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Dynamics of compact denatured states of glutaminyl-tRNA synthetase probed by bis-ANS binding kinetics

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Abstract

Bis-ANS binds to native glutaminyl-tRNA synthetase (GlnRS) with a fast and a slow phase. The rate constant of the slow phase is independent of bis-ANS concentration suggesting a slow conformational change in the pathway of bis-ANS binding. Aging of GlnRS causes a large decrease of the slow phase amplitude with concomitant increase of the fast phase amplitude. Several other large, multi-domain proteins show similar patterns upon aging. The near UV-CD spectra of the native and the aged GlnRS remain similar. Significant changes in far UV-CD, acrylamide quenching and sulfhydryl reactivity, are seen upon aging, suggesting disruptions in native interactions. Refolding of GlnRS from the urea-denatured state rapidly produces a state that is very similar to the equilibrium molten globule state. Bis-ANS binds to the molten globule state with kinetics similar to that of the aged state and unlike that of the native state. This suggests that the slow binding phase of bis-ANS, seen in native proteins, originate from relatively high energy barriers between the native and the more open states. Thus bis-ANS can be used as a powerful probe for large amplitude, low-frequency motions of proteins.

Keywords: Aging; GlnRS; bis-ANS; Denaturation/kinetics

Abbreviations: Bis-ANS, 1,1′-bi(4-anilino)naphthalene-5,5′-disulfonic acid; Tempol, (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl); DTNB, dithio-bis-(2-nitrobenzoic acid); CD, circular dichroism; RNAse A, Ribonuclease A; IDH (NADP), NADP-dependent isocitrate dehydrogenase; LADH, liver alcohol dehydrogenase; BSA, bovine serum albumin; GluRS, glutamyl-tRNA synthetase; GlnRS, glutaminyl-tRNA synthetase

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1. Introduction

Folding and unfolding of smaller proteins are often characterized by two-state transition behavior [1], although increasingly the existence of compact denatured states of the molten globule type is being reported. Compact denatured states in proteins can be generated by varying pH, temperature and concentrations of various kinds of denaturants, such as, urea and guanidine hydrochloride [2–4]. The characterization of these compact denatured states in proteins had a tremendous impact on folding studies [5–7]. Less is known about non-native states of large multidomain proteins, although it has been suggested that majority of the proteins in the cell belong to this class [8].

Denaturation of proteins often involves changes in solvent accessibility of hydrophobic regions. One of the most important probes, for studying protein denaturation is bis-ANS [9]. Bis-ANS binds to accessible hydrophobic areas of proteins, which results in fluorescence enhancement and blue shift of emission maximum. Previously, most investigators assumed that bis-ANS binding to proteins is rapid. Recently, Fink and co-workers have shown that the binding of bis-ANS to DnaK is biphasic. One of the phases is rapid, which is over within the dead time of manual mixing and the other is very slow and strongly temperature-dependent with a half-life ranging from minutes to hours [10]. This has now been confirmed in other proteins [11]. They have hypothesized that binding of bis-ANS to a more open state of the protein that is in equilibrium with the native state and gradual conversion results in slow binding kinetics.

In this article we have explored bis-ANS binding kinetics to two compact denatured states of GlnRS, molten globule and aged, and its structural implications. Prasad et al. [12] and Sarkar et al. [11] have reported an aged state of tubulin, which has increased bis-ANS fluorescence compared with the native state. GlnRS is a five-domain protein of molecular weight 65 kDa. The N-terminal half of the protein consists of alpha-beta-alpha architecture of familiar Rossman fold. The C-terminal half has two domains which are all beta type and is devoid of helices. One major advantage of GlnRS, is that unlike tubulin, it is a monomer and thus is devoid of added complications of protein monomer–dimer equilibrium. We demonstrate that kinetics of bis-ANS binding is a sensitive monitor of large amplitude slow motions in proteins, which are thought to play important roles in protein function.

2. Experimental

2.1. Materials

Bis-ANS was purchased from Molecular Probes Inc. (Eugene, OR). All other chemicals were of analytical grade.

2.2. Methods

2.2.1. Enzyme purification

Glutaminyl-tRNA synthetase was purified according to Bhattacharyya et al. [13]. Enzyme assays were done as described in the same article.

2.2.2. Aging protocol

The isolated protein, as described above, is kept frozen at −80°C. They were thawed and the enzyme was dialyzed against 0.1 M Tris–HCl buffer (pH 7.5) at 4°C before use. This state is designated as the native state. The aging was always carried out in solutions of 750 μl volume [0.1 M Tris–HCl buffer (pH 7.5), containing 15 mM $MgCl_2$] in Pyrex glass vials. The aging process was performed by incubating 2 μM protein solution at 37°C for 3 h. The vials were then withdrawn, the contents were transferred to a cuvette, equilibrated at 25°C. It was then mixed with 10 μM bis-ANS and the fluorescence measured. The fluorescence was measured in a Hitachi F3010 spectrofluorometer. The chamber was maintained at 25°C by circulating water from a constant temperature water bath. The excitation and emission wavelengths were 450 and 490 nm, respectively. The band passes were 5 nm each.

