)	
5.8–4.7		−1.81 ± 0.22 (13)
4.2–2.9	1.53 ± 0.30 (12)	
4.1–2.5		−0.91 ± 0.16 (12)
2.9–2.5	0.28 ± 0.36 (5)	

[a] N, number of data points. For experimental details, see Figures 2 and 3.

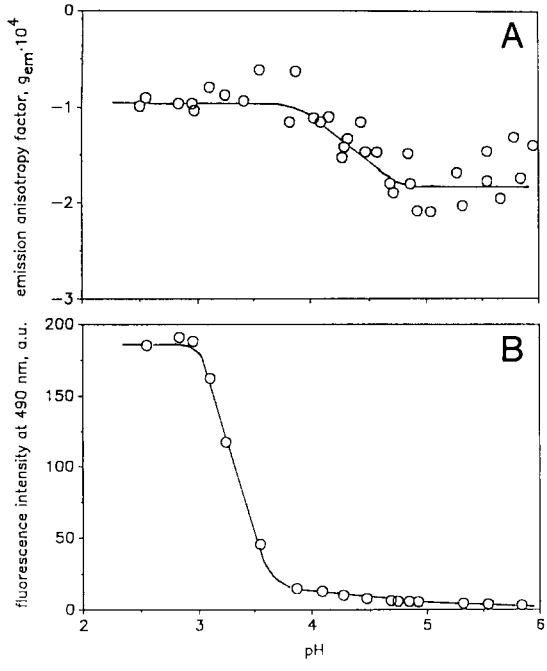

Fig. 3. pH dependence of ANS emission anisotropy factor g_{em} at 490 nm (**A**) and ANS fluorescence intensity at 490 nm (**B**) of the ANS–bovine α-lactalbumin complex in 2.7 mM $CaCl_2$. Excitation at 365 nm.

did not coincide with the transition to the MG-state (A-state), in which ANS binding is enhanced. The difference between the ANS-g_{em} values in the two pH ranges ($2.5 < pH < 4.1$ and $4.7 < pH < 5.8$) was statistically significant (Table 1). These CPL measurements detected a conformational change associated with the weak fluorescence of ANS under conditions that do not show the dramatic increase of the ANS quantum yield. Comparison of Figures 2 and 3 shows that the pH-dependent transition detected by ANS-g_{em} coincided with the first transition detected by Trp-CPL (pK_{a1} 4.9). Yet the second pH-dependent conformational transition detected by Trp-CPL at pH 2.9 was not detected by ANS-CPL. Interestingly, ANS-CPL was not reduced to zero even at a pH as low as 2.5. This indicates residual asymmetry of the environment of the ANS chromophore in its excited electronic state when bound to α-LA in the A-state of α-LA. CPL of free ANS in 95% ethanol solution was measured as above. There was no detectable CPL signal from the solution of ANS.

Discussion

Origin of intrinsic α-LA CPL

The CPL spectra of several proteins (staphylococcal nuclease, antibodies, antibody fragments, and azurin) were reported. No dependence of their g_{em} on emission wavelength was found (Steinberg et al., 1974; Jaton et al., 1975; Schlessinger et al., 1975a, 1975b). g_{em} of apo-glyceraldehyde-3-phosphate dehydrogenase as well as of human serum albumin and chicken pepsinogen showed significant wavelength dependence (Schlessinger & Levitzki, 1974; Steinberg et al., 1974). The values of the emission anisotropy factors are expected to be constant across the fluorescence spectrum, when the fluorescence is contributed by a single electronic transition in a single chromophore (Steinberg et al., 1974). There are four tryptophan residues in α-LA that can be excited at 280 nm. The fluorescence of α-LA seems to be almost completely dominated by its tryptophan residues in the spectral range of 320–340 nm. This was due to the low tyrosine contribution to total protein fluorescence in this range (Burstein, 1977; Lakowicz, 1983; Permyakov, 1993). Ostrovsky et al. (1988) showed that at least two tryptophan residues contribute a significant share of the total emission of α-LA. Sommers and Kronman (1980) concluded that three of the four tryptophan residues in α-LA contribute significantly to the total emission (Trp 28, Trp 108, and Trp 123 contribute about 20, 20, and 50%, respectively). Therefore, it was reasonable to assume that the g_{em}-values in the range of 320–340 nm reflect contributions from all three tryptophan residues. Contributions from individual tryptophan residues to the total g_{em}-value can vary, and could not be determined from the data presented here.

