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Use of fluorescence decay times of 8-ANS-protein complexes to study the conformational transitions in proteins which unfold through the molten globule state

Vladimir N. Uversky [a,*], Stefan Winter [b], Günter Löber [b]

[a] *Institute of Protein Research, Russian Academy of Sciences, 142292 Pushchino, Moscow Region, Russia*
[b] *Institute for Molecular Biotechnology, 100813, D-07708, Jena, Germany*

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Abstract

The conformational transitions starting with the native protein, passing the molten globule state and finally approaching the unfolded state of proteins was investigated for bovine carbonic anhydrase B (BCAB) and human α-lactalbumin (α-HLA) by means of fluorescence decay time measurements of the dye 8-anilinonaphthalene-1-sulphonic acid (8-ANS). Stepwise denaturation was realized by using the denaturant guanidinium chloride (GdmCl). It was shown that 8-ANS bound with protein yields a double-exponential fluorescence decay, where both decay times considerably exceed the decay time of free 8-ANS in water. This finding reflects the hydrophobic environment of the dye molecules attached to the proteins.

The fluorescence lifetime of the short-time component is affected by protein association and can be effectively quenched by acrylamide, indicating that 8-ANS molecules preferentially bind at the protein surface. The fluorescence lifetime of the long-time component is independent of the protein and acrylamide concentration and may be related to protein-embedded dye molecules.

Changes of the long lifetime component upon GdmCl-induced denaturation and unfolding of BCAB and α-HLA correlate well with overall changes of the protein conformation. The transition from native protein to the molten globule state is accompanied by an increase of the number of protein-embedded 8-ANS molecules, while the number of dye molecules located at the protein surface decreases. For the transition from the molten globule to the unfolded state was the opposite behaviour observed.

Keywords: Molten globule; Protein denaturation and unfolding; 8-ANS; Fluorescence decay time

1. Introduction

The protein self-organization is one of the most interesting and intriguing problems of molecular biology which has attracted considerable attention of many researchers starting from the classical works of Anfincen's group [1]. One of the crucial questions is how protein molecules can achieve their rigid tertiary structure specified on the atomic level and avoiding a large number of alternatives. One of the possible solutions of this problem is given in the so-called frame-work model of protein folding [2], which suggests the sequential mechanism of the

* Corresponding author.

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formation of different structural levels of a protein-molecule secondary structure, folding pattern and rigid tertiary structure — and, hence, has predicted the existence of at least two partly folded intermediates within the protein folding pathway. Although this model was confirmed experimentally in many details (for review see [3–12]), the structural properties of kinetic and equilibrium intermediates — premolten globule and molten globule states — are of substantial interest up to now (see [5] and the references therein).

Another side of consideration relates to the possible physiological role of non-native states of protein molecules [13–15]. This opens a quite new area of investigations and requires quite new technologies for the description of structural properties of protein molecules in complex systems.

It has been established long ago that the interaction of hydrophobic fluorescent probes such as 1-anilino-8-naphthalenesulfonic acid (8-ANS) with the exposed hydrophobic sites on the surface of native protein molecules results in a considerable increase of the dye fluorescence intensity and a blue shift of its fluorescence spectrum [16]. Considerable increase of ANS fluorescence intensity was also observed upon the pressure-induced denaturation of chymotrypsinogen and lysozyme [17], which was interpreted in terms of plurality of pressure-denatured forms of these proteins. On the other hand, the transition of the protein molecule into the molten globule state (see [5] for a recent review) leads to a dramatic increase of the fluctuation ability of side chains and, as a consequence of this, to the appearance of large hydrophobic surfaces accessible for the solvent. All this allows to use successfully 8-ANS for the visualization of the process of the molten globule formation upon protein folding in vitro [18,19].

Unfortunately, the use of hydrophobic dyes for studies of the conformational transitions of proteins in organic solvent systems was questioned by the outcome that both usual parameters of the 8-ANS fluorescence spectrum, i.e. intensity and position of the fluorescence maximum, change strongly, not only upon interaction of the probe molecules with proteins, but also by altering the properties of the solvent, such as, for example, the dielectric constant [16].

The present report is aimed to the elaboration of structural approaches which allows to study the interaction of 8-ANS dye with protein molecules independent on the solvent system used. To this end the fluorescence decay time in 8-ANS-protein complexes under different experimental conditions and at different steps of the in vitro protein unfolding process were studied.

