Solvation Dynamics in the Molten Globule State of a Protein

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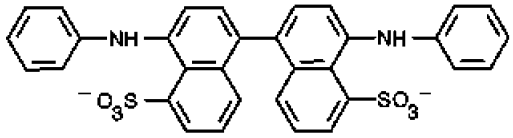
Solvation dynamics in the molten globule state of a protein, glutaminyl-tRNA synthetase (GlnRS), has been studied using both a noncovalent probe (bis-ANS) and a covalent probe 4-(*N*-thioacetylamino)-phthalimide. In the native state of GlnRS, bis-ANS exhibits an average solvation time ($\langle\tau_S\rangle$) of 1400 ps, which is 12 times longer than that for the covalent probe (120 ps). The difference in the solvation times for the two probes in the native state of the protein is ascribed to different locations of the probes. The covalent probe resides close to the protein surface and experiences fast relaxation of the water molecules. The noncovalent probe penetrates deeper inside the protein and displays slower relaxation in the buried region. In the molten globule state, $\langle\tau_S\rangle$ is 400 ps for the noncovalent probe and 250 ps for the covalent probe. Evidently, in the molten globule state, $\langle\tau_S\rangle$ is much longer than that the longest component of the solvation dynamics (∼1 ps) in bulk water. This shows that, in the compact molten globule state, the protein retains considerable residual structure.

1. Introduction

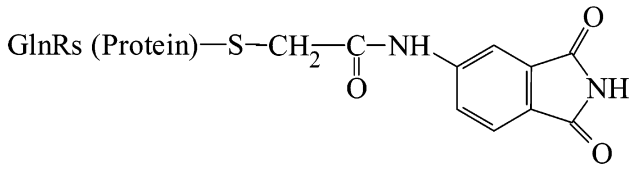
Relaxation of water molecules is essential to solvate the hydrophilic groups in a protein.[1] Thus water plays a fundamental role in structure, dynamics, and biological function of a protein.[1] Precise information on the solvent relaxation at a selected site of a protein has been obtained recently, using tryptophan as an intrinsic solvation probe for single-tryptophan proteins.[2−5] A second approach has been to label a single, selected site of a protein with a covalent probe such as acrylodan,[6] 4-(*N*-thioacetylamino)-phthalimide,[7] and dansyl group.[8] The third approach is to use a noncovalent probe whose location is inferred indirectly.[8−12]

According to these studies, the dynamics of the water molecules in the hydration shell of a protein displays a dramatically slow component. In bulk water, the longest component of solvation dynamics is ≈1 ps and a major part of solvation occurs in less than 0.1 ps.[13] In contrast, solvation dynamics in an aqueous solution of a protein often exhibits a component in the 100−1000 ps time scale. In a protein, the solvation time depends markedly on the location of the probe. Subtilisin Carlsberg (SC) contains a single tryptophan residue buried 4−5 Å below the protein surface. Zewail and co-workers reported that solvation dynamics of SC exhibits a component of 38 ps, which is markedly slower than the longest component of solvation dynamics of tryptophan in bulk water (1.1 ps).[2] However, when the same protein is labeled such that the probe (dansyl) resides 7 Å outside the protein surface, the 38 ps component vanishes and solvation dynamics becomes bulk-water-like.[2] Buzady et al. showed that solvation dynamics of the single tryptophan residue in human serum albumin (HSA) exhibits a component of 5 ns.[5] We have earlier studied solvation dynamics of a probe 4-(*N*-thioacetylamino)-phthalimide, (TAP, Scheme 1) covalently attached to a sulfhydryl group in a cleft approximately 8 Å below the surface of a protein, glutaminyl RNA synthetase (GlnRS).[7] In this case, solvation dynamics is described by two components−40 and 580 ps.