2.2.3. Stopped-flow measurements

The fast phase of the kinetics of association of

bis-ANS with native and aged GlnRS was measured in SX18.MV kinetic spectrometer from Applied Photophysics Limited (UK). Samples were excited at 360 nm and the emission was measured at 480 nm. The dead-time of the instrument was 2 ms. The large syringe contained the protein solution at 5 μM and the smaller syringe contained bis-ANS solution in the same buffer at 100 μM. The two solutions were mixed at a ratio of 10:1. The final concentrations were 4.5 and 10 μM. The temperature was 25 ± 1°C. The first part of the fluorescence build-up curve as a function of time was fitted to a second order rate equation using Sigma Plot to obtain the second order rate constant.

2.2.4. Non-linear least squares fit

The fluorescence increase was fitted to a single exponential with a zero-time offset (rapid binding component) by a non-linear least squares fit procedure described by Sengupta et al. [14]. In this procedure, three parameters, zero-time fluorescence value (F_o), rate constant (k) and the fluorescence value at infinite time (F_{inf}) were varied systematically to produce the best fit.

2.2.5. Determination of initial and equilibrium binding

Initial and equilibrium binding of bis-ANS were determined by mixing the native protein or the aged protein with different concentrations of bis-ANS and measuring the fluorescence intensity as a function of time. The data were then fitted to a single exponential with a zero time offset and the F_o and F_{inf} values were extracted. The F_o and F_{inf} values were then plotted against total bis-ANS concentration. The aging was performed as described above. The kinetics of binding was determined in solution conditions as described above.

2.2.6. Activation energy

The rate of aging at each temperature (37, 35, 34 and 33°C) was determined as described in the aging protocol, except the incubation temperature was varied. F_o and F_{inf} were calculated by non-linear least squares fit as described above. The fraction of native protein at time t is defined as

$$[(F_o/F_{inf})_\infty - (F_o/F_{inf})_t]/[(F_o/F_{inf})_\infty - (F_o/F_{inf})_{zero}]$$

where the subscripts, zero, t and ∞ refer to times at 0, t and infinity. Since F_{inf} values for the aged and the non-aged proteins are very similar, F_o/F_{inf} ratio is a direct measure of the aged population.

2.2.7. CD spectroscopy

Far UV CD spectra were obtained in a 1-mm pathlength cuvette, whereas the near UV spectra were recorded in 10-cm pathlength cuvette. The spectra were recorded in 0.1 M Tris–HCl (pH 7.5) containing 15 mM $MgCl_2$ at ambient temperature, which was 25 ± 1°C. The protein concentration was 2 μM in each case. Spectra were signal averaged to improve the signal-to-noise ratio. The spectrum of the buffer was subtracted from each spectrum.

2.2.8. Quenching of tryptophan fluorescence

The quenching was carried out in 0.1 M Tris–HCl buffer (pH 7.5) containing 15 mM $MgCl_2$. Small volumes of 2 M acrylamide solution were added and the fluorescence measured. The excitation wavelength was 295 nm and the emission wavelength was 340 nm. The bandpasses were 5 nm. The fluorescence values were corrected for dilution effect and no significant inner filter effect is seen in this range of acrylamide concentration.

2.2.9. Sulfhydryl reactivity

The aging was carried out in 0.1 M potassium phosphate buffer (pH 7.5) as described. The sulfhydryl reactivity was measured in 0.1 M potassium phosphate buffer (pH 7.5) at a final DTNB concentration of 1 mM and protein concentration of 2 μM. The absorbance was measured at 412 nm in a Shimadzu UV-160 double beam spectrophotometer. A similar measurement was done in 0.1 M Tris–HCl (pH 7.5) containing 15 mM $MgCl_2$, which yielded similar results.

2.2.10. Refolding studies

GlnRS was incubated with 8 M urea for 18 h. The refolding was initiated by diluting the protein into 0.1 M Tris–HCl (pH 7.5) containing 15 mM $MgCl_2$. The spectral measurements were then conducted as a function of time as described above.

