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# Effect of Amino Acid Substitutions at the Subunit Interface on the Stability and Aggregation Properties of a Dimeric Protein: Role of Arg 178 and Arg 218 at the Dimer Interface of Thymidylate Synthase

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***ABSTRACT*** **The significance of two interface arginine residues on the structural integrity of an obligatory dimeric enzyme thymidylate synthase (TS) from *Lactobacillus casei* was investigated by thermal and chemical denaturation. While the R178F mutant showed *apparent* stability to thermal denaturation by its decreased tendency to aggregate, the Tm of the R218K mutant was lowered by 5°C. Equilibrium denaturation studies in guanidinium chloride (GdmCl) and urea indicate that in both the mutants, replacement of Arg residues results in more labile quaternary and tertiary interactions. Circular dichroism studies in aqueous buffer suggest that the protein interior in R218K may be less well-packed as compared to the wild type protein. The results emphasize that quaternary interactions may influence the stability of the tertiary fold of TS. The amino acid replacements also lead to notable alteration in the ability of the unfolding intermediate of TS to aggregate. The aggregated state of partially unfolded intermediate in the R178F mutant is stable over a narrower range of denaturant concentrations. In contrast, there is an exaggerated tendency on the part of R218K to aggregate in intermediate concentrations of the denaturant. The 3 Å crystal structure of the R178F mutant reveals no major structural change as a consequence of amino acid substitution. The results may be rationalized in terms of mutational effects on both the folded and unfolded state of the protein. Site specific amino acid substitutions are useful in identifying specific regions of TS involved in association of non-native protein structures. Proteins 1999;34:356–368.**


**Key words: dimeric enzyme stability; arginine residues in protein–protein interactions; aggregation of multimeric enzymes; thymidylate synthase**

## INTRODUCTION

Recent studies on the stability of dimeric proteins *vis-a-vis* monomers have led to the general conclusion that the former are, on an average, 4–13 kcal/mol ($\Delta G_U$ ($H_2O$) for denaturation) more stable than monomeric proteins[1] Increased stability in the case of dimers seems to stem from the fact that additional surfaces are buried upon subunit association.[2,3] Point mutations which alter the hydrophobicity, size, or charge of crucial amino acids involved in critical interactions have resulted in substantial destabilization, sometimes even leading to dissociation of many dimeric proteins and protein complexes.[4–7] Analyses of protein–protein interfaces observed in crystal structures indicate that in contrast to protein interiors, interfaces contain a large fraction of polar and charged residues, suggesting that in addition to the hydrophobic interactions,[8–12] hydrogen bonding, salt bridges,[13–16] and water mediated interactions[17–20] may be important at these sites. Arginine, in contrast to lysine, is known to occur in abundance at the interface of dimeric proteins and at the contact site of antigen-antibody or protease-inhibitor complexes.[20–22] Buried ionic interactions between subunits are thought to confer higher stability to oligomeric enzymes from thermophilic organisms.[14] Arginine at protein interfaces is found to be invariably involved in hydrogen bonding networks.[2,3] It has also been shown that the guanidinium group of Arg is involved in unique hydrogen bond interactions with the backbone peptide carbonyl group, especially at the subunit interface of multimers.[23] These interactions have been hypothesized to be critical for the stability of such proteins and have been tested and verified in a few instances.[24] For example, replacing Lys at the dimer interface of D-xylose isomerase by an Arg results in the stabilization of the enzyme against thermal inactivation.[24]

Thymidylate synthase (TS) is a highly conserved, obligatory dimeric enzyme, which has been structurally characterized from various sources.[25–28] *Lactobacillus casei* TS consists of 316 residues in each subunit, with the interface formed by the back to back stacking of six stranded β sheets from each subunit. The catalytic site is situated in a large cavernous site between the subunits.[25] The buried interface area is ~ 2,368 Å$^2$/ subunit surface and involves

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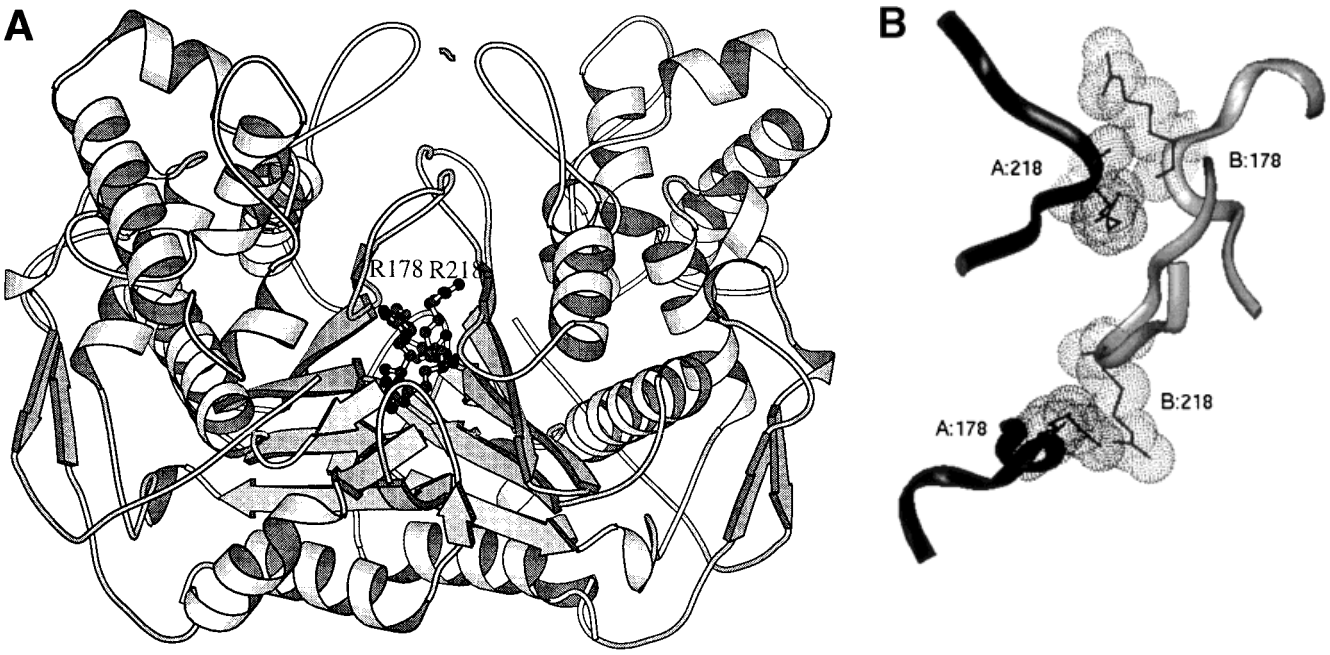


Fig. 1. TS interface highlighting R178 and R218: **A.** Molscript[63] representation of the TS dimer. Shown in ball and stick representation are the sites of mutation. **B.** Ribbon diagram showing the interface contacts made by residues R178 and R218.

~ 40 residues. The *L. casei* enzyme has five Arg residues at the dimer interface, four of which (Arg23, Arg178, Arg179, and Arg218) are conserved in the proteins from as many as 27 sources.[29] From the crystal structure of *L. casei* TS (Figure 1), it is evident that Arg178 and Arg218 make extensive contacts at the subunit interface. Arg178 establishes two hydrogen bond interactions and one ionic interaction, while Arg218 is engaged in one hydrogen bond. Interestingly, several atoms of Arg178 and Arg218 from opposite subunits are within a distance of 3.5 Å. A quartet of Arg residues are present at the dimer interface of the enzyme constituting the phosphate binding pocket.[25] Earlier studies on the effect of mutations at these sites have been confined primarily to the in vivo complementation of activity by monitoring the growth of thy$^-$*Escherichia coli* host strains, in a medium devoid of thymine.[29] These studies have established the importance of both Arg178 and Arg218 in the catalytic activity of the enzyme. It is pertinent to note that Arg178 at the active site of one subunit is contributed by the other symmetry-related subunit and is probably one of the reasons why TS is an obligatory dimer.[25] Many Arg178 mutants fail to complement the growth of thy$^-$ *E. coli* strains, indicating that the residue is critical for enzymatic activity. Substitutions for Arg178 that allow complementation of thy$^-$ *E. coli* include basic and other hydrophilic substitutions.