SCHEME 1: (a) Structure of Bis-ANS and (b) Protein Labeled with 4-(*N*-thioacetylamino)-phthalimide (TAP)



(a)



(b)

In a photon echo study on a noncovalent probe eosin bound to lysozyme, Fleming et al. detected a slow component of 530 ps that is absent in the case of eosin in bulk water.[8] We have reported earlier that the solvation dynamics of a noncovalent probe (DCM) bound to HSA shows two components−600 ps and 10 ns.[12]

The slow relaxation in the vicinity of a protein is also detected in NMR (NOE[14a] and NMRD[14b,c]) studies and in dielectric relaxation.[15] The dielectric relaxation times of an aqueous solution of a protein span a wide range and may be classified as reorientation of bulk water (about 10 ps), reorientation of the entire protein molecule (10−50 ns), and relaxation of water associated with the protein (10 ps to several hundred ps).[15b,16]

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To explain the slow relaxation in a protein, Nandi and Bagchi[16] proposed that the water molecules in the vicinity of a protein ("biological water") consist of two sets of water molecules−bound and free. The bound water molecules are completely immobilized while the free water molecules are bulk-like. According to Nandi and Bagchi the slow component of solvent relaxation arises from a dynamic equilibrium between bound and free water. More recently, several groups have carried out large-scale computer simulations on solvation dynamics in micelles,[17] reverse micelles,[18] and liquid interfaces.[19] These simulations also reveal a slow component of solvation.

In contrast to the native states, there are very few studies on solvation dynamics in the non-native or denatured state of a protein. Peon et al. reported that in the sweet protein monellin, the slowest component of solvation dynamics increases about 3 times from 16 ps in the native state to 56 ps on denaturation by 6 M guanidine hydrochloride.[3] The slow dynamics in denatured Monellin has been attributed[1a] to Rouse chain dynamics for a homopolymer.[20] According to this model, an unfolded protein resembles a polymer. For such a polymer, the solvation energy fluctuations display a multiexponential decay, $\Sigma a_i \exp(-t/\tau_i)$. According to Rouse chain dynamics, the eigen values of normal modes of polymer, λ_i ($= 1/\tau_i$) is given by[1a,20]

$$\lambda_i = 3D_0\left(\frac{i\pi}{Nb}\right)^2 \qquad (1)$$

where, N is the number of monomers and $i = 1, 3, 5 \ldots N-1$. D_0 denotes translational diffusion coefficient of a monomer, and b^2 is the mean square bond length. For monellin, $N = 50$ and, from eq 1, τ_i varies from 10 ps (for $i = 49$) to 30 ns (for $i = 1$).[1a,20] In the case of α-chymotrypsin, in the enzymatically active (native) state the solvation dynamics of a noncovalent probe is much faster than that in the inactive (acid denatured) state.[21] Dutta et al. reported that the average solvation time ($\langle\tau_s\rangle$) of coumarin 153 increases from 330 ps in the native state of lysozyme to 7250 ps for lysozyme denatured by sodium dodecyl sulfate (SDS).[22] When the disulfide bonds of lysozyme are destroyed by adding dithiothreitol to the lysozyme−SDS complex, $\langle\tau_s\rangle$ is found to be 1140 ps.[22]

In a non-native state, a protein is considered to be much less structured and much more exposed to bulk water. The slow dynamics in the denatured protein compared to a native state is apparently contrary to this. Several other recent studies also suggest the presence of considerable structure in the unfolded states.[23]

The transition from an unfolded state of protein to the native (folded) state may involve many intermediates. The molten globule state is one of the most important folding intermediates.[24] In the molten globule state the protein loses the tertiary structure while retaining a compact structure.[25] Such compact denatured states (e.g., molten globule)[24] have attracted a lot of recent attention because of their role in many neurodegenerative diseases.[24,25] A recent NMRD study indicates that the water molecules in the molten globule state exhibit a considerably long relaxation dynamics of a few ns.[18b] The elucidation of the origin of such slow dynamics may shed light on the structure and dynamics of the compact denatured states of proteins. Time dependent fluorescence Stokes shift provides information on slow water molecules in the vicinity of a probe and hence, in principle, may help to correlate dynamics with the structure.

