*J. Mol. Biol.* (1994) **235**, 89–94

# Thermal Stabilization of Thymidylate Synthase by Engineering Two Disulfide Bridges Across the Dimer Interface

**Rajesh S. Gokhale[1], Sanjay Agarwalla[1], V. S. Francis[2], 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, CA 94143-0448, U.S.A.*

Thermal inactivation of oligomeric enzymes is most often irreversible and is frequently accompanied by precipitation. We have engineered two symmetry related disulfide bridges (155–188′ and 188–155′) across the subunit interface of *Lactobacillus casei* thymidylate synthase, at sites chosen on the basis of an algorithm for the introduction of stereochemically unstrained bridges into proteins. In this communication, we demonstrate a remarkable enhancement in the thermal stability of the covalently cross-linked double disulfide containing dimeric enzyme. The mutant enzyme remains soluble and retains secondary structure even at 90 °C, in contrast to the wild-type enzyme which precipitates at 52 °C. Furthermore, the mutant enzyme has a temperature optimum of 55 °C and possesses appreciable enzymatic activity at 65 °C. Cooling restores complete activity, in the mutant protein, demonstrating reversible thermal unfolding. The results suggest that inter-subunit crosslinks can impart appreciable thermal stability in multimeric enzymes.

*Keywords:* thymidylate synthase; thermal stabilization; engineered disulfides; precipitation; dimeric enzyme

The engineering of disulfide bonds has been explored as a means of imparting thermal stability to globular protein structures (Wetzel, 1987; Creighton, 1988) and also as a probe of protein folding pathways (Clarke & Fersht, 1993) and internal motions in proteins (Careaga & Falke, 1992). In the case of monomeric proteins or in situations where intrasubunit disulfides have been introduced in multimers, modest stabilization has been achieved, with more appreciable gains observed for multiple disulfide bonded mutants (Sowdhamini & Balaram, 1993; Matsumura *et al.*, 1989). Ironically, in some cases crosslinking has led to decreases in thermal stability (Agarwalla *et al.*, unpublished results; Mitchison & Wells, 1989; Villafranca *et al.*, 1987). Rationalization of the effects of engineered disulfide bonds on protein stability has been obscured by the difficulties of separating the influences of the crosslink on the native and unfolded states and assessing the relative importance of enthalpic and entropic contributions made by the disulfide to these states (Doig & Williams, 1991; Tidor & Karplus, 1993). The thermal denaturation of multimeric proteins generally involves the concomitant processes of polypeptide chain unfolding and subunit dissociation (Jaenicke, 1987). Thermal inactivation of oligomeric enzymes is most often irreversible and frequently accompanied by precipitation (Wetzel, 1992).

Thermal stabilization can, in principle, be achieved by reinforcing the subunit contact interface, rendering it more "sticky", by the introduction of additional interactions. Covalent bridging by means of multiple, engineered disulfide bonds should impede subunit dissociation. There have been few attempts at analyzing the effect of disulfide crosslinks across protein–protein interfaces (Shirakawa *et al.*, 1991; Scrutton *et al.*, 1988; Sauer *et al.*, 1986). We have engineered two disulfide bridges across the dimer interface of the enzyme, *Lactobacillus casei* thymidylate synthase (TS†)

† Author to whom all correspondence should be addressed.

† Abbreviations used: TS, thymidylate synthase; WT, wild-type; TSM, thymidylate synthase mutant.