2. Materials and methods

2.1. Materials

Carbonic anhydrase B (from bovine blood erythrocytes), α-lactalbumins (from bovine and human milk) and bovine milk β-lactoglobulin were purified by N.V. Kotova (Institute of Protein Research, Russian Academy of Sciences). Lysozyme (hen egg white) was purchased from Boehringer (Mannheim, Germany).

8-Anilinonaphthalene-1-sulfonic acid (8-ANS) Mg salt was from Serva (Heidelberg, Germany). Guanidinium chloride was from Merck (Darmstadt, Germany).

The concentration of proteins in solutions was determined spectrophotometrically. For decay time measurements the protein concentration ranged from 0.1 to 0.5 mg/ml, with the exception of special cases, marked in the text. A molar ratio [8-ANS]/[protein] = 10 was used if not mentioned otherwise. All measurements were made at 23°C.

2.2. Equipment

Fluorescence lifetimes were measured by means of a SLM 48000 MHF Fourier transform spectrofluorimeter (SLM Instruments, IL, USA). The 325 nm line of an helium–cadmium laser (Plazma, Ryazan, Russia) was used for fluorescence excitation. Since the laser emission at 325 nm is not very intense, its use was restricted on measurements of fluorescence decay characteristics only in solutions with relatively high fluorescence intensities.

Steady-state measurements of 8-ANS and tryptophan fluorescence were performed by using a SFM-25 spectrofluorimeter (Tegimenta, Switzerland). Absorption spectra were recorded with the aid of a Cary

4E UV-VIS spectrophotometer (Varian, Mulgrave, Australia)

2.3. Methods

2.3.1. Fluorescence decay time measurements

The cw-laser light was modulated by a Pockel's cell driven by a multi-harmonic high frequency generator. The desired frequencies, starting with 5 MHz, were superimposed with their harmonics up to 250 MHz and applied to the Pockel's cells. Thus the sample was excited by a light pulse of definite shape which was determined by the superimposed frequencies. The fluorescence light emitted by the sample in consequence is intensity modulated, but it's Fourier spectrum differs from that of the exciting light. The fluorescent sample shows an impulse response containing a phase shift φ and a reduction of the modulation depth M for each frequency of the Fourier spectrum.

The complex Fourier transform of the impulse response, $I(t)$, contains a real (R) and an imaginary (I) component, given by

$$R = \int_0^\infty I(t)\cos(\omega t)\mathrm{d}t \tag{1}$$

$$I = \int_0^\infty I(t)\sin(\omega t)\mathrm{d}t \tag{2}$$

Here sin (ωt) and cos (ωt) represent the harmonic excitation of the sample with circular frequency ω.

Assuming a multiple lifetime population in the sample the exponential decay law is defined by the equation

$$I(t) = \sum_i a_i \exp\left(-\frac{t}{\tau_i}\right) \tag{3}$$

where τ_i and a_i are lifetime and weighing factor for component i, respectively.

Substituting $I(t)$ in Eqs. (1) and (2) and solving the integral in the real part of the Fourier transform gives

$$R = \sum_i \frac{f_i}{1 + \omega^2\tau_i^2} \tag{4}$$

where the fractional contribution f_i of the component i is determined as $f_i = a_i\tau_i$.

Derivation of the imaginary part gives

$$I = \sum_i \frac{f_i\omega\tau_i}{1 + \omega^2\tau_i^2} \tag{5}$$

The quantities, observable in measurements, phase (φ) and modulation (M) are derived from the transform components by the definitions:

$$\varphi = a\tan\frac{I}{R} \text{ and } M = \sqrt{R^2 + I^2} \tag{6}$$

The fluorescence lifetime can be obtained by substituting I and R in (6) with their corresponding Eqs. (4) and (5), resulting in the *phase lifetime equation:*

$$\tau_\varphi = \frac{\tan\varphi}{\omega}$$

and the *modulation lifetime equation:*

$$\tau_M = \frac{1}{\omega}\sqrt{(1/M^2 - 1)}$$

The curves were obtained for phase and modulation at all frequencies used in the Fourier analysis. The curves were fitted by a least square algorithm. Phase and modulation curves, obtained for the 8-ANS-protein complexes, can be fitted well ($\chi^2 = 5$ to 7) with the use of two lifetimes τ_1 and τ_2 and their corresponding amplitudes a_1 and a_2 in all the cases studied. Values of a_1 and a_2 were normalized in sum to 100%. Using a single exponential decay model for the curve fitting leads to much worse results ($\chi^2 = 30$ to 50). Attempts to fit the data with three exponents were unsuccessful. Only two components were accepted by the fitting program, giving τ_1 and τ_2 with their corresponding amplitudes a_1 and a_2, whereas the third amplitude was calculated to be zero.