With this end in view, we report on the solvation dynamics in the molten globule state of a protein, GlnRS.[26−28] It has been shown earlier that GlnRS forms a molten globule in the presence of 1.9 M urea.[28] In this work, we have studied solvation dynamics in the molten globule state of GlnRS using both a covalent probe (TAP, Scheme 1) and a noncovalent probe, bis-anilinosulfonate (bis-ANS, Scheme 1).[29] The inferred location of the covalent probe (TAP) is discussed in detail in a recent publication.[7]

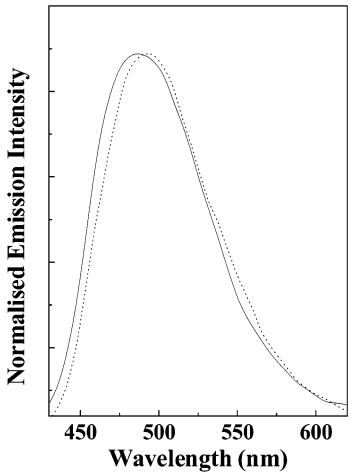

Figure 1. Steady-state emission spectra of bis-ANS in 100 mM tris buffer (pH = 7.5) solution in the presence of GlnRS without (—) and with 1.9 M urea (·····).

2. Experimental Section

Bis-ANS (Molecular Probes) was used as received. The procedures for isolation, purification, and labeling GlnRS with the covalent probe are described in detail elsewhere.[7,27−28] The steady-state absorption and emission spectra were recorded in a JASCO 7850 spectrophotometer and a Perkin-Elmer 44B spectrofluorimeter, respectively. To study the molten globule state, the protein in 1.9 M urea was allowed to stand for 10−12 h. Bis-ANS is known to disturb the native state if more than one probe binds to the protein.[29] Thus, native protein concentration (20 μM) was kept deliberately greater than the probe concentration (16 μM). In the molten globule state, the protein concentration was kept low (5 μM), as at higher concentrations protein aggregation may occur.

For lifetime measurements, the GlnRS labeled with the covalent probe was excited at 300 nm by the second harmonic of a rhodamine 6G dual jet dye laser with DODCI as saturable absorber (Coherent 702-1) synchronously pumped by a CW mode locked Nd:YAG laser (Coherent Antares 76s). The emission was collected at magic angle polarization using a Hamamatsu MCP photomultiplier (2809U). Our time correlated single photon counting (TCSPC) setup consists of Ortec 935 QUAD CFD and Tennelec TC 863 TAC. The data are collected with a PCA3 card (Oxford) as a multichannel analyzer. The typical fwhm of the system response is about 50 ps. In the case of bis-ANS, the samples were excited at 405 nm using a picosecond diode (IBH nanoled). In this case the system response is about 80 ps. In a tris buffer solution absorption and emission spectra of bis-ANS remain unchanged on addition of 1.9 M urea.

3. Results

3.1. Steady-State Spectra. The emission spectra of bis-ANS in a tris buffer solution (pH = 7.5) containing GlnRS are shown in Figure 1 ($\lambda_{ex} = 405$ nm). It is readily seen that in the absence of urea the emission maximum of bis-ANS is at 488 nm. Upon incubation in 1.9 M urea, GlnRS forms a molten globule state and its emission maximum undergoes a red shift by 5 nm to

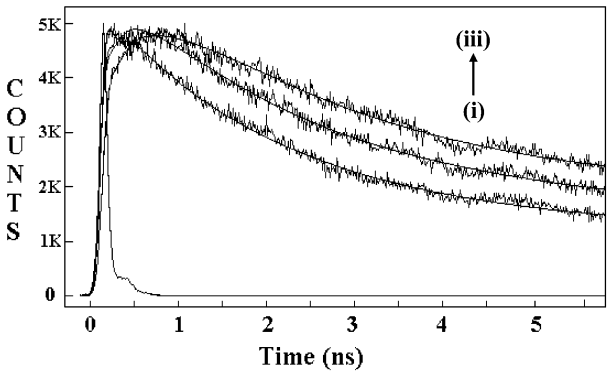

Figure 2. Fluorescence decays of 16 μM bis-ANS in 100 mM tris buffer (pH = 7.5) solution in the presence of 20 μM GlnRS at (i) 445 nm, (ii) 475 nm, and (iii) 580 nm.

493 nm. The red shift in the emission maximum suggests that in the molten globule state of GlnRS the probe (bis-ANS) experiences a microenvironment which is more polar than that in the native protein itself. This suggests that in the molten globule state the probe becomes more exposed to bulk water compared to the native state of the protein. In a tris buffer solution, absorption and emission spectra of bis-ANS remain unchanged on addition of 1.9 M urea in the absence of GlnRS.