0022-2836/94/010089-06 $08.00/0

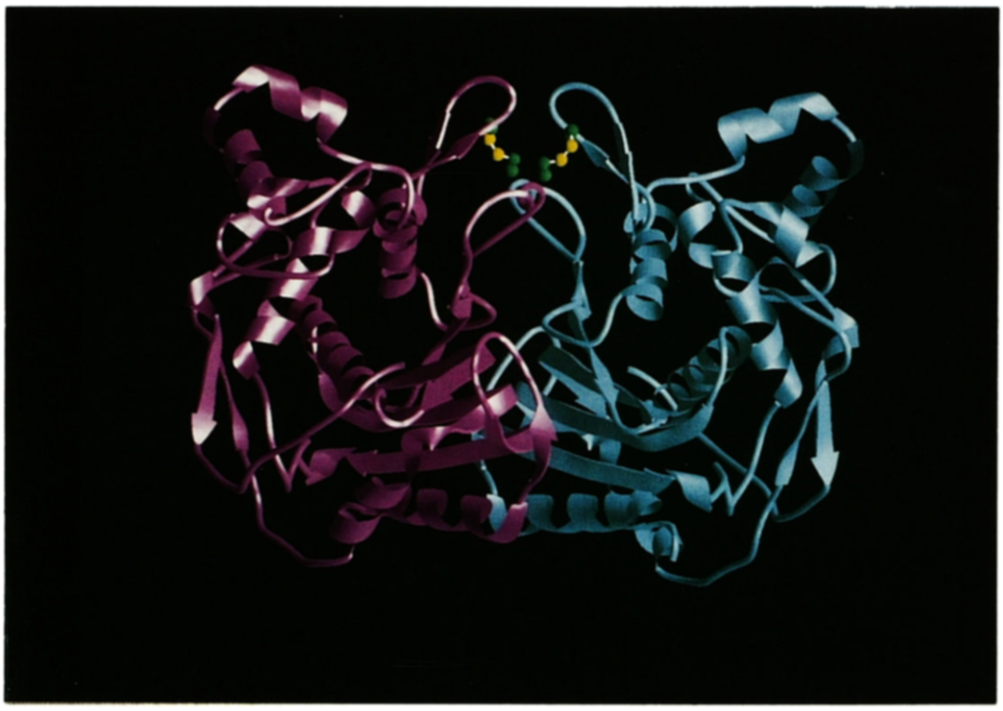

**Figure 1.** Ribbon drawing of the *L. casei* thymidylate synthase dimer viewed perpendicular to the 2-fold axis generated using the program Ribbons 2.0 (Carson, 1987) on a Silicon Graphics IRIS4D/310VGX. The 2 monomers are identified by the blue and cyan colors of the polypeptide backbone. The modeled disulfide bonds between residues 155–188′ and 188–155′ are indicated. $C^{\alpha}$ and $C^{\beta}$ atoms are shown in green and S atoms in yellow. Disulfide bridges were generated using the MODIP program (Sowdhamini *et al.*, 1989) and have the following stereochemical parameters:

$$\tau_{s-s} = 1{\cdot}87\ \text{Å},\ \chi_{ss} = 114°,\ \chi_i^1 = -94°,\quad \chi_i^2 = -150°,\ \chi_j^1 = -97°,\ \chi_j^2 = 98°.$$

In the ribbon representation residues 91 to 118 are deleted, as these are disordered in the structure.

(E.C.2.1.1.45), leading to a considerable enhancement in thermal stability. Such intersubunit crosslinks might be expected to impede dissociation and promote the maintenance of interfacial interactions at higher temperatures.

An examination of the 2·3 Å resolution crystal structure of *L. casei* TS (Hardy *et al.*, 1987) using the disulfide modeling program MODIP (Sowdhamini *et al.*, 1989) revealed that stereochemically unstrained disulfide bridges could be constructed across positions 155 (Thr)–188′(Glu) and the symmetry-related positions (across the 2-fold axis of the dimer) 188(Glu)–155′ (Thr). Alternative disulfide pairing patterns retaining the native dimer structure are ruled out by excessively large interresidue distances. Figure 1 shows a view of the dimeric enzyme with the modeled disulfide bridges.

A triple mutant containing Cys residues at positions 155 and 188 and a Thr residue at position 244 was constructed. The wild-type thymidylate synthase (TSWT) contains two Cys residues at positions 198 and 244. The former is an essential thiol which is the active site nucleophile. Cys244 is at a peripheral position and is non-essential for activity. The enzymatic activity of the C244T mutant is indistinguishable from TSWT, while mutations at Cys198 lead to a complete loss of activity (Climie *et al.*, 1990). The mutant protein T155C/E188C/C244T obtained by expression of the corresponding gene in *Escherichia coli*, was isolated in the reduced form ($TSM_{red}$), as judged by thiol estimation (Ellman, 1959) and mobility on SDS-PAGE. Oxidation to the double disulfide form ($TSM_{ox}$) could be effected by aerial oxidation at pH 8·8 or by a 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) catalyzed process at pH 8·0. Figure 2 compares the electrophoretic mobility of the oxidized and reduced mutants. The anomalous mobility of the crosslinked dimer, prompted an independent determination of the molecular weight. Electrospray mass spectrometry, gel filtration and analytical ultracentrifugation confirmed the dimeric nature of the protein. In the presence of reducing agents like dithiothreitol (DTT), the mobility on SDS-PAGE was identical for TSWT and $TSM_{red}$.

