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FOR THE RECORD

Disulfide engineering at the dimer interface of *Lactobacillus casei* thymidylate synthase: Crystal structure of the T155C/E188C/C244T mutant

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Abstract: The crystal structure of a covalently cross-linked *Lactobacillus casei* thymidylate synthase has been determined at 2.8 Å resolution. The sites for mutation to achieve the bis-disulfide linked dimer were identified using the disulfide modeling program MODIP. The mutant so obtained was found to be remarkably thermostable. This increase in stability has been reasoned to be entirely a consequence of the covalent gluing between the two subunits.

Keywords: dimer interface; disulfide engineering; stability; thymidylate synthase

Disulfide cross-links are an important stabilizing element in the structures of many globular proteins. Disulfide engineering has been advanced as an approach to stabilizing structures of proteins that lack such covalent cross-links in the native state (Sauer et al., 1986; Wells & Powers, 1986; Wetzel, 1987; Scrutton et al., 1988; Shirakawa et al., 1991; Wakarchung et al., 1994), and the energetic contributions have been evaluated (Tamma & Privalov, 1998). In the case of multimeric proteins, disulfide cross-linking of subunits affords mutant structures that are prevented from dissociation. Such a device permits a study of the chain unfolding process without the attendant complications of concomitant chain dissociation. We have been exploring the potential use of intersubunit disulfide cross-links in the model proteins *Lactobaccilus casei* thymidylate synthase (ts) (Gokhale et al., 1994) and *Plasmodium falciparum* triosephosphate isomerase (Gopal et al., 1999). In the case of ts, a mutant (T155C/E188C/C244T), bearing two intersubunit cross-links (155–188′, 188–155′, Fig. 1A,B) has been shown to be remarkably stabilized against thermal denaturation and chaotrope induced aggregation (Gokhale et al., 1994; Agarwalla et al., 1996). In this paper we describe the crystallographic characterization of the two engineered disulfide bridges and the dimer interface in the mutant enzyme. Gokhale et al. (1994) reported that the oxidized triple mutant remained soluble and retained secondary structure even at 90 °C, in contrast to the wild-type enzyme that precipitates at 52 °C. Furthermore the bis-disulfide cross-links abolished the aggregation observed for the wild-type enzyme at moderate concentrations of the chaotropes, urea, and guanidinium chloride. The

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Table 1. *Summary of crystallographic data and refinement statistics*

Total number of reflections measured	44,495
Average number of times each intensity was estimated	3.69
Total number of unique reflections	11,008
Number of unique reflections in the resolution range 10–2.8 Å with $I/\sigma(I) > 2.0$	10,115
Completeness in the resolution range 10–2.8 Å with $I/\sigma(I) > 2.0$	85.4%
Completeness in the last resolution shell 3.0–2.8 Å	73.0%
R-merge[a]	9.07%
Initial R^b-factor for the WT ts model in the cell of T155C/E188C/C244T	31.3%
Initial free R^b-factor for the WT ts model in the cell of T155C/E188C/C244T	30.2%
Final R^b-factor for reflections with $I/\sigma(I) > 2.0$ in the range 10.0–2.8 Å	20.3%
Final R^b-factor for all reflections in the range 10.0–2.8 Å	23.5%
Final free R^b-factor for reflections with $I/\sigma(I) > 2.0$ in the range 10.0–2.8 Å	25.6%
Final free R^b-factor for all reflections in the range 10.0–2.8 Å	25.2%
Number of nonhydrogen atoms in the model	2,587
RMS deviation in bond angles (°)	1.913
RMS deviation in bond lengths (Å)	0.015

[a]Defined as $\Sigma|\langle I\rangle - kI_h|/\Sigma\langle I\rangle \times 100$.
[b]Defined as $\Sigma|F_o - kF_c|/\Sigma F_o \times 100$.

