Protein Science (1996), 5:270–277. Cambridge University Press. Printed in the USA.

Covalent tethering of the dimer interface annuls aggregation in thymidylate synthase

SANJAY AGARWALLA,[1] RAJESH S. GOKHALE,[1] DANIEL V. SANTI,[2] AND P. BALARAM[1]

[1] Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, India
[2] Department of Biochemistry and Biophysics, University of California in San Francisco, San Francisco, California 941143-0448

(RECEIVED August 9, 1995; ACCEPTED November 15, 1995)

Abstract

Thymidylate synthase (TS), a dimeric enzyme, forms large soluble aggregates at concentrations of urea (3.3–5 M), well below that required for complete denaturation, as established by fluorescence and size-exclusion chromatography. In contrast to the wild-type enzyme, an engineered mutant of TS (T155C/E188C/C244T), TSMox, in which two subunits are crosslinked by disulfide bridges between residues 155–188′ and 188–155′, does not show this behavior. Aggregation behavior is restored upon disulfide bond reduction in the mutant protein, indicating the involvement of interface segments in forming soluble associated species. Intermolecular disulfide crosslinking has been used as a probe to investigate the formation of larger non-native aggregates. The studies argue for the formation of large multimeric species via a sticky patch of polypeptide from the dimer interface region that becomes exposed on partial unfolding. Covalent reinforcement of relatively fragile protein–protein interfaces may be a useful strategy in minimizing aggregation of non-native structures in multimeric proteins.

Keywords: intermolecular disulfides; protein aggregation annulment; size-exclusion chromatography; thymidylate synthase

Protein aggregation and precipitation are problems that frequently confound studies of protein refolding from concentrated denaturant solutions (Jaenicke, 1987; Fields et al., 1992; DeYoung et al., 1993b). Insoluble inclusion body formation during overexpression of proteins in heterologous systems is probably a consequence of association of non-native structures (Schein, 1989). Association and precipitation of proteins have also been implicated in the pathology of amyloid formation in Alzheimer's disease (Come & Lansbury, 1994) and in the neurodegenerative disorders caused by prions (Prusiner, 1991; Gasset et al., 1992). Folding intermediates have also been linked to genetic diseases in vivo (Bychkova & Ptitsyn, 1995). In vitro unfolding experiments suggest that partially unfolded structures of polypeptides show a marked tendency to self associate, as exemplified in studies of human growth hormone (Brems et al., 1986) and apomyoglobin (DeYoung et al., 1993a). The formation of large soluble aggregates by partially unfolded proteins provides an opportunity to examine the structural prerequisites for association of non-native structures. Multimeric proteins are generally prone to aggregation upon perturbation of subunit interactions (Jaenicke, 1987; Garel, 1992). In this report, we demonstrate that the wild-type dimeric enzyme, *Lactobacillus casei* thymidylate synthase, forms large soluble aggregates in urea solutions at denaturant concentrations of 3.3–5 M. In sharp contrast, aggregation is completely abolished in an engineered mutant that contains two disulfide crosslinks across the dimer interface, which preclude subunit dissociation.

L. casei TS is a homodimer ($M_r \sim 70$ kDa) in which the two subunits are noncovalently associated. The dimer interface of TS is composed primarily of a five-stranded β-sheet, which forms a part of a conserved core (Hardy et al., 1987). The dimeric structure of this enzyme is obligatory for its activity, because each of the two active sites contains amino acid residues contributed by the other subunit (Pookanjanatavip et al., 1992). Studies employing absorption spectroscopy have shown that wild-type TS undergoes a cooperative unfolding transition between 3.5 and 5.5 M urea. Refolding and subunit reassociation from the urea denatured state is achieved by dilution with potassium phosphate buffer containing 0.5 M potassium chloride (Perry et al., 1992). Early analysis using gel filtration and ultracentrifugation established the aggregation of TS in urea, a

Reprint requests to: P. Balaram, Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, India; e-mail: pb@mbu.iisc.ernet.in.

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, aminoethylaminonaphthalenesulfonyl; DTNB, 5,5′-dithio*bis*(2-nitrobenzoic acid); DTT, dithiothreitol.

process that appeared to be dependent on phosphate concentration and temperature (Reinsch et al., 1979). Despite the importance of aggregation and precipitation, little is currently understood about these non-native structures. To investigate the structural requisites and to analyze the contribution of subunit disassembly to aggregation, it is important to design mutants that annul aggregation. The present study demonstrates that aggregation is abolished in a mutant enzyme T155C/E188C/C244T, which contains two symmetrical intersubunit bridges, 155–188′ and 188–155′. The results establish that perturbations at the dimer interfaces are essential for protein aggregation.

Results

Aggregation of wild-type enzyme

During the course of a detailed spectroscopic analysis of TS unfolding in urea and guanidinium chloride, the presence of an equilibrium intermediate has been observed (unpublished results). Analysis of this stable equilibrium intermediate revealed a population of aggregated species. Concentration/dependent unfolding of TSWT with urea is shown in Figure 1. Denaturation profiles are monitored using excitation energy transfer from the seven tryptophans located on the protein to an active site-labeled AEDANS fluorophore. TSWT contains two thiol groups at Cys 198 and Cys 244. In chemical labeling experiments, only Cys 198 is reactive and no labeling is obtained at Cys 244 (see the Materials and methods). Because the active site in TS is located at the dimer interface, the spectroscopic label at this position is expected to report directly the changes that accompany subunit disruptions. Comparison of the unfolding profiles at three different protein concentrations ranging from 0.1 to 0.9 μM suggest that there is indeed a dramatic concentration dependence between 3 and 6 M urea. Although a largely two-state transition is observed at the lowest concentration, there is clear evidence for a stable intermediate species at the highest protein concentration (0.9 μM).