Arg218 could not be replaced by any amino acid without compromising enzymatic activity.[29] This residue, apart from establishing a hydrogen bond with the substrate dUMP, also interacts with peptidyl carbonyl oxygen of both Arg178' (residue from the opposite subunit) and Pro196. The guanidinium group of Arg218 is 3.5 Å away from the catalytic Cys198 and its role seems to be to stabilize the reactive thiolate form of Cys198 via ion pairing.[29]

Two mutants of TS, R178F and R218K, were studied for the following reasons. R218K was the only mutant which was found to complement the activity of thy$^-$ *E. coli* strains among all the other substituents tested at this position. Although the purified R178F mutant has no catalytic activity in vitro, this protein was found to form functional heterodimers with yet another inactive mutant C198A.[30] These features and the fact that they could be obtained in homogenous form in good yields indicated that the proteins R178F and R218K were probably well-folded and "native like." Thermal stability of the two mutants and their stability to chaotropes like urea and GdmCl were assessed by various spectroscopic techniques and gel electrophoresis. The crystal structure of the R178F mutant has been determined at 3 Å resolution to aid in the interpretation of the biophysical results. It was earlier reported from this laboratory that partially unfolded *L. casei* wild type TS (wt TS) aggregated under various denaturing conditions[31,32] and precipitated irreversibly upon heat denaturation. Heat and chaotrope-induced aggregation was completely abolished in an intersubunit disulfide cross-linked mutant leading to the suggestion that a segment of the interface was probably involved in the aggregation process.[31,33] The effect of critical Arg replacements on the unfolding process of TS was therefore investigated with the aim of assessing the role of interface segments in the non-native association of the unfolding intermediate.

## MATERIALS AND METHODS

Sephadex-G100 was obtained from Pharmacia (Sweden), while hydroxyapatite was from Bio-rad (USA). Polyethyleneimine, GdmCl were obtained from Sigma Chemicals (USA). Sodium chloride and ammonium sulfate were obtained from E. Merck (India). Yeast extract and Tryptone were obtained from Hi-Media (India). Glycerol was procured from Qualigens (India). Commercial grade urea was recrystallized from 95% ethanol. GdmCl was obtained from Gibco BRL.

### Purification of TS

Thy$^-$ *E.coli* strains harboring *L. casei* TS clone in PUC vectors were grown in Luria or Terrific broth for 16 h at 37°C on a shaker. Cells were harvested by centrifugation at 5,000 rpm for 10 min at 4°C. The pellet was washed in 25 mM phosphate buffer, pH 6.9 and was resuspended in 50 mM Tris-HCl, pH 6.5 containing 1 mM EDTA. The suspension was French-pressed twice at 3000 psi. The lysate so obtained was centrifuged at 10,000 rpm for 20 min at 4°C in a Sorvall fixed-angle rotor to remove unlysed cells and cell debris. The supernatant was diluted using 25 mM phosphate buffer pH 6.9 and TS was subsequently purified according to published protocols.[33]

### Purification of Mutants of TS

TS interface mutants R178F and R218K were purified essentially as described for wt TS with a few modifications.

After cell disruption using a French press, the lysate was centrifuged and the supernatant was diluted using phosphate buffer, pH 6.9 containing 1mM EDTA. To this, polyethyleneimine neutralized to pH ~7, by the addition of HCl, was added to a final concentration of 1% in the presence of 0.25 M NaCl. The suspension was allowed to stand on ice for 20 min and centrifuged at 10,000 rpm for 40 min at 4°C, following which $(NH_4)_2\ SO_4$ was added to 90% saturation. After standing overnight at 4°C, the suspension was centrifuged. The pellet was briefly washed with 90% $(NH_4)_2\ SO_4$, resuspended in a small volume of 25 mM phosphate buffer, pH 6.9 and desalted on a Sephadex-G50 column. Void volume fractions containing TS were collected and fractionated over a hydroxyapatite column as described for wt TS. Fractions containing mutants of TS were pooled, dialyzed against double-distilled water, and lyophilized. Samples were stored at −20°C until further use.

### Spectroscopic Studies

Circular Dichroism spectra of TS and the mutants in their native state in phosphate buffer, pH 6.9 were recorded using a JASCO J500A spectro-polarimeter attached to a DP501N data processor. Far UV-CD was recorded in the wavelength region 250–200 nm in a 1mm cuvette using a protein concentration of 3.8μM, and near UV in the region 250–300 nm, using a 5 mm pathlength cuvette. Protein concentrations used were wt TS-6.09μM, R178F and R218K-6.57μM. Fluorescence spectra were recorded on a Hitachi 650–60 spectrofluorometer using excitation and emission bandpass of 5nm.

### Activity of TS

The activity of R178F and R218K were determined under standard conditions as described for wt TS.[34] Refolding of the R178F mutant after urea induced unfolding was determined by monitoring the appearance of enzymatic activity by formation of hybrid dimers with another inactive deletion mutant V316 Am.[35] A mixture of 2.5 μM V316Am and 1.5 μM of R178F were denatured in 6.5 M urea in a total volume of 100 μl containing 20 mM phosphate, 1mM DTT, and 0.1 mM of EDTA for 1hr at 4°C. Refolding was achieved by rapid 10-fold dilution using 900 μl of 20 mM phosphate containing 0.5 M KCl, 1 mM DTT, and 0.1 mM EDTA. Activity of 200 μl of the reaction mixture was measured after 1.5 h under standard TS assay conditions in a total volume of 1ml. dUMP and methylenetetrahydrofolate were present at 200 μM concentration. The activities measured are (i) R178F and V316Am (denatured and refolded, with the concentration of R178F in the assay mixture being 0.03 μM) the activity measured is 3.073 (μmol dTMP/sec). (ii) R178F and V316Am (taken in 1 ml of 20mM phosphate containing 0.5 M KCl and 0.65 M urea, the concentration of R178F in the assay mixture being 0.03 μM) the activity measured is 0.69 (μmol dTMP/sec). (iii) R178F and V316Am (taken in 20 mM phosphate containing 0.5 M KCl, the concentration of R178F in the assay mixture being 0.03 μM) no activity was detected. (iv) R178F alone and V316Am alone in similar conditions showed no detectable activity.