Although dipole moments of the indole chromophore in the ground and excited electronic states are different (Mataga et al., 1964), no measurable Trp-CPL signal was detected for *N*-acetyl-L-Trp-amide or for a number of linear Trp-containing peptides (Schlessinger et al., 1974). This indicates that Trp-g_{em} did not originate from an intrinsic asymmetry of the indole electronic configuration. In contrast, Trp-containing cyclo-dipeptides showed Trp-g_{em} of about 2×10^{-4} in a viscous solution (zero at low viscosity) and in an asymmetrical viscous polymer solution (cellulose acetate in dioxane). Thus, in proteins, Trp-g_{em} originates mainly from a conformationally induced asymmetry of the electronically excited indole chromophore.

In the N-state, at pH above 5, Trp-CPL of α-LA seems to be a probe of the ordered local conformation around its nonquenched tryptophan residues. In the A-state, at pH below 3, the lack of any Trp-CPL signal reflects a loss of the asymmetry induced by the environment of the tryptophan residues. At present, it is impossible to estimate the relative contribution of individual tryptophan residues to the total CPL signal. Even in the extreme case, where the Trp-CPL signal is dominated by the contribution of a single residue, and where ANS-CPL detects the same transition at one or more of the binding sites, this is an indication of conformational transition in at least two parts of the protein molecule (see also below).

N → A transition

This transition in α-LA has been studied by near-UV CD (Robbins & Holmes, 1970; Kuwajima et al., 1975, 1976, 1980), intrinsic tryptophan fluorescence (Sommers & Kronman, 1980; Permyakov et al., 1981, 1985; Lala & Kaul, 1992), and differential spectrophotometry (Kuwajima et al., 1980) techniques. The single transition in the pH range of 3–4 was detected by each one of these techniques. On the basis of far-UV CD measurements, the secondary structure of α-LA was found to be unchanged between pH 7 (N-state) and pH 2 (A-state) (Kuwajima et al., 1985). This discrepancy between the near-UV (aromatic side chains) and the far-UV (backbone) CD data was one of the main arguments for defining the A-state of α-LA as an MG-state (Ptitsyn, 1987; Kuwajima, 1989).

The conformational state dependence of the emission anisotropy factor g_{em} could be due to a variation in the chromophore conformation and/or an asymmetric perturbation by its environment (Steinberg et al., 1974). Tryptophan residues in un-

folded short peptides or small model compounds had zero g_{em} values in the pH range of 2.5–5.5. The linear polarization and fluorescence lifetimes of tryptophan residues in α-LA was the same at pH 5 and pH 2 (Dolgikh et al., 1981, 1985). This indicated that the magnitude and orientation of the tryptophan dipole moments in the two states were not changed. Hence, the change of chirality properties of the tryptophan residues in α-LA in response to changes of pH was caused mainly by changes in the asymmetry of their environments. The parameters that can affect g_{em} are wave functions of the excited electronic states A′ and B′ (Equation 2), which are sensitive to environmental fields. Therefore, the only factor that can affect Trp-CPL within the pH range of 2.5–5.5 is the asymmetry of the environment of the tryptophan residue that produces the asymmetrical perturbations of the tryptophan chirality.

The pK_a values in the range of 2.9–3.3 were lower than the pK_a values of 3.5–3.8 that were observed for α-LA from tryptophan fluorescence data (Sommers & Kronman, 1980; Permyakov et al., 1981) and also from differential spectrophotometry and CD data for bovine and goat α-lactalbumins (Kuwajima et al., 1980). Permyakov et al. (1981) showed that the pK_a was shifted to lower values when the Ca^{2+}/α-LA ratio was increased from 1 to 50 at Ca^{2+} concentrations of 0.024 and 1.32 mM, respectively. In the present experiments, the Ca^{2+} concentration in the protein solution was 2.7 mM. Hence, the reduced values of the p$K_a(g_{ab})$ in our data evidently correspond to those obtained at the higher Ca^{2+} concentration.

Permyakov et al. (1981, 1985) used computer simulation in a study of the protonation transition of α-LA in the pH range of 2.3–5. They suggest that Ca^{2+} can be exchanged with protons of three carboxylate groups with pK_a 5.0 ± 0.1. Bratcher and Kronman (1984) also found a sharp decrease in the Ca^{2+} binding to α-LA at a pH below 5. Guanidine hydrochloride, which perturbs the α-LA molecular structure, induced a shift of pK_a of the CD-detected transition from 3.8 to about 4.5 in α-LA (Kuwajima, 1977). This shift of the pK_a resembles the shift of pK_a found after Ca^{2+}–α-LA dissociation. The conformational transition with a pK_a of 4.5–4.9, which was found in the present study, coincides with a weakening of the Ca^{2+}–α-LA interaction, which leads to a partially disordered α-LA structure.