3. Results

3.1. Bis-ANS binding kinetics to native and aged GlnRS

The kinetics of Bis-ANS binding to other proteins in the native state has been studied previously [10,11]. Biphasic kinetic behavior was observed, with one rapid and another slow component. Fig. 1 shows the kinetics of fluorescence increase of bis-ANS at 25°C with the native and aged GlnRS. The bis-ANS fluorescence increase with the native protein as a function of time shows biphasic behavior similar to that observed for DnaK [10]. In contrast, the aged protein shows rapid binding, with most of the binding being complete by the dead time of the manual mixing. This clear difference in binding kinetics is suggestive of a significant structural change in GlnRS upon aging.

The inset shows the shift of emission maximum as a function of time. Immediately upon mixing the native protein with bis-ANS, the emission maximum is 502 nm, which gradually shifts to 492 nm in the limit. In contrast, the aged protein shows an emission maximum of 491 nm immediately upon mixing with bis-ANS, which remains, more or less, invariant with time. It is well known that emission maximum of bis-ANS is directly related to the hydrophobicity of its environment. Thus, in the native state, gradual shift of bis-ANS emission maximum to the blue suggests a slow conformational change somewhere in the bis-ANS binding pathway. We have also fitted the slow part of the fluorescence rise in the native protein to the first order rate equation at different bis-ANS concentration. The first order rate constants are independent of bis-ANS concentration. This again is strongly suggestive of a slow conformational change being the rate-determining step in the bis-ANS binding pathway. The nature of such processes is discussed in the discussion section.

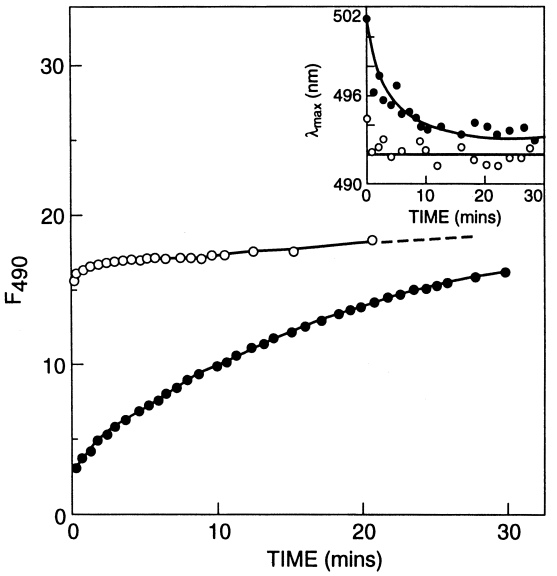

Fig. 1. Kinetics of bis-ANS binding to the native and the fully aged GlnRS at 25°C. The excitation and emission wavelengths were 450 and 490 nm, respectively. The band passes were 5 nm each. The solution conditions were 0.1 M Tris–HCl (pH 7.5), containing 15 mM $MgCl_2$. (●) The fluorescence increase as a function of time for the native; (○) fully aged glutaminyl-tRNA synthetase (2 μM). The inset shows the bis-ANS emission maximum shift as a function of time during bis-ANS (10 μM) binding to native and aged GlnRS. All the conditions were same as above, except the excitation and the emission bandpasses, which were 10 nm and 1.5 nm, respectively. (●) The emission maximum shift for the native and (○) for the aged GlnRS.

Since a large increase in initial binding of bis-ANS occurs upon aging, we have explored the binding kinetics of this rapid binding class using stopped-flow measurements. GlnRS and bis-ANS was mixed in a stopped-flow apparatus at final concentrations of 4.5 and 10 μM, respectively, and fluorescence was monitored upon mixing. The fluorescence increase profile is shown in Fig. 2 for the native and the aged protein. The fluorescence increase profile in case of native protein when fitted to a second order reaction kinetics yields a second-order rate constant of $1.48 \pm 0.284 \times 10^6$ M^{-1} s^{-1}. This rate constant is several orders of

magnitude slower than many small molecule association rate constants, suggesting even bis-ANS binding to fast sites perhaps involve protein conformational changes. The kinetics of fluorescence increase that occurs upon mixing of the aged protein with bis-ANS is similar but the amplitude is at least 10-fold larger. When fitted to a second-order kinetic equation, it yields a rate constant of $3.22 \pm 0.386 \times 10^6$ M^{-1} s^{-1}, similar to that of the native protein. The increase in amplitude, however, is consistent with large increase in F_o value observed upon aging and is consistent with the interpretation that upon aging some of the slow binding sites are converted to fast binding sites.