### Dansylation of wt TS and its Interface Mutants

The proteins were dansylated essentially as described in Gokhale et al., (1996).[31]

### Crystallization and Data Collection

Crystals of the R178F mutant of *L. casei* TS were obtained using the hanging drop method. The reservoir solution contained 50 mM potassium phosphate buffer pH 5.8–6.2. A 6 μL drop containing 13–15 mM ammonium sulphate in 50 mM potassium phosphate buffer pH 5.8–6.2 and 10 mg/ml protein in a hanging drop placed on a pre-siliconized cover-slip was equilibrated with the reservoir solution. The crystals grew over a period of three days to sizes of 0.6 × 0.3 × 0.2 mm. The crystals belong to the hexagonal space group $P6_122$, with a = b = 79.18 Å, c = 244.80 Å or a = b = 79.30 Å, c = 230.89 Å. The crystals having c = 230.89 Å (Form A) invariably undergo transformation during the course of data collection, whereas those of c = 244.80 Å (Form B) do not. The asymmetric unit of the crystal is compatible with one monomer (Matthews coefficient 2.8212 Å$^3$/dalton, assuming a molecular weight of 34kD per monomer). Both the crystal forms have the same external morphology. The data were collected at 23 ± 2°C on an MAR image plate attached to a Rigaku RU-200 rotating anode X-ray generator. The data were processed using the DENZO and SCALEPACK suite of programs. Crystals of the R218K mutant could not be obtained even after extensive trials as the protein precipitated after one

day of setting up the crystallization droplet. wtTS coordinates were used to initiate refinement of the mutant structure using the program X-PLOR.[36] The resulting electron density map and model were examined using the graphics program O.[37]

### Denaturation of wt and Interface Mutants of TS

#### *Thermal denaturation*

Thermal denaturation of wt TS and its mutants was monitored by measuring Rayleigh scattering from dilute protein solutions. Proteins in 25 mM phosphate buffer, pH 6.9 were heated for 10 min at different temperatures in the cuvette chamber, using a thermostatted cell assembly and an attached circulating water bath. The scatter intensity was monitored at 400 nm using a 90° geometry on a Hitachi 650–60 spectrofluorometer.

#### *Denaturation by GdmCl*

TS, R178F or R218K were treated with 0–4 M GdmCl in 25 mM phosphate buffer, pH 6.9 for 1 hr at 25°C and structural changes in the protein were monitored by fluorescence spectroscopy and circular dichroism. Fluorescence of the extrinsically labeled dansyl group was followed either by direct excitation at 340 nm or by exciting the tryptophans in TS at 280 nm (for details see Gokhale *et al*., 1996[31]).

Tertiary and secondary structural changes in the presence of GdmCl were monitored by recording the CD spectra in the near (300–250 nm, using a 0.2 mm cuvette ) and far UV region (250–200 nm, using a 5mm cuvette) respectively, in a JASCO J-500A spectropolarimeter.

#### *Denaturation by urea*

Tertiary and secondary structural changes in the presence of urea were monitored by recording the CD spectra in the near- (300–250 nm, using a 0.2 mm cuvette ) and far-UV region (250–200 nm, using a 5mm cuvette) respectively, in a JASCO J-500A spectropolarimeter.

### Electrophoresis

Native-gel electrophoresis under non-reducing conditions in the presence or absence of 4.5 M urea at 10°C was performed using the Laemmli[62] continuous buffer system at pH 8.8.

## RESULTS

### Comparison of wt TS and the Interface Mutants Under Non-Denaturing Condition

The two arginine interface mutants were compared with the wt enzyme for their activity, absorption spectra, intrinsic fluorescence properties, and secondary and tertiary structure content in 25 mM phosphate buffer, pH 6.9. These parameters are sensitive probes of structural changes and may be used for gross comparisons of structural features of proteins.

The three proteins wt TS, R178F, and R218K have almost identical UV absorption spectra in the region 300–250 nm. The fluorescence emission maxima for all three proteins are observed at 341nm, with R218K showing a small increase in the relative quantum yield (data not shown). The CD spectra in the near and far UV region for wt TS and the two mutants are shown in Figure 2. Interestingly R218K shows a significant reduction in the near UV CD band at 290 nm and an enhancement in the far UV CD band at 220 nm. Similar characteristics in the CD spectra of mutants have been observed in other cases, more specifically in the N-terminal domain of λ repressor,[38] wherein cavity-creating mutants were reported to have decreased α helical content. The CD data suggest that R218K may have a less well-packed interior with perturbations in the environment of the aromatic side chains, which make a major contribution to the CD signal at 290 nm. When assayed for their enzymatic activity under standard conditions used for wt TS, the R178F

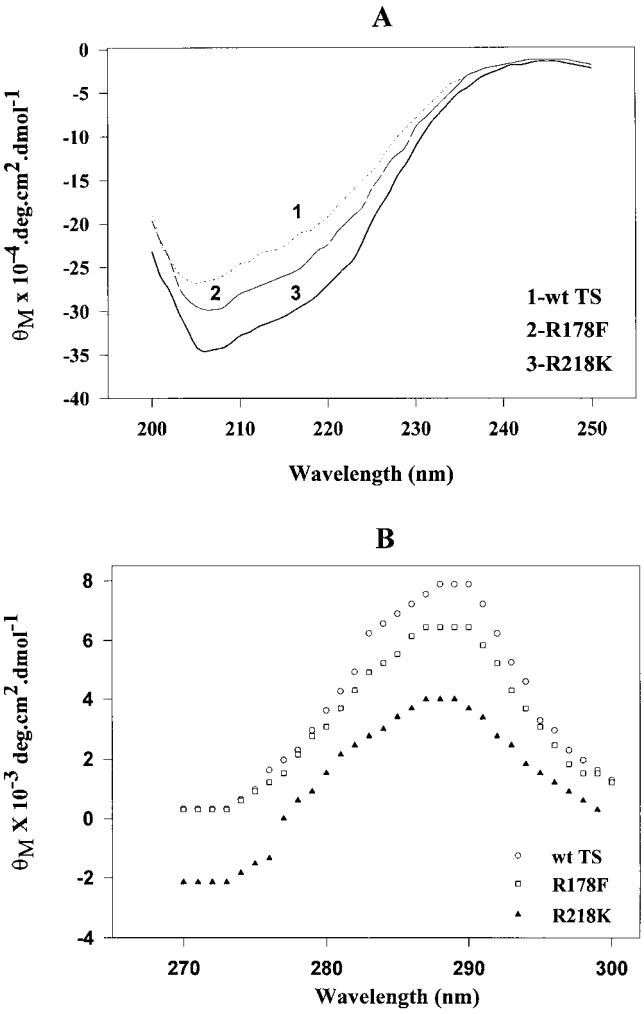


Fig. 2. Spectroscopic studies of TS and its mutants in aqueous medium: All spectra were recorded in 25 mM phosphate buffer, pH 6.9. **A.** Secondary CD: Spectra were recorded in the near-UV region between 250–200 nm in a 1mm cuvette using a protein concentration of 3.8 μM. Spectra averaged over four scans are shown. **B.** Tertiary CD: Spectra were recorded in the near-UV region between 250–300 nm using 5 mm cuvette. Concentration of proteins were: wt TS-6.09 μM, R178F and R218K-6.57μM. Spectra averaged over four scans are shown.

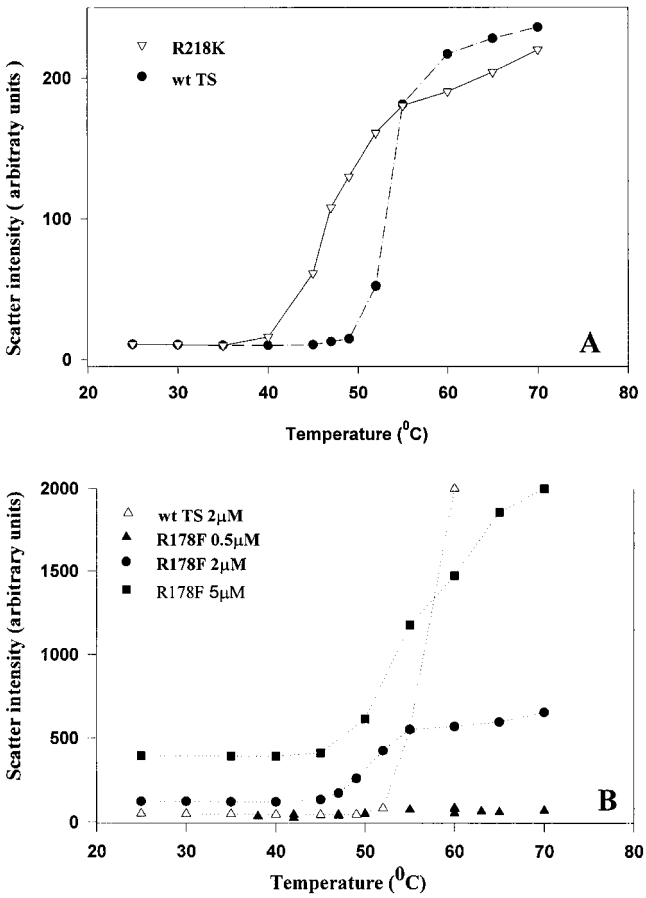


Fig. 3. Effect of temperature on the stability of TS and its mutants: **(A)** wt TS and R218K and **(B)** R178F in 25 mM phosphate buffer, pH 6.9 were heated at each temperature for ten minutes. Light scattering intensity read at 400 nm using a Hitachi fluorimeter at 90° geometry was plotted against temperature. Scattering intensity of wt TS at 5μM concentration at 52°C and above, goes out of the instrument range.

