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Study of the "Molten Globule" Intermediate State in Protein Folding by a Hydrophobic Fluorescent Probe

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SYNOPSIS

Binding of the hydrophobic fluorescent probe, 1-anilino-naphthalene-8-sulfonate (ANS), to synthetic polypeptides and proteins with a different structural organization has been studied. It has been shown that ANS has a much stronger affinity to the protein "molten globule" state, with a pronounced secondary structure and compactness, but without a tightly packed tertiary structure as compared with its affinity to the native and coil-like proteins, or to coil-like, α-helical, or β-structural hydrophilic homopolypeptides.

The possibility of using ANS for the study of equilibrium and kinetic molten globule intermediates is demonstrated, with carbonic anhydrase, β-lactamase, and α-lactalbumin as examples.

INTRODUCTION

One of the main difficulties in experimental investigations of folding and unfolding of proteins is the reliable identification and structural description of the intermediate states (see, for example, Refs. 1 and 2). The study of properties of the intermediate states formed at equilibrium folding and unfolding of globular proteins shows that several methods sensitive to different levels of protein structure must be used for a good structural description of these intermediates.[1-9] The multiparametric studies of equilibrium folding and unfolding of several globular proteins have elucidated the class of thermodynamically stable intermediates between the native and fully unfolded states. The common general properties of these intermediates are the presence of a pronounced secondary structure, a high compactness without a rigid packing inside a molecule, and a substantial increase of fluctuations of side chains as well as of larger parts of a molecule.[2-9] Due to these properties this class of intermediate states has been called the "molten globule" intermediate state (see, for example, Refs. 2, 9, and 10).

It has been found that the intermediates with similar properties accumulate at the early stage of refolding kinetics of some proteins.[11-13] This fact is of special interest as it means that there can be a general kinetic pathway for protein folding. Unfortunately, only a very restricted number of structural methods can be used for investigation of the fast (with a time of $\sim 10^{-2}$ s) processes of protein folding and for a structural description of short-living kinetic intermediates. Nevertheless, there is significant progress in these studies using such structural methods as CD,[14] electron spin resonance spectroscopy,[13] and nmr.[15,16]

In this paper we propose a much simpler method for identification of both the equilibrium and kinetic intermediate states of proteins, which are similar to the molten globule intermediate state. This method is based on a much stronger affinity of a fluorescent hydrophobic probe (1-anilino-naphthalene-8-sulfonate or others) to the molten globule intermediate state of proteins as compared with the native or the fully unfolded states. This affinity is due to the absence of rigid packing of hydrophobic clusters in this state and, hence, due to a greater accessibility of the protein hydrophobic core for a solvent. The for-

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mation of a rigid, tightly packed interior of a protein molecule with a low accessibility of protein hydrophobic clusters to a solvent leads to a decrease of 1-anilino-naphthalene-8-sulfonate (ANS) affinity for a protein molecule. The same phenomenon take place at disruption of a loosely packed hydrophobic core at transition from the molten globule state to a fully unfolded state.

EXPERIMENTAL

Samples

Carbonic anhydrase B from bovine blood erythrocytes, bovine and human α-lactalbumins from milk, and apo-cytochrome C from horse heart were prepared by V. E. Bychkova and N. V. Kotova (Institute of Protein Research, Pushchino). Carbonic anhydrase from human blood erythrocytes was prepared according to Ref. 17. β-Lactamase PC1 from *Staphylococcus aureus* was given by Prof. R. Pain (Newcastle upon Tyne, England). Polyacrylamide gel electrophoretic tests showed 98–99% purity of each protein preparation. Polylysine ($\sim$ 30,000 daltons; Sigma), polyglutamic acid (15,000–50,000 daltons; Sigma) were used without additional purification.

Solvents

All buffers were prepared with bidistilled water. Tris, glycine (Serva) and ANS (Fluka) were used without additional purification. Urea and guanidine hydrochloride (GuHCl) were purified by a twice recrystallization from water and from ethanol.

Procedure

Absorbance and fluorescence measurements were done with a Specord-M40 spectrophotometer (Carl Zeiss Jena, GFR) and a SPF-1000^{CS} spectrofluorimeter (Aminco, USA), respectively. The polypeptide conformational states, the coil-like and α-helical or the β-structural ones, were tested by CD spectra measured with a Mark-III-S spectropolarimeter (Jobin Ivon, France).

All measurements were carried out at 25°C. The protein concentration was 0.03–0.8 mg/mL. Fluorescence of ANS was excited at the maximum of the excitation spectra.

Kinetic measurements were done with a Durrum (USA) stopped-flow apparatus (dead time 2 ms) or with stopped-flow device schematically represented in Figure 1. We developed this device on the basis of an instrument constructed earlier.[18] It allows us to operate with any optical spectrometer with a 12 $\times$ 12 $\times$ 45 mm cell holder.