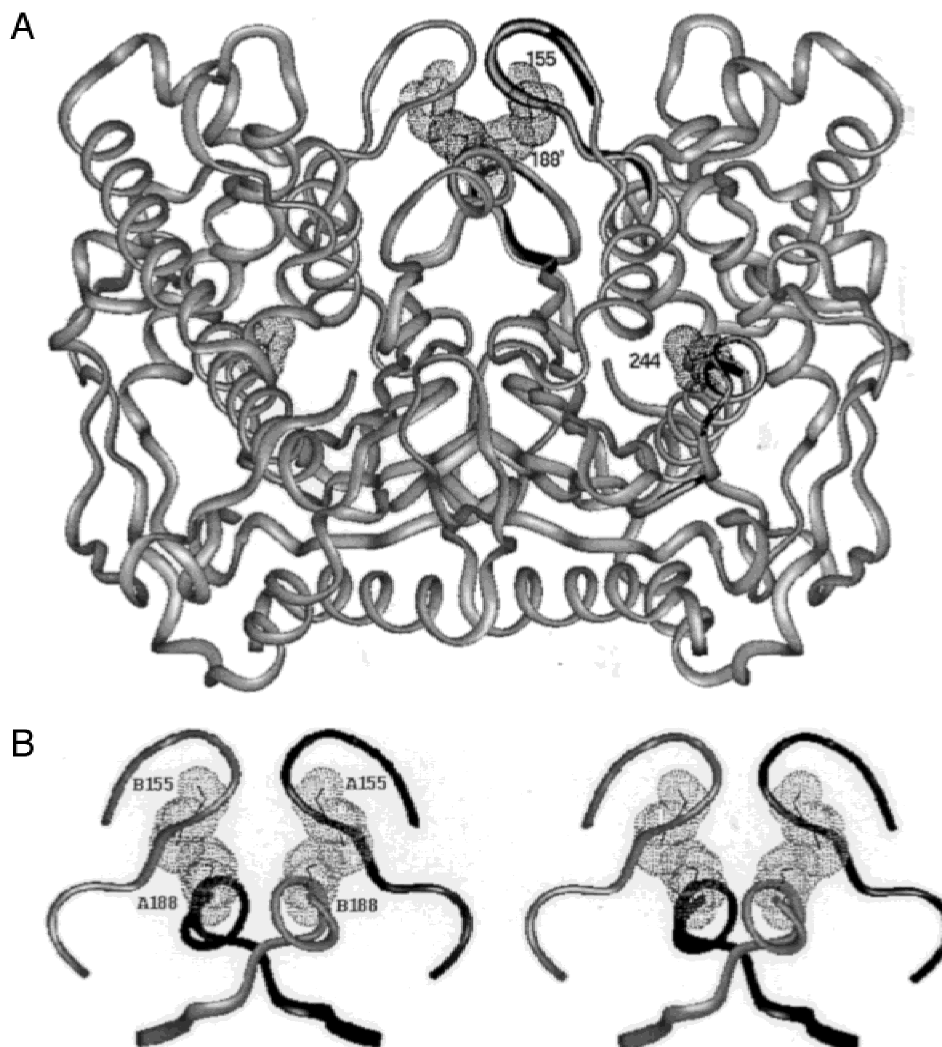

Fig. 1. A: Ribbon drawing of the molecule of *L. casei* ts prepared using InsightII (Biosym Technologies Inc., San Diego, California). The sites of mutation, residues Cys155 and Cys188, are indicated. The two monomers are drawn in different shades. **B:** Stereo view of the dimer interface at the site of mutation in the two subunits.

reduced triple mutant retained full enzymatic activity, while the oxidized form showed only 15% of the wild-type activity (Gokhale et al., 1996).