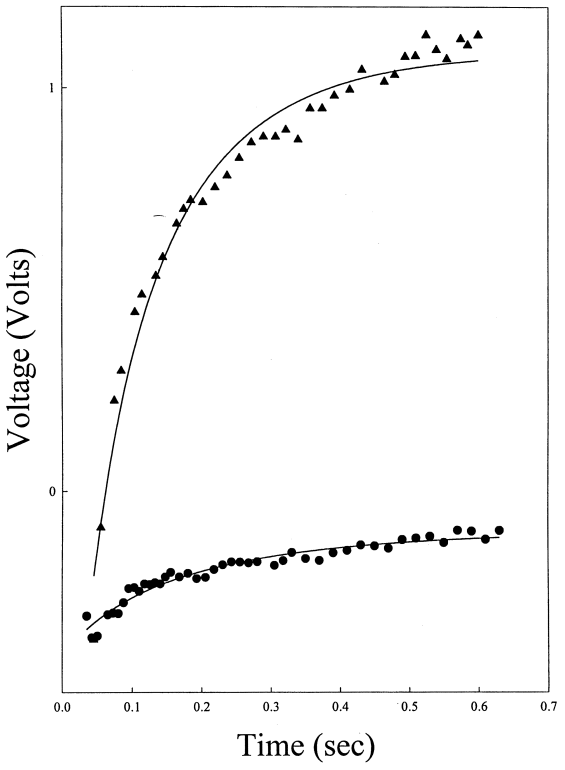

Fig. 2. Bis-ANS binding to native (●) and aged (▲) GlnRS in a stopped-flow kinetic spectrometer. Samples were excited at 360 nm and the emission was measured at 480 nm. The final concentrations were 4.5 and 10 μM for GlnRS and bis-ANS, respectively. The temperature was 25 ± 1°C. The solution conditions were 0.1 M Tris–HCl (pH 7.5) containing 15 mM $MgCl_2$. The solid lines are the best-fit lines.

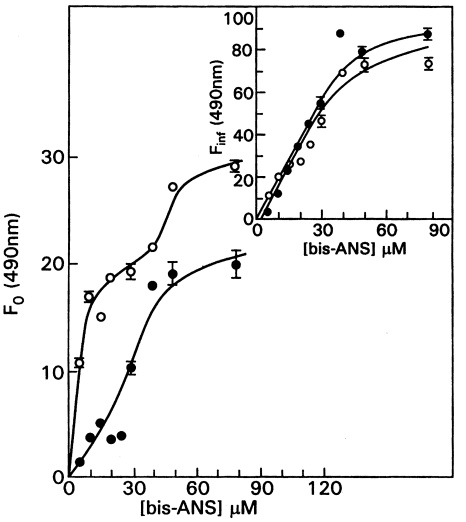

Fig. 3. Fluorescence intensity of the rapid binding component vs. bis-ANS concentration for the native (●) and the aged (○) glutaminyl-tRNA synthetase. The fluorescence increase was fitted to a single exponential with a zero time component by a non-linear least squares fit procedure. The zero-time components obtained from the non-linear least squares fit at various bis-ANS concentrations are plotted against bis-ANS concentration. The solution conditions and the spectroscopic parameters are the same as in Fig. 1. The inset shows the plot of F_{inf} values vs. bis-ANS concentration. The F_{inf} values were obtained from the same non-linear least squares fit procedure described above. The error bars reflect the standard error determined from four independent measurements. The solid lines are drawn to indicate approximate biphasic nature of F_o increase as a function of bis-ANS concentration.

3.2. Equilibrium binding parameters of bis-ANS to the native and the aged state

A significant difference in the kinetics of binding raises the question, whether the equilibrium binding of bis-ANS is same for the native and the aged state. Determination of a precise binding isotherm of bis-ANS is difficult because of multiple binding stoichiometry and possible quantum yield difference of different binding sites. F_{inf} values, however, are an indirect measure of overall bis-ANS binding at equilibrium at a specified bis-ANS concentration. The inset of Fig. 3 shows the plot of F_{inf} vs. bis-ANS concentration for the native and the aged GlnRS. Although, there are some differences, the plots are remarkably simi-

lar, suggesting roughly similar equilibrium binding of bis-ANS to the native and the aged state. Fig. 3 shows a plot of F_0 values at different bis-ANS concentrations. In contrast to the approximate invariance of F_{inf} values, F_0 values increase very significantly, in biphasic manner, for the aged protein, indicating that change in the kinetics of bis-ANS binding in Fig. 1 is the predominant consequence of the aging process. The biphasic nature of F_o increase may be a consequence of different classes of bis-ANS binding sites.

3.3. Activation energy of the aging process

Since the proportion of the rapid bis-ANS binding increases dramatically with aging, some function of F_{inf}/F_o ratio may be used to measure kinetics of the aging process. To measure the rate of aging quantitatively, a quantitative measure of aging is required. We assume the F_{inf}/F_o value of the freshly isolated protein at 25°C as the value of this parameter for 100% native protein, whereas the F_{inf}/F_o value of protein aged for 3 h as the value for 100% aged protein. We also make an assumption that values in between are directly related to the degree of aging. The first assumption is fully justified since the native protein shows similar F_{inf}/F_o value in several different preparations and after 3 h of aging no further change in F_{inf}/F_o value is seen at all temperatures. The justification of the second assumption is less straightforward, but is likely to hold because F_{inf} is roughly constant throughout the aging process and hence the ratio is only dependent on F_o. Based on these assumptions, we use F_{inf}/F_o as a quantitative measure of aging.