All fluorescence decay measurements were performed under the emission at 470 nm. In a few cases measurements were also done at $\lambda_{em} = 520$ nm. No visible changes in decay parameters evaluated at different wavelength were observed.

2.3.2. Fluorescence quenching by acrylamide

The acrylamide-induced quenching of 8-ANS fluorescence in complexes of this dye with BCAB and lysozyme was studied by monitoring the fluorescence decay time measurements at various quencher

concentrations. The quenching efficiency was determined as the bimolecular quenching rate constant k_q from the Stern–Volmer equation for dynamic fluorescence quenching:

$$\frac{\tau_0}{\tau} = 1 + k_q \tau_0 [Q] \quad (7)$$

where τ_0 and τ are the lifetimes in the absence and in the presence of a definite quencher concentration $[Q]$, respectively. It is necessary to underline that the acrylamide-induced changes of 8-ANS fluorescence lifetime showed the same pattern as the fluorescence intensity.

Results of fluorescence quenching experiments are presented as Stern–Volmer plots (τ_0/τ vs. $[Q]$).

2.3.3. Shift of a tryptophan fluorescence spectrum

The shift of the tryptophan fluorescence spectrum was registered as

$$A = \frac{I_{370}^{\mathrm{Trp}}}{I_{310}^{\mathrm{Trp}}} \quad (8)$$

where (I_{310}^{Trp}) and (I_{310}^{Trp}) are values of the tryptophan fluorescence intensity at 370 and 310 nm, respectively, excited at 295 nm.

3. Results

3.1. Characterisation of the fluorescence decay in 8-ANS-protein complexes

It has been shown earlier that the fluorescence decay of free 8-ANS in aqueous solutions is a monoexponential process with a characteristic fluorescence lifetime of 250 ps [20]. As already mentioned above, the laser line which was used as a light source is rather weak. This excludes fluorescence lifetime measurements in solutions with low quantum yield. Unfortunately, this was the case for free 8-ANS in water, whose quantum yield amounts to 0.004 [16]. On the other hand, interaction of this dye with solvent exposed hydrophobic clusters of protein molecules results in a considerable increase of its fluorescence intensity (for example, the quantum yield of probe molecules when bound to apomyoglobin is 0.98, or, in other words, its fluorescence intensity increase 245 times in comparison with the correspondent value obtained for the free probe molecules in water [16]). This means that fluorescence decay parameters for the 'bound' form of 8-ANS can be measured with the SLM 48000 MHF Fourier transform spectrofluorimeter.

In order to obtain information about changes of 8-ANS fluorescence lifetime upon interaction of the

Table 1
Parameters of fluorescence decay in 8-ANS-protein complexes

Protein	Conformational state, conditions	Protein concentration (mg/ml)	Fluorescence decay parameters			
			τ_1 (ns)	I_1(%)	τ_2 (ns)	I_2 (%)
Lysozyme	N: pH 2.5, 25°C,	0.23	10.7	76	2.8	24
	0.05 M Na–*P*	1.07 [a]	10.1	58	5.4	42
β-Lactoglobulin	N: pH 2.5, 25°C,	0.14	11.4	82	2.3	18
	0.05 M Na–*P*	0.95	11.8	69	4.9	31
BCAB	MG: pH 3.5, 25°C,	0.31	17.3	95	2.3	5
	0.05 M Na–*P*;	1.56 [a]	16.9	69	5.8	31
	MG: 1.8 M GdmCl,	0.26	15.9	92	2.8	8
	pH 6.8, 25°C	1.15	16.4	64	4.4	36
α-HLA	MG: pH 2.0, 25°C,	0.24	15.9	88	3.1	12
	0.05 M Na–*P*;	1.34 [a]	15.3	61	6.1	39
	MG: 2.0 M GdmCl,	0.29	14.1	69	2.5	31
	pH 6.8, 25°C	1.48 [a]	14.4	41	5.2	59
α-BLA	MG: pH 2.0, 25°C,	0.19	15.7	82	2.5	18
	0.05 M GdmCl	1.21	16.1	54	4.2	46

[a] Indicates the cases in which considerable opalescence was observed.

dye with protein molecules the measurements were done by using five different proteins. Two of them — lysozyme and β-lactoglobulin — can bind 8-ANS in the native state, while three remainder proteins — BCAB, human and bovine α-lactalbumins (α-HLA and α-BLA) — possess large affinity to 8-ANS after transformation to the molten globule state [18,19].