L. casei ts is a dimer of identical subunits having 316 residues each. The two subunits are related by a twofold axis of symmetry. The dimer interface in ts is extensive and consists of interactions made by three distinct segments of the polypeptide chain corresponding to residues 17–38, 174–190, and 201–220 (Prasanna et al., 1998). The lower part of the interface seen in Figure 1 consists of a sandwich formed by the five-stranded β-sheet structures, which also accommodates a cavernous active-site. The upper part of the interface harbors fewer intermolecular contacts with residues 151–160 being primarily involved. Inspection of Figure 1 suggests that these segments may in fact correspond to a more fragile region. Application of the disulfide modeling program MODIP (Sowdhamini et al., 1989) to the 2.8 Å structure of *L. casei* ts (Hardy et al., 1987) yielded the sites of mutation (residues 155 and 188) where strain free, symmetry related disulfide bridges could be introduced. Covalent cross-links between residues 155–188′ and 188–155′ should impede dissociation of interactions in this region. The novel properties of the bis-disulfide bridged mutant provided the impetus for crystallographic characterization of the interface. These sites were away from the active sites. The chance of alternative pairing of disulfides was ruled out by excessively large distances. Disulfide bridges were generated using MODIP with the following stereochemical parameters $r_{s\text{-}s}$ = 1.87 Å, $\chi_{s\text{-}s} = 114°$, $\chi_i^1 = -94°$, $\chi_i^2 = -150°$, $\chi_j^1 = -97°$, $\chi_j^2 = 98°$. Cysteine-244 was mutated to a threonine to circumvent complications due to thiol disulfide exchange in the protein. The C244T mutant has properties identical to the wild-type enzyme. The procedure for the mutagenesis, expression, and purification of the enzyme has been described earlier (Gokhale et al., 1994). Crystals of the ts mutant were obtained by the hanging drop method in 50 mM phosphate buffer pH 5.8–6.2, 50 mM ammonium sulfate. Crystals appeared after 3 days and grew to sizes of 0.7 × 0.3 × 0.3 mm. The mutant crystallized in the space group $P6_122$ with the cell parameters $a = b = 78.3$ Å, $c = 245.3$ Å. The asymmetric unit of the crystal is compatible with one monomer (Matthews coeffi-

cient 2.76 Å/Da assuming a molecular weight of 34 kDa per monomer). Diffraction data were collected on a Siemens Nicolet area detector system. Data were processed using the XENGEN program (Howard et al., 1987). Data collection and refinement statistics are given in Table 1. The structure was solved using the Molecular Replacement program AMoRe (Navaza, 1994) using the polyalanine model of the PDB 4TMS coordinates (Hardy et al., 1987) and the structure was refined using X-PLOR (Brünger, 1992). As the disulfide was introduced across the dimer interface, the dimer was generated and the refinement was carried out with the dimer coordinates in the space group $P6_1$. This was essential as X-PLOR did not accept a disulfide specification between symmetry related subunits. The initial map calculated was examined for the electron density corresponding to the two disulfide cross-links using O (Jones et al., 1991; Fig. 2A,B). The stereochemical parameters for the two disulfides observed in the crystal structure are (155–188′) $r_{s\text{-}s} = 2.03$ Å, $\chi_{s\text{-}s} = -98.48°$, $\chi_i^1 = -62.88°$, $\chi_i^2 = -62.83°$, $\chi_j^1 = -53.14°$, $\chi_j^2 = -94.69°$; (188–155′) $r_{s\text{-}s} = 2.13$ Å, $\chi_{s\text{-}s} = -103.16°$, $\chi_i^1 = -68.58°$, $\chi_i^2 = -61.06°$, $\chi_j^1 = -54.35°$, $\chi_j^2 = -91.01°$. It may be noted that the dimer has been used as the asymmetric unit in the space group $P6_1$ in the refinement protocol contributing to the lack of symmetry between the two disulfide bridges, whereas wild-type *L. casei* ts is refined in the space group $P6_122$ with the monomer in the asymmetric unit and exact crystallographic symmetry relating the two subunits. While exact two-fold symmetry should lead to identical stereochemical parameters for the two disulfide bridges, the small differences observed are a consequence of the refinement protocol which is based on a dimer in the asymmetric unit. An independent refinement with strict non-crystallographic symmetry imposed between the monomers did not significantly alter the refinement statistics.

The remarkable stability of this mutant enzyme has been interpreted to be a consequence of covalent reinforcement of a potentially fragile region of the interface, which may be involved in non-native aggregate formation by partially unfolded structures (Gokhale et al., 1994). Covalent cross-linking of the labile portion of the interface has presumably made the dimer packing more rigid.