Fig. 4 shows the plot of degree of aging (see Section 2) vs. time at four different temperatures. As can be seen from the plot the rate of aging slows down tremendously with only a small change in temperature. The degree of aging vs. time plot can approximately be fitted to a first order process. The inset shows the Arrhenius plot of natural log of first order rate constants thus derived vs. the inverse of temperature. The derived activation energy is 180.1 kcal/mol. Clearly the high activation energy is consistent with the observation that

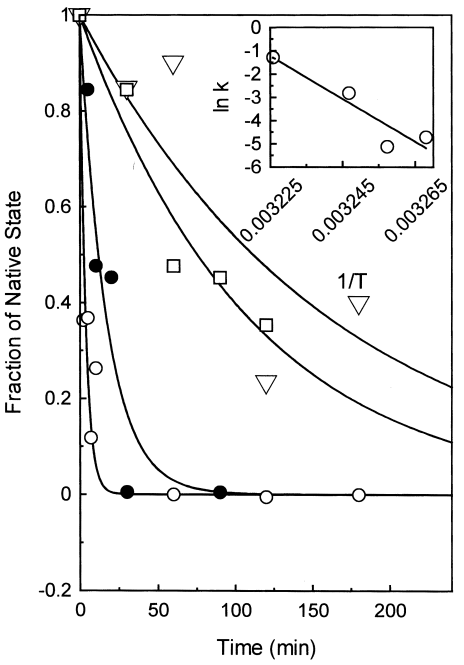

Fig. 4. Kinetics of aging of glutaminyl-tRNA synthetase at various temperatures. The aging was performed at 37°C (○), 35°C (●), 34°C (▽) and 33°C (□) at 2 μM protein concentration as described in Sections 2.1 and 2.2. The aging was performed in 0.1 M Tris–HCl (pH 7.5) containing 15 mM $MgCl_2$. At designated time points, 750 μl solution was withdrawn, mixed with 10 μM bis-ANS at 25°C and fluorescence increase determined. F_o and F_{inf} were then determined by non-linear least squares fit as described in the legend of Fig. 3. In this protocol, fraction of native protein is given by the value $[(F_o/F_{inf})_\infty - (F_o/F_{inf})_t]/[(F_o/F_{inf})_\infty - (F_o/F_{inf})_{zero}]$, where the subscripts, zero, t and inf refer to times at 0, t and infinity. The inset shows the plot of natural log of rate constants of aging at various temperatures vs. $1/T$.

no significant aging takes place at approximately 25°C. High activation energies of similar magnitudes have been observed in many processes that involve unfolding of a large part of a macromolecular structure. For example, a duplex to hairpin transition in a 14-mer DNA has activation energy of 96 kcal/mol [15]. Similarly, proton exchanges in BPTI and yeast $tRNA^{Phe}$ has been found to have activation energies in the range of 60–70 kcal/mol [16,17]. We thus suggest, unfolding and refolding of a significant part of the protein structure on the solid surface may be

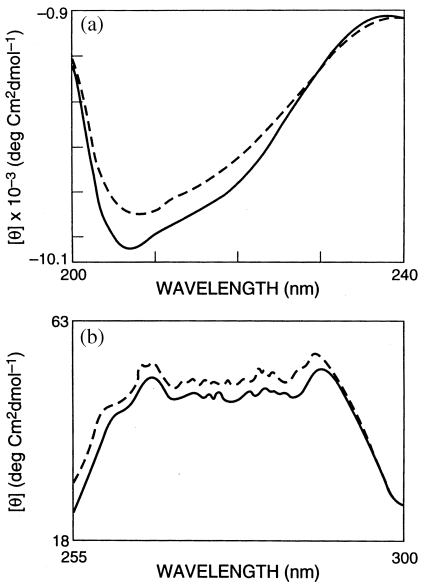

Fig. 5. (a) Far UV and (b) near UV CD spectra of aged (---) and native (—) GlnRS. Far UV CD spectra were obtained in a 1-mm pathlength cuvette, whereas the near UV spectra were recorded in a 10-cm pathlength cuvette. After 3 h of aging, as discussed above, the spectra were recorded in 0.1 M Tris–HCl (pH 7.5) containing 15 mM $MgCl_2$ at ambient temperatures, which were 25 ± 1°C. The protein concentration was 2 μM. Spectra were signal averaged to improve the signal-to-noise ratio. The spectrum of the buffer, was subtracted from each spectrum.

responsible for conversion of the native state to the aged state.

