Covalent Reinforcement of a Fragile Region in the Dimeric Enzyme Thymidylate Synthase Stabilizes the Protein against Chaotrope-Induced Unfolding†

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Received December 8, 1995; Revised Manuscript Received March 11, 1996°

ABSTRACT: Urea and guanidinium chloride induced unfolding of thymidylate synthase, a dimeric enzyme, and engineered interface mutants have been monitored by circular dichroism, fluorescence, and size-exclusion chromatography. Equilibrium unfolding studies show biphasic transitions, with a plateau between 3.5 and 5 M urea, when monitored by far-UV CD and fluorescence energy transfer employing an (aminoethylamino)naphthalenesulfonyl (AEDANS) label at the active site residue, Cys198. AEDANS was also specifically incorporated at position Cys155 in the mutant protein T155C. Direct excitation of this extrinsic fluorophore in the wild type protein (labeled at Cys198) and mutant T155C (labeled at Cys155) showed remarkable differences in the unfolding profiles. C155 AEDANS has a transition centered at 3.5 M urea, which is in contrast to Cys 198 AEDANS (5.5 M urea). Unfolding studies monitored by following intrinsic fluorescence of Trp residues which are located in a small structural domain suggest that this region of the protein is intrinsically fragile. The stable equilibrium intermediate is identified to be an ensemble of partially unfolded aggregated species by gel filtration studies. The chaotrope-induced denaturation of TS appears to proceed through a partially unfolded intermediate that is stabilized by aggregation. Dissociation and loss of structure occur concomitantly at high denaturant concentrations. Introduction of two symmetrically positioned disulfide bridges across the dimer interface in the triple mutant T155C/E188C/C244T (TSMox) stabilized the protein against denaturant-induced unfolding. Aggregate formation was completely abolished in the mutant TSMox, which also enhanced the overall structural stability of the protein. Structural reinforcement of the fragile interface in thymidylate synthase results in dramatic stabilization toward chaotrope-induced unfolding.

Multimeric enzymes provide an attractive system for investigating spontaneous self-assembly of protein structures and for examining regulatory interactions between subunits. Quaternary structure constitutes one of the most complex levels of structural organization in biological macromolecules. Analysis of the unfolding transitions of multimeric proteins may be useful in establishing the hierarchy of events that occur during denaturation (Garel, 1992; Jaenicke, 1987). Equilibrium unfolding studies have so far provided considerable information on stable intermediate states in small, monomeric proteins. To delineate the transition states and folding pathways in these proteins, equilibrium and kinetic parameters of refolding of the wild type proteins are compared with engineered mutants (Fersht, 1994; Zitwewitz & Matthews, 1993; Shortle, 1992). Experimental studies have unequivocally established that the native state of protein is reached via formation of folding intermediates (Pittsyn, 1995; Kuwajima, 1989; Kim & Baldwin, 1990). In an important study Peng and Kim (1994) showed that the native-like tertiary fold is preserved in a molten globule state. Under certain conditions one can enrich equilibrium inter-

† This work was supported by the Council of Scientific and Industrial Research, India (P.B.), and United States Public Health Service Grant CA14394 from the National Cancer Institute (D.V.S.). R.S.G. and S.A. were supported by Senior Research Fellowships from the Council of Scientific and Industrial Research, India.
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° Abstract published in Advance ACS Abstracts, May 1, 1996.

† Abbreviations: TS, thymidylate synthase; TSWT, thymidylate synthase wild type; TSMox, thymidylate synthase mutant oxidized (T155C/E188C/C244T); TSMred, thymidylate synthase mutant reduced (T155C/E188C/C244T); GdmCl, guanidinium chloride; AEDANS, (aminoethylamino)naphthalenesulfonyl; βME, 2-mercaptoethanol.
solutions using fluorescence, gel filtration, and ultracentrifugation established aggregation in denaturant solutions, a process which was dependent on phosphate concentration and temperature (Reinsch et al., 1979). A more recent report employing absorption spectroscopy has shown that wild type thymidylate synthase (TSWT) undergoes a cooperative transition between 3.5 and 5.5 M urea. Refolding and subunit association from the urea-denatured state are achieved by rapid dilution with buffer containing 0.5 M potassium chloride (Perry et al., 1992). Since the earlier reports of denaturation of TS appeared fragmentary, a detailed investigation of the unfolding of TSWT was undertaken. In order to dissect the processes of dimer dissociation and chain unfolding during denaturation, the unfolding behavior of a bisdisulfide cross-linked mutant, T155C/E188C/C244T (TSMox), was compared with TSWT. The bisdisulfide mutant differs significantly from the wild type enzyme in that it does not precipitate at high temperature, in contrast to TSWT which has a precipitation temperature of 52 °C. Furthermore, the covalently cross-linked mutant showed a remarkable thermal stability as monitored by the assay of enzymatic activity and circular dichroism (Gokhale et al., 1994).