The temperature dependence of the specific activities of TSWT, and reduced ($TSM_{red}$) and oxidized ($TSM_{ox}$) forms of the mutant T155C/E188C/C244T are compared in Figure 3. A sharp fall in activity is observed for TSWT at temperatures above 40 °C, with the complete abolition of activity at 52 °C. In the reduced mutant ($TSM_{red}$), the loss of activity is more gradual, with residual activity being obtained at 55 °C. The oxidized mutant ($TSM_{ox}$) differs dramatically from its reduced counterpart and TSWT. Enhancement of activity is observed up to 60 °C and appreciable activity is retained at 65 °C. Indeed the temperature

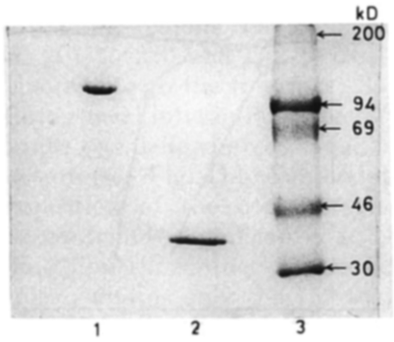


**Figure 2.** Mobility of the T155C/E188C/C244T mutant protein on 10% (w/v) SDS-PAGE using Laemmli's dissociating buffer system (Laemmli, 1970). Samples (10 $\mu$g) were loaded on the gel in sample buffer in the presence and absence of a reducing agent 2-mercaptoethanol (5%). Lane 1, mutant, non-reducing conditions. Lane 2, mutant, reducing conditions. Lane 3, molecular mass markers: 200 kDa, myosin; 94 kDa, phosphorylase *b*; 69 kDa, bovine serum albumin; 46 kDa, ovalbumin; 30 kDa, carbonic anhydrase. Protein bands were visualized by staining with Coomassie brilliant blue.

*Mutagenesis and protein purification:* Plasmid pSCTS9 (synthetic TS gene in pUC18) DNA was restricted with the appropriate restriction endonucleases and the linear DNA purified by gel electrophoresis on 1% (w/v) low melting agarose. The oligonucleotides used were annealed by mixing equimolar amounts of the respective synthetic oligonucleotides. E188C mutations were obtained by excising the segment limited by *Sal*I and *Nco*I, followed by replacement of the synthetic DNA duplex having a Cys codon (TGT) at position 188. Cys155 and Cys188 were constructed by replacing the fragment containing the *Bss*HII and *Cla*I sites with a synthetic DNA duplex that had a Cys codon (TGT) at positions 155 on a Cys188 mutant. C244T mutations in the double mutants were made by removal of the segment limited by *Nci*I and *Xho*I and inserting a synthetic duplex DNA that contained a Thr codon (ACT) at this position. The ligation product was transformed into *E. coli* DH5$\alpha$ cells. The mutations were confirmed by sequencing double-stranded DNA. DNA from such transformants was used to transform *E. coli* $\chi^{2913}$ (thy$^-$). All the protocols were as used by Climie & Santi (1990). Cells were grown in 1 litre of Luria broth containing 50 $\mu$/ml ampicillin at 37°C for 10 h and then harvested by centrifugation. Cells were resuspended in 20 ml of 100 mM Tris·HCl, EDTA (pH 7·4) and lysed using a French press. The supernatant after centrifugation was loaded onto a hydroxyapatite column, which was eluted with a linear gradient from 25 mM potassium phosphate (pH 6·9) to 350 mM potassium phosphate (pH 6·9). TS-containing fractions were identified by SDS-PAGE, pooled and precipitated by 43% (w/v) ammonium sulfate. This was further purified by gel filtration on a G-100 column. TS was dialyzed against distilled water and lyophilized. The double disulfide dimer is obtained by incubating the protein at 25°C in 1·5 M Tris·HCl (pH 8·8) for 3 days. Disulfide crosslinks can be formed in 25 mM Tris·HCl (pH 8·0) in 6 h in the presence of DTNB. Slow oxidation gives a single band on SDS-PAGE. Electrospray mass spectrometry of $TSM_{ox}$ was carried out at the UCSF mass spectrometry facility yielding a molecular mass of 72 kDa (The calculated molecular mass of monomeric *L. casei* TS based on the sequence is 36,618 Da). Analytical gel filtration was performed on a LKB 2135-360 pre-packed column (7·5 mm × 600 mm) using 25 mM potassium phosphate buffer (pH 6·9) and a flow rate of 0·1 ml/min, using an LKB 2150 HPLC pump. The WT non-covalent dimer and $TSM_{ox}$ eluted at identical positions. Analytical ultracentrifugation performed in 25 mM potassium phosphate buffer (pH 6·9) yielded a molecular mass of 71 kDa for $TSM_{ox}$.