The solutions are injected into the observation cell (1) by a pneumatic drive (2) through thermostated syringes (3) with a 1 : 5 volume ratio. The syringes are connected by filling valves (4) and thermostated flexible tubes (5) with 2 $\times$ 6 hole tangential mixer (6) placed under the cell (1). The mixer (6) and the cell (1) are combined inside the thermostated block (7) with external dimensions of 12 $\times$ 12 $\times$ 50 mm. The reagents are collected by the syringe (8). The recording system includes a personal computer, a digital oscilloscope, a plotter, and five amplifiers with good possibilities for time constants. The cell has a volume of 90 μL. The dead time of attachment is 20 ms as measured by the reaction of $K_3Fe(CN)_6$ with ascorbic acid.[19] The temperature interval extends from 4 to 45°C. This stopped-flow attachment was combined with a spectrofluorimeter described earlier[20] and monitors several parameters (such as absorbance, intensity of fluorescence at different wavelengths, and polarization of fluorescence). Comparative measurements of the test reactions[19] with the described attachment and Durrum apparatus have shown no essential differences.

RESULTS AND DISCUSSION

Hydrophobic fluorescent probes have been long used for the study of exposed hydrophobic sites in proteins.[21,22] Binding of a probe to a protein leads usually to drastic changes of the probe fluorescent parameters. Fluorescence intensity increases several

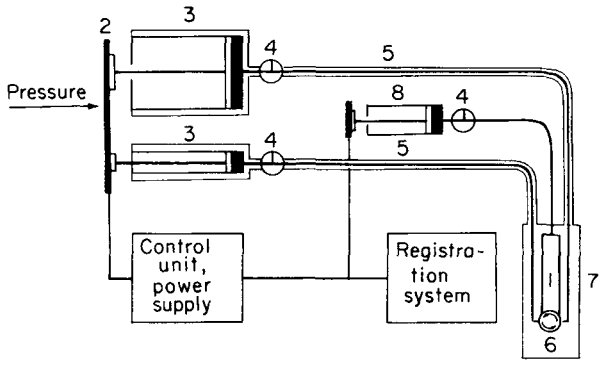

Figure 1 Scheme of the stopped-flow device for monitoring millisecond kinetic processes (see explanation in the text).

times while a spectrum shifts to a shorter wavelength.[21,22] This monitors probe binding (and, consequently, the existence of accessible hydrophobic sites) by the fluorescent technique. These probes also can be used to investigate hydrophobic core formation during folding of globular proteins. Interest in such information is conditioned by the essential role that the hydrophobic core plays in the protein structure and its stabilization. However, the interaction between the probe and proteins may be due not only to the hydrophobic, but also to the electrostatic forces, as all water-soluble probes have charged or polar groups (for example, ANS has a sulfonyl group). Moreover, one cannot exclude interaction of the probe with individual hydrophobic groups of proteins that are not involved in hydrophobic clusters or with secondary structure elements without hydrophobic clusters. All these circumstances lead to the necessity of studying the interaction of the hydrophobic probe (ANS in our case) with polypeptide chains of different compositions and conformations.

Binding of ANS to Homopolypeptides in the Random Coil, α-Helical, and β-Structural Conformations

To clarify the level of electrostatic interactions of ANS with polypeptides in the absence of hydrophobic side chains, we investigated the binding of ANS to polyglutamic acid and polylysine. It is known that polyglutamic acid has a random-coil conformation at neutral and alkaline pH when it is negatively charged, while polylysine has this conformation at neutral and acid pH when it is positively charged.

Figure 2 represents the fluorescence spectra of ANS in the presence of coil-like polylysine (at pH 5.0) and polyglutamic acid (at pH 8.0) as well as in the absence of polypeptides. One can see that at these conditions the fluorescence intensity of ANS practically does not depend on the presence of coil-like homopolypeptides with positively or negatively charged side chains.

The binding of a hydrophobic probe to a homopolypeptide with a secondary structure has been investigated before.[23,24] In these studies 2-*p*-toluidinyl-naphthalene-6-sulfonate (TNS) was used as a hydrophobic fluorescent probe and polylysine as a homopolypeptide. The authors have shown that TNS has almost no affinity to a coil-like or α-helical conformations of polylysine, while it binds appreciably to a β-structural conformation. This has been ascribed to the greater hydrophobicity of the β-structure as compared with the α-helix.[24]