mutant was confirmed to be inactive,[30] while the specific activity of R218K was ~15% of wt TS (specific activity of R218K was found to be 0.189 U/mg of protein, as compared to a value of 1.35 U/mg in the case of wt TS). These results indicate that the mutant proteins posses a largely "native-like" fold.

### Heat Denaturation of TS and its Mutants

In order to investigate the consequence of the amino acid substitution on the thermal stability of TS, the two Arg mutants in 25 mM phosphate buffer, pH 6.9 were heated for 10 min, at different temperatures and the light-scattering intensity was recorded at 400 nm. Wild type TS was included for comparison. As seen from Figure 3**,** there was a rapid loss in the stability of wt TS at 52°C, presumably due to structural changes leading to protein aggregation.[33] R218K has an apparent Tm which is 5°C lower than that of the wt protein (Tm of R218K = 47°C). The sharp transition characteristic of wt structure is lacking in this mutant, seemingly indicative of a complex unfolding process.[38] Under the same experimental conditions, R178F remained in solution up to 85°C. The effect of temperature on the stability of R178F was further assessed as a function of protein concentration. As can be seen from Figure 3, the Tm of R178F decreased when the protein concentration is increased from 0.5 μM to 2 μM. However at 5 μM, like the wt protein, the structure melts at ~52°C. Even at this protein concentration, instead of the characteristic sharp transition, there was a gradual increase in the light-scattering intensity with temperature. This absence of an "all or none" phenomenon is once again indicative of a complex unfolding process[38] and is probably due to the accumulation of an unfolding intermediate(s). It is possible that this intermediate in the case of R178F is not "sticky enough" to form strong non-native contacts at low protein concentrations although at high protein concentrations, aggregation is observed.

The relative solubility of R178F as compared to wt TS was subsequently tested by calculating the amount of protein that remains in solution after heat denaturation. Fifty-five (55) μM wt TS and R178F in 25 mM phosphate buffer, pH 6.9 were heated at 55°C or at 85°C for 15 min each and centrifuged at 10,000 rpm for 5 min. Residual protein in solution was quantified by measuring the absorbance of the supernatant solutions at 278 nm. About 30% of R178F could be recovered from the supernatant after heating 55 μM protein solution while in the case of wt TS, hardly any protein remained in the soluble fraction. No further loss in protein was observed after heating R178F to 85°C. The results described above establish that R178F is significantly more stable at high temperatures.

### Chemical Denaturation

In order to investigate the effect of single amino acid substitution at the interface of TS on a) subunit affinity, b) stability of tertiary and secondary structure of TS, and c) the role of dimer interface in the aggregation of the unfolding intermediate,[32,33] equilibrium denaturation of the mutant proteins in GdmCl and urea were followed by various spectroscopic techniques. In the case of wt TS refolding from 8 M urea solutions can be accomplished to yield almost 70% of original enzymatic activity.[30,31] In the case of R178F, refolding from denaturant solutions has been monitored by formation of an active heterodimer with a TS mutant V316 Am (an inactive deletion mutant lacking the C-terminal Val 316 residue[35]). Both R178F and V316 Am form inactive homodimers. Under standard assay conditions, no activity is detected. No activity is detectable in a mixture of R178F and V316 Am in 20 mM phosphate buffer containing 0.5 M KCl at varying protein concentrations. If, however, a mixture of the two inactive mutants is denatured in 6.5 M urea at 4°C and then rapidly refolded by dilution, appreciable activity is observed with an apparent Kcat of 1.7 $s^{-1}$ (μmol of dTMP formed per sec/μmol of R178F). Under these conditions, the kcat value of wt TS that has not been taken through the unfolding/refolding cycle is 5.8 $s^{-1}$. Chemical denaturation is thus reversible for both wt and mutant enzymes.

### Dansyl Fluorescence

TS has a single reactive thiol—the catalytic Cys198 in each subunit. Wild type TS and the two interface mutants were therefore labeled with IAEDANS.[33] The position of Cys 198 in the context of the quaternary and the tertiary structure of the protein was utilized in two ways to monitor the stability of the two mutants. 1) By virtue of its location at the dimer interface, the fluorescence of the dansyl moiety is likely to reflect the changes in the quaternary structure of the TS variants. This is an important probe in monitoring the subunit affinity of the mutant proteins. Using this extrinsic label, spectral changes were followed by direct excitation of the fluorophore at 340 nm. 2) The absorption spectra of the dansyl fluorophore and the emission spectra of the tryptophan residues in TS overlap and the transfer of energy between the fluorophore can be used to monitor the unfolding of the polypeptide chain pertaining to a specific region of the protein. Distances between sulfur atoms of Cys 198 and the centroid of the indole ring of the tryptophan side chains (seven Trp per subunit) in *L. casei* TS indicate that Trp residues and the Cys within the same subunit are separated by distances of about 9–17 Å (except for Trp 90, which is at distance of 26.8 Å)[31]. The corresponding distances between the two residues across the subunit interface range between 19–50 Å. It became obvious that due to distance constraints, transfer of energy is more likely between residues within each subunit. Thus by exciting the Trp in the protein at 280 nm, the efficiency of energy transfer between Trp and the dansyl groups can be monitored to provide information on subunit unfolding.[32,33]

When probed either by direct excitation at 340 nm or at 280 nm, it was found that complete denaturation of wt TS and its interface mutants was achieved at about the same concentration of GdmCl (Figure 4). However distinct differences were apparent in the early stages of denaturation at low concentrations of GdmCl. In the case of R178F and more distinctly in the case of R218K, the fluorescence intensities decrease over a narrow range of GdmCl (0–1M GdmCl) as compared to wt TS. It has been demonstrated that it is the partially unfolded wt dimer that aggregates in the concentration range of 1.5–2 M GdmCl, prior to chain unfolding. Thus, the structural changes in R218K preceding those between 0.8–2 M GdmCl are likely to involve alterations in the dimeric structure of the protein. Since the probe used here involves the modified Cys present at the dimer interface, it is quite possible that intersubunit interactions are destabilized in R218K and to a lesser extent in R178F at such low concentrations of GdmCl.