In the present study we report unfolding of TS in urea and GdmCl solutions using the following probes: (i) Far- and near-UV circular dichroism (CD) as monitors of polypeptide secondary and tertiary structures, respectively. (ii) Intrinsic tryptophan fluorescence as a reporter of the environment of seven Trp residues in TS that cluster in one domain of the structure. (iii) Fluorescence energy transfer from Trp residues to the active site Cys198 of TSWT, which serves as a probe of the integrity of the tertiary structure of the protein. (iv) Size exclusion chromatography to establish the presence of aggregation at intermediate denaturant concentrations. The bisdisulfide mutant (TSMox) assists in separating the effects due to dissociation from the unfolding process. These results identify the fragile region in the structure of TSWT, which upon partial unfolding at intermediate urea and GdmCl concentrations forms aggregates involving interface segments. This associated species retains appreciable secondary structure although the tertiary interactions appear to have been diminished, suggestive of an “aggregated molten globule” state. Two inter-subunit disulfide cross-links impart appreciable stability to the protein by covalently locking weakly interacting regions of the dimer interface.

EXPERIMENTAL PROCEDURES

Procedures for protein isolation, purification, mutagenesis, and characterization of the mutant protein have been described earlier (Gokhale et al., 1994). All the reagents used were of analytical grade. Urea was recrystallized from boiling ethanol and dried in a vacuum oven.

Unfolding Studies. All denaturation experiments were carried out in 25 mM potassium phosphate buffer, pH 6.9, 0.5 mM EDTA (buffer A). Protein samples were incubated for 1 h at the desired denaturant concentration before the measurements were made in order to ensure equilibrium. Preliminary experiments with varying incubation times indicated that equilibrium was achieved within a few minutes.

Fluorescence. Emission spectra were recorded on a Hitachi 650-60 spectrofluorimeter. Intrinsic tryptophan fluorescence intensity was monitored at varying concentrations of urea. Protein samples (1 µM) were excited at 280 nm, and the emission intensity at 340 nm was followed. Excitation and emission band passes of 5 nm were used.

Labeling of the Active Site Cysteine 198 by 5-(2-(Homo- doacetyl)amino)ethyl)aminonaphthalen-1-sulfonic Acid (1,5-IAEDANS). 150 µM protein was dissolved in 100 µL of 100 mM Tris-HCl, pH 8.0, 1 mM EDTA buffer. 1,5-IAEDANS was added to it to a final concentration of 1.5 mM and incubated for 30 min in the dark. Cysteine was added to a final concentration of 6 mM and incubated for 30 min to quench the reaction. Free label was removed by passing through a Sephadex G-15 gel filtration column equilibrated with buffer A. The extent of labeling was estimated using an extinction coefficient of 6100 M⁻¹ cm⁻¹ at 337 nm for 1,5-IAEDANS (Hudson & Weber, 1973). TS contains two thiol groups at Cys 198 and Cys 244. In the labeling experiments, only Cys 198 was found to be reactive and no label was obtained at Cys 244. About 1.6 mol of label per mole of enzyme dimer was obtained, a value which is in accordance with the previous reports of thiol labeling in TS (Bradshaw & Dunlap, 1993). In the presence of the substrate deoxyuridine monophosphate, proteins could not be labeled, indicating that the only reactive thiol is the active site Cys 198 residue. A similar extent of labeling was obtained for the C244T mutant of TS, further confirming that the label is present only at Cys 198.

AEDANS Labeling at Position Cys155. The fluorophore was specifically incorporated at position 155 by specifically blocking the active site Cys198 with FdUMP, a well-known mechanism-based inhibitor of TS (Santi & McHenry, 1972). 200 µM TSWT was incubated with 450 µM FdUMP (reaction volume 100 µL) at 25 °C for 1 h. FdUMP modification of the active site nucleophile C198 was monitored by measuring its specific activity. A 10-fold molar excess of IAEDANS was added to this mixture, and the reaction was incubated at room temperature for 2 h. The excess label was quenched and then separated through a gel filtration column.