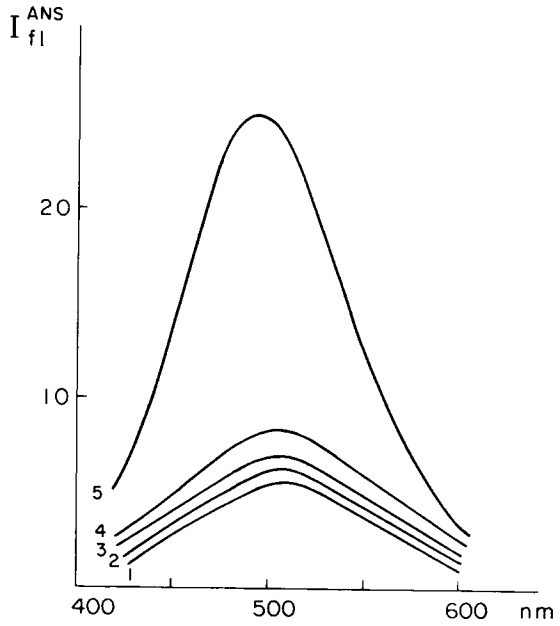

Figure 2 Fluorescence spectra of (1) free ANS (250 μM) at pH 5.0 or pH 11.0; (2) ANS (250 μM) in the presence of coil-like polyglutamine acid (H_2O, pH 8.0) or polylysine (H_2O, pH 5.0); (3) ANS (250 μM) in the presence of helical polylysine (H_2O, 0.15 M NaCl, pH 11.0); (4) ANS (250 μM) in the presence of helical polyglutamic acid (H_2O, 0.15 M NaCl, pH 4.0); and (5) ANS (250 μM) in the presence of β-structural polylysine (H_2O, 0.15 M NaCl, pH 11.0, after incubation for 30 min at 56°C and cooling up to 25°C). The ratio of molar concentrations [ANS]/[amino acid residues] = 1/1.

We have carried out analogous investigations with ANS and polylysine under our conditions, and have obtained similar results (see Figure 2). In particular, we did not observe a sufficient affinity of the ANS probe to the α-helical conformation of polylysine, while the β-structural conformation of polylysine binds ANS more strongly (Figure 2). Moreover, we did not observe a sufficient affinity of ANS to the α-helical conformation of polyglutamic acid (see Figure 2). It is noteworthy that ANS does not affect the polypeptide conformations as tested by CD measurements (not shown).

Thus, random and α-helical polypeptides in the absence of hydrophobic side chains have no considerable affinity to the hydrophobic ANS probe at a ratio of 1 molecule of ANS per 1 amino acid of homopolypeptide. The effect of electrostatic interactions on ANS binding is probably negligible in these cases. However, the β-structural polypeptides can bind the hydrophobic probe even in the absence of hydrophobic side chains. To explain this fact, we propose the same interpretation as given earlier,[24]

i.e., the β-structure is more hydrophobic than the α-helix in the absence of hydrophobic side chains.

Binding of ANS to the Proteins in the Native, Fully Unfolded, and Molten Globule Conformations

It is known that globular protein polypeptide chains include both hydrophilic (easily soluble in water) and hydrophobic (poorly soluble in water) side chains. Moreover, the secondary structure of proteins forms clusters from hydrophobic side chains that, in turn, form the hydrophobic core of globular proteins. Usually the hydrophobic core of the native water-soluble globular proteins is well protected from the solvent by the rigid packing of side chains (i.e., due to the rigid tertiary structure). This feature of globular proteins results in their low affinity to hydrophobic probes such as ANS.[21] In particular, carbonic anhydrase B and α-lactalbumin belong to this class of proteins (see Figure 3A). But some of the native proteins have a stronger affinity to ANS because they have hydrophobic sites exposed to the solvent (for example, active centers, sites for heme or substrate binding).[21,22] The *S. aureus* β-lactamase is an example of such proteins (Figure 3B; see also Table I).

The presence of hydrophobic side chains in disordered polypeptide chains (such as fully unfolded proteins) does not affect the ANS binding strongly. Figure 3A represents the fluorescence spectrum of ANS in the presence of apo-cytochrome C at pH 5.6 as well as in the presence of some proteins at high concentrations of strong denaturants. Under these conditions proteins practically have no secondary structure and are not compact.[9,3,25] Therefore they do not bind ANS.

The affinity of ANS to the protein molecule increases significantly when the rigidity of protein tertiary structure is disrupted, while the secondary structure and compactness is retained, i.e., when the molten globule intermediate state is formed.[2,9] In this state the hydrophobic core of proteins becomes more accessible for ANS than in the native state. Figure 3B and C represents spectra of ANS in the presence of the acid molten globule states of bovine carbonic anhydrase B (at pH 3.6)[2,4,8] and bovine α-lactalbumin (at pH 2.0),[5,9] as well as in the presence of neutral molten globule states (at a moderate con-