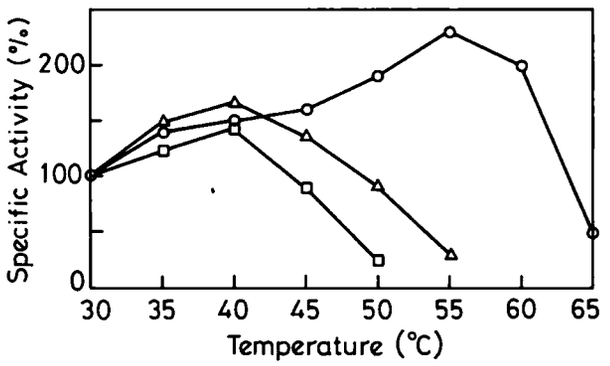


**Figure 3.** Temperature dependence of enzymatic activity of wild-type and mutant thymidylate synthase. Specific activities ($\mu$mol dTMP formed min$^{-1}$ mg protein$^{-1}$) were determined in the presence of 600 $\mu$m deoxyuridine monophosphate (dUMP) and 150 $\mu$M 5,10-methylenetetrahydrofolate ($CH_2$-$H_4$ folate) after enzymes were incubated in the assay buffer (without 2-mercaptoethanol) for 15 min. 2-mercaptoethanol used in the preparation of $CH_2$-$H_4$ folate was removed by lyophilization. This $CH_2$-$H_4$ folate was used immediately for the assay. (□) TSWT; (△) $TSM_{red}$ (○) $TSM_{ox}$. The activity at 30°C was taken as 100%. TS activity was assayed spectrophotometrically by continuously monitoring the increase in absorbance at 340 nm due to the conversion of methylene tetrahydrofolate to dihydrofolate ($\varepsilon_{340}$ = 6400 M$^{-1}$ cm$^{-1}$) (Santi & Sakai, 1971). The specific activity of $TSM_{ox}$ is approximately 15% of the activity of TSWT (see the text).

optimum for $TSM_{ox}$ is about 55°C, whereas a value of 40°C is observed for the other two. It may be noted that the specific activity of $TSM_{ox}$ is approximately 15% of the specific activity of TSWT at 30°C. The specific activity of TSWT and $TSM_{red}$ are almost identical at 30°C. The diminished activity of the covalently bridged mutant may be a consequence of the restrictions placed on the movements of the polypeptide chains during catalysis. Large conformational changes have been suggested to accompany substrate and cofactor binding in the case of thymidylate synthase. These changes have been observed in the crystal structure of a ternary complex of *E. coli* TS (Montfort *et al.*, 1990). Attempts to oxidize the $TSM_{red}$ mutant in the presence of the substrate deoxyuridine monophosphate (dUMP) failed (data not shown), suggesting that substrate binding results in the movement of thiol groups to positions which are stereochemically unsuitable for disulfide formation.

Figure 4 shows the far-UV CD spectra of TSWT and $TSM_{ox}$. A dramatic loss in ellipticity is observed between 50 and 55°C for TSWT, whereas the intensity of the CD bands of $TSM_{ox}$ remain largely unaltered up to 90°C. A notable change at 90°C is the enhancement of ellipticities at 218 nm and 209 nm