3.2. Time-Resolved Studies. *3.2.1. Solvation Dynamics of bis-ANS in the Native State of GlnRS.* In the absence of urea (i.e., in the native state of the protein), in a tris buffer solution (pH = 7.5) containing 20 μM GlnRS and 16 μM bis-ANS, the fluorescence decays of bis-ANS are found to be markedly dependent on the emission wavelength (Figure 2). For example, at 445 nm (blue end) the fluorescence decay is triexponential with three decay components of 40 ps (42%), 1.4 ns (33%), and 7.8 ns (25%), while at 580 nm (red end) the decay of time constants 850 ps and 7.9 ns is preceded by a distinct rise with a time constant of 700 ps. The wavelength dependence of the fluorescence decays is a clear signature of solvation dynamics occurring in this system. Following the procedure given by Fleming and Maroncelli,[30] the time-resolved emission spectra (TRES) were constructed using the parameters of best fit to the fluorescence decays and the steady state emission spectrum. The TRES clearly show a time dependent Stokes shift of the emission spectrum of bis-ANS bound to GlnRS (Figure 3). The solvation dynamics is described by the decay of the solvent correlation function $C(t)$, defined as,

$$C(t) = \frac{\nu(t) - \nu(\infty)}{\nu(0) - \nu(\infty)} \qquad (2)$$

where $\nu(0)$, $\nu(t)$, and $\nu(\infty)$ are the peak frequencies at time 0, t, and ∞, respectively. The decay of $C(t)$ is shown in Figure 4, and the decay parameters are summarized in Table 1. It is readily seen that in the solution of GlnRS the decay of $C(t)$ is biexponential, with a fast component of 170 ps (45%) and a slow component of 2400 ps (55%). The average solvation time ($\langle\tau_s\rangle = \sum a_i\tau_i$) is found to be 1400 ps. The total Stokes shift for bis-ANS bound to native GlnRS is found to be 750$\pm$50 cm^{-1}.

3.2.2. Solvation Dynamics of bis-ANS in the Molten Globule State of GlnRS. To study solvation dynamics in the molten globule state of GlnRS, 5 μM GlnRS and 10 μM bis-ANS were incubated in 1.9 M urea. Under this condition, the fluorescence decays of bis-ANS once again become wavelength dependent. In this case, at the blue end (445 nm) the decay is fitted to a triexponential with three components of 90 ps (40%), 1.85 ns (26%), and 8 ns (34%). However, at the red end (580 nm), bis-

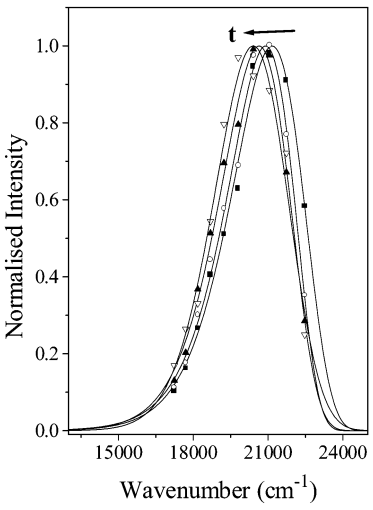

Figure 3. Time-resolved emission spectra of 16 μM bis-ANS in 100 mM tris buffer (pH = 7.5) solution in the presence of 20 μM GlnRS at 5 ps (■), 150 ps (○), 1000 ps (▲), and 9000 ps (▽).

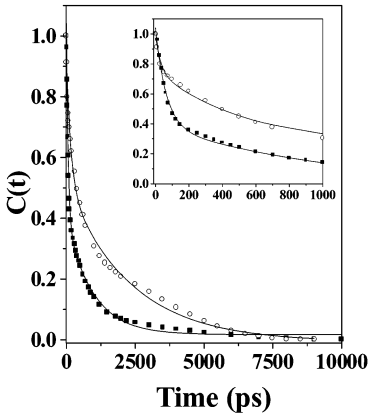

Figure 4. Decay of response function, $C(t)$, of bis-ANS in the presence of GlnRS without urea (○) and with 1.9 M urea (■) in tris buffer (pH = 7.5). The points denote the actual values of $C(t)$ and the solid line denotes the best fit to a biexponential decay. The initial parts of the decays of $C(t)$ are shown in the inset.