Figure 2 shows a plot of the emission wavelength maximum of the AEDANS probe, located at the active site Cys 198 residue, as a function of urea concentration. In the native protein, an emission maximum of 479 nm is observed, suggestive of a relatively hydrophobic location of the probe. In the completely unfolded state, obtained at 8 M urea (data not shown), a fluorescence maximum of 490 nm is obtained, indicating appreciable exposure of the fluorescent label to the solvent. At intermediate urea concentrations of 3–5 M, an interesting dependence of emission wavelength with the protein concentrations is observed. At the highest protein concentration (0.9 μM), an appreciable blue shift of the emission maximum is observed (from 479 to 472 nm). This blue shift of the AEDANS probe decreases with decreasing protein concentration, suggesting that formation of protein aggregates results in a large decrease in the polarity of the probe environment.

Size-exclusion chromatography

Wild-type TS

Figure 3A shows profiles of gel permeation experiments on TSWT carried out on an LKB TSK-G3000 SW column (exclusion limit = 3×10^5 Da) at urea concentrations ranging from 0 to 6 M. The permeation properties of TSK gel have been shown to be unaffected by high denaturant concentrations (Corbett & Roche, 1984). Native TS elutes at 16.3 mL. With increasing urea concentration (up to 3 M), there was a small decrease in retention volume indicative of a predenaturation swelling of the protein. With further increase in urea concentrations, two distinct species (peaks A, B) are observed, between 3.3 and 3.8 M urea. Peak B appears at a dramatically reduced retention volume of 10.2 mL, which is the void volume of the column, indicative of a vastly increased hydrodynamic radius for the protein. The transition from peak A to peak B is effectively complete by 4 M urea. These results conclusively prove that TSWT forms soluble aggregates at these intermediate urea concentrations. Indeed, an early analysis of TS in urea solutions established the presence

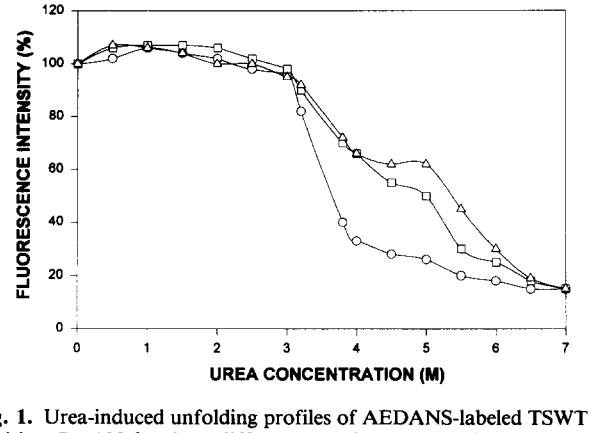

Fig. 1. Urea-induced unfolding profiles of AEDANS-labeled TSWT at position Cys 198 for three different protein concentrations at pH 6.9, 25 °C. Fluorescence spectra were recorded by exciting Trp residues at 280 nm and monitoring the AEDANS emission spectrum at 480 nm through energy transfer. Emission intensity at 0 M urea was taken to be 100%. Protein concentrations are (○) 0.1 μM; (□) 0.45 μM; (△) 0.9 μM.

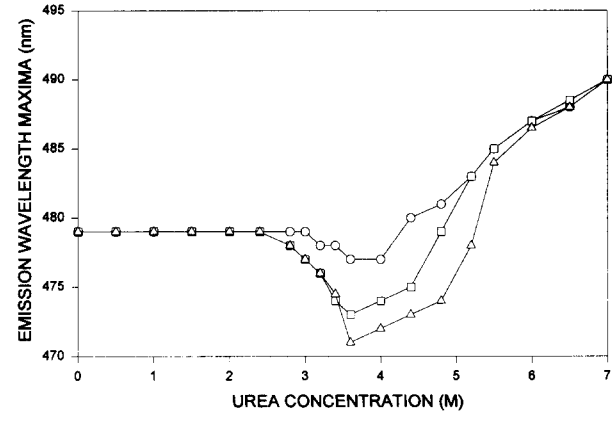

Fig. 2. Dependence of the emission maximum of the fluorophore AEDANS, labeled at position Cys 198 in TSWT, as a function of urea concentration for three different protein concentrations. (○) 0.1 μM; (□) 0.45 μM; (△) 0.9 μM at pH 6.9, 25 °C. Measurements were carried out by excitation through energy transfer from Trp residues (λ_{ex} = 280 nm).

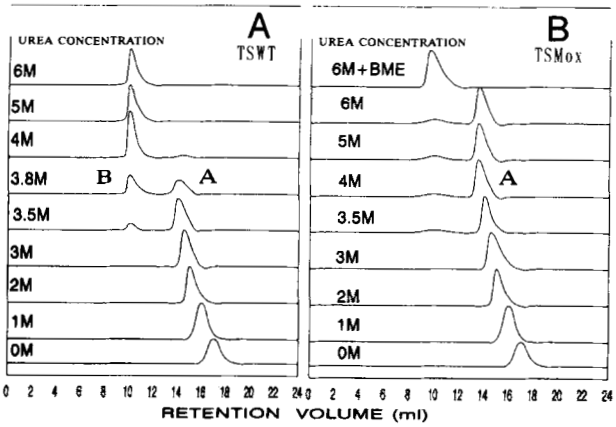

Fig. 3. Chromatographic profiles of (**A**) TSWT and (**B**) TSMox conducted on a TSK-G 3000 SW HPLC column at various urea concentrations. Column was equilibrated with 25 mM potassium phosphate buffer, pH 6.9, and the desired urea concentration. Peak A corresponds to dimer and peak B is assigned to the aggregated species. The topmost profile in B corresponds to the elution profile of TSMox in 6 M urea containing 10 mM β-mercaptoethanol. All the other profiles are in the absence of reducing agent. Protein concentration, 6.5 μM. Detection was at 280 nm.