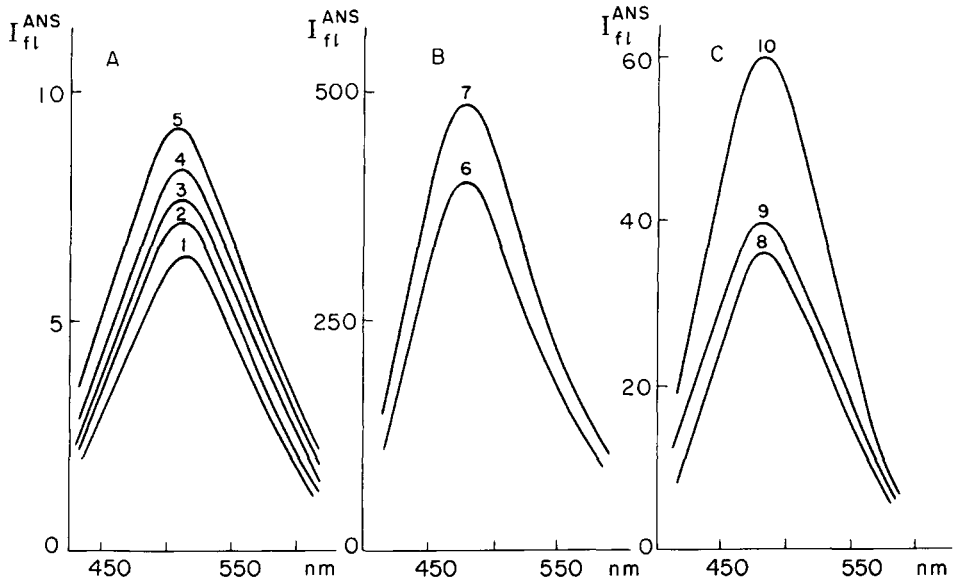

Figure 3 Fluorescence spectra of 250 μM ANS in the presence of the following: (A) 1—native (0.05 M Tris HCl, pH 8.0) and 2—unfolded (0.05 N Tris HCl, pH 8.0, 8.5 M urea) bovine carbonic anhydrase; 3—unfolded (0.05 M Tris HCl, pH 8.0, 6.0 M Gu HCl) and 4—native (0.05 M Tris HCl, pH 8.0) bovine α-lactalbumine; 5—coil-like apocytochrome C (0.05 M KCl, pH 5.6). (B) 6—molten globule state of bovine α-lactalbumine (H_2O, 0.1 M NaCl, pH 2.0) and 7—of the bovine carbonic anhydrase (0.1 M Gly HCl, pH 3.6). (C) 8—native (0.05 M Na phosphate, pH 7.0) *S. aureus* β-lactamase; 9—molten globule state of the bovine carbonic anhydrase (0.05 M Tris HCl, pH 8.0, 1.9 M Gu HCl) and 10—of the *S. aureus* β-lactamase (0.05 M Na phosphate, pH 7.0, 0.8 M Gu HCl). The ratio of molar concentrations [ANS]/[protein] = 250/1.

Table I Parameters (n: the Number of Binding Sites; K_{ass}: the Association Constant Values) of the ANS Affinity to Proteins in Various Conformational States

Protein	Conditions	Conformational State[a]	n	$K_{ass} \times 10^{-4}$ (M^{-1})
Bovine carbonic anhydrase	pH 8.0	N	Unmeasurable	
	pH 3.6	MG	2.0 ± 0.2	4.2 ± 0.6
	pH 8.0 and 1.9M GuHCl	MG	2.0 ± 0.2	1.3 ± 0.4
	pH 8.0 and 6.0M GuHCl	U	Unmeasurable	
S. aureus β-lactamase	pH 7.0	N	2.0 ± 0.2	2.5 ± 0.5
	pH 7.0 and 0.8M GuHCl	MG	3.0 ± 0.3	1.3 ± 0.5
	pH 7.0 and 4.0M GuHCl	U	Unmeasurable	

[a] N: native; MG: molten globule; U: unfolded.

centration of GuHCl) of bovine carbonic anhydrase B (at pH 8.0, 1.9M GuHCl)[3] and of the *S. aureus* β-lactamase (at pH 7.0, 0.7M GuHCl).[26] It can be seen that the ANS fluorescence in the presence of the molten globule states of the proteins (Figure 3) is greater than in the presence of the unfolded or native proteins even in the case of the β-lactamase, which binds ANS in the native state (Figure 3C and Table I).