Further analysis of the spectra indicate that wt TS unfolds by an "all or none" phenomenon with no evidence for the formation of an intermediate. In contrast, a stable intermediate inferred from the plateau in the transition region was stable between GdmCl concentrations of 0.8–2 M and 1.2–1.8 M, in the case of R218K and R178F, respectively (Figure 4A). When tryptophan was the donor of excitation energy, R218K was seen to unfold via a stable

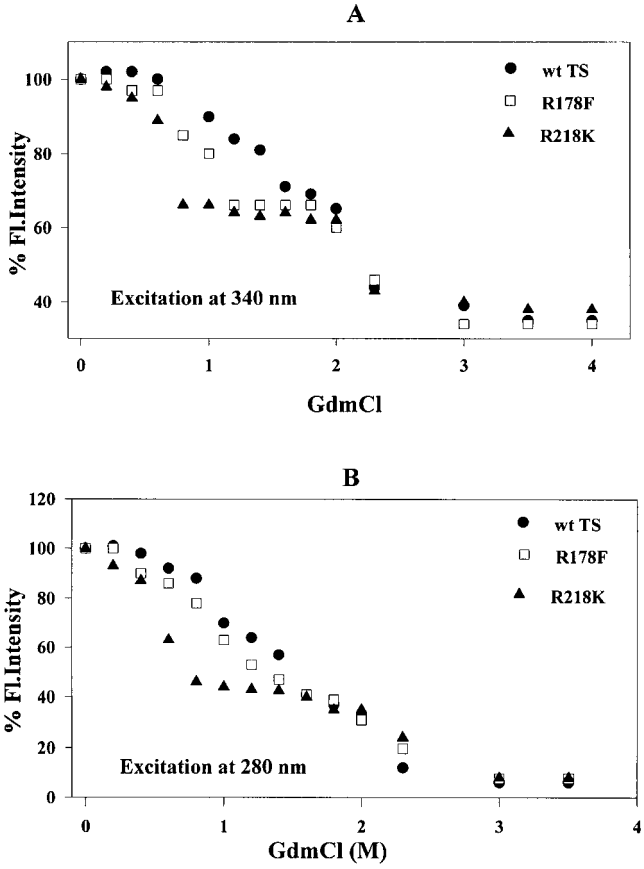


Fig. 4. GdmCl denaturation of dansylated wt TS and its mutants: After incubation for 1 hour at 25°C in varying concentrations of GdmCl in 25 mM phosphate buffer, pH 6.9 fluorescence intensity of dansylated wt TS, R178F, and R218K (0.9 µM) were monitored as a function of GdmCl. Fluorescence intensity at the respective emission maxima in the absence of GdmCl was considered as 100%. Samples were excited **(A)** at 340nm and **(B)** at 280nm.

intermediate, which accumulates between 0.8–1.6 M GdmCl (Figure 4B). While an intermediate was detectable over a very narrow concentration range of GdmCl in the case of R178F, none was apparent in the case of wt TS. It is interesting to note that an unfolding intermediate is well stabilized in the R218K mutant in the presence of GdmCl as compared to either the wt protein or R178F. Comparing the unfolding profiles of the three proteins obtained by energy transfer, it appears that the interactions between the dansyl and the tryptophan domains are far more sensitive in the case of R218K as compared to either the wild type protein or R178F. R218K seems to undergo greater perturbations in structure at low denaturant concentrations.

### Tertiary Structural Changes (CD studies)

The effect of interface mutations on the stability of different hierarchial structures of TS in the presence of denaturants was further investigated by CD studies. CD spectra of wt TS and the mutants in the presence of GdmCl were recorded in the near UV region. Tertiary interactions

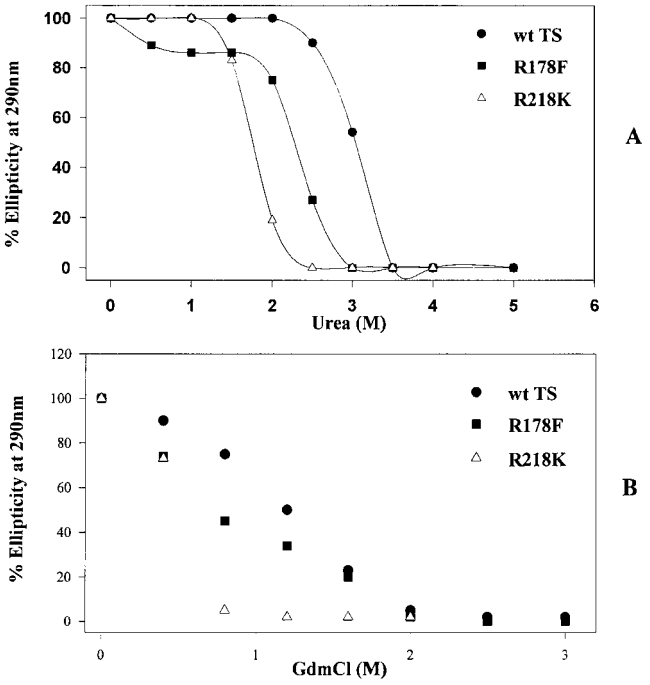


Fig. 5. Tertiary structural changes of wt TS and its mutants in GdmCl and urea TS and mutants (7μM) were incubated with varying concentration of **(A)** 0–3 M GdmCl and **(B)** 0–4M urea in 25 mM phosphate buffer, pH 6.9 for 1 h at 25°CD. Spectra were scanned in the near-UV region using a 5 mm cuvette. Ellipticity values at 290 nm for proteins in the absence of GdmCl is considered 100%.

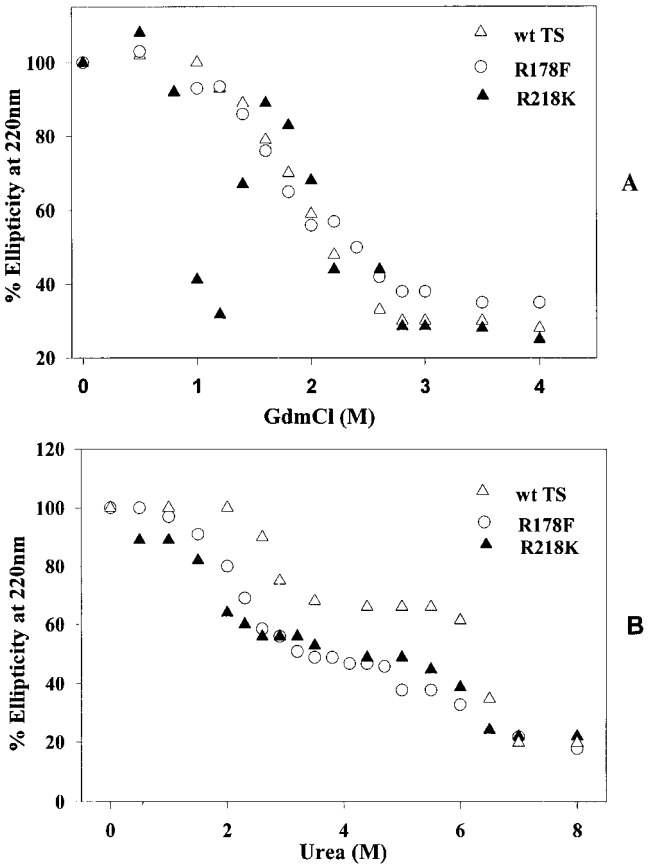


Fig. 6. Secondary structural changes in TS and its mutants following denaturation in GdmCl and urea. **A.** TS (3.16μM) and its mutants R178F (12.7μM) and R218K (10μM) were incubated in GdmCl ranging in concentrations from 0–4 M in 25 mM phosphate buffer, pH 6.9 for 1 h at 25°C. CD spectra of the mutants were scanned between 250–200 nm using a 0.2 mm cuvette and that of wt TS in a 1mm cuvette. Ellipticity value at 220 nm in the absence of GdmCl was taken as 100% in each case and the relative values at every GdmCl concentration was plotted against the respective GdmCl concentration. **B.** wt TS at 8.7μM, R178F at 7μM and R218K at 6.14μM were subjected to denaturation in the presence of 0–8 M urea for 1h and the CD spectra were recorded using a 0.2 mm cuvette.

in wt TS and R178F are lost completely by around 2.0 M GdmCl. While 0.8 M GdmCl brings about 50% loss in the band intensity of R178F at 290 nm, 1.2 M GdmCl is required to bring about the same effect in wt TS. The CD band at 290 nm was completely abolished by 0.8 M GdmCl in the case of R218K, indicating that the tertiary interactions in this protein are significantly more unstable in the presence of GdmCl (Figure 5A). Similar results were obtained when all three proteins were denatured in urea (Figure 5A). The relative stability's of the tertiary interactions in wt TS and the two Arg mutants followed the same order as in GdmCl solutions. The CD band at 290 nm was lost by 3.5 M in the case of wt TS, by 3 M in R178F and 2.5 M in R218K by urea. These two results illustrate the fact that quaternary interactions in TS are important for the integrity of the non-local interactions within the subunit.