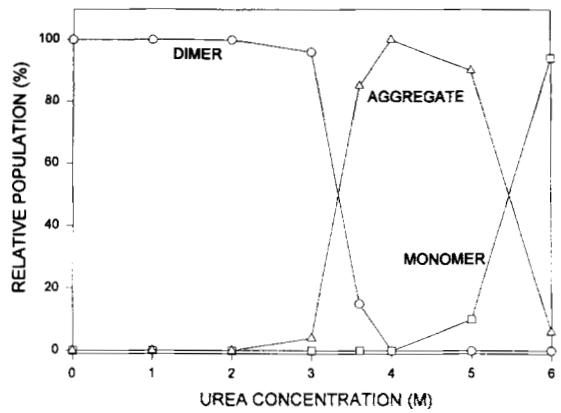

Fig. 4. Relative populations of different quarternary states of TSWT observed on a Superose-6 gel filtration column at different urea concentrations. All the conditions used were identical to those used for TSK-G 3000 SW HPLC column. Three species observed during the course of experiment were (○) dimer, (△) aggregate, and (□) monomer.

of protein aggregates using gel filtration and analytical ultracentrifugation (Reinsch et al., 1979).

In order to further examine the nature and dimension of the protein species formed at urea concentrations greater than 3 M, the size-exclusion chromatography experiments were performed on a Pharmacia Superose-6 FPLC column, which has a higher exclusion limit of 4×10^7 Da. Figure 4 shows the relative population of different species observed at varying urea concentrations by Superose-6 gel filtration. There are three species observed during the course of the experiment. Native dimer (elution volume 16.4 mL) begins to convert into aggregates (elution volume 9.3 mL) in 3 M urea with the transition being completed by 4 M urea. A new peak appears (14.9 mL) in 5 M urea, which is the only remaining species at 6 M urea. Assignment of this peak was done based on a comparison with the covalently crosslinked mutant (see below).

Crosslinked bisdisulfide mutant (T155C/E188C/C244T)

Two symmetry-related disulfide bridges (155–188′ and 188–155′) have been engineered across the subunit interface of TS, at sites chosen on the basis of an algorithm, MODIP (Sowdhamini et al., 1989), for the introduction of stereochemically unstrained bridges into proteins. The design, purification, and characterization of the mutant TSMox (T155C/E188C/C244T) have been described previously (Gokhale et al., 1994). The results of gel filtration experiments on the covalently crosslinked dimer performed on TSK-G 3000SW are shown in Figure 3B. The protein in the absence of urea elutes at 16.4 mL (peak A), at a position identical to the wild-type enzyme. Unlike TSWT, this mutant protein shows only one peak at all the urea concentrations studied. Small changes observed in the retention volume of peak A at increasing urea concentrations probably correspond to unfolding and solvation of the dimer. A very low-intensity peak observed at a position close to the wild-type species B arises from a small amount of the non-crosslinked dimer. Addition of 10 mM 2-mercaptoethanol to the mutant enzyme at 6 M urea results in the complete disappearance of peak A, with the concomitant appearance of peak B in the void volume of the column, a profile similar to that of wild-type enzyme at 6 M urea. The results were identical on a Superose-6 column also. There were no peaks observed in the void volume, as well as at the 14.3 mL peak, as observed in the case of TSWT. Under reducing conditions at 6 M urea, TSMox behaves like wild-type protein and elutes at 14.3 mL. Because disulfide crosslinking abolishes monomerization, this 14.3-mL peak in TSWT can be assigned to solvated unfolded monomer. Interestingly, the data in Figure 1 also establish that there is no protein concentration dependence after 6 M urea.

It is noteworthy in the gel filtration experiments that the native dimer has a greater retention volume than the unfolded monomer. Although this appears counterintuitive at first glance, the likely interpretation is that the unfolded monomer in denaturant solutions has a much greater hydrodynamic volume than the compact folded dimer. Similar observations have been noted earlier in studies of pyruvate decarboxylase (Pohl et al., 1994). Studies of TSWT and TSMox in GdmCl solutions yielded qualitatively similar results to studies of the same in urea solutions. Subunit dissociation appears to be complete by 4 M GdmCl, with an aggregated state observed between 1.5 M and 2 M GdmCl concentrations.

Molecular size of TSWT aggregate

In order to quantitate the results, the Superose-6 gel filtration column was equilibrated with proteins of known Stokes radii. The calibration plot obtained using the equation $K_d^{1/3} = A - Ba$ (where a is the Stokes radii and A and B are constants, $K_d = V_e - V_o/V_i - V_o$, where V_e is the elution volume and V_i and V_o are the included volume and void volume of the column, respectively) was used to calculate the Stokes radii of various species observed in the gel filtration experiment (Fish et al., 1969). The calculated Stokes radii for native TSWT, for TSWT in 4 M GdmCl, and TSMox in 4 M GdmCl were 29 Å, 57 Å, and 75 Å, respectively (Table 1). A plot of log molecular weight versus log of Stokes radii of GdmCl denatured proteins (Fish et al.,

Table 1. *Molecular weights for TS species*

TS	Quaternary state	R_s (Å)	Estimated M_r (Da)	Expected M_r (Da)
Native TS	Dimer	29	70,000	72,000
TSWT (4 M GdmCl)	Monomer	57	34,500	36,000
TSMox (4 M GdmCl)	Dimer	75	59,000	72,000
TSWT (2 M GdmCl)	Aggregate	320	>800,000	–