3.4. Conformational properties of the aged state

In order to determine if the structural changes that accompany the aging process, also lead to changes of other parameters that are characteristic of the native state, we have measured CD, sulfhydryl reactivity and fluorescence quenching in the native and the aged state. Fig. 5a,b shows the near and far UV CD of the native and the aged protein. Both the far and the near UV CD are similar indicating only a small change of secondary and tertiary structures. Similarity of near UV CD suggests that this state does not belong to the molten globule family [18,19]. A modest change in far UV CD suggests a limited conformational change upon aging involving changes in secondary structure. The Stokes radius of the fully aged protein, measured by high performance size exclusion chromatography, remains identical to that of the native protein [19] (data not shown).

Fig. 6 shows the reaction of sulfhydryl groups with DTNB for the native and the aged GlnRS. Previously, we have shown that in the native protein, DTNB reacts with three sulfhydryl groups, of which reaction with one is virtually instantaneous and another is also complete within couple of minutes [20]. A similar reaction profile is seen with native GlnRS here. In contrast, the aged enzyme shows four reactive sulfhydryl groups of which three reacts very rapidly. The reaction profiles of the remaining group is similar to the slower reacting sulfhydryl groups in the native GlnRS. Curve-fitting suggests that the slow reacting sulfhydryl in the aged state reacts approximately twice as fast than in the native state (analysis not shown). This suggests that one buried unreactive sulfhydryl group in the native protein becomes completely exposed. In addition, there

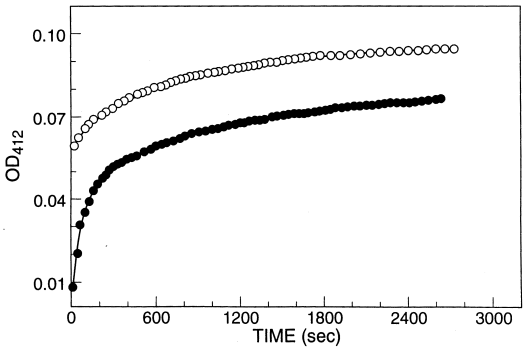

Fig. 6. Reaction of DTNB with the aged (○) and the native (●) glutaminyl-tRNA synthetase. The DTNB reaction was initiated by adding DTNB to a final concentration of 1 mM. The solution conditions were 0.1 M potassium phosphate (pH 7.5), at 25°C. The protein concentration was 2 μM. The absorbance was monitored at 412 nm. The aging was also carried out in 0.1 M potassium phosphate (pH 7.5) as described in Section 2. The solid lines are the best-fit lines to kinetic equation describing two classes of sulfhydryls having different rate constants.

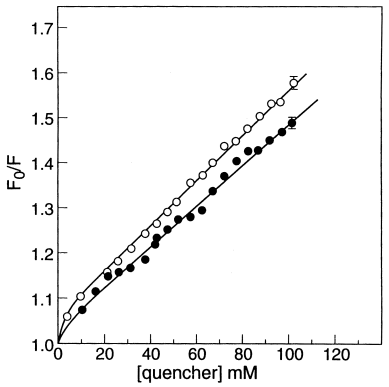

Fig. 7. Stern–Volmer plot of acrylamide quenching of tryptophan fluorescence of the native (●) and the aged (○) glutaminyl-tRNA synthetase. The protein concentration in each case was 2 μM and the solution conditions were 0.1 M Tris–HCl (pH 7.5), containing 15 mM $MgCl_2$. Excitation was at 295 nm and emission was at 340 nm. The bandpasses were 5 nm each. The temperature was 25°C.

may also be significant changes of reactivity of a slower reacting sulfhydryl group.

Collisional quenching has been widely used to assess the accessibilities of fluorophores in proteins [21]. Fig. 7 shows the Stern–Volmer plot of acrylamide quenching of tryptophan fluorescence of the native and the aged GlnRS. Although the Stern–Volmer plot does not differ greatly, the aged protein shows significantly enhanced quenching. Thus, it appears that one or more tryptophan residues in GlnRS become more accessible to collisional quenchers upon aging.

We have also measured the aminoacylation activity of the aged GlnRS. At 37°C, the activity of the aged enzyme (95% of the native) is similar to the native enzyme. Since one may argue that under the assay conditions, the aged protein may reconvert to the native protein, we have performed the assay at 25°C where the conversion rate is virtually zero. Even at this temperature, the aged protein shows similar activity (93% of the native) to the native enzyme and both are approximately 25% active when compared to their activity at 37°C. The linearity of the assays at both temperatures also argues against a re-conversion under assay conditions (data not shown).