## Secondary Structural Changes

The stability of the secondary structural elements in GdmCl or urea followed by circular dichroism in the far UV region were found to be similar for wt TS and the Arg mutants. The unfolding pathway, as monitored by this technique could be described by a two-state mechanism for both wt TS and R178F. Interestingly, the R218K mutant visibly precipitated out of solution between 0.8 and 1.2 M GdmCl, which was also reflected in the dramatic reduction in CD signal at these GdmCl concentrations. Upon further additions of GdmCl, the protein re-dissolved and, surprisingly, the unfolding curve traced the same path as that of wt TS and R178F (Figure 6A). This probably indicates that the intermediate which undergoes aggregation is not drastically altered in structure. Thus the intermediate and the aggregate are compact, with the structure closer to native TS rather than the denatured state. It is to be noted that an equilibrium unfolding intermediate of wt TS stable over a very narrow range of GdmCl, deduced by the non-overlap of various unfolding transition curves was reported earlier.[32,33] Using gel filtration, this intermediate was shown to form large molecular weight aggregates.[33]

When the two mutant proteins and wt TS were treated with urea and their secondary structural changes followed as a function of the denaturant, unfolding was found to proceed via the accumulation of at least a single stable equilibrium intermediate (Figure 6B). The initial phase was centered between 0–2.0 M in the case of wt TS and 0–1 M in the case of R178F and R218K. The plateau correspond-

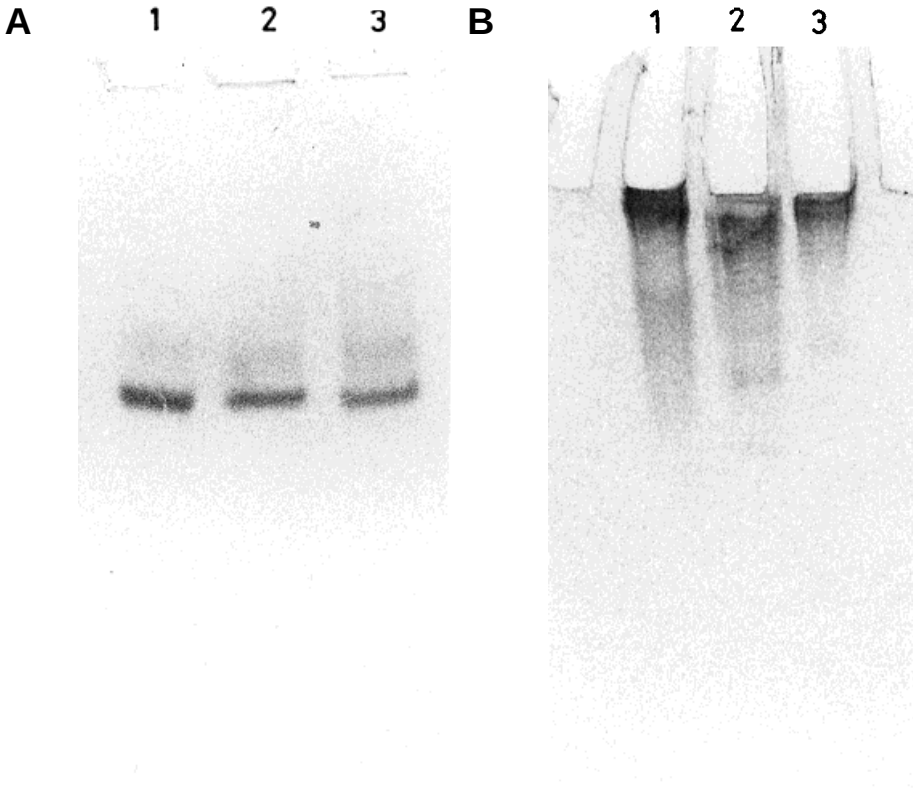


Fig. 7. Native-PAGE of TS and its dimer interface mutants under non-reducing conditions and in the presence of 4.5 M urea. **A.** TS, R178F, and R218K (30 µg) in 25 mM phosphate buffer, pH 6.9 containing 0.5 mM EDTA were resolved on a 5–15% gradient gel at 4°C. **B.** 30 µg of wt TS R178F and R218K were incubated in the presence of 4.5 M urea in 25 mM phosphate buffer, pH 6.9 (in a volume of 50 µl) containing 0.5 mM EDTA for 1 h at 25°C. Samples were analyzed on a 5–15% gradient gel containing 4.5 M urea at pH 8.8 at 4°C. Gel was stained with Coomassie brilliant blue G.

ing to the transition began at 3.5 M and extended up to 6 M in the case of wt TS. In the case of R178F and 218K, the intermediates were detectable at lower concentrations of urea and found to be stable between 2.5–4.6 M urea in the former and between 2.3–6 M urea in the latter. Interestingly, in contrast to GdmCl, urea stabilizes an unfolding intermediate over a large concentration range probably due to the preferential interaction of the unfolding intermediate with urea. Such preferential interactions between the unfolding intermediate and a denaturant are not uncommon.[39,40]

The ellipticity at all urea concentrations was marginally larger in magnitude for the wt TS when compared to either of the mutants (Figure 6B). Furthermore, the occurrence of the unfolding intermediate in the case of the two mutants at low concentrations of urea implies that bonding interactions between the subunits are destabilized as a consequence of amino acid substitution. This is consistent with other spectroscopic evidence presented earlier (see above).

### Analysis of the Qaternary Structure of wt TS and the Mutants

A stable unfolding intermediate of wt TS in urea solutions (3–5 M) has been reported.[33] The protein was shown to aggregate in the concentration range of 3.5–5.5 M urea by gel electrophoresis and gel filtration techniques before complete unfolding of the polypeptide chain occurs.[31,33] Based on these results, it was deduced that the partially-unfolded equilibrium intermediate forms large molecular weight aggregates. The associated intermediate exhibits molten globule-like properties with loss of the near-UV CD signal and quenching of tryptophan fluorescence while appreciable secondary structure is retained as monitored by the ellipticity at 222 nm. Most interestingly, an intersubunit disulfide cross-linked mutant protein failed to aggregate under similar conditions implying that disulfide bridging has probably tethered a fragile region of the interface responsible for the aggregation of the partially unfolded wt TS dimer.[32] As followed by far-UV CD, an unfolding intermediate implied by the presence of a plateau in the transition region is seen to accumulate over a similar concentration range of urea in the two mutants. To further deduce the role of interface segments in TS aggregation, wtTS, R178F, and R218K were incubated for 1 h with or without 4.5 M urea and analyzed on a 5–15% acrylamide gradient gel containing the same concentration of urea. As compared to the protein treated as control (Figure 7A), aggregates of very high molecular weight in the case of wt TS and R218K were found to be retained

within the wells of the gel (Figure 7B). Interestingly, the R178F mutant migrated into the running gel with no obvious retention within the wells (Figure 7B). The mobility, however, was retarded, indicative of an aggregate. It is to be noted that the plateau corresponding to the intermediate was found to be less stable in R178F, as analyzed by circular dichroism spectroscopy in the far-UV region (Figure 6B). It is possible that the aggregate is less stable in this mutant or the aggregate size is smaller. It is to be recalled that the R178F mutant had a decreased tendency to aggregate upon heat dentauration.