Is there a preMG-state in α-lactalbumin?

Two conformational transitions were detected in the proton titration of α-LA. The first transition, within the pH range of 4–5, was not detected by the CD measurement. It was detected only by measurements of CPL of both the tryptophan residues and the bound ANS (nonbound ANS did not show any CPL signal). The other transition in the pH range of 2.9–3.7 caused perturbations that were detected both by Trp-CD and Trp-CPL. These results lead to the conclusion that, within the pH range of 2.5–6, α-LA can exist in three different pH-dependent states. The state stabilized below pH 3 was defined as an MG-state (Ptitsyn, 1987; Kuwajima, 1989). The state stabilized above pH 5 is the native state, and we suggest that the state stabilized between pH 3.7 and 4.0 could be defined as a "premolten globule" state.

An important question is whether the pH-dependent transition detected by the CPL signal can be explained by changes in tryptophan quenching by ionizable side groups (Burstein, 1977; Permyakov, 1993). This is probably not the case, because the CPL signal is the ratio of the circularly polarized part of the fluorescence to the total fluorescence intensity. Therefore, by definition, it does not depend on the fluorescence intensity, which is affected by the quenching. Therefore, the intermediate (preMG) state was not an artifact caused by internal quenching of tryptophan fluorescence. The reduction of the g_{em}-factor evidently reflected a partial loss of the induced asymmetry of the tryptophan environment. Indeed, changes of conformational asymmetry could be indirectly caused by a change in the ionization state of a side chain. But there could not be a direct influence of the side-chain ionization on Trp-CPL of α-LA. Changes of ionization state of functional groups in the close environment of tryptophan residues affect the quantum yield of their fluorescence (Burstein, 1977; Permyakov, 1993). However, no changes of quantum yield (intensity) of α-LA were found in the pH range of 4–6 at room temperature (Kronman et al., 1964; Sommers & Kronman, 1980; Permyakov et al., 1981; Lala & Kaul, 1992). This indicates that there are no ionizable quencher groups with a pK_a in the range of 4–6 in the environment of tryptophan residues in α-LA.

Figure 4 shows a summary of the transitions detected by the three approaches used in the present study. It clearly shows the two states and the intermediate preMG-state. The transitions reflect two levels of local asymmetry of the environments of the chromophores. In the N → preMG transition, only a partial change in the tryptophan environment took place. Indeed, the CD and CPL measurements probe the asymmetry of the local environment of a chromophore. Tryptophan residues in α-LA are not clustered in one site: according to Sommers and Kronman (1980), Trp 28 and Trp 108 are situated close to one another (about 4 Å), and Trp 123 is located 15–25 Å from the first pair. (The average diameter of the α-LA molecule is about 18–20 Å as detected by X-ray diffraction, light scattering, and centrifugation [Kuwajima, 1989].) In addition, there is no evidence that α-LA has only one specific ANS-binding site. Therefore, it is reasonable to assume that both Trp-CPL and bound ANS-CPL reported the asymmetry in several parts of the α-LA molecule, and to expect that real conformational transitions reflected in the CPL measurements were not restricted to a single site.

Kuwajima measured spectral parameters of α-LA in guanidine hydrochloride solutions and deduced the existence of an inter-

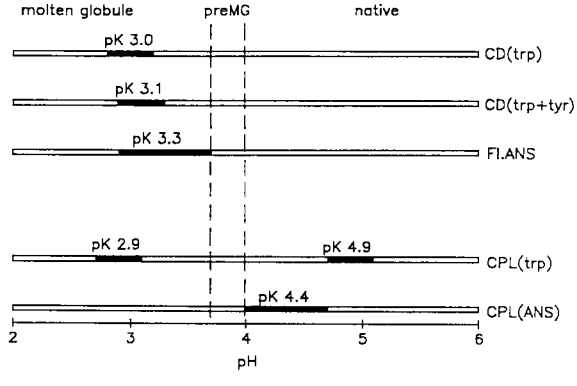

Fig. 4. Summarized scheme of the pH-dependent transitions of the absorption anisotropy factor g_{ab} at 272 and 292 nm (CD(Trp+tyr) and CD(Trp), respectively), ANS fluorescence (Fl.ANS), tryptophan and ANS emission anisotropy factor g_{em} (CPL(Trp) and CPL(ANS), respectively) of bovine α-lactalbumin and its ANS complex.