1970) was used to estimate molecular weight of species observed. The estimated molecular weight for TSWT at 4 M GdmCl was 34,500 Da, which is in close agreement with the reported molecular weight of the monomer ~36,000 Da. The molecular weight estimated for the crosslinked dimer was 59,000 Da, which was slightly lower than the expected 70,000 Da. This may be due to the fact that two polypeptide chains are linked by disulfides (Tanford et al., 1974). Using similar calculations, the species corresponding to peak B in Figure 3A was estimated to have a very large Stokes radii of 320 Å (Table 1) and molecular mass of the order of 800,000 Da, indicative of aggregates formed by greater than 20 monomeric units. Although the Stokes radii and molecular mass estimated for the large aggregated species in the denaturant solutions by gel filtration may be susceptible to error, it is nevertheless clear that the aggregated TSWT species observed in the range of 3.3–5 M urea or 1.5–2 M GdmCl are clusters of several protein molecules. The high molecular weight TSWT aggregates formed at intermediate denaturant concentration, then undergo a further transition to solvated monomeric species at higher denaturant concentrations. All these transitions correspond to slow equilibria and, therefore, discrete species are detected on the time scale of the gel filtration experiment.

Urea PAGE

Further support for protein aggregation is obtained using urea PAGE. Figure 5 shows the results of gel electrophoresis experiments conducted in the absence and presence of urea (4 M and 8 M) for TSWT and TSMox. The two proteins migrate closely in the absence of urea (Fig. 5, lanes A and B). In lane A, TSWT shows a minor smearing of the band. The reason for such a behavior is not clearly understood, although this was reproducible and appeared to correlate with the concentration of protein. It should be stressed that, on SDS-PAGE, both TSWT and TSMox behave identically, yielding sharp well-focused bands (Gokhale et al., 1994). In lane B, TSMox migrates slower than TSWT. Interestingly, even on SDS-PAGE, TSMox moves as a 105-kDa species (instead of 70 kDa). Electrospray mass spectrometry and analytical ultracentrifugation confirmed the dimeric nature of the protein (Gokhale et al., 1994). Proteins with disulfides are known to show anomalous behavior in gel electrophoresis experiments (Creighton, 1992). At 4 M urea, TSWT barely enters the gel and is seen at the top of the 5% crosslinked polyacrylamide gel (lane C), confirming the formation of aggregates with an effective molecular mass of several hundred thousand daltons. In contrast, under these conditions, the mutant TSMox (lane D) shows a major band and a minor band, both of which have a considerable mobility in the gel. The two bands, which are somewhat diffuse, presumably arise from states in

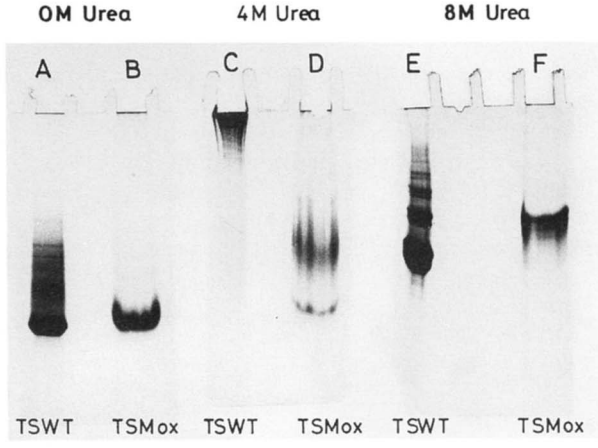

Fig. 5. PAGE of TSWT and TSMox at different urea concentrations. Lane A, TSWT no urea; lane B, TSMox no urea; lane C, TSWT 4 M urea; lane D, TSMox 4 M urea; lane E, TSWT 8 M urea; lane F, TSMox 8 M urea. Lanes A/B, C/D, E/F correspond to three independent experiments.

slow equilibrium at urea concentrations close to the midpoint of the unfolding transition. It has been noted earlier in transverse urea gradient gel experiments that a slight spreading of the protein bands occurs in the vicinity of the transition (Goldenberg & Creighton, 1984). Lanes E and F compare the mobilities of TSWT and TSMox at 8 M urea. It is noteworthy that the TSWT once again shows considerable mobility and the major species moves ahead of the crosslinked mutant. The observation of several higher molecular weight species suggests that, even at extreme denaturant concentrations, a heterogeneous range of aggregated species are still present, although their molecular mass and abundance is much less than that in 4 M urea. These bands can also be a consequence of differential urea interactions with unfolded states of TS.

Although it is difficult to structurally characterize the various species observed during the course of these experiments, it is clear that several species with varying stabilities are present during the unfolding of the protein by denaturant. What is important in this experiment is to compare the TSWT and TSMox mobilities across the three different electrophoretic runs. The experiments unambiguously show that, in the absence of urea, both the wild-type and mutant proteins have identical behavior. At 4 M urea, wild-type protein forms large aggregates that do not enter the gel, whereas the mutant exists as a partially unfolded dimer. At 8 M urea, the major TSWT species is an unfolded monomer, which has a greater electrophoretic mobility than the unfolded crosslinked dimeric form of the mutant enzyme.