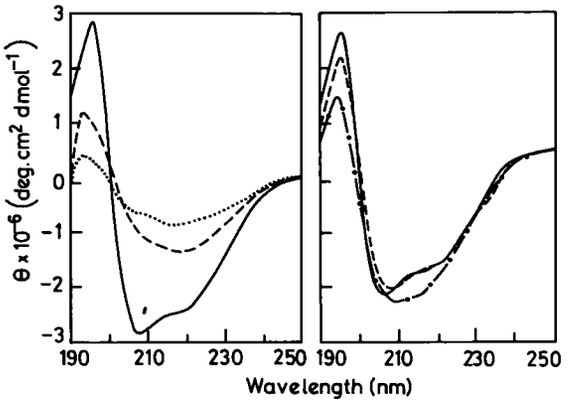


**Figure 4.** CD spectra of TSWT (left) and $TSM_{ox}$ (right). Measurements were made in 25 mM potassium phosphate buffer (pH 6·9), 1 mM EDTA using a JASCO J500A and thermostatted cells over the temperature range 30 to 90 °C. Spectra shown here are recorded at 30 °C (————), 50 °C (- - - -), 55 °C (. . . . .), 90 °C (-●-●-).

resulting in a flattening out of the spectrum. This may correspond to a retention of secondary structure at the crosslinked β-sheet interface, while melting out of helices may occur. The reduced protein $TSM_{red}$ shows a loss of CD ellipticity at 60 to 65 °C. The change in percentage molar ellipticities at 208 nm as a function of temperature is plotted in Figure 5 (top panel) for all the three proteins. In the case of TSWT and $TSM_{red}$, visible precipitation occurs at the melting temperatures, whereas clear solutions of $TSM_{ox}$ were obtained even at 90 °C. Figure 5 (bottom panel) shows a plot of light scattering intensity *versus* temperature for the three proteins, clearly demonstrating that $TSM_{ox}$ remains soluble even at high temperatures in contrast to its non-crosslinked analogs.

The formation of two disulfide crosslinks across the subunit interface in TS has resulted in dramatic stabilization of the overall structure of the protein, with CD studies providing evidence for the retention of secondary structure at temperatures as high as 90 °C. In two previous studies of engineered intersubunit disulfides involving the DNA-binding proteins λ-repressor (Sauer *et al.*, 1986) and λ-cro (Shirakawa *et al.*, 1991) proteins, stabilization of structure has been demonstrated by CD/NMR studies. In both cases a loss of secondary and tertiary structure is observed by 65 °C. Functional activity at higher temperatures has not been reported, presumably because of the problem of double-stranded DNA melting. In the case of the dimeric enzyme glutathione reductase, engineering of an intersubunit crosslink (75–75′) yielded a fully active enzyme (Scrutton *et al.*, 1988). However, no changes were observed in the melting temperature. The absence of any thermal stabilization may be a consequence of the fact that appreciable conformational flexibility is possible at the dimer interface. The lone covalent crosslink acts as a flexible tether and is presumably unable to restrict interdomain movements.

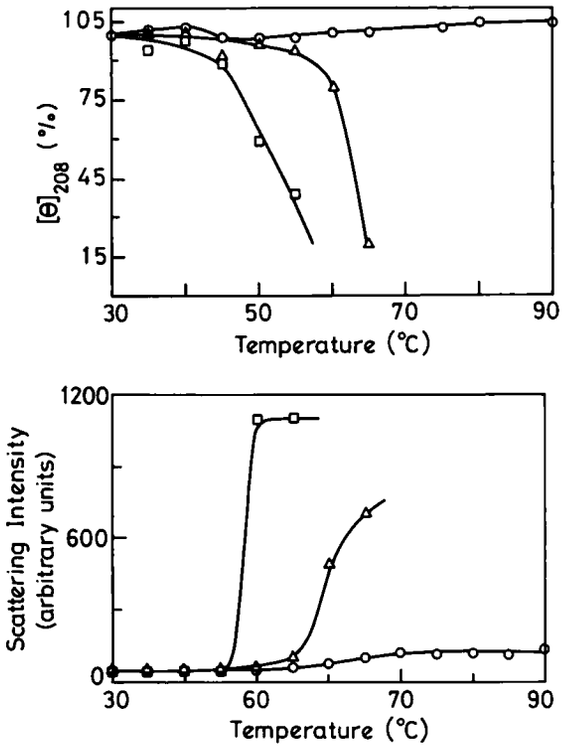


**Figure 5.** (Top). Dependence of CD ellipticity at 208 nm as a function of temperature. Protein concentrations were 3 μM in 25 mM potassium phosphate buffer (pH 6·9), 1 mM EDTA. (□) TSWT; (△) $TSM_{red}$; (○) $TSM_{ox}$. The ellipticity at 30 °C is taken as 100%. (Bottom). Dependence of the Rayleigh scattering intensity on temperature. Samples were equilibrated at the desired temperatures for 15 min in 25 mM potassium phosphate buffer (pH 6·9), 1 mM EDTA. Measurements were made on a Hitachi 650–60 fluorimeter using a 90° geometry. Samples were excited at 400 nm and scattering intensity recorded at 400 nm, using a band-pass of 5 nm for both monochromators. (□) TSWT; (△) $TSM_{red}$; (○) $TSM_{ox}$.