ANS in the molten globule state of GlnRS exhibits decay components of 1.7 ns and 7.6 ns with a distinct rise of 560 ps. The TRES clearly show a time dependent Stokes shift of emission. The decay of $C(t)$ for bis-ANS bound to the molten globule state of GlnRS is found to be biexponential with a very fast component of 60 ps (63%) and another of 960 ps (37%), with an average solvation time of 400 ps (Figure 4, Table 1). The total Stokes shift is observed to be 500$\pm$50 cm^{-1}.

It is well known that the molten globule states of a protein are prone to aggregation. A protein aggregate may have dynamics very different from the monomer. To verify that under this condition (5 μM) GlnRS does not aggregate, we have measured the intensity of the scattered light as a function of time after addition of urea to 5 μM solution of GlnRS for several hours. No increase in the intensity of the scattered light was observed. This indicates that at this protein concentration (5 μM) no aggregation takes place.

3.2.3. Solvation Dynamics of 4-(N-thioacetylamino)-Phthalimide Covalently Bound to GlnRS in the Presence of 1.9 M Urea. Solvation dynamics in the molten globule state of GlnRS was also studied using a covalent probe, 4-(*N*-thioacetylamino)-phthalimide (TAP, Scheme 1). In a 100 mM potassium phosphate (KP) buffer solution (pH = 7.5) and in the presence of 1.9 M urea, emission decays of the probe covalently bound

TABLE 1: Decay Parameters of $C(t)$ in Different Systems

probe	system	$\Delta\nu^a$ (cm^{-1})	a_1	$\tau_1{}^a$ (ps)	a_2	$\tau_2{}^a$ (ps)	$\langle\tau\rangle^{a,b}$ (ps)
16 μM bis-ANS	20 μM GlnRS	750	0.45	170	0.55	2400	1400
10 μM bis-ANS	5 μM GlnRS + 1.9 M urea	500	0.63	60	0.37	960	400
60 μM 4-AP derivative	60 μM GlnRS	1330	0.85	40	0.15	580	120
60 μM 4-AP derivative	60 μM GlnRS + 1.9 M urea	700	0.77	50	0.23	900	250

[a] $\pm$10%. [b] $\langle\tau\rangle = a_1\tau_1 + a_2\tau_2$.

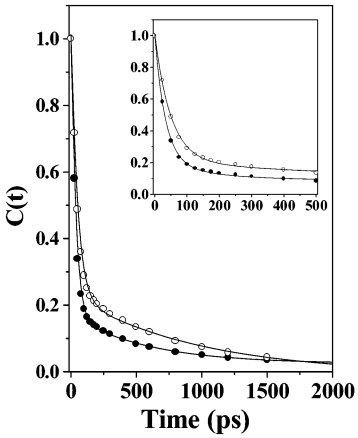

Figure 5. Decay of response function, $C(t)$, of 4-(*N*-thioacetylamino)-phthalimide covalently bound to GlnRS in the absence of urea (●) and in the presence of 1.9 M urea (○) in phosphate buffer (pH = 7.5). The points denote the actual values of $C(t)$, and the solid line denotes the best fit to a biexponential decay. The initial parts of the decays of $C(t)$ are shown in the inset.

to GlnRS show a marked wavelength dependence. At the blue end (410 nm), a fast decay component of 400 ps (23%) and a long component of 6 ns (77%) exist. However, at the red end (540 nm), the decay of 6.5 ns is preceded by a distinct rise of 50 ps. The decay of $C(t)$ is found to be biexponential with a very short component of 50 ps (77%) and another component of 900 ps (23%), with the average solvation time 250 ps (Figure 5, Table 1). The total Stokes shift is observed to be 700$\pm$50 cm^{-1}. We have earlier studied solvation dynamics of the covalent probe bound to GlnRS (Scheme 1) and found that the average solvation time is 120 ps.[7] For comparison, the decay of $C(t)$ for the covalent probe in the native state of GlnRS is also included in Figure 5. Evidently, the average solvation time in the presence of 1.9 M urea, i.e., in the molten globule state, is about two times longer than that in the native state.