Noncovalent and reversible nature of TSWT aggregates

TSWT has two free thiols groups at Cys 198 and Cys 244. To address the question of whether aggregation could be mediated by disulfide crosslinking involving these residues, gel filtration experiments were carried out at 4.5 M urea in the absence and presence of 1 mM dithiothreitol. TSWT showed aggregation irrespective of the presence or absence of the reducing agent (data not shown). The fact that aggregate formation is a consequence

of noncovalent interactions is also confirmed by SDS-PAGE (Fig. 6). Samples of TSWT and TSMox were incubated in 4.5 M urea and were run on SDS-PAGE under both reducing and nonreducing conditions. It is noteworthy that TSWT showed identical mobility under both the conditions, with no bands corresponding to higher molecular weight species. TSMox had an identical mobility to the wild-type enzyme in the presence of reductant, whereas in the absence of a reducing agent, it had significantly lower mobility owing to the covalent crosslinking of the two subunits.

The aggregate process observed at the intermediate denaturant concentrations is reversible. Although the refolding from 6 M urea is achieved quantitatively, renaturation from these intermediate concentrations can be accomplished only up to 80% levels (unpubl. work). Moreover, this refolding is concentration dependent, and the best yields are obtained at lower temperatures, suggestive of aggregation mediated by hydrophobic patches from protein.

Disulfide crosslinking as a probe of dimer integrity

TS mutant protein (T155C/E188C/C244T) is expressed in the reduced form in *Escherichia coli*. Disulfide formation can be forced by 5,5′-dithio*bis*(2-nitrobenzoic acid) crosslinking to form *bis*-disulfide bridges (155–188′, 188–155′) across the interface. Single mutants T155C and E188C independently do not form any intersubunit crosslinks, i.e., 155–155′ and 188–188′ (unpubl. results), underlying the importance of appropriate stereochemistry required for disulfide formation. In this experiment, disulfide crosslinking was used to investigate the perturbations at the dimer interface of the protein. TSMred protein was incubated at varying urea concentrations (0–6 M) for 1 h, after which disulfide crosslinking was carried out using DTNB. Samples were then loaded on the SDS-PAGE under *nonreducing* conditions. Figure 7 shows the densitometric scan analysis of the gel. At 0 M urea, the majority of the protein is in the crosslinked dimeric form, with approximately 16% in the monomeric state.

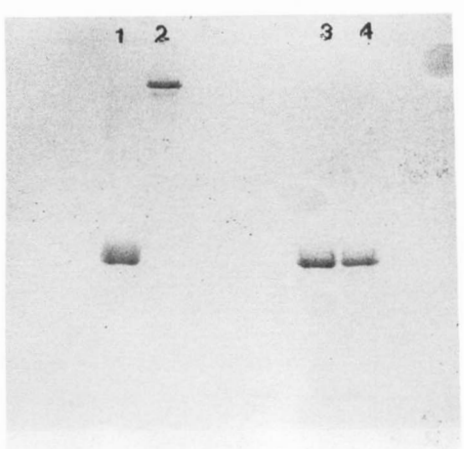

Fig. 6. SDS-PAGE of TSWT and TSMox under reducing condition (1 mM β-mercaptoethanol) and nonreducing conditions, after samples were incubated at 4.5 M urea in 25 mM potassium phosphate buffer, pH 6.9, a condition where aggregation of TSWT is established. Lane 1, TSWT nonreducing buffer; lane 2, TSMox nonreducing buffer; lane 3, TSWT reducing buffer; lane 4, TSMox reducing buffer.

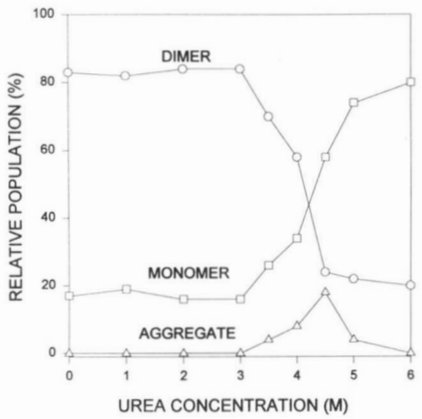

Fig. 7. Densitometric scan of a SDS-PAGE gel showing relative populations of species observed with varying urea concentrations. TSMred samples were incubated at different urea concentrations and crosslinked using DTNB. Samples were run under nonreducing conditions. (○) dimer; (□) monomer; (△) aggregate.

This number gives the basal level of crosslinking, under the given conditions. This ratio of dimer to monomer is maintained up to 3 M urea. At 3.5 M urea, new bands appear on the gel with reduced mobility. These high molecular weight species observed are disulfide crosslinked aggregates, because these bands are absent on the *reducing* SDS-PAGE gel. Bands corresponding to covalently crosslinked aggregates are observed upon incubation in urea up to 5.5 M. At 6 M urea, 80% of the protein is in the monomeric form, whereas 20% is in the dimeric state. There are no aggregates observed at 6 M urea. The observation of disulfide-bonded aggregates in the case of TSMred requires that the thiol residues 155 and 188 undergo dramatic changes of environment at intermediate denaturant concentrations. Aggregate formation must then be preceded by profound structural disruption of the interface. It is noteworthy that crosslinking is effective in the associated species, because disulfide formation requires proximity and appropriate stereochemistry of the two Cys residues (Srinivasan et al., 1990). Crosslinking of aggregated species at 3–5 M urea has been shown in the case of rhodanase using dimethyl suberimidate (Horowitz & Butler, 1993).

Discussion

Association of proteins at relatively low denaturant concentrations may occur via partially unfolded intermediates that serve as aggregation competent structures (Havel et al., 1986). Aggregation involving non-native structures is likely to be mediated by specific interactions (London et al., 1974). For example, in the case of bovine growth hormone, a segment responsible for aggregation has been identified as an amphipathic helix spanning residues 109–133 (Lehrman et al., 1991). In the case of insulin, two critical residues, Pro B28 and Lys B29, have been identified that can alter its self-association (Brems et al., 1992). There has been some debate on the nature of the associating species, with several reports favoring *partially* unfolded intermediates as the aggregation competent structures. However, in the apomyglobin case, recent studies suggest that *completely* unfolded species are responsible for aggregation (DeYoung et al., 1993a). The complete abolition of aggregation in the *bis*-disulfide

mutant of TS, suggests that elements of the dimer interface may be involved in promoting protein association. Equilibrium unfolding experiments with TSWT establish that a partially unfolded dimeric intermediate is stabilized by aggregation (unpubl. results). It is likely that partial unfolding and disruption of interactions at the dimer interfaces could yield an intermediate structure that associates via sticky patches.