Table 1 presents the fluorescence lifetime of 8-ANS measured for all five proteins in different conformational states and under different experimental conditions. It is clearly seen that the interaction of 8-ANS with the protein molecule results in a double-exponential fluorescence decay kinetic. It is necessary to underline that values of both components exceed considerably those of the decay of free 8-ANS in water. Indeed, as it follows from Table 1, even the lowest value of the short lifetime component of 8-ANS in the presence of proteins is one order of magnitude larger than the corresponding value measured for free 8-ANS in water. Such an increase of fluorescence lifetime is usually explained by the changes of the dye microenvironment and, most likely, by its transfer to a less polar surrounding. The change of the fluorescence decay characteristics is indicative for the formation of adducts with the hydrophobic part of the protein molecule. The existence of two decay components in the fluorescence of 8-ANS molecules bound with the protein reflects the existence of at least two different dye–protein adducts with rather different probe environments. Table 1 also shows that the relative amplitudes of the different lifetimes change drastically upon raising the protein concentration: The amplitude of the long-lifetime component decreases upon increasing protein concentration, while for the short-lifetime component the opposite effect is observed.

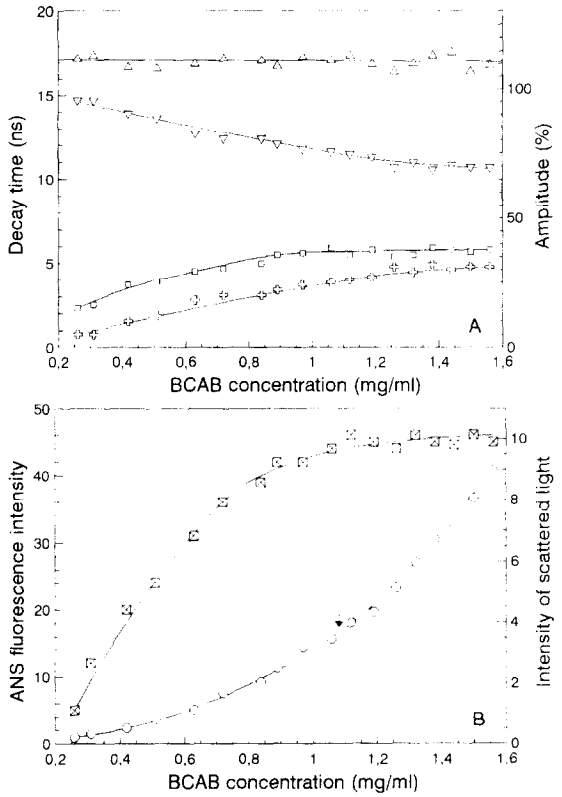

Fig. 1. Changes of the fluorescence parameters in 8-ANS-BCAB complexes caused by increasing protein concentration. (A) Changes of the decay parameters: □ and +, the short lifetime component and its amplitude, respectively; △ and ▽, the long lifetime component of 8-ANS fluorescence and its amplitude, respectively. (B) □, changes of the intensity of scattered light, measured as fluorescence intensity at $\lambda_{em} = \lambda_{ex} = 325$ nm; ○, changes of the 8-ANS fluorescence intensity at $\lambda_{em} = 470$ nm. The 8-ANS concentration was kept equal to 0.13 mg/ml at pH 3.6.

3.2. The short lifetime component of 8-ANS fluorescence decay is due to probe molecules which are attached to the protein surface and are accessible to water

3.2.1. Dependence on protein association

To understand the nature of 8-ANS decay components their dependencies on protein and 8-ANS concentrations were studied. Results of this investigations are presented in Table 1 and Figs. 1 and 2. As it follows from Table 1 the short lifetime component of the fluorescence decay of 8-ANS depends strongly on the protein concentration and increases more than twice with the increase of the protein content (Fig. 1A). Such a concentrational dependence of the short lifetime component may be due to protein association. As a confirmation of this assumption Fig. 1B represents the dependence of scattered light intensity (measured as fluorescence intensity at $\lambda_{em} = \lambda_{ex} =$ 325 nm) on protein concentration. The level of the scattered light increases considerably with the increase of protein concentration. Such behaviour is usually attributed to the protein association process. Indeed, starting from the point marked at the figure

by an arrow, the protein solution even started to become opalescent (see Fig. 1B). A similar decay time effect was observed for solutions with moderate protein concentration (0.25 mg/ml) upon increase of the amount of 8-ANS (see Fig. 2A). Fig. 2B shows that in this case the short lifetime component increases practically in the same manner as it does upon the increase of the protein concentration. We are tempted to conclude that both experiments have changes in common in the environment of the dye molecules. In other words, such changes can be referred to those probe molecules, which interact with the superficial hydrophobic clusters of a protein