The covalent intersubunit links engineered in the mutant T155C/E188C/C244T completely abolished the heat-induced aggregation process that is observed in TSWT. While appreciable enzymatic activity is observed in the mutant enzyme even at 65 °C, the loss of activity at higher temperatures may be a consequence of local structural disorganization of the active site, although diminished substrate binding cannot be ruled out. Furthermore, in $TSM_{ox}$, total activity is regained upon cooling from 70 °C to 30 °C, suggesting that the thermal unfolding transition is reversible, in marked contrast to TSWT and $TSM_{red}$, where thermal denaturation is irreversible.

The crystal structure of *L. casei* TS shows that monomers form dimer contacts primarily between two five-stranded beta sheets that are related by a unique right-handed twist, with a dihedral angle of 28° between strand directions (Hardy *et al.*, 1987). A similar arrangement has also been observed in *E. coli* TS (Matthews *et al.*, 1989). Analysis of the interresidue contacts (using a contact limit of 3·5 Å) at the dimer interface in *L. casei* TS reveals four

distinct, interacting regions. The segments 18 to 37, 153 to 188, 201 to 220 and 252 to 261 from each monomer contribute to the interface. Based on the number of interfacial interactions, the structure can be divided into two defined areas. The "lower" part of the structure (see Fig. 1) accommodates a much greater proportion of the contacts and may be expected to contribute significantly to the binding energy. The "upper" contact region between the two molecules contains only the 153 to 188 region. It is in this "weaker" region of the protein that we have engineered two intersubunit disulfide crosslinks. Presumably, covalent bridging of an intrinsically fragile region of the interface confers appreciable thermal stabilization, as evidenced by studies on the triple mutant T155C/E188C/C244T. The results suggest that covalent crosslinking across protein-protein contact surfaces may provide an attractive means of stabilizing multimeric structures in general. In the case of a dimeric protein like TS, unfolding and dissociation presumably precede thermally induced precipitation, which is a consequence of aggregation of non-native structures. Structural reinforcement of the dimer interface may thus provide a useful strategy for avoiding irreversible precipitation, a desirable goal of many protein engineering studies. Interestingly, symmetrical disulfide bridging of a dimeric structure has been observed in the case of human interleukin-5 (Milburn *et al.*, 1993).

We are grateful to Dr V. Prakash for the analytical ultracentrifugation and the UCSF mas spectrometry facility for the mass spectral analysis. Figure 1 was generated using facilities of the Bioinformatics center supported by the Department of Biotechnology. We thank Debasis Mohanty for helping to generate Figure 1. This research was supported by grants from the United States Public Health Service, the National Cancer Institute (D.V.S.) and the Council of Scientific and Industrial research, India (P.B.). R.S.G. and S.A. were supported by senior research fellowships from CSIR, India.

## References

Careaga, L. C. & Falke, J. J. (1992). Thermal motions of surface α-helices in the D-galactose chemosensory receptor. Detection by disulfide trapping. *J. Mol. Biol.* **226**, 1219–1223.

Carson, M. (1987). Ribbon models of macromolecules. *J. Mol. Graphics*, **5**, 103–106.

Clarke, J. & Fersht, A. R. (1993). Engineered disulfide bonds as a probe for folding pathways of barnase: increasing the stability of proteins against rate of denaturation. *Biochemistry*, **32**, 4322–4329.

Climie, S. & Santi, D. V. (1990). Chemical synthesis of the thymidylate synthase gene. *Proc. Nat. Acad. Sci., U.S.A.* **87**, 633–637.

Climie, S., Ruiz-Perez, L., Gonzalez-Pacanowska, D., Prapunwattana, P., Cho, S.-W., Stroud, R. & Santi, D. V. (1990). Saturation site-directed mutagenesis of thymidylate synthase. *J. Biol. Chem.* **265**, 18776–18779.