The highest affinity of ANS to the molten globule intermediate states of globular proteins identifies these intermediates at equilibrium unfolding and folding of proteins. Figure 4 represents the change of ANS fluorescence at equilibrium unfolding of the bovine and human carbonic anhydrases B (which do not bind ANS in the native state) as well as of the β-lactamase (which binds ANS in the native state) by a decrease of pH (Figure 4A) or by an increase of a GuHCl concentration (Figure 4B). This figure shows that ANS fluorescence achieves the maximum mainly under those conditions under which the molten globule population is maximal (see known data[2-4,8,26,27]). Thus, the existence of the maximum of ANS fluorescence under unfolding or

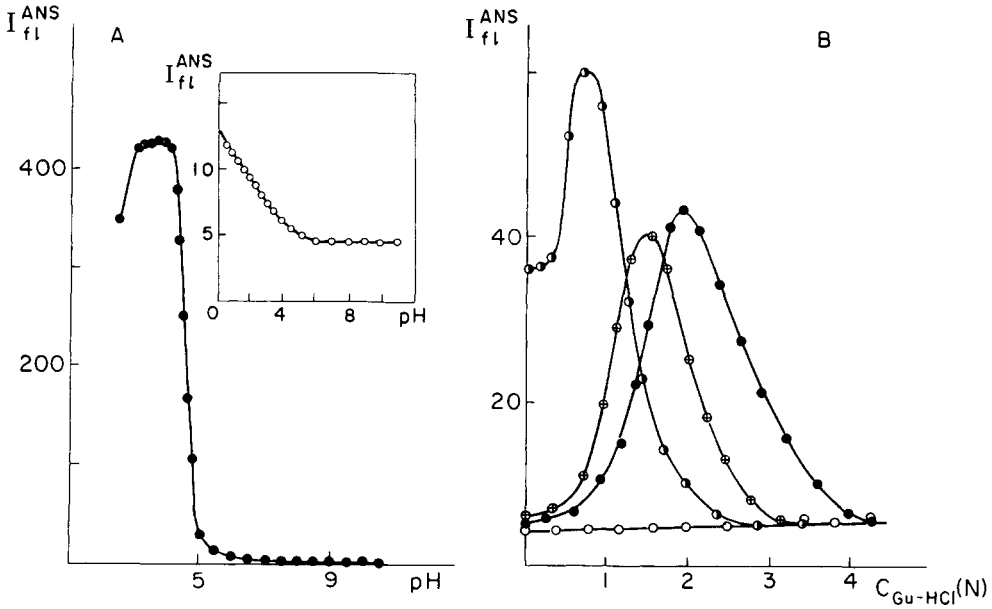

Figure 4 Change of the fluorescence intensity at 480 nm of 250 μM free ANS (— ○ —) and in the presence of bovine carbonic anhydrase B (— ● —), human carbonic anhydrase B (— ⊕ —), and *S. aureus* β-lactamase (— ◑ —) at a decrease of pH (A) and increase of the Gu HCl concentration (B). The other conditions shown on Figure 3.

refolding of the proteins indicates the possible accumulation of transient intermediates with properties of the molten globule state.

However, it is surprising that the ANS fluorescence intensity in the presence of the acid molten globule states is greater than in the presence of the neutral molten globule states (Figures 3 and 4). Similar results have been obtained recently with molten globule intermediate states of β-lactamase I from *Bacillus cereus*.[28] In this paper the authors interpret this result as a stronger affinity of ANS to acid molten globule possible due to additional electrostatic interactions of ANS with protein the molecule.[28] To clarify this effect, we carried out titration experiments to determine the number of ANS binding sites and the association constant values for the molten globule state under various pH using the Scatchard plot method. Figure 5 represents the result of these experiments for bovine carbonic anhydrase B (as an example). It can be seen that when only one ANS molecule is bound with the protein molecule (at high excess of the protein under ANS; Figure 5A and B, curves 2 and 4) the ANS fluorescence in the case of the acid molten globule (curve 2) is higher than in the case of the molten globule, which realized at neutral pH and moderate GuHCl concentration (curve 4). This means that here the quantum yield of ANS bound to the molten globule state at acid pH is higher than at neutral pH. In fact, as shown in Fig. 5A, B, and C, and Table I, the number of ANS binding sites is the same for the acid and neutral molten globule states of the bovine carbonic anhydrase, and the ANS association constant values are different only twofold. On the other hand, the fluorescence of ANS itself in solution increases only twofold at a change of pH from neutral values to acid [Figure 4A (insert)]. What is the reason of such large differences between the quantum yields of the ANS bound to acid and to neutral molten globule states of the carbonic anhydrase? Here it should be noted that the molten globule state can be readily associated due to its increased hydrophobicity. In some cases[28,29] the possibility of acid molten globule association was observed. Under comparatively low protein concentrations such associates can be labile and soluble. However, the en-