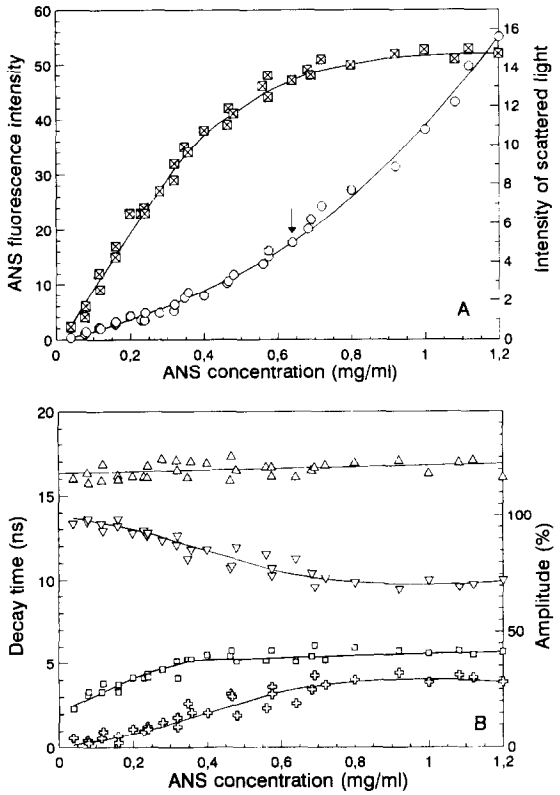

Fig. 2. Changes of the fluorescence parameters in 8-ANS-BCAB complexes caused by increasing ANS concentration. (A) □, changes of the intensity of scattered light, measured as fluorescence intensity at $\lambda_{em} = \lambda_{ex} = 325$ nm; ○, changes of the 8-ANS fluorescence intensity at $\lambda_{em} = 470$ nm. (B) Changes of the decay parameters: □ and +, the short lifetime component and its amplitude, respectively; △ and ▽, the lifetime component of 8-ANS fluorescence and its amplitude, respectively. The protein concentration was kept equal to 0.25 mg/ml at pH 3.6.

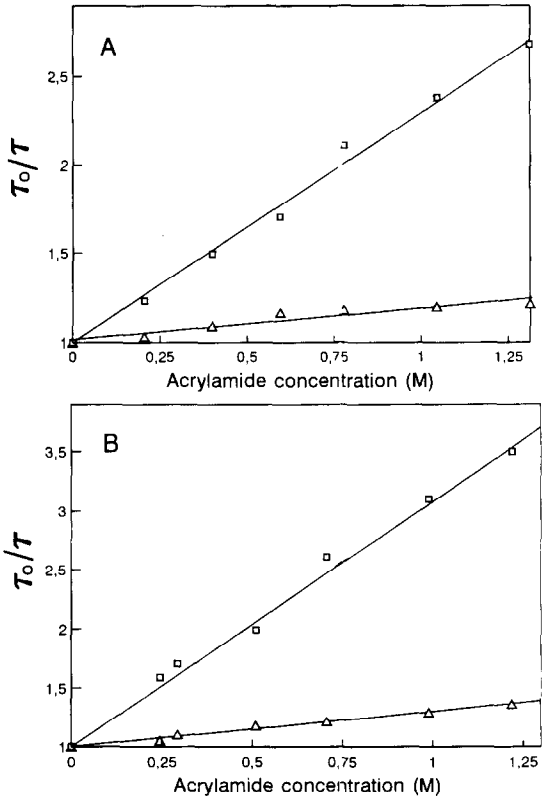

Fig. 3. Stern–Volmer plots for the quenching of 8-ANS fluorescence in protein–dye complexes by acrylamide. □, short lifetime component; △, long lifetime component. (A) Results for 8-ANS complexed with BCAB at pH 3.6. (B) Results for the probe–lysozyme complex at pH 2.0.

molecule, reached either by protein association processes or by an enhancement of the surface-bound dye molecules at higher dye concentrations.