3.5. Aging of other proteins

To see if the aging changes bis-ANS binding kinetics in other proteins, we have measured bis-ANS binding kinetics to the native and the aged state in a similar manner in other proteins. Table 1 shows the F_{inf}/F_o ratio for a number of proteins in the native state and after 3 h of incubation at 2 μM concentration at 37°C. The smaller and medium sized proteins like ribonuclease A, liver alcohol dehydrogenase and larger 'hard' protein such as BSA (stabilized by 17 intra-chain disulfide links) shows a low F_{inf}/F_o ratio in the native state and little change upon prolonged

Table 1
Changes in Bis-ANS binding characteristics of several proteins upon aging[a]

Protein	Subunit molecular weight (Da)	$(F_{inf}/F_o)_{Native}$	$(F_{inf}/F_o)_{aged}$	Remarks
Ribonuclease A	12 000	1.0	1.0	Small –S–S– linked
LADH	40 000	1.3	1.2	
IDH (NADP)	45 000	1.5	1.1	Two domains
BSA	66 000	1.0	1.1	17 intra-chain –S–S– bonds
GluRS	54 000	3.3	1.4	Multi-domain
GlnRS	64 000	4.5	1.2	Multi-domain

[a] Surface catalyzed aging and bis-ANS binding was carried out as described in the legend of Fig. 1. The fluorescence increase was fitted to a single exponential with a zero-time offset (rapid binding component) by a non-linear least square-fit procedure to extract the F_o and F_{inf} [19].

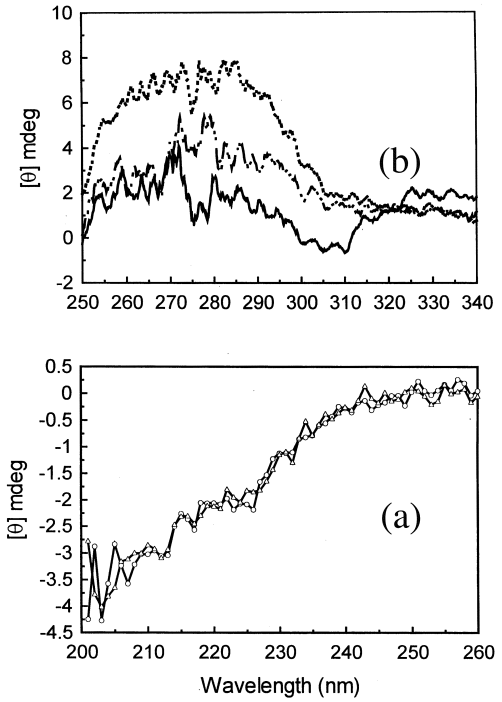

Fig. 8. (a) Far UV Circular dichroism and (b) near UV circular dichroism spectra of GlnRS as a function of refolding time. In (a) the solid line represents the native GlnRS at concentration and the dashed line represents the refolded protein at 3 min from initiation. The pathlength was 1 mm. In (b) the dashed-dot line (top at 280 nm) represents the native, the solid line (bottom) represents the refolded GlnRS at 3 min and the dashed represents the refolded GlnRS at 7 min. The protein concentration here was also 1 μM. The pathlength was 10 cm. The solution conditions were same as in the legend of Fig. 5.

incubation, as well. Larger 'soft' proteins such as, GlnRS, GluRS and tubulin ([11]; Sarkar and Bhattacharyya, unpublished observation) show a significantly higher F_{inf}/F_o ratio in the native state and large decline upon prolonged incubation.

3.6. Bis-ANS binding properties of folding intermediates

In an attempt to understand the origin of different bis-ANS binding kinetics, we have explored the bis-ANS binding properties of folding intermediates of GlnRS. We have chosen a transient folding intermediate so that the solution conditions may be kept similar to that used in the study of the native and the aged state. Fig. 8a,b shows the far UV CD spectra and near UV circular dichroism spectra of denatured GlnRS after refolding is initiated, as a function of time. Both near and far UV CD spectra gain within a few minutes, suggesting structure formation. Table 2 shows the emission maxima and fluorescence intensity values as a function of refolding time. Emission maximum returns to native-like values whereas the fluorescence intensity remains like unfolded, much lower than the native values. Lower near UV-CD and fluorescence intensity and native-like far UV-CD and emission maximum is the characteristic of the previously characterized equilibrium molten globule state [19].

However, the light scattering value increases over a period of time indicating continuing aggregation (Table 2). The protein remains biologically inactive within the time-period investigated (data not shown). This behavior is suggestive of a folded molten-globule type intermediate that forms relatively rapidly followed by aggregation. This type of behavior has been observed in some proteins. We have examined the bis-ANS binding behavior of the folding intermediate(s) as a function of refolding time. The F_{inf}/F_o values of bis-ANS fluorescence as function of refolding time is invariant with time and is very similar to aged GlnRS. We may conclude that the molten globule type folding intermediate(s) that forms, do not have native-like bis-ANS binding kinetics.