Figure 8 provides a schematic representation of such an aggregation process that, in principle, could result in the formation of both open and closed aggregates. Such aggregation is reminiscent of the formation of micellar structures by amphipathic molecules. In the proposed model, partial unfolding and disruption of intersubunit interactions at the dimer interface yields an intermediate structure that is now poised for aggregation by means of a sticky patch of polypeptide, exposed to the solvent. In the case of TS, the dimer interface can be divided into two regions of interacting surfaces as indicated in the projection drawn in the Figure 9, which also shows the position of the interfacial bonds in the covalently bridged mutant. The top region, which contains the segment from residues 155–188, has significantly fewer interactions than the lower region, which comprises the segment 18–37, 200–220, 254–259 (Hardy et al., 1987). Interactions involving the 153–188 segment are presumably weaker than those obtained in the other region of the interface. The TS dimer interface contains several polar residues, and both electrostatic and hydrogen bonding interactions contribute appreciably to dimer stability. In the region under consideration, Thr 155, Ser 156, Glu 163, Arg 178, Arg 179, and Glu 188 participate in polar contacts. Recent calorimetric studies of the interaction of urea and GdmCl with proteins suggest that the denaturant molecules bind to proteins at sites that permit several hydrogen bonding interactions (Makhatadze & Privalov, 1992). Invasion of dimer interface by urea and GdmCl at intermediate denaturant concentration may weaken intersubunit contacts at regions involving clusters of polar interactions. A plausible model for aggregation might then involve swiveling of the TS dimer about a noncovalent hinge located in the stronger region of the interface, after denaturant-induced breakdown of attractive interactions in the weaker top region. Although Figure 8 is actually an exaggerated version of events accompanying the aggregation process, this illustration emphasizes the role of interface segments.

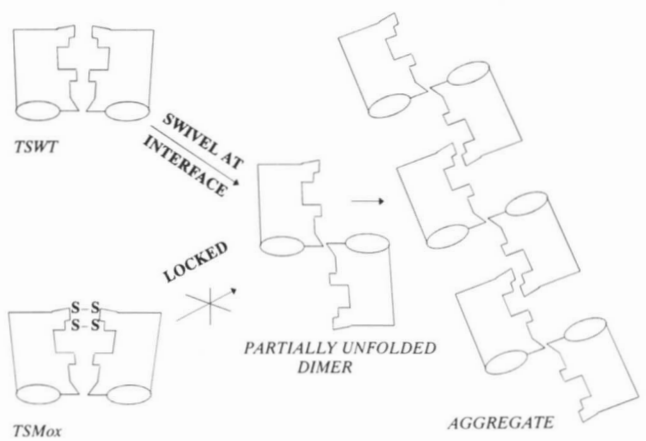

Fig. 8. Schematic of the mode of aggregation of dimeric proteins with fragile, sticky interfaces. The protein dimer is viewed perpendicular to the twofold axis. The engineered disulfide bridges are marked in the mutant protein, which prevents swiveling motion at the interface. Crevices between the two semi-cylinders indicate the interfacial interactions.

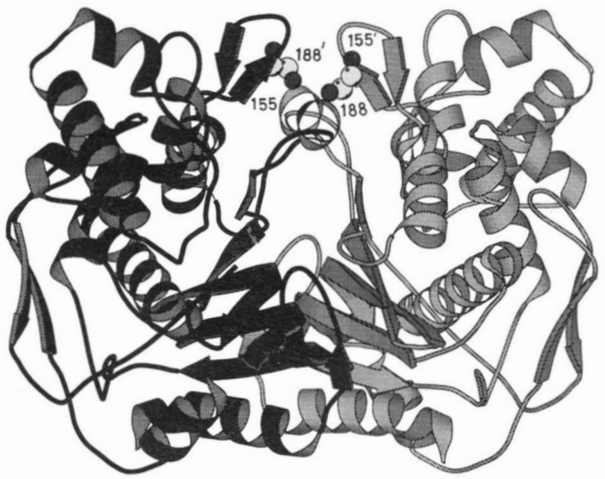

Fig. 9. Ribbon diagram of the *L. casei* thymidylate synthase dimer showing the location of symmetrically disposed intersubunit disulfide bridges (155–188′ and 188–155′). Side chains of these cysteine residues are superimposed on the ribbon in different ball sizes. C^{β} atoms are small shaded circles, whereas the sulfur atoms are large dotted circles. MOLSCRIPT (Kraulis, 1991) was used to generate the ribbon drawing with the coordinate set 4TMS (Brookhaven Protein Data Bank). Disulfide bridges were generated using the program MODIP (Sowdhamini et al., 1989).