3.2.2. *Effective quenching by acrylamide*

As the value of the short lifetime component is strongly dependent on the protein concentration, this component may be attributed to dye molecules interacting with more or less solvent accessible hydrophobic surface clusters of the protein molecule. Direct evidence of this suggestion was obtained by investigations of acrylamide-induced quenching of the 8-ANS fluorescence. Fig. 3 presents Stern—Volmer plots of 8-ANS in the presence of BCAB (pH 3.6) and lysozyme (pH 2.0). It is clearly seen that the short lifetime component can be quenched

quite effectively by acrylamide, reflecting an accessibility of the corresponding probe molecules to the quencher and, as a consequence of this, to the surrounding solvent.

3.3. The long-lifetime component characterizes 8-ANS molecules attached to protein parts not accessible to water

3.3.1. The long-lifetime component of the fluorescence decay in 8-ANS-protein complexes is independent on the protein association

As it follows from Table 1 and Figs. 1 and 2, the long lifetime component of the fluorescence decay upon 8-ANS-protein interaction does not depend on the protein concentration, and, consequently, is not affected by protein association. This means that in this case the decay component is determined by protein-embedded 8-ANS molecules which are practically completely isolated from the environmental solvent.

3.3.2. The long lifetime component is much less accessible to the acrylamide quenching

Fig. 3 presents an impressive confirmation of the suggestion mentioned above. Indeed, when compared with the short lifetime component, those 8-ANS molecules which show the longer fluorescence lifetime possess much less accessibility to the quencher molecules. The corresponding bimolecular quenching rate constant k_q can be determined by Eq. (7) from the initial slopes of the curves presented in Fig. 3 and amounts to 6.5×10^6 M^{-1} s^{-1} and 1.5×10^7 M^{-1} s^{-1} for BCAB and lysozyme, respectively, whereas the short lifetime probe molecules are characterized by respectively, k_q values of 3.7×10^8 M^{-1} s^{-1} and 4.7×10^8 M^{-1} s^{-1}. In other words, the long lifetime component of 8-ANS fluorescence in the presence of proteins is less quenched by more than one order of magnitude.

3.4. The long lifetime component of the 8-ANS fluorescence is higher for the molten globules

As it follows from the analysis of data presented in Table 1, the long lifetime component of 8-ANS fluorescence in its complexes with the molten globules exceeds roughly 1.5 times the corresponding values observed for the dye after interaction with native proteins. It seems likely that changes of the decay time upon protein denaturation and unfolding can be correlated with an overall structural rearrangement of the protein molecule. In order to check this assumption the GdmCl-induced conformational transitions in BCAB and α-HLA were studied by using the fluorescence decay of 8-ANS in 8-ANS-protein complexes.

3.5. Studies of denaturant-induced conformational transitions in proteins by using 8-ANS fluorescence decay in dye–protein complexes

3.5.1. GdmCl-induced denaturation and unfolding of proteins at neutral pH

It is known that carbonic anhydrase and α-lactalbumin at neutral pH unfold by GdmCl in two separate steps, or, in other words, unfolding goes through the formation of the equilibrium molten globule state [6,21]. Accumulation of this intermediate can be easily visualized because the 8-ANS fluorescence intensity passes through the maximum [19]. The position of such a maximum correlates well with the maximal population of the molten globule state and is observed at 1.8 M and 2.0 M GdmCl for carbonic anhydrase and α-lactalbumin, respectively. As it follows from Figs. 4 and 5, overall changes of the protein conformation upon denaturation and unfolding are accompanied also by considerable changes of the 8-ANS fluorescence lifetime. This is

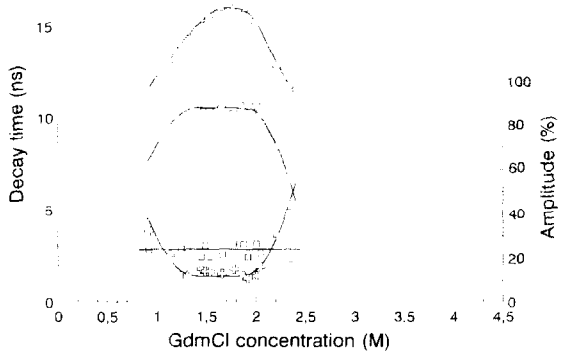

Fig. 4. Changes of the fluorescence decay parameters of the complexes 8-ANS-BCAB, connected with GdmCl-induced denaturation (N → MG transition) and unfolding (MG → U transition) of this protein at pH 6.8. □ and +, short lifetime component and its amplitude, respectively; △ and ▽, the long lifetime component of 8-ANS fluorescence and its amplitude, respectively.