4. Discussion

Different conformational states of proteins are of great interest in the study of folding processes of proteins. Several classes of compact denatured states have been characterized of which molten globules are the most prominent ones. In this study, we have shown that aged glutaminyl-tRNA synthetase differ from the native state in the kinetics of binding of an apolar probe, bis-ANS. The aged protein appears to be compact by many structural criteria, such as, native-like secondary

Table 2
Spectroscopic properties of refolded GlnRS

Time (min)	F_{340}	λ_{max} (nm)	F_{inf}/F_o	Light scattering at 340 nm[a]
Native	161.1	338.6	4.5	
0[b]	67.08	340.0	1.1	2232
3				3766
5				3949
10	70.1	339.4		4169
15				4298
20				4395
30			1.15	4502
Denatured	60.9	351.4		

[a] The light scattering measurements were made in a Hitachi spectrofluorometer. The excitation and emission wavelength was set at 340 nm with band passes were 5 nm each. The final protein concentration was 0.75 μM.
[b] Refers to measurements taken immediately after dilution.

structure, hydrodynamic radius as measured by gel filtration, etc. The major but not the only difference being the disappearance of the slow binding phase, i.e. the drastic reduction of the energy barrier of bis-ANS binding in the aged protein. We have also shown that under refolding conditions, GlnRS rapidly produces a molten globule state which slowly aggregates. This molten globule state has bis-ANS binding property similar to that of the aged state.

In the native proteins, the protein interiors are hydrophobic and inaccessible [22,23]. Interior amide proton exchanges are very slow [24,25] and tryptophans situated in the interiors are non-quenchable by collisional quenchers. Thus, the access to the protein interior may be controlled by protein 'breathing' to 'open states' [26].

$$P_{CL} \underset{k_2}{\overset{k_1}{\leftrightarrows}} P_{OP} \overset{k_b}{\rightarrow} P \cdot L$$

where P_{CL} is the closed state, P_{OP} is the open state, PL is the ligand bound state and ks are the rate constants. Two limiting cases can be observed. If the forward rate constant (k_1) is sufficiently small (smaller than $k_b[L]$), the rate of $\{P \cdot L\}$ production will be controlled by the rate of conversion of closed to the open form of the protein. If P_{OP} concentration is sufficiently small, the rate of formation of ligand protein complex in the open state may become small enough, despite rapid equilibration of the first step. However, in the later case, the ligand binding kinetics will still be proportional to ligand concentration, whereas in the former case it will be independent of ligand concentration as observed. Since, the slow phase of bis-ANS binding is independent of bis-ANS concentration, it is likely that bis-ANS binds to an open state of the protein with slow conversion of the native state to the putative bis-ANS/open state complex. A similar hypothesis has been advanced by Fink et al. [10]. Why the conversion to the open state is so slow, remains to be understood. Like Fink et al., we have also observed that decreasing temperature and increasing the concentration of a viscous solvent like glycerol, decreases the rate of the slow phase drastically (Mandal and Roy, unpublished observation). Such slow and strongly temperature-dependent rate processes are indicative of a large activation energy barrier between the native and the open state. Such large activation energy is required for significant displacement of large number of atoms. We thus hypothesize that large amplitude correlated motions are necessary to convert relatively compact native state (P_{CL}) into a more open state (P_{OP}).

Aging may increase the binding rate of the ligand (bis-ANS) by increasing the rate of open state formation, i.e. aging reduces the activation energy barrier between the native state and the

open state. This would be possible if some of the interactions that bind the domains are removed during the aging process. Native-like near and far UV CD spectra in the aged state suggest only modest structural alterations. Structural alterations of one or few surface loops and/or the realignment of the domain–domain interactions may lead to a looser structure capable of converting rapidly to the open state and hence rapid bis-ANS binding. It is instructive that the molten globule state also binds bis-ANS rapidly. This is consistent with the notion that the molten globule state is relatively flexible and open.

5. Conclusion

We thus conclude that the native state is compact and largely impenetrable to large apolar probes such as bis-ANS and only route of bis-ANS binding is through large amplitude slow motions, opening the protein interior. These large amplitude slow motions are thought to play roles in protein functions. Compact denatured states, such as aged and molten globule, are capable of binding bis-ANS rapidly thereby indicating that an intact native structure is a necessary pre-condition for such motion. Thus, bis-ANS is a useful probe for studying these large amplitude slow motion in proteins which are otherwise very difficult to study.

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