The specificity of molecular interactions involved in aggregate formation has been noted in early classical studies of ribonuclease A (Crestfield et al., 1962) and is reminiscent of a recently proposed model for domain swapping as a general mechanism for the evolution for the oligomeric proteins (Bennet et al., 1994). Covalent bridging at interfaces in oligomeric proteins with relatively weak interface may provide a general approach to stabilization under denaturing conditions (Gokhale et al., 1994). The use of disulfide bridging as described in the paper necessarily requires the availability of a high-resolution three-dimensional structure. If the Cys residues are introduced at stereochemically inappropriate positions, the resulting covalent crosslink may indeed destabilize native interface interactions. The positioning of the interface disulfide bridges may also be important, with reinforcement of weak regions being likely to result in enhanced multimer stability. Covalent bridging may also enhance the effective native noncovalent interactions at interfaces, because the absence of dissociation would increase the "effective concentration" of interacting residues. Such effects have indeed been suggested in the case of intramolecular interactions in globular proteins (Creighton, 1983). The molecular characterization of aggregated species, in cases where interfaces are perturbed at low denaturant concentration, may provide useful models for defining the structural pre-requisites for aggregation-prone segments in proteins. From a practical viewpoint, rational design of mutant proteins that display vastly diminished aggregation tendencies can have biotechnological applications.

Materials and methods

Procedures for protein isolation, purification, mutagenesis, and characterization of mutant protein have been described earlier

(Gokhale et al., 1994). All the reagents used were of analytical grade or better and were purchased from Bio-Rad, Sigma, and Merck. Urea was recrystallized from boiling ethanol to remove residual impurities. Iodo *N*-(acetylaminoethyl)-5-napthylamine-1-sulfonic acid (1,5-IAEDANS) was purchased from Molecular Probes.

Fluorescence studies

For IAEDANS labeling, 4.5 mg protein was dissolved in 200 μL, 100 mM Tris-Cl, pH 8.0, 1 mM EDTA. 1,5-IAEDANS was added to a final concentration of 1.5 mM and incubated for 30 min in the dark. Cysteine (6 mM) was added to quench the reaction. The reaction mixture was passed through a Sephadex G-15 gel filtration column equilibrated with 25 mM potassium phosphate buffer, pH 6.9, to remove excess label. TSWT contains two cysteines at positions Cys 198 and Cys 244. The labeling in TSWT was selectively achieved at Cys 198. Cys 244 is not accessible for labeling. This was confirmed by labeling the mutant C244T of TS, which yielded identical extent of labeling. The stoichiometry of labeling was calculated by using an extinction coefficient of 6,300 M^{-1} cm^{-1} at 336 nm for the AEDANS and 10,700 M^{-1} cm^{-1} at 278 nm for the protein. About 1.6 mol label per mol enzyme was obtained. These results are in close agreement with the earlier reports of the extent of thiol labeling of TSWT (Bradshaw et al., 1979). Dansylated protein was incubated at different urea concentrations in 25 mM potassium phosphate buffer, pH 6.9, for 1 h before recording the spectra. Fluorescence spectra were recorded on a Hitachi 650-60 spectrometer by exciting the protein at 280 nm and monitoring dansyl emission excited by energy transfer. Excitation and emission band pass were 5 nm.

Size-exclusion chromatography

Gel filtration experiments were carried out on an LKB Ultrospec TSK-G 3000 SW column (manufacturers exclusion limit 3×105 for proteins) using an LKB 2150 HPLC pump or on a Pharmacia Superose-6 column (manufacturers' exclusion limit 4×10^7 Da for proteins) using a Pharmacia FPLC system. For experiments performed on the TSK-G 3000 SW column, 6.5 μM protein was incubated at the required concentration of urea for 1 h in 25 mM potassium phosphate buffer, pH 6.9 (1 h of equilibration time has been standardized using spectroscopic methods). Fifty microliters of this sample was injected onto the column equilibrated at the same urea concentration and buffer (flow rate 0.1 mL/min, detection 280 nm). For experiments on the Superose-6 column, 3 μM protein was incubated for 1 h at the required urea or GdmCl concentration in 25 mM potassium phosphate buffer. 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).

PAGE

Electrophoresis was conducted on a 5–20% polyacrylamide gradient gel in absence or in presence of 4 M and 8 M urea. The reservoir contained 25 mM Tris-glycine buffer, pH 8.8, and the required amount or urea. The ratio of acrylamide to *N,N*-methylene-*bis*-acrylamide was 30:0.8 (w/w); 30 μM protein was incubated for 1 h in 25 mM potassium phosphate buffer, pH 6.9, containing 0 M, 4 M or 8M urea. Glycerol was added to a final concentration of 10% (v/v) and 30 μL of this sample was loaded onto the gel. SDS-PAGE were run on 10% acrylamide gels as described by Lammeli (1970).

Acknowledgments

We thank Dr. M.K. Mathew (NCBS, Bangalore) for use of the FPLC system. We also thank Mr. Gautham Nadig (MBU) for helping to generate the TS picture using MOLSCRIPT. 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.). S.A. and R.S.G. were supported by Senior Research Fellowships from the Council of Scientific and Industrial Research.

References

Bennet MJ, Choe S, Eisenberg D. 1994. Domain swapping: Entangling alliances between proteins. *Proc Natl Acad Sci USA 91*:3127–3131.

Bradshaw TP, Dunlap BR. 1979. Characterization of the covalent chromatography of thymidylate synthase on thiopropyl-sepharose 6B. *Biochim Biophys Acta 1163*:165–175.

Brems DN, Alter LA, Beckage MJ, Chance RE, DiMarchi RD, Long HB, Pekar AH, Shields JE, FranK JH. 1992. Altering the association properties of insulin by amino acid. *Protein Eng 5*:527–533.

Brems DN, Plaisted SM, Kauffman EW, Havel HA. 1986. Characterization of an associated equilibrium folding intermediate of bovine growth hormone. *Biochemistry 25*:6539–5643.

Bychkova VE, Ptitsyn OB. 1995. Folding intermediates are involved in genetic diseases? *FEBS Lett 359*:3–5.