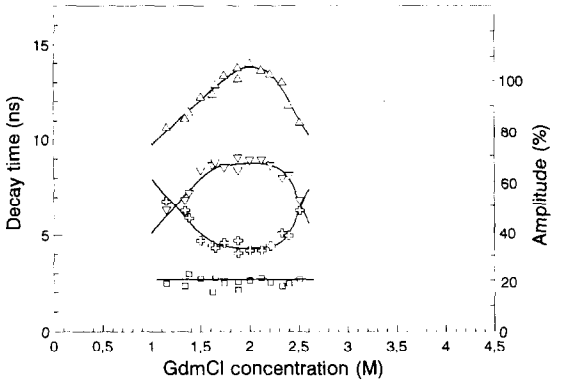

Fig. 5. Changes of the 8-ANS fluorescence decay parameters caused by GdmCl-induced conformational rearrangement of human α-lactalbumin at neutral pH: □ and +, short lifetime component and its amplitude, respectively; △ and ▽, long lifetime component of 8-ANS fluorescence and its amplitude, respectively.

especially the case for the long lifetime component. The dependence of this parameter on the GdmCl concentration passes through the maximum, which practically coincides with the point of maximum population of the molten globule state. Such behaviour can reflect the appearance and disappearance of the protein-embedded 8-ANS molecules upon the conformational transitions: native protein (N)–molten globule state (MG)–unfolded (U) protein.

Figs. 4 and 5 also show that the amplitudes of both fluorescence decay components ensure GdmCl-induced changes. For example, with the increase of the GdmCl concentration the contribution of the long lifetime component increases, reaches the plateau and eventually decreases. The changes in the amplitudes of different components of the dye fluorescence decay can be attributed to the changes of the number of probe molecules participating in the formation of different complexes. This means, that for the N → MG transition the number of protein-embedded 8-ANS molecules increases, while the number of dye molecules located at the protein surfaces and accessible to the solvent molecules decreases. For the MG → U transition the opposite behaviour was observed. Finally, it is necessary to underline that the value of the short lifetime component of 8-ANS remains practically independent of the applied GdmCl concentration in the range from 1 M to 2.5 M.

3.5.2. Guanidinium chloride-induced unfolding of the 'pure' molten globule

It is known that the equilibrium molten globule state can be obtained not only at moderate concentration of strong denaturants, but also under other mild denaturing conditions — low or high pH value or moderately high temperatures (for reviews see [4,5]). In many cases denaturant-induced N → MG and MG → U transitions are not well separated. Sometimes it is possible to obtain the 'genuine' molten globule states by the decrease of pH. One of the examples of such 'genuine' or 'pure' molten globule state is the acidic form of carbonic anhydrase B, accumulated at pH 3.6 [19,22–24].

Results obtained for the changes of the fluorescence lifetime parameters of 8-ANS-molten globule complexes upon GdmCl-induced unfolding of this intermediate state are presented in Fig. 6, panel A. Since in this case the protein molecule is already denatured (the molten globule state exists even in solution without denaturant), the high values of the long lifetime component and its amplitude are not surprising. It reflects the fact that all complexes between 8-ANS and protein molecules are formed starting from the early beginning of the transition. As it follows from Fig. 6A, increasing GdmCl concentration results in the decrease of the long lifetime component. The value of short lifetime component is independent of the concentration of denaturant while its amplitude increases upon the unfolding of the molten globule.

For comparison, Fig. 6B shows the usual unfolding curve characterizing the MG → U transition in this protein. This transition was monitored by the red-shift of the maximum of the tryptophan fluorescence spectrum. Changes in this parameter are connected with changes of the environmental polarity of the tryptophan residue. As BCAB contains 8 tryptophan residues distributed more or less stochastically within the protein molecule, the transition curve monitored by the tryptophan fluorescence reflects global rearrangements of the protein structure. Indeed, it has been established that in bovine carbonic anhydrase B the main change in the position of the maximum tryptophan fluorescence occurs at the MG → U transition [24].