Fluorescence spectra of AEDANS-labeled protein were recorded at a protein concentration of 0.45 µM. Spectra were recorded by excitation at 280 nm (energy transfer through Trp residues) and 340 nm (direct dansyl absorption). Fluorescence emission intensity of AEDANS-labeled protein was monitored at 480 nm.

Circular Dichroism. CD spectra were recorded on a JASCO J500A spectropolarimeter equipped with a DPN 501 data processor. Far-UV CD spectra were recorded using a 0.1 mm path length cuvette and a protein concentration of 7 µM for the urea denaturation experiments. Near UV-CD spectra were recorded in the region of 250–300 nm using a protein concentration of 8.1 µM and 5 mm path length. CD band intensities are expressed as molar ellipticities.

Size Exclusion Chromatography. Gel filtration experiments were carried out on a Pharmacia Superose-6 column (manufacturer’s exclusion limit, 4 × 10⁷ Da for proteins) using a FPLC system. 3 µM protein was incubated for 1 h at the required urea or Gu-HCl concentration in buffer A. 100 µL of this sample was injected on the column equilibrated with 25 mM potassium phosphate buffer pH 6.9, 150 mM sodium chloride, and the required denaturant.
concentration (flow rate, 0.2 mL/min, detection, 280 nm). Three distinct peaks were obtained during the experiment. Native dimeric TS eluted at 16.5 mL, and aggregated TS at intermediate denaturant concentrations eluted in the void volume at 7.4 mL (in GdmCl solutions) or at 10 mL (in urea solutions). Unfolded monomeric TS eluted at 14.2 mL (4 M GdmCl) and 14.9 mL (6 M urea). Fractions of the individual species were calculated using the relative areas of the chromatogram peaks.

RESULTS AND DISCUSSION

Urea-Induced TSWT Unfolding. *L. casei* thymidylate synthase contains seven tryptophans, as shown in Figure 1, in which the spatial location of Trp residues are mapped on the structure. Trp63 is located on helix B, Trp82 in the loop between helix C and helix D, residues 85 and 90 on helix D, Trp150 on the helix G, Trp153 in the loop between helix G and helix H, and Trp185 on the loop between strand V and helix I [the notation for the helix and strand segments follows the original description of the crystal structure (Hardy et al., 1987)]. The changes in intrinsic tryptophan fluorescence intensity as a function of urea concentration are shown in Figure 2. An approximate two-state transition is observed between 3 and 5 M urea with the transition midpoint at the denaturant concentration of 4.2 M. The profile is qualitatively similar to that reported earlier by following the absorption band of Trp at 294 nm (Perry et al., 1992). It may be noted that the transition observed in the present study occurs at slightly lower urea concentration. The tryptophan fluorescence data provides an incomplete picture of the unfolding, since only the changes in the local environment of these residues are probed. Interestingly, in TS the Trp residues are clustered in one region of the protein (Figure 1). Four of these Trp residues are located on helices, and the other three are on the loops connecting secondary structural elements. This unfolding profile thus primarily reflects the stability of helices in this region.

Circular dichroism studies, on the other hand, provide more global information about the structure. The near-UV CD spectra of TSWT with the increasing denaturant concentrations are shown in Figure 3. The changes in the near- and far-UV CD molar ellipticity at 290 and 220 nm, respectively, are plotted as a function of urea concentration (Figure 4). When the aromatic side chain CD bands, resulting primarily from the Trp residues (290 nm) are monitored, a sharp transition is observed at 3.5 M urea, with complete abolition by 4 M urea. In contrast, the unfolding transition monitored by the peptide backbone CD bands is distinctly "non-two-
state”, with a partially structured intermediate observed in the region of 4–5 M urea. The plateau in the unfolding profile is indicative of a stable intermediate species that is predominantly populated over the urea concentration range of 4–5 M urea. The two transitions are centered at 3.6 and 5.8 M urea. These results are considered in greater detail subsequently when a comparison is made with engineered mutants.