mediate A‡ in the A→N transition (Kuwajima, 1977; Kuwajima et al., 1981, 1989). Kuwajima (1989) assumed that the A-state is a general MG-related state, which can be reached either at low pH or at neutral pH under some specific conditions. A‡ was defined as a "critical activated state" with a reduced Ca^{2+} binding constant. An intermediate state in the N→A transition was also proposed by Kronman and Bratcher (1984) for apo-α-LA. Lala and Kaul (1992) proposed that this intermediate is preMG, but they studied apo-α-LA (no Ca^{2+}), whereas the first transition found here depends on Ca^{2+} dissociation. It is proposed here that the preMG-state detected in the present study is related to the intermediate state proposed by Kuwajima and coworkers for Ca^{2+}-α-LA. Kuwajima et al. (1989) noted that there was no way to observe the A‡ state directly and therefore they had to adopt indirect parameters for characterizing this state. In the present study, the preMG-state was directly demonstrated by the CPL measurements.

The preMG-state should, in fact, precede the full transition to MG-state, in which the tertiary structure is significantly lost. Therefore, we propose that the intermediate state detected by the CPL measurements is a preMG-state. The preMG-state is characterized by a reduced Ca^{2+} binding and reduced local and nonlocal interactions that lead to a less tight 3D structure with concomitant increases in fine symmetrical fluctuations on the nanosecond time scale.

Advantages of CPL measurements in folding research

CPL spectroscopy as a tool for conformational studies of proteins was introduced by Steinberg and his coworkers (1978a). In the present study, the application of the CPL method to the problem of the MG-states and the high sensitivity and selectivity of this technique were shown. This sensitivity originates from the nature of CPL as a physical effect that can detect changes in the environment of chromophores. The selectivity of CPL measurements in the presence of several different luminescent chromophores originates from two steps of spectral resolution, first by the excitation (absorption) spectrum, and second by the emission spectrum, whereas the CD spectrum can be resolved by the absorption only.

The susceptibility of chromophores to environmental changes is higher in the excited electronic state than in the ground electronic state because of several reasons: a higher dipole moment, an enhanced chemical reactivity, and a change in the orbital symmetry (Calvert & Pitts, 1966; Barltrop & Coyle, 1975). Therefore, it enabled detection of the intermediate state, which was not found by techniques based on the susceptibility of chromophores in the ground electronic state. This extra sensitivity of the excited electronic state of tryptophan and ANS, as compared with CD and differential spectrophotometry, enabled the detection of the preMG-state of α-LA.

Materials and methods

Materials

Commercial preparations of α-LA (Sigma, type I and III) and ANS were used without additional purification. The α-LA powder was dissolved in 5–10 mM phosphate buffer containing 2.3–2.7 mM $CaCl_2$. This concentration of $CaCl_2$ provided Ca^{2+} saturation of α-LA (Ca^{2+}/protein ratio of 66–84).

Absorption, fluorescence, and CD measurements

The absorption of each solution was measured with an AVIV 17DS (Aviv Associates, Lakewood, New Jersey) computerized spectrophotometer. For α-LA solutions, a molar extinction coefficient ϵ_{280} of 28,500 M^{-1} cm^{-1} was used according to E_{280} = 2.01 mL/mg (Kronman & Andreotti, 1964) and MW = 14,200 (Kuwajima, 1989). The CD data were obtained with a Cary-60 recording spectropolarimeter equipped with a model 6002 CD attachment and are presented here as $g_{ab} = \Delta\epsilon/\epsilon$, where $\Delta\epsilon = [\theta]/3{,}300$ is the CD. The absolute error of ellipticity θ measurements was 0.8 mdeg, with a solution optical density of 0.9 at 280 nm (1-cm pathlength) that corresponds to a 32 μM protein concentration. The steady-state fluorescence of ANS and its protein mixtures were measured with a ISS GregPC computerized spectrofluorimeter (ISS, Urbana, Illinois) at an excitation of 365 nm. A 3 × 3 mm quartz cuvette was used. Both excitation and emission bandwidths were 4 nm.