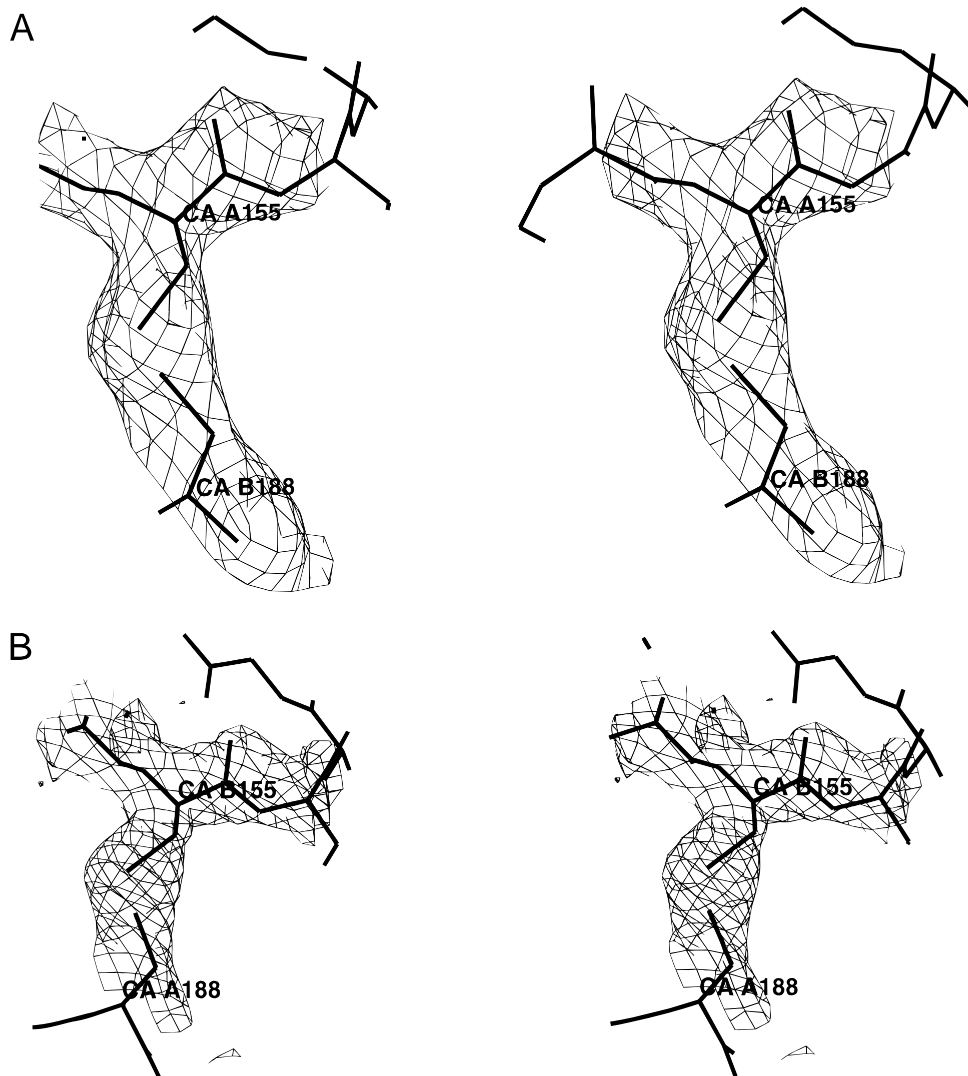

Fig. 2. A: $2F_o - F_c$ electron density maps at the oxidized site 155–188′. The disulfide density and approximate orthogonal geometry about the S-S bond are depicted. **B:** The symmetry related site 188–155′.

The RMS deviation between corresponding Cα atoms of the mutant and wild-type 4TMS structures, including segments of high temperature factors, is 0.49 Å. Inspection of the crystal structure does not reveal any significant change in the dimer interface interactions as a consequence of disulfide cross-linking. The observed structural results suggest that enhanced stability is almost entirely a consequence of covalently gluing the two subunits. The present study thus emphasizes the utility of the disulfide modeling program MODIP for identifying sites for introduction of disulfide bridges by mutagenesis.

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