In order to see if the critical concentration of urea required to dissociate the mutant dimer was any lower than that required in the case of wt TS, all three proteins were analyzed on a 6-M urea polyacrylamide gel as described above. The results indicate that, the aggregates begin to break down but there is no indication for the presence of a monomeric species in the case of either mutants or the wt TS (data not shown).

### Crystal Structure Analysis

It is clear that while the Phe substitution for R178 has resulted in the stabilization of the mutant against thermal denaturation, the Lys substitution for R218 has resulted in destabilization. The effect observed could be due to the alteration either in the native structure of the proteins or in the stability of their unfolded states. To obtain an insight into the effect of amino acid substitution on the three-dimensional structure, attempts were made to crystallize R178F and R218K. While diffraction-quality crystals could be obtained for R178F, the R218K mutant failed to crystallize under similar conditions. Two hexagonal crystal forms of the R178F mutant were obtained which differed only in the length of the "c" axis (Form A, c = 230.89 Å; Form B, c = 244.8 Å). Several Form A crystals were observed to transform to Form B crystals during data collection a feature that has been noted in earlier studies. It has also been observed in substrate bound complexes of wt TS.[41] Structures were determined using a Form A crystal which remained untransformed during data collection and Form B crystals. Table I gives the details of the crystallographic data and refinement of the structures. The structures of the dimeric enzyme in the two forms were practically identical but for minor changes in crystal symmetry related contacts. Figure 8 shows a view of the vicinity of residue 178 in the R178F mutant structure. The excellent superposition obtained for residues in the vicinity of Arg178 in the mutant and the wt TS clearly suggests that replacement of Arg178 by a bulky hydrophobic residue like Phe does not result in an appreciable perturbation of the structure near the site of mutation.

**TABLE I. Summary of Data Statistics and Refinement Statistics for the Two Crystal Forms**

| | Form A/ Form B |
|---|---|
| Total number of reflections measured | 14424/32492 |
| Average number of times each intensity was estimated | 2.43/4.05 |
| Total number of unique reflections | 6945/8024 |
| Number of unique reflections in the resolution range 10–3.0Å with $I/\sigma(I) > 2.0$ | 3924/4112 |
| Completeness in the resolution range 10–3.0 Å with $I/\sigma(I) > 2.0$ | 64.1%/81.9% |
| Completeness in the last resolution shell 3.14–3.0Å | 60.53%/61.26% |
| R-merge[a] | 11.9%/9.1% |
| Initial R[b]-factor for the 4TMS model in the cells of Form A/Form B | 27.0%/29.9% |
| Initial free R[b]-factor for the 4TMS model in the cells of Form A/Form B | 31.4%/31.4% |
| Final R[b]-factor for reflections with $I/\sigma(I) > 2.0$ in the resolution range 10.0–3.0 Å | 23.3%/22.2% |
| Final free R[b]-factor for reflections with $I/\sigma(I) > 2.0$ in the resolution range 10.0–3.0 Å | 27.1%/26.7% |

[a] $R_{merge} = \Sigma|F_o - kF_c|/\Sigma F_o \times 100$.
[b] R factor $= \Sigma|\langle I\rangle - I_h|/\Sigma\langle I\rangle \times 100$, where $I_h$ is the intensity of a measurement and $\langle I\rangle$ is the average of the measurements for reflection h. 4TMS is the PDB code for wtTS.[25]

## DISCUSSION

Arginine 178 and arginine 218 are key residues at the dimer interface of TS. They are absolutely-conserved active site residues as well. A classification of the TS interface based on segmentation similar to that reported by Jones and Thornton,[3] identifies 17–38, 174–190, and 201–220 of the polypeptide chain as the regions involved in holding the subunits together. Segments 174–190 and 201–220 contribute ~ 37 and 21% of the buried surface area at the interface. Arg178 and Arg218 are a part of the TS interface which is maximally populated with atoms of residues within 4 Å distance across the subunits (Figure 1). The contribution of these arginines to the stability of TS is therefore of critical interest. Moreover it is increasingly believed that the instability of the isolated subunits of a multisubunit protein is due to the exposure of the previously buried interface region to the aqueous environment.[2,21] Since most oligomeric proteins that undergo aggregation do so when they are only partially unfolded and the aggregates themselves are very compact entities, specific regions of the subunit interface may have an important role in the association process.

### Effect of Interface Mutations on the Thermal Stability of TS

The results presented here indicate that the R178F mutant is apparently more stable to thermal denaturation as monitored by heat-induced precipitation, when compared to wt TS. The crystal structure does not provide any immediately obvious reason for the enhancement in the stability of this mutant or for its lower tendency to aggregate. One probable reason is that the Arg at position 178 in wt TS is situated very close to an Arg at position 23. By changing this residue to a Phe, no new favorable interaction is introduced; rather an unfavorable proximity

**TABLE II. Effect of Arg Substitution at the Interface of Multimeric Proteins**

| Protein | Mutation | Parameter measured | Effect | Reference |
|---|---|---|---|---|
| D-Xylose isomerase homo tetramer from actinoplanes missouriensis | K23R Inter dimer interface (loop) | Heat inactivation at 84°C | Half-life increases about 6-fold | Mrabet et al., 1992[24] |
| | K309R and K319R (solvent exposed side of a helix) | | 1.6- and 1.4-fold increase in stability | |
| | K309R/K319R/K323R | | 2.8-fold reduction in stability | |
| Human Cu, Zn Superoxide dismutase (homo dimer) | K9R (not involved in intersubunit interaction) | Enzyme inactivation at 85°C | 6-fold increase in half life | Mrabet et al., 1992[24] |
| Glyceraldehyde 3-phosphate dehydrogenase from Bacillus subtilis. (homo tetramer) | wt protein | Temperature stability as measured by enzyme activity | Enzyme inactivation at 50°C | Mrabet et al., 1992[24] |
| | G281K (inter dimer interface) | | 72°C: (Half life 5 min) | |
| | G281-R | | 75°C: (Half-life 18 min) | |
| Superoxide dismutase B. Xenopus laevis (dimer) | K3R | Near the dimer interface | Decreases the unfolding rate of the protein: more stable than wt enzyme | Folcarelli et al., 1996[61] |
| | K67R | Solvent accessiblility | Monomerization in the μM range | |
| | K3R/K67R | | (Monomeric species active) | |

of two charged residues is removed. It is tempting to suggest that R178 is important for promoting non-native protein association (which is substantiated by the protein-concentration-dependent aggregation of this mutant) observed during the thermal precipitation of TS. Removal of this residue may then impede aggregate formation resulting in the *apparent* stability of the folded structure of the mutant protein. Protein aggregation may be kinetically controlled, a feature which complicates interpretation for the structural effects of mutations on the folded and the unfolded states. Nevertheless, it is pertinent to note that mutational effects can influence the properties of both the folded and unfolded state of a protein; mutational effects on the unfolded state of staphylococcal nuclease being a particularly relevant example.[42] The irreversibility of thermal unfolding precludes a detailed thermodynamic analysis.

Analysis of the protein crystal structure data have suggested that Arg residues may play an important part in protein–protein interaction surfaces. Indeed, Arg has a far greater propensity than lysine to occur at interfaces.[2,3] It is therefore not surprising that Arg178 in TS may in fact be a key component of an "aggregation competent" segment. The results on the unfolding studies of the mutant proteins presented here lend credence to this view. Denaturation by GdmCl as well as by urea indicate that as compared to the wt protein, local interactions (at the level of quaternary and tertiary structure) are probably more fragile in the Phe mutant. The apparent stability of R178F to heat-induced aggregation and the proposition based on denaturation studies that the interface mutation is destabilizing in nature can be reconciled by postulating that the amino acid substitution influences the association properties of the unfolding intermediate to a significant extent and has only a limited effect on the stability of the native dimer per se. The absence of appreciable local changes in the mutant crystal structure favors this interpretation.