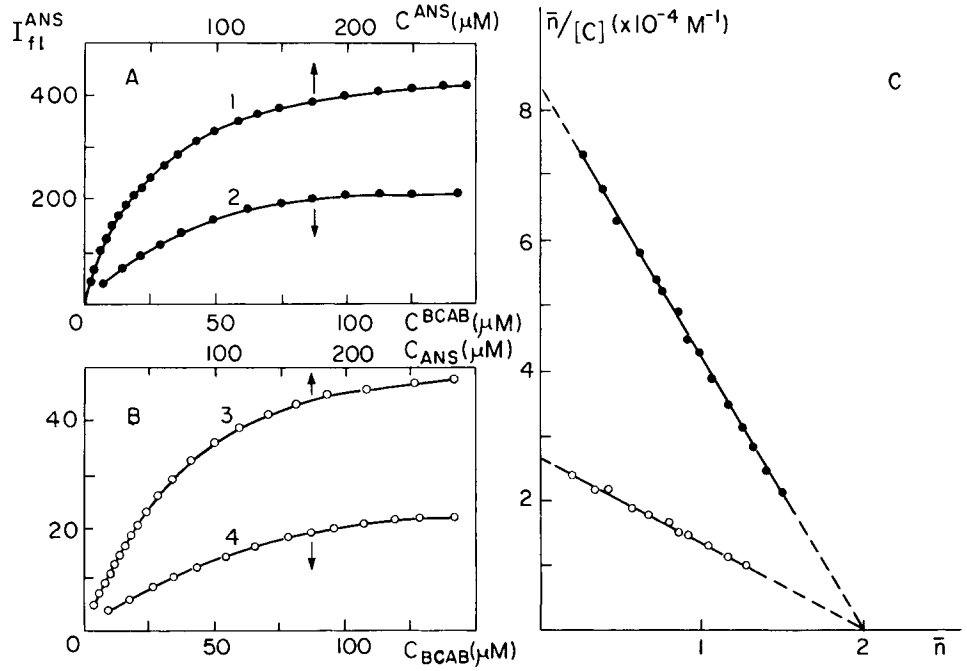

Figure 5 Parameters of the ANS binding: (— ○ —) to neutral (0.05*M* Tris HCl, pH 8.0, 1.9*M* Gu HCl) and (— ● —) to acid (0.1*M* Gly HCl, pH 3.6) molten globule states of the bovine carbonic anhydrase B (BCAB). (A) 1—titration curves of 1 μ*M* BCAB by ANS and 2—of 1 μ*M* ANS by BCAB at pH 3.6; (B) 3—titration curves of 1 μ*M* BCAB by ANS and 4—of 1 μ*M* ANS by BCAB at pH 8.0, 1.9*M* Gu HCl. (C) Scatchard plots; $\bar{n}$ is the moles of ANS bound per mole of BCAB; [*C*] is the free ANS concentration expressed as moles/liter.

vironment of the ANS molecules that are inside these associates must be less polar than in the case of monomers and hence the ANS quantum yield must be higher.[21,22] To check this idea, we measured the dependence of the ANS intensity of the acid molten globule state of the carbonic anhydrase on the dilution of this solution by a buffer without ANS and protein. Figure 6 represents this dependence in a logarithmic scale. It can be seen that the dependence is not linear. Extrapolation of the final linear part of this dependence (under strong dilution of the initial solution 100- to 1000-fold) to a zero dilution of the protein and ANS indicates that the intensity of ANS is one order less than the initial intensity. The insert in Figure 6 represents the titration curves of the acid and neutral molten globule states of the carbonic anhydrase with ANS at a concentration of the protein two orders lower than before (see Figures 4 and 5). Under extremal low protein concentration ($\sim 10^{-8}M$), the difference between the fluorescence of the ANS bound to acid and neutral molten globules is practically the same, as can be expected only from the pH dependence of

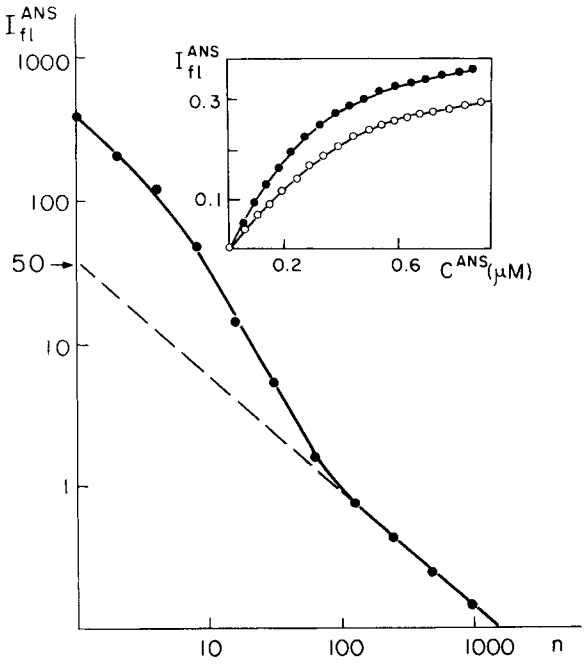

Figure 6 Dependence of the fluorescence intensity of the ANS solution in the presence of the acid molten globule of bovine carbonic anhydrase B (250 μM ANS, 1 μM protein, 0.1 M Gly HCl, pH 3.6) on the times (n) of the dilution by buffer (0.1 M Gly HCl, pH 3.6). Insert shows the titration curves of the 0.005 μM protein by ANS at pH 3.6 (— ● —) and at pH 8.0, 1.9 M Gu HCl (— ○ —).

the ANS fluorescence itself [Figure 4A (insert)]. Thus, the quantum yield of ANS bound to the molten globule state at some conditions can be considerably increased under the formation of the soluble associates. Nevertheless, from our point of view this circumstance cannot be a principal obstacle for using ANS as a useful probe for the determination of the molten globule intermediate state's possible existence.