4. Discussion

The location of the covalent probe, 4-(*N*-thioacetylamino)-phthalimide, (TAP, Scheme 1) in the protein GlnRS is inferred fairly accurately.[7] GlnRS is a multidomain protein containing several sulfhydryl groups. Using X-ray crystallography, Rould et al.[26] studied the reactivity of various cysteines of GlnRS toward heavy atoms (Au, Hg, etc). They showed that cysteine 206 is the most reactive sulfhydryl and most probable site for heavy atom labeling. It is highly exposed and resides near the surface of the protein. Under the conditions of our experiment, only one probe molecule is attached to the protein. Based on previous reactivity studies[26,27] we expect that the probe is attached to the most reactive cysteine 206 but binding to other sites, which stay at a depth 5$-$8 Å from the surface of the protein, cannot be totally ruled out.[7] In a previous publication we have given a detailed picture of the site 206 in GlnRS generated using the RASMOL program.[7] The sulfur atom of cysteine 206 is situated in a cleft at a depth $\approx$8 Å from the surface of the protein.[7] A simple MM2 calculation indicates that the length of the linker ($-CH_2CO-$) between the S atom of the protein and the amino nitrogen of the probe (aminophthalimide) is about 3 Å. If we subtract the distance of about 3 Å from the sulfur to the amino nitrogen, the nitrogen atom is situated at a depth of 5 Å from the protein. Thus about half of the probe aminophthalimide moiety (diameter $\approx$ 8 Å) remains buried inside the protein and the other part sticks partially out of the protein, and hence the probe reports of dynamics at or near the surface of the protein. We have reported earlier that in the native state of GlnRS the average solvation time is 120 ps.[7] In the present work, we found that upon incubation in 1.9 M urea, GlnRS forms a molten globule and the average solvation time increases to 250 ps.

The location of the noncovalent probe (bis-ANS) within GlnRS is known less precisely. Since the concentration of probe is less than that of GlnRS, it may be assumed that at most one bis-ANS binds to each protein and the probe reports property of only one location. The average solvation time of bis-ANS (1400 ps) is nearly 12 times longer than that of the covalent probe. The difference in the average solvation time reported by bis-ANS and 4-(*N*-thioacetylamino)-phthalimide indicates that in the native state of GlnRS the two probes reside in different locations. The slower dynamics reported by bis-ANS corresponds to a buried location. Fluorescence quenching studies indicate that bis-ANS bound to GlnRS is virtually nonquenchable.[31] This also indicates that bis-ANS is buried deep inside and is inaccessible to the quencher.[31] It may be noted that in the case of α-chymotrypsin the location of a noncovalent probe (ANS) both in the native and the denatured state has been ascertained using X-ray crystallography.[32] Unfortunately, such clear-cut information on the location of the noncovalent probe (bis-ANS) in the molten globule state of GlnRS is not available.

We will now try to estimate the binding energy of the water molecules at the locations around the two probes. According to Nandi and Bagchi,[16] the magnitude of the slow component of solvent relaxation depends on the difference between the free energy of the bound (G_b°) and the free water molecules (G_f°), with $G_b^\circ < G_f^\circ$. In the limit of very high binding energy i.e., $|\Delta G_{bf}^\circ|$, the slow component of solvation (τ_{slow}) is given by[1a,14]

$$\tau_{slow} \approx k_{bf}^{-1} \tag{3}$$

where k_{bf} is the rate constant for bound-to-free interconversion,

$$k_{bf} = \left(\frac{k_B T}{h}\right) \exp\left(\frac{-(|\Delta G_{bf}^\circ| + \Delta G^*)}{RT}\right) \tag{4}$$

where ΔG^* is the activation energy for the conversion of free-to-bound water molecules. From eqs 3 and 4 and using the average solvation times ($\langle\tau_s\rangle = \tau_{slow}$), one may calculate the free energy difference ($|\Delta G_{bf}^\circ|$) between the bound and free water molecules in different systems. Using $\Delta G^* \approx 900$ cal mol^{-1}, in the native state of GlnRS, $|\Delta G_{bf}^\circ|$ is calculated to be 3.0 kcal mol^{-1} in the case of the covalent probe (4-(*N*-thioacetylamino)-phthalimide) and 4.5 kcal mol^{-1} in the case of the noncovalent probe (bis-ANS). The difference in ΔG_{bf}° suggests that bis-ANS goes deep inside the protein where the

bound water molecules are held much more tightly compared to the immediate environment of the covalent probe, which monitors relatively loosely held water molecules.