In the absence of the crystal structure of R218K, no direct structural interpretation is possible for the properties of this mutant. Nevertheless, taking a cue from results obtained in other systems wherein it is documented that replacement of Arg at dimer interfaces by Lys is in general destabilizing in nature (Table II), we believe that the instability of R218K mutant may be due to similar reasons. Lys at this position in TS is probably unable to mimic the optimum interactions provided by R218 in native TS. Support for this view is derived from CD studies in the near-UV region which indicate that the protein interior is less well-packed in this mutant. A similar mutation R176K in bacteriophage T4 TS resulted in the blue shift of the fluorescence maxima indicating a change in the environment of tryptophan residues in the protein.[43] Furthermore, attempts to crystallize R218K under conditions used for the wt enzyme and R178F mutant resulted invariably in the precipitation of the protein. It is appropriate to point out that single amino acid substitutions at subunit interfaces have been shown to alter the aggregation properties of many multimeric protein[44–47] and in several cases are responsible for pathological disorders.[48–50]

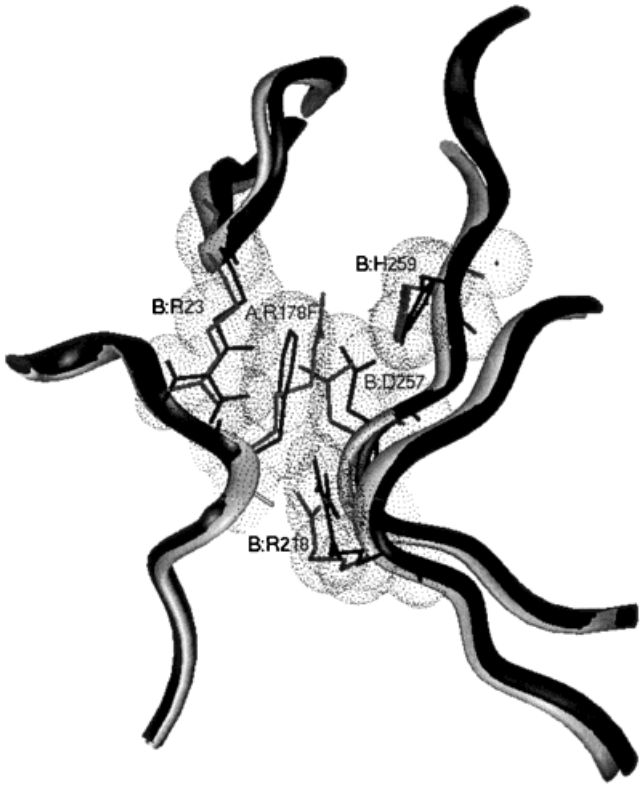


Fig. 8. Comparison of the crystal structure of R178F with wtTS at the site of mutation. All the residues which make contact with the Phe at position 178 are shown. There was clear electron density for the Phe confirming the rotamer shown in the model.

### The Influence of Arginine Interface Mutations on the Stability of the Quaternary and Tertiary Structure of TS

Two independent observations suggest that Arg replacements have resulted in the local destabilization of the quaternary structure in TS:

1. The interface mutants showed distinct differences in their behavior in the initial phase of denaturation when fluorescence changes of their respective dansylated derivatives were followed. It may be recalled that the dansyl fluorophore is attached to *Cys198* present at the dimer interface of TS. Hence, it is very likely that the fluorescence properties of this group will be modulated by changes in the quaternary interactions at least in the vicinity of the fluorophore. The two interface mutants were found to be more susceptible to structural perturbations at low concentrations of GdmCl (Fig. 4). In the case of wt TS, it was previously demonstrated that this region of the transition curve is independent of protein concentration.[31,32] Moreover the concentration of the denaturants at which complete unfolding of the two interface mutants take place is identical to that of the wt protein. It is therefore presumed that the structural perturbations brought about by the denaturants may be confined to a restricted region of the dimer interface of the two mutants. Very low concentrations of GdmCl have been found to destabilize subunit interactions or disrupt interdomain interactions.[51,52,53]

The labile nature of the intersubunit interactions is further confirmed by the finding that the unfolding intermediates in the case of the mutants appear at lower concentration of GdmCl than that observed for wt TS. These observations suggest that the interface mutations have a destabilizing effect on the quaternary structure of the mutant proteins.

2. While the environment of the tryptophan is the earliest to be perturbed in the presence of either urea or GdmCl, even in wt TS, the concentration of the denaturant required to bring about 50% loss in the CD ellipticity at 290 nm is substantially lower in the case of R218K. The stability of tertiary interactions in the case of R178F appears to fall between that of wt TS and R218K. Optimal subunit association may be generally crucial to the stability of the tertiary fold of multimeric proteins.[54,55] Arg218 belongs to that segment of the TS interface which is involved extensively in tertiary interactions within the subunit.[25]

Although the two mutants bring about significant changes in the quaternary and tertiary structure of the proteins as well as in their stability to heat denaturation (Fig. 3), they do not seem to drastically alter overall stability. Similar results have been reported in the case of lactate dehydrogenase, wherein mutants differing considerably in stability to high temperature exhibit almost identical free energy of stabilization upon chemical denaturation.[56] Moreover, the problem of protein aggregation in TS (an off-pathway reaction) may mask the true affinity between the subunits. So far no conditions have been found under which the unfolding intermediate of TS does not aggregate. It is interesting to note that the rate of heterodimer formation or intersubunit association of a few of the inactive mutants of *E. coli* TS when combined with R126E (*L. casei* R178) mutant were found to be significantly altered. The specific activity recovered ranged from 0.02 U/mg of protein in the case of R126E/C146S (*E. coli* C146 corresponds to *L. casei* C198) heterodimer to 3.86 U/mg of protein in the case of R126E/Y94A.[57]

### Aggregation Competent Segments in TS

TS has been shown to form high molecular weight aggregates under diverse conditions including high temperature,[33] intermediate concentrations of urea and GdmCl, [31,32] and in the presence of large excess of synthetic interface peptides.[58] While the nature of the aggregates formed under these diverse conditions is unknown, it is of interest to examine whether specific segments on the protein are responsible for non-native aggregate formation. An important point to be stressed is that aggregation may in fact involve specific interactions, as has been demonstrated in the literature for proteins like human growth hormone and serpins.[44,59,60] Earlier studies in this laboratory revealed that a covalently-bridged bis-disulfide mutant which contains two cross-links across the dimer interface (155–188' and 155'–188) exhibited a significantly high thermal melting temperature.[33] Denaturant-induced

aggregation was also shown to be abolished in this mutant leading to the suggestion that segments at the dimer interface may in fact contribute to protein aggregation.[31] The studies on the two interface mutants of TS, presented here establish that the aggregation process induced by denaturants or high temperature is indeed sensitive to interface mutation. Further experiments are necessary in order to identify specific residues involved in non-native aggregation. One possible strategy could be to use site directed mutagenesis, as exemplified in the case of R178F, which showed a decreased tendency to aggregate upon heating. It is noteworthy to point out in this regard that suppressor mutants counteract the tendency of the unfolding intermediates of the *tsf* mutants of tail spike protein of *Salmonella typhimurium* to aggregate, leading to the suggestion that there are "grammar sequences" in a polypeptide chain, which have a tendency to rescue unfolding intermediates from aggregation.[60]

The results presented here tend to suggest that amino acid replacements at the interface of a dimeric protein can indeed affect stability and association properties, albeit in a manner not easily rationalized in detailed structural terms.

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