Circular polarization of fluorescence

The instrument used to measure CPL was based on a design described elsewhere (Steinberg & Gafni, 1972; Steinberg, 1978b). Figure 5 shows a general scheme of the instrument after slight modifications. The light of the 100 W mercury lamp was passed through a double monochromator (Jarell-Ash 82-440) and was focused on the sample cell through a two-lens system and depolarizer. The emitted light of luminescence was passed throughout a cut-off glass filter, and its circularly polarized portion was modulated by a photoelastic modulator (Morvue PEM-FS3). The cut-off filter was a Pyrex glass that transmitted light in the near-UV above 300 nm for α-LA intrinsic fluorescence measurements or a Schott KV408 glass filter for the ANS fluorescence measurements. The modulator head was equipped with polarizing film as recommended by Steinberg and Gafni (1972). The fluorescence collected by the quartz lens, was passed through the emission monochromator (Jarell-Ash 82-410) and was monitored by the photomultiplier (EMI 9863QB/350), which was controlled by the high-voltage power supply (Fluke, model 412B). The photocurrent consisted of direct-current (d.c.) and alternating-current (a.c.) components, which corresponded to nonpolarized (f) and circularly polarized (Δf) portions of the total fluorescence. The total photocurrent was preamplified and the d.c. part was registered by a Vdc voltmeter resulting in a f-value. The a.c. part was amplified by the lock-in amplifier (EG&G PARC model 5209) controlled by the phase of the reference signal from the modulator power supply (Morvue C-3 Control Unit). The d.c. signal from the lock-in amplifier was the Δf value. The d.c. signals from the both photomultiplier-preamplifier and the lock-in amplifier were applied to a PCL-711 data acquisition board (Advantech) within an IBM PC with PCLS-702 Labtech Acquire software. The signals were treated statistically, resulting in $g_{em} = 2\Delta f/f$. A $2\Delta f/f$-value was calibrated by means of a special calibrating device (Steinberg & Gafni, 1972) using a fluorescein solution. A 5-mm-pathlength cuvette was used for CPL measurements. Tryptophan and ANS fluorescence was excited at 280 and 365 nm, respectively (optical density about 0.5). The excitation wavelength bandwidth was

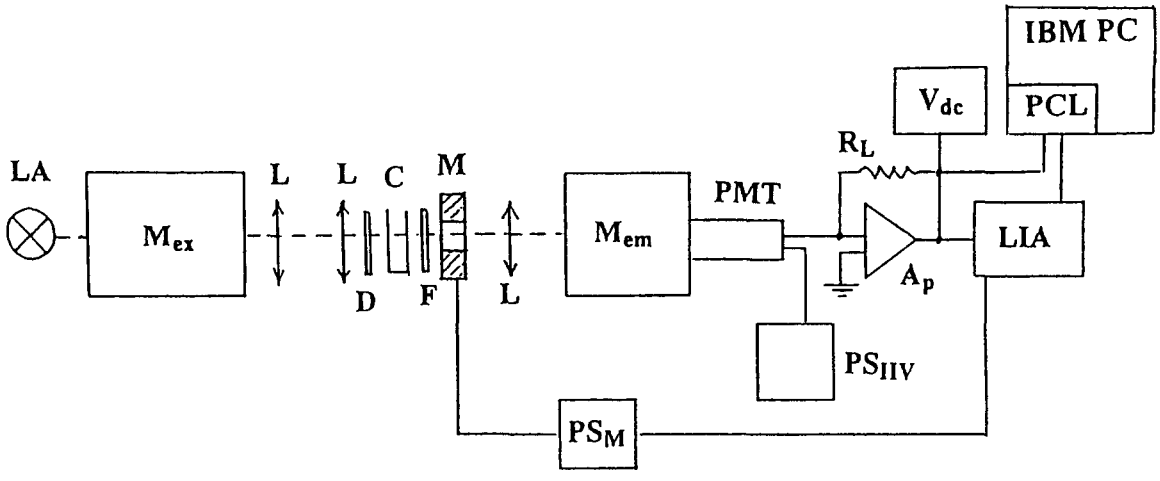

Fig. 5. Scheme of the CPL-machine. LA, mercury lamp; Mex, excitation double monochromator; L, lenses; D, depolarizer; C, cell; F, cut-off filter; M, photoelastic modulator head; PSM, power supply for photoelastic modulator head; Mem, emission monochromator; PMT, photomultiplier tube; RL, load resistor; PSHV, high voltage power supply for PMT; LIA, lock-in amplifier, Vdc, d.c. voltmeter; PCL, data acquisition board inserted into IBM PC computer; Ap, preamplifier.

20 nm, and the emission monochromator bandwidth was 13 nm. All measurements were made at room temperature (18–22 °C). The measurement time of 2 min prevented the photochemical destruction of the samples.

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