In contrast to the native state, in the molten globule state of the protein the difference in solvation times reported by the two probes is much smaller (250 ps for the covalent probe and 400 ps for the noncovalent probe). These components correspond to $|\Delta G^{\circ}_{bf}| = 3.4$ kcal mol^{-1} for the covalent probe and 3.7 kcal mol^{-1} for the noncovalent probe. This shows that in the molten globule state binding energy of water molecules around the two probes are quite close.

It is important to note that there is a large difference in average solvation time and ΔG°_{bf} for the two probes in the native state. However, in the molten globule state the difference is much less. It is possible that the location of the noncovalent probe may be different in the native and in the molten globule state. An alternative explanation is as follows. The molten globule state involves greater penetration of water into the protein structure so that there is no deeply buried location with a long solvation time as reported in the native state. This may be the reason for the shortening of the solvation time in the case of bis-ANS in going from the native to the molten globule state.

The increase in solvation time for the covalent probe during the transition from native (120 ps) to molten globule state (250 ps) is intriguing. However, one should note that solvation time in a completely denatured protein has been found to be longer than that in a native state.[3,21−22] To explain this it has been proposed that a denatured protein resembles an extended polymer, and hence the slow dynamics in a denatured protein has been attributed[1a] to Rouse chain dynamics of a polymer.[20] In a molten globule, the protein contains wrong contacts but the protein as a whole may not be considered as an extended polymer. However, even in a molten globule a small region of the protein, particularly the surface region, may open up and resemble an extended polymer. For a probe (TAP) which resides near the surface of a protein, the transition from the native to the molten globule state may thus result in an increase in the average solvation time. Since the covalent probe (TAP) resides near the surface of the protein, the increase in solvation time during the transition from native to molten globule state may be attributed to Rouse chain dynamics of the polymer at the surface of the protein in the molten globule state.

The most important finding of the present work is obviously the rather long average solvation time in the molten globule state. The prevailing notion about the molten globule state has been that in the molten globule state the protein loses its tertiary structure and the solid packing.[24] This should cause relatively fewer conformational restraints and, consequently, faster solvent relaxation. However, as shown in the present work, the solvation dynamics in the molten globule state is considerably slower than that in bulk water (<1 ps) for both the noncovalent probe (bis-ANS, 400 ps) and the covalent probe (TAP, 250 ps). The slow dynamics in the molten globule state indicates a considerable amount of residual structure and restricted movement of the water molecules. The slow dynamics in the molten globule state observed in the present work is consistent with the NMRD studies.[14b]

5. Conclusion

The present work clearly demonstrates that the solvation dynamics in the molten globule state of GlnRS is quite slow. The slow solvation dynamics in the protein hydration shell in the non-native protein conformations is consistent with a recent NMRD study on molten globules.[2a] In the native state, the marked difference in the solvation times of the two probes has been assigned to different locations of the probes and the difference in the binding energy of the water molecules.[16] The noncovalent probe, bis-ANS, resides in a deeply buried location and hence exhibits a solvation time 12 times longer compared to that of the covalent probe, 4-(*N*- (thioacetylamino)-phthalimide, which resides near the surface of the protein. In the molten globule state, when the protein loses its tertiary structure, solvation times of the two probes differ by only about a factor of 1.5.

In protein−macromolecule and protein−ligand interactions, the interacting surface has to be dehydrated before the ligand or the macromolecule binds to it. Elucidation of the protein−ligand and protein-macromolecule interactions requires deeper knowledge about the nature of the local hydration. A detailed understanding of the structure and dynamics of the hydration layer of a protein may have profound implications in molecular recognition and drug design.

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