Creighton, T. E. (1988). Disulfide bonds and protein stability. *BioEssays*, **8**, 57–63.

Doig, A. J. & Williams, D. H. (1991). Is the hydrophobic effect stabilizing or destabilizing in proteins? The contribution of disulfide bonds to protein stability. *J. Mol. Biol.* **217**, 389–398.

Ellman, G. L. (1959). Tissue sulphydryl groups. *Arch. Biochem. Biophys.* **82**, 70–77.

Hardy, L. W., Finer-Moore, J. S., Montfort, W. R., Jones, M. O., Santi, D. V. & Stroud, R. M. (1987). Atomic structure of thymidylate synthase: target for rational drug design. *Science*, **235**, 448–455.

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

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)*, **277**, 680–685.

Matsumura, M., Signor, G. & Matthews, B. W. (1989). Substantial increase of protein stability by multiple disulfide bonds. *Nature (London)*, **342**, 291–293.

Matthews, D. A., Appelt, K. & Oatley, S. J. (1989). Stacked beta-bulges in thymidylate synthase account for a novel right-handed rotation between opposing beta-sheets. *J. Mol. Biol.* **205**, 449–454.

Milburn, M. V., Hassel, A. M., Lambert, M. H., Jordan, S. R., Proudfoot, A. E. I., Graber, P. & Wells, T. N. C. (1993). A novel dimer configuration revealed by the crystal structure at 2·4 Å resolution of human interleukin-5. *Nature (London)*, **263**, 172–176.

Mitchinson, C. & Wells, J. A. (1989). Protein engineering of disulfide bonds in subtilisin BPN'. *Biochemistry*, **28**, 4807–4815.

Montfort, W. R., Perry, K. M., Fauman, E. B., Finer-Moore, J. S., Hardy, L., Maley, F. & Stroud, R. M. (1990). Structural, multiple site binding, and segmental accommodation in thymidylate synthase on binding dUMP and an antifolate. *Biochemistry*, **29**, 6964–6977.

Santi, D. V. & Sakai, T. T. (1971). Inhibition of thymidylate synthase by 5-formyl-and-5-hydroxymethyl-2′-deoxyuridylate. *Biochem. Biophys. Res. Commun.* **42**, 813–817.

Sauer, R. T., Hehir, K., Stearman, R. S., Weiss, M. A., Jeitler-Nilsson, A., Suchanek, E. G. & Pabo, C. O. (1986). An engineered intersubunit disulfide enhances the stability and DNA binding of the N-terminal domain of λ-repressor. *Biochemistry*, **25**, 5992–5998.

Scrutton, N. S., Berry, A. & Perham, R. N. (1988). Engineering of an intersubunit disulfide bridge in glutathione reductase from *Escherichia coli*. *FEBS Letters*, **241**, 46–50.

Shirakawa, M. S., Matsuo, H. & Kyogoku, Y. (1991). Inter-subunit disulfide-bonded λ-cro protein. *Protein Eng.* **4**, 545–552.

Sowdhamini, R. & Balaram, P. (1993). *Protein Structure and Stability in Thermostability of Enzymes* (Gupta, M. N., ed.), pp. 2–23, Narosa Publishing House, Delhi.

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

Tidor, B. & Karplus, M. (1993). The contribution of cross-links to protein stability: a normal mode analysis of the configurational entropy of the native state. *Proteins: Struct. Funct. Genet.* **15**, 71–79.

Villafranca, J. E., Howell, E. E., Oatley, S. J., Xuong, N. & Kraut, J. (1987). An Engineered disulfide bond in dihydrofolate reductase. *Biochemistry*, **26**, 2182–2189.

Wetzel, R. (1987). Harnessing disulfide bonds using protein Engineering. *Trends Biochem. Sci.* **12**, 478–482.

Wetzel, R. (1992). Principles of protein stability. 2 Enhanced folding and stabilization of proteins by suppression of aggregation *in vitro* and *in vivo*. In *Protein Engineering, A Practical Approach* (Rees, A. R., Sternberg, M. J. E. & Wetzel, R., eds.), pp. 191–219, IRL Press, Oxford.

*Edited by A. R. Fersht*

*(Received 6 July 1993; accepted 15 September 1993)*