Using ANS in Kinetic Studies of Globular Protein Refolding

We used the ANS technique in kinetic studies of globular protein refolding previously.[13,30,31] In these publications we concentrated attention only on the existence of a maximum of the ANS affinity to a protein molecule during its refolding. This maximum indicates an accumulation of the kinetic intermediate states with the highest affinity to ANS, i.e., intermediates with properties of the molten globule state (see Figure 4 and Table I). However, as the papers were very brief, it was impossible to describe all the experimental details of the ANS technique for refolding kinetic studies. In this part of our study we present experimental details and methodology of the ANS technique for study of protein refolding kinetics.

First. What time does it take for ANS to bind to the molten globule state? Figure 7A (insert) represents the stopped-flow experiment on mixing of the molten globule state (bovine carbonic anhydrase at pH 8.0 and 1.9 M GuHCl; see Figure 4B) with the ANS solution. One can see that the ANS binding to the molten globule state occurs within a dead time of the stopped-flow experiment (i.e., within 0.002 s). This means that the change of the ANS fluorescence during protein refolding is apparently due to conformational changes of the protein chain.

Second. Figure 7A and B shows the refolding kinetics of bovine carbonic anhydrase and human α-lactalbumine in the presence of ANS with presentation of the free ANS fluorescence level (both proteins in the unfolded and native states do not bind ANS; see Figures 2, 3A, and 4B). In both cases the increase of the ANS affinity to the protein chain has two stages. One occurs within a dead time of the stopped-flow experiment (within 0.02 s), while another can be recorded (Figure 7). It has been shown previously for bovine carbonic anhydrase B that the recorded process of increase of the ANS affinity to

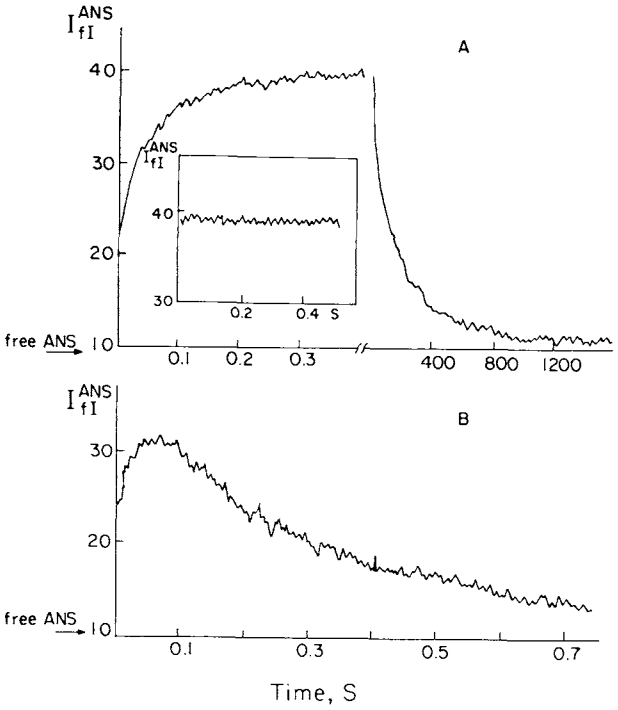

Figure 7 Kinetics of fluorescence intensity change of ANS in the presence of bovine carbonic anhydrase (A) and of human α-lactalbumine (B) upon their refolding. Final concentration of protein $10^{-6}M$ and ANS 500 $\times 10^{-6}M$. Refolding of both protein from the fully unfolded state (0.05M Tris HCl, 8.5M urea, pH 8.0, 500 $\times 10^{-6}M$ ANS) was initiated by a sixfold dilution with the stopped-flow device (see Figure 1) to the native conditions (0.05M Tris HCl, 1.4M urea, pH 8.0, 500 $\times 10^{-6}M$ ANS) at 25°C. The insert indicates the result of a twofold dilution of the molten globule-like intermediate state of bovine carbonic anhydrase (0.05M Tris HCl, 1.9M Gu HCl, pH 8.0; see Figure 4) with buffer (0.05M Tris HCl, 1.9M Gu HCl, pH 8.0, $10^{-3}M$ ANS) by the Durrum stopped-flow apparatus (dead time is 2 ms). The final protein concentration was $10^{-6}M$.

the protein chain has an intrinsic time $t_{1/2}$ = 0.04 s, which is practically the same as for the process of protein compactization ($t_{1/2}$ = 0.03 s).[13] The same result was also obtained for β-lactoglobulin.[32] This fact allows us to propose that formation of the hydrophobic core of the globular proteins proceeds simultaneously with the formation of the protein compactness.[13,32] But what does the superfast increase of the protein chain affinity to ANS mean (within 0.02 s; see Figure 7A and B)?