To delineate the unfolding of a relatively large protein such as thymidylate synthase, it is necessary to place spectroscopic markers at different locations in the structure. Appropriately positioned probes should yield information on relative stabilities of the different regions of the protein structure. Fluorescence energy transfer experiments between two distinct domains are ideally suited for this purpose. We have used the Trp and AEDANS groups as a donor/acceptor pair to provide an energy transfer probe. The AEDANS group is located at the active site residue Cys198 (see Figure 1). TSWT also has a free thiol at position Cys244, which however, remains inert during the chemical labeling experiments (see Experimental Procedures). Figure 5 shows the changes in AEDANS fluorescence intensity as a function of urea concentration by direct excitation ($\lambda_\text{ex}$) 340 nm) and indirect excitation by energy transfer from Trp residues ($\lambda_\text{ex}$) 280 nm). The unfolding transition of TSWT, when monitored by direct excitation of the extrinsic fluorophore shows a cooperative transition centered around 5.5 M urea. In sharp contrast, when energy transfer from Trp residues is monitored, two distinct transitions centered at around 3.3 and 5.2 M urea are observed. There is very little change in the fluorescence energy transfer efficiency between 3.5 and 5 M urea, suggesting a stable equilibrium intermediate at these denaturant concentrations. These observations parallel those made using the backbone CD bands as a probe. It is noteworthy that in the case of direct excitation there is substantial fluorescence intensity even at high urea concentration (>7 M), whereas the energy transfer from Trp residues is totally lost. This may be a consequence of local structure persistent even at 7 M urea. It is pertinent to note that NMR studies of proteins at high urea concentrations have provided evidence for appreciable residual structure (Wüthrich, 1994).

**Comparative Studies of TSWT Unfolding in GdmCl Solutions.** Analysis of TSWT unfolding was also carried out in guanidinium chloride (GdmCl) solutions in order to compare the effects of two denaturants, which may differ in
their detailed mode of interactions with the protein (Makhadze & Privalov, 1992). Unfolding profiles of TSWT in GdmCl solutions obtained using fluorescence and CD approximate to a two-state transition and do not show any indication of stable intermediates over wide range of denaturant concentration. A detailed analysis of the unfolding curves shows transitions at distinct denaturant concentrations when determined using different optical probes. This is suggestive of the presence of a stable equilibrium intermediate(s) during unfolding (Cantor & Schimmel, 1980). Figure 6 depicts the fraction of the unfolded form of TSWT as a function of GdmCl concentration, monitored using three different spectroscopic probes, namely, Trp-AEDANS energy transfer, direct AEDANS excitation, and far-UV CD. The errors estimated in the determination of the unfolding curves are less than 5%. The denaturation curves obtained by following Trp-AEDANS energy transfer show a gradual unfolding with the transition centered around 1.8 M GdmCl. The AEDANS emission intensity followed upon direct excitation does not detect any structural change up to 1.6 M GdmCl, above which a sharp transition takes place. The unfolding curve obtained by monitoring ellipticity at 220 nm displays a transition after 1.4 M GdmCl, suggesting that the secondary structure is perturbed prior to alterations in the environment of the AEDANS moiety. It is clear that the unfolding transition in GdmCl occurs at significantly lower concentrations than in urea solutions. This is not surprising since the former is known to be a more powerful denaturant (Pace, 1986). The absence of a plateau in the GdmCl-induced unfolding process may be due to the fact that the intermediate species is not stable over a substantial window of denaturant concentration. This may be contrasted with the case of urea in which a distinctive stable intermediate species is observed (see Figures 4 and 5). The non-coincidence of unfolding profiles obtained using different unfolding spectroscopic probes has generally been interpreted as evidence for equilibrium intermediates.