As it follows from GdmCl-induced unfolding of 'genuine' molten globule (acid form of BCAB, see

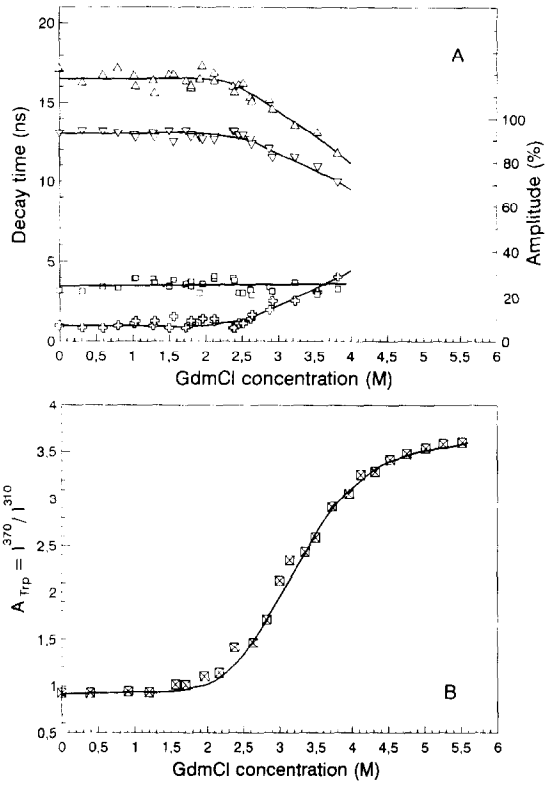

Fig. 6. GdmCl-induced unfolding of 'genuine' molten globule — acid form of BCAB — monitored by (A), changes of 8-ANS fluorescence decay parameters in 8-ANS-molten globule complexes. □ and +, short lifetime component and its amplitude, respectively; △ and ▽, long lifetime component of 8-ANS fluorescence and its amplitude, respectively. (B) Unfolding was monitored by the red-shift of the tryptophan fluorescence spectrum.

Fig. 6) a good correlation exists between the changes of the fluorescence lifetime and overall changes of the structure of the protein molecule. Changes in the fluorescence lifetime of 8-ANS-protein complexes indicate quite reasonably the processes connected with global transformations of the protein structure.

4. Discussion

The experimental findings given above promote time-resolved spectroscopy as a valuable tool for the study of conformational transitions in proteins. The reason for this conclusion follows from observations that, on the one hand, global conformational rearrangements of protein molecules involve all structural levels and, on the other hand, fluorescence decay parameters are highly sensitive to the microenvironment of the fluorophoric groups. Nevertheless, the fluorescence lifetime of the most sensitive intrinsic protein fluorophore tryptophan shows a rather complicated behaviour of its fluorescence decay. Thus, free tryptophan dissolved in water displays double-exponential decay kinetics [25–29]. Since time-resolved intrinsic fluorescence studies in proteins show that the fluorescence lifetime of a tryptophan residue varies by a factor 100 in different proteins [28] this method was successfully applied for the description of dynamic characteristics of the barstar molten globule state [30]. Hence, the interpretation of the results in practically all cases studied are complicated what is mainly due to the multi-exponential character of the fluorescence decay even in single-tryptophan proteins [28,31–40] or in the completely unfolded state of single-tryptophan proteins [41].

Results presented in this paper show one of the possible ways to apply the time-resolved fluorescence spectroscopy to investigations of global conformational rearrangement of protein molecules, avoiding practically all the difficulties mentioned above by using external rather than internal fluorescence labels. There is indeed a good chance to find among a large number of available external labels a proper one with quite definite physico-chemical properties necessary to respond on the corresponding folding phenomenon.

As an example, 8-ANS used in the present paper has an uni-exponential fluorescence decay in the nonbound state, independent on the solvent used. Its interaction with the protein molecule results in the appearance of double-exponential fluorescence decay in the nonbound state, independent on the solvent used. Its interaction with the protein molecule results in the appearance of double-exponential decay kinetics. Both decay components exceed the fluorescence lifetime of free 8-ANS in water considerably, reflecting a preferred attachment of the 8-ANS molecules to the hydrophobic regions of the protein molecule. A simple analysis of the data presented allows to conclude that the short lifetime component of 8-ANS refers to interaction with the surface of the protein molecule, while a long lifetime component refers to

the protein-embedded 8-ANS molecules. The most important finding of our studies is the correlation of changes in the long lifetime component with overall conformational changes of the protein molecule observed upon its denaturation and unfolding.

All this allows to look forward to the application of time-resolved fluorescence spectroscopy to analyze protein conformational transitions by using external fluorescence labels with large optimism.

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