As noted previously[23,24] (Figure 2), β-structure formation in polypeptide chains even in the absence of hydrophobic side chains leads to a perceptible ANS affinity. Certainly, this affinity can be stronger due to the existence of hydrophobic side chains in proteins. Moreover, it is very likely that the α-helical conformation of protein chains with hydrophobic side groups can bind hydrophobic probes, as in this case where hydrophobic closters can be formed. But under the equilibrium conditions it is impossible at least now to realize the well-populated intermediates of proteins with pronounced secondary structure and without hydrophobic core, i.e., without compactness[27] probably due to the low stability of these intermediates under denaturating conditions. Nevertheless, such kind of intermediates can be well populated during protein refolding under strong native conditions, i.e., kinetically. Moreover, the experimental study of refolding kinetics of some proteins under strong native conditions shows that a large part of the protein secondary structure forms very fast (within 0.01 s), while the formation of the rigid tertiary structure of proteins proceeds more slowly (see, for example, Ref. 14). There are experimental data showing that the native structure formation of the globular protein occurs from the preceding compact intermediates with pronounced secondary structure (molten globule state).[11,13,30,32] Therefore it is reasonable to assume that the process of superfast (within 0.02 s) increase of the ANS affinity to the protein molecule (see Figure 7A and B) reflects the superfast secondary structure formation. To support the conclusions above, it must be noted that the formation of the complete secondary structure during refolding of the carbonic anhydrase and human α-lactalbumin under our final conditions (see Figure 7) occurs within 0.01 s and does not change further, as was monitored by the far-uv CD stopped-flow technique[32] (Semisotnov, and Kuwajima, in press).

Third. We did not observe the influence of ANS on refolding of studied proteins.[13,30,31] In all the cases during protein refolding, after an increase of the protein affinity to ANS, we observed a decrease of this affinity (see also Figure 7A and B).[13,30,31] The decrease of the protein affinity to ANS during its refolding (see Figure 7A and B) indicates a rigid packing of the hydrophobic core and usually occurs simultaneously with the restoration of other parameters (tryptophan fluorescence, absorbance, activity, ^{1}H-nmr, near-uv CD).[13,32,33] However, in the same cases the decrease of the protein affinity to ANS can be faster than the restoration of near-uv CD or activity.[13] This can mean that a rigid hydrophobic core of a protein can be formed earlier than a rigid surface.[13]

CONCLUSIONS

The experimental data presented in this paper show that the hydrophobic fluorescent probe (ANS) has a different affinity to various conformations of polypeptide chains (see Table I). This probe is not practically bound to coil-like and α-helical polypeptides without hydrophobic side chains or to fully unfolded proteins, as well as to a number of native proteins. On the other hand, ANS has an appreciable affinity to the β-structural conformation of a polypeptide chain (even in the absence of hydrophobic side groups) and to some native proteins. Moreover, the possibility of ANS binding to the α-helical conformations of the protein polypeptide chains with hydrophobic side groups cannot be excluded. Nevertheless, the results of this study show a very good correlation of the highest protein affinity to ANS with the formation of the molten globule intermediates. This allows us to record the accumulation of the molten globule-like intermediates during the equilibrium and kinetic processes of protein folding. At the same time it is noteworthy that not only formation of the molten globule-like intermediates can lead to the increase of the ANS fluorescence. Native proteins can also lose the possibility of ligand or substrate binding as result of a change in the conditions. In such cases, increase of the ANS affinity to the protein without a lose of the rigid packed tertiary structure can be registrated (for example, myoglobin and apomyoglobin[21]). If the protein can bind ANS in the native state, the unspecific association and even the pH decrease can also lead to the increase of the ANS fluorescence.

We cannot exclude cases when the number of the ANS binding sites and the association constants in the molten globule state will be the same as in the native state. In these cases it will be impossible to observe the decrease of the ANS fluorescence during protein refolding. Nonpredictable results can be obtained if the molten globule states form strong associates.

Nevertheless, the use of ANS can be very effective for the study of the loosely packed molten globule formation as well as formation of the rigid, packed hydrophobic core during protein refolding, and allows us to work with a simple technique (fluorescent spectroscopy) and at very low protein concentrations.

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