Subunit Dissociation of TSWT. Size exclusion chromatography studies were performed with TSWT to identify the quaternary state of the protein at various denaturant concentrations. Figure 7a shows the chromatographic profiles of TSWT at 0, 4, and 6 M urea concentrations. It can be observed that the protein elutes at three different retention volumes (marked A, B, and C in Figure 7a). Analysis of gel chromatography studies carried out at high denaturant concentration can sometimes be difficult to interpret due to the effect of the denaturant on the column matrix. To confirm the nature of the protein species, comparative studies were carried out with the mutant protein TSMox. Engineering of two disulfide bridges (155–188′ and 188–155′) across the dimer interface links the two subunits covalently preventing dissociation (Agarwalla et al., 1996). Chromatographic profiles of TSMox at varying denaturant concentration do not show any dramatic changes in the retention volumes (Figure 7b), as observed for TSWT. There is a steady decrease in retention volume due to the solvation and unfolding of the dimer. Addition of 10 mM 2-mercaptoethanol to the mutant protein, reduces the disulfide bonds (TSMred); the protein then behaves as wild type enzyme (Figure 7b). On the basis of these observations, the peak B, that elutes in the void volume of the column, can be assigned to aggregated protein species and the peak C can be assigned to the solvated unfolded monomer. Interestingly the unfolded monomer elutes before the folded dimer. The relative populations of species of TSWT observed on gel filtration employing varying concentrations of urea and GdmCl are shown in Figure 8. Protein aggregates are observed in both the denaturants, although the aggregated species in GdmCl is present over a very narrow range of denaturant concentration. It is clear from the gel filtration that the formation of aggregated species precedes the formation of unfolded monomers. Indeed, protein aggregates has been observed in several systems (Havel et al., 1986; Horowitz & Butler, 1993) and may be a general feature due to the tendency of partially unfolded states to interact with one another in order to cover up the exposed hydrophobic surfaces.

Studies of TSWT, TSMred, and TSMox in Urea Solutions. The changes in secondary and tertiary interactions in the three proteins were monitored by circular dichroism studies. The far-UV CD spectra of the mutant protein TSMred are identical to that observed for TSWT, indicating that the thiol interface mutations do not perturb the overall structure of the protein. There is a small 4% decrease in the molar ellipticity observed for the covalently bridged dimer TSMox, as compared to the TSWT. The changes in the molar ellipticity of these proteins at 220 nm are plotted as a function of urea concentration in Figure 9. TSWT and TSMred show a biphasic transition with a plateau between 3.5 and 5 M urea. The TSMred unfolding curve shows a shift in the midpoint of the transition by 0.4 M toward higher urea concentration, suggesting a marginal stabilization of the protein, in comparison to TSWT. In contrast, for the disulfide-bridged dimer TSMox, there is no discernible plateau in the unfolding profile. A broad transition is observed at about 5.5 M urea which may be compared to the second transition of TSWT and TSMred. Interestingly, the changes in Θ220, which are a reflection of secondary structure content, are much smaller for TSMox compared to TSWT and TSMred. This is strongly suggestive of stabilization of secondary structure by disulfide bridging. It is noteworthy that the dimeric interface of TS is composed of two interacting five-stranded β-sheets. Covalent bridging across the interface prevents the inter-subunit dissociation, which in turn, may contribute to the stabilization of sheet.
The loss of ellipticity in the near-UV CD spectra with increasing urea concentration for the three proteins is plotted in Figure 10. The near-UV CD band at 290 nm shows a remarkable shift in the unfolding transition for the TSMox protein. Whereas TSWT and TSMred have lost the 290 nm band by 4 M urea, TSMox shows a marginal 30% decrease.

**Figure 7:** Changes in quaternary state of wild type thymidylate synthase and mutants observed at different concentrations of urea in a gel filtration experiment. Column was equilibrated with 25 mM potassium phosphate buffer, pH 6.9, 150 mM NaCl, and the required concentration of urea. 100 µL of 3 µM protein solution was loaded on the column after preincubating the protein at appropriate urea concentration for 1 h. Flow rate was 0.2 mL/min, and detection was at 280 nm. Three typical states of TSWT protein are shown in panel (a) marked A, B, and C for folded dimer, partially unfolded aggregate, and unfolded monomer, respectively. Panel b shows the profiles for the inter-subunit cross-linked mutant protein TSMox. Panel c shows the effect of reducing disulfide bridges in the mutant protein, TSMred, on its quaternary state.

**Figure 8:** Relative populations of all the species observed by gel filtration chromatography at varying concentrations of denaturants urea and GdmCl. The relative populations are calculated by determining the area under the curve for each species. The number of data points used for generating this graph are labeled on the X-axis.
The transition in the case of TSMox is complete by 7 M urea. The results in Figure 10 strongly suggest that even tertiary interactions have been stabilized by intermolecular disulfide bridging. Inhibition of dissociation also appears to strengthen the folded states of individual subunits.