Come JH, Lansbury PT Jr. 1994. Predisposition of prion protein homozygotes to Creutzfeldt–Jacob disease can be explained by a nucleation-dependent polymerization mechanism. *J Am Chem Soc 116*:4109–4110.

Corbett RJT, Roche RS. 1984. Use of high speed size-exclusion chromatography for the study of protein folding and stability. *Biochemistry 23*: 1888–1894.

Creighton TE. 1983. An empirical approach to protein conformation stability and flexibility. *Biopolymers 22*:49–58.

Creighton TE. 1992. *Protein folding*. W.H. Freeman & Co.

Crestfield AM, Stein WH, Moore S. 1962. On the aggregation of bovine pancreatic ribonuclease. *Arch Biochem Biophys*(Suppl 1):217–233.

DeYoung LR, Dill KA, Fink AL. 1993a. Aggregation and denaturation of apomyoglobin in aqueous urea solutions. *Biochemistry 32*:3877–3886.

DeYoung LR, Fink A, Dill KA. 1993b. Aggregation of globular proteins. *Acc Chem Res 26*:614–620.

Fields GB, Alomo DOV, Stigler D, Dill KA. 1992. Theory of aggregation of proteins and copolymers. *J Phys Chem 96*:3974–3981.

Fish WW, Mann KG, Tanford C. 1969. The estimation of polypeptide chain molecular weights by gel filtration in 6 M guanidine hydrochloride. *J Biol Chem 244*:4989–4994.

Fish WW, Reynolds JA, Tanford C. 1970. Gel chromatography of proteins in denaturing solutions. *J Biol Chem 245*:5166–5168.

Garel JR. 1992. Folding of large proteins. In: Creighton TE, ed. *Protein folding*. W.H. Freeman & Co. pp 405–454.

Gasset M, Baldwin MA, Lloyd DH, Gabriel JM, Holtzmann DM, Cohen F, Fletterick R, Prusiner SB. 1992. Predicted α-helical regions of the prion protein when synthesized as peptides form amyloids. *Proc Natl Acad Sci USA 89*:10940–10944.

Gokhale RS, Agarwalla S, Francis VS, Santi DV, Balaram P. 1994. Thermal stabilization of thymidylate synthase by engineering two disulfide bridges across the dimer interface. *J Mol Biol 235*:89–94.

Goldenberg DP, Creighton TE. 1984. Gel electrophoresis in studies of protein conformation and folding. *Anal Biochem 138*:1–18.

Hardy LW, Finner-Moore JS, Montford WR, Jones MO, Santi DV, Stroud RM. 1987. Atomic structure of thymidylate synthase: Target for rational drug design. *Science 235*:448–455.

Havel HA, Kauffman EW, Plaisted SM, Brems DN. 1986. Reversible self-association of bovine growth hormone during equilibrium unfolding. *Biochemistry 25*:6533–6538.

Horowitz PM, Butler M. 1993. Interactive intermediates are formed during the urea unfolding of rhodanase. *J Biol Chem 268*:2500–2504.

Jaenicke R. 1987. Folding and association of proteins. *Prog Biophys Mol Biol 49*:117–237.

Kraulis PJ. 1991. MOLSCRIPT: A program to produce both detailed and schematic plots of protein structures. *J Appl Crystallogr 24*:946–950.

Lammeli UK. 1970. Cleavage of structural proteins during the assembly of the bacteriophage T4. *Nature 277*:680–685.

Lehrman RS, Tuls JL, Havel HA, Haskell RJ, Putnam SD, Tomich CSC. 1991. Site-directed mutagenesis to probe protein folding: Evidence that the formation and aggregation of a bovine growth hormone folding intermediate are dissociable processes. *Biochemistry 30*:5777–5784.

London J, Skrzynia C, Goldberg ME. 1974. Renaturation of *E. coli* tryptophanase after exposure to 8 M urea. Evidence for the existence of multinucleation centeres. *Eur J Biochem 47*:409–415.

Makhatadze GI, Privalov PL. 1992. Protein interactions with urea and guanidinium chloride, a calorimetric study. *J Mol Biol 226*:491–505.

Perry KM, Pookanjanatavip M, Zhao J, Santi DV, Stroud RM. 1992. Reversible dissociation and unfolding of the dimeric protein thymidylate synthase. *Protein Sci 1*:796–800.

Pohl M, Grotzinger J, Wollmer A, Kula M. 1994. Reversible dissociation and unfolding of pyruvate decarboxylase from *Zymomonas mobilis*. *Eur J Biochem 224*:651–661.

Pookanjanatavip M, Yuthavong Y, Greene PJ, Santi DV. 1992. Subunit complementation of thymidylate synthase. *Biochemistry 31*:10303–10309.

Prusiner SB. 1991. Molecular biology of prion diseases. *Science 252*:1515–1518.

Reinsch JW, Smith LL, Dunlap RB. 1979. Denaturation of thymidylate synthase from amethopterin-resistant *Lactobacillus casei*. *Cancer Biochem Biophys 3*:57–64.

Schein CH. 1989. Production of soluble recombinant protein in bacteria. *Biotechnology 7*:1141.

Sowdhamini R, Srinivasan N, Shoichet B, Santi DV, Ramakrishnan C, Balaram P. 1989. Stereochemical modelling of disulfide bridges. Criteria for introduction into proteins by site-directed mutagenesis. *Protein Eng 3*:95–103.

Srinivasan N, Sowdhamini R, Ramakrishnan CR, Balaram P. 1990. Conformation of disulfide bridges in proteins. *Int J Peptide Protein Res 36*:147–157.

Tanford C, Nozaki Y, Reynolds JA, Makino S. 1974. Molecular characterization of proteins in detergent solutions. *Biochemistry 13*:2369–2376.