Analysis of TS Unfolding Using a Fluorescent Label at Position Cys155. A dramatic stabilization of the mutant protein TSMox toward denaturation suggested that a dimer interface may be critically involved in the early stages of the unfolding process. The TS mutant T155C was chosen to position a thiol at the interface, which could then be labeled with an AEDANS reporter group. The catalytic residue Cys198 lies on a β-strand that forms a part of the interface. However, this residue does not make any contacts with the neighboring subunit. Figure 11 shows that there is a dramatic difference in the unfolding profiles obtained using reporter groups at positions 155 and 198. In the latter case a clear transition centered at 5.5 M urea is observed. In contrast, when the label is positioned at residue 155, the perturbations are observed immediately. This suggests that the environment around the residue is affected even at very low denaturant concentrations. In both cases there is a 5 nm blue shift in the emission maximum at intermediate urea concentrations (3.5–5 M), suggestive of the burial of

the fluorophore in protein aggregates. The relative sensitivity of the Cys 155 AEDANS label to low urea concentrations suggests that interface perturbations involving this segment probably occur in the very early stages of urea-induced unfolding. Inspection of the structure shown in Figure 1 reveals relatively few inter-subunit contacts in the “top” region of the interface which contains the residue 155. The “lower” segment of the interface has a very substantial number of inter-subunit contacts and is presumably significantly robust. The stabilization of TS to denaturants imparted by disulfide bridging across the weaker region of the interface is clearly understandable, if this segment is indeed prone to unfold upon perturbation.

CONCLUSIONS

In considering strategies to stabilize proteins, in general, it is necessary to identify structural regions which are in fact most susceptible to environmental influences. Inspection of protein three-dimensional structures does not provide a conclusive method for identifying “weak spots” in protein folds. Experimental investigations of the structural events that occur upon unfolding provide an opportunity to identify segments that are most labile even at low denaturant concentrations. Protein unfolding/refolding studies in urea and GdmCl solutions have focused on the identification of equilibrium and kinetic intermediates. In the case of kinetic studies, considerable effort has been directed toward establishing the time scales on which secondary and tertiary structure formation occurs. Equilibrium studies have largely centered around characterization of intermediates for which the all-encompassing term “molten globule” has been coined (Okazaki et al., 1994; Hayer-Hartl et al., 1994; Peng & Kim, 1994). For rational approaches toward understanding the protein folding problem, structural information on the species populated at different stages in the unfolding studies may be particularly relevant (Redfield et al., 1994). The present study provides an example in which disulfide engineering has resulted in imparting appreciable stabilization toward chaotrope-induced denaturation. The sites for reinforcement do indeed appear to correspond to protein segments that are
casei thymidylate synthase as shown (Scheme 1). D N and Cys 155 are located in a small domain of structure which contains perturbations of this segment, since the seven Trp residues Trp fluorescence and the behavior of AEDANS label at which harbors the fewest inter-subunit contacts. The intrinsic cross-links have been introduced into a region of the interface clue to potentially weak segments. Interestingly, the covalent reagents form via an intermediate dimeric state. Monomerization probably occurs via a D* to M_U pathway such that the aggregate corresponds to an off-pathway process. The possibility of the collapse of the aggregated state directly to the unfolded monomers (broken equilibrium) can also be considered. However, our concentration dependent unfolding studies show no aggregate formation at very low concentrations. Interestingly, refolding from the aggregated state is achieved at lower efficiency than that from the completely unfolded, monomeric state (unpublished results), suggesting that the aggregate is not an obligatory intermediate in the refolding pathway. A similar model has indeed been proposed for bovine growth hormone (bGH) where studies have shown that the formation of this bGH folding intermediate and its aggregation are separate processes; however, in this case, formation of a monomeric intermediate precedes aggregation (Brems et al., 1988). The soluble aggregates of TSWT are in contrast to insoluble aggregates of apomyoglobin, which are formed in the denatured state and do not require the involvement of an intermediate (De Young et al., 1993).

The loss of the near-UV CD and quenching of tryptophan fluorescence of the aggregated intermediate are indicative of diminished tertiary structure, while appreciable secondary structure is still retained (ellipticity at 220 nm). These observations are consistent with the “molten globule-like” state for the intermediate. ANS binding experiments did not show any enhanced dye binding at intermediate denaturant concentrations. It may, however, be noted that molten globule states of proteins do not necessarily demonstrate enhanced ANS binding as exemplified by barnase mutants (Sanj & Fersht, 1993). Multimeric proteins like creatinase (Schumann & Jaenicke, 1993), tumor necrosis factor (Hlodan & Pain, 1994), and triosephosphate isomerase (unpublished results) have been shown to retain their quaternary state in the molten globule form. For large proteins, generalized terms like “molten globule” may indeed be structurally uninformative. It is easy to envisage situations in which well-organized local domains of structure are retained in the equilibrium intermediate which are characterized by the presence of secondary and tertiary structure. It is almost certain that the use of spectroscopic techniques with much higher degrees of resolution, like NMR, will result in identification of such situations. The term molten globule may indeed be a better descriptor of the methodology used to identify the equilibrium intermediate rather than a satisfactory structural description of the state itself. Even with the limited resolution of fluorescence, there is a possibility of varying the position of the probe within the protein structure by introducing single cysteine residues with site-directed mutagenesis. CD, on the other hand, is intrinsically limited to providing low-resolution global information. At the present time, structural interpretations of unfolding profiles

most susceptible to perturbation by denaturants. The possibility of engineered disulfides having global effect on protein stability must also be considered.

The studies described in this report attempt to separate the processes of chain unfolding and subunit dissociation in urea and GdmCl solutions for wild type L. casei thymidylate synthase (TSWT). Figure 12 summarizes the key results of the unfolding study. Unfolding profiles using two distinct spectroscopic probes, namely, CD ellipticity at 220 nm and fluorescence energy transfer from the clustered Trp residues to an active site dansyl label, reveal the presence of an equilibrium intermediate that is stable over a urea concentration range of 3.5–5 M. Gel filtration experiments establish that this intermediate is composed of associated protein species. Monomeric species are exclusively observed only at concentrations greater than 6 M urea. The studies clearly demonstrate that aggregation precedes subunit dissociation. The unfolding studies on covalently cross-linked engineered mutant, containing two inter-subunit disulfide bridges, TSMox, abolish aggregation at intermediate denaturant concentrations. The unfolding profiles show classical two-state behavior. The reduced mutant behaves in a manner similar to wild type enzyme. These results provide strong circumstantial evidence for the involvement of the dimer interface segments in the aggregation process. The fragile region of the interface is presumably disrupted, permitting the protein—protein association via aggregation competent segments. The crystal structure does provide, in principle, a clue to potentially weak segments. Interestingly, the covalent cross-links have been introduced into a region of the interface which harbors the fewest inter-subunit contacts. The intrinsic Trp fluorescence and the behavior of AEDANS label at position 155 provide further evidence for early structural perturbations of this segment, since the seven Trp residues are located in a small domain of structure which contains Cys 155.

These studies suggest the pathway for the unfolding of L. casei thymidylate synthase as shown (Scheme 1). D_N and D* are the native folded dimer and partially unfolded dimer, respectively, while A corresponds to an aggregated state and M_U is the unfolded monomer. The equilibria indicated by solid arrows would correspond to a situation in which aggregates form via an intermediate dimeric state. Monomerization probably occurs via a D* to M_U pathway such that the aggregate corresponds to an off-pathway process. The distribution of the collapsed state directly to the unfolded monomers (broken equilibrium) can also be considered. However, our concentration dependent unfolding studies show no aggregate formation at very low concentrations. Interestingly, refolding from the aggregated state is achieved at lower efficiency than that from the completely unfolded, monomeric state (unpublished results), suggesting that the aggregate is not an obligatory intermediate in the refolding pathway. A similar model has indeed been proposed for bovine growth hormone (bGH) where studies have shown that the formation of this bGH folding intermediate and its aggregation are separate processes; however, in this case, formation of a monomeric intermediate precedes aggregation (Brems et al., 1988). The soluble aggregates of TSWT are in contrast to insoluble aggregates of apomyoglobin, which are formed in the denatured state and do not require the involvement of an intermediate (De Young et al., 1993).

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need to be validated by studies on appropriately designed mutants. Identification of Achilles’ heels in protein structures provides a means of engineering stability, as exemplified in these studies.

ACKNOWLEDGMENT

We are grateful to M. K. Mathew, National Centre for Biological Science (TIFR) Bangalore, for the use of FPLC system and to Gautham Nadig for help in generating Figure 1.

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