Mutagenesis and crystallographic studies of *Zymomonas mobilis* tRNA-guanine transglycosylase to elucidate the role of serine 103 for enzymatic activity

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Received 29 April 1999

Abstract The tRNA modifying enzyme tRNA-guanine transglycosylase (TGT) is involved in the exchange of guanine in the first position of the anticodon with preQ$_1$ as part of the biosynthesis of the hypermodified base queuine (Q). Mutation of Ser^{90} to an alanine in *Escherichia coli* TGT leads to a dramatic reduction of enzymatic activity (Reuter, K. et al. (1994) Biochemistry 33, 7041–7046). To further clarify the role of this residue in the catalytic center, we have mutated the corresponding Ser^{103} of the crystallizable *Zymomonas mobilis* TGT into alanine. The crystal structure of a TGT(S103A)/preQ$_1$ complex combined with biochemical data presented in this paper suggest that Ser^{103} is essential for substrate orientation in the TGT reaction.

Key words: Queuine; Modified nucleoside; tRNA; Crystal structure; Catalytic mechanism

1. Introduction

Eubacterial tRNA-guanine transglycosylase (TGT, EC 2.4.2.29) [1] catalyzes the first step of the posttranscriptional modification in the anticodon loop of cognate tRNAs (Asn, Asp, His, Tyr), resulting in the exchange of guanine-34 at the wobble position by the queuine precursor 7-aminomethyl-7-deazaguanine (preQ$_1$). This precursor is converted to the hypermodified base queuine (Q-base: 7-(4,5-cis-dihydroxy-1-cyclo-penten-3-ylamino-methyl)-7-deazaguanine) in subsequent enzymatic steps [2]. Queuine is found at the wobble position of tRNAs in most organisms with the exception of yeast and archaebacteria [3–5]. In eukaryotes, the Q-base is a nutrient and the replacement of guanine-34 with queuine is carried out directly in one single enzymatic step [6]. The exact biological function of queuine in tRNA is not yet fully understood, but it seems to play a role in the fine tuning of protein biosynthesis in eubacteria [7] and in different cellular events such as development, differentiation, aging and cancer in eukaryotes [8,9]. In Shigellae, which are the causative agents of dysentery and effect some 500 000 infant deaths per year, the enzymatic activity of TGT is a prerequisite for pathogenicity [10]. This prompted us to launch a detailed biochemical and structural study of this enzyme, since it might be a useful target for the design of a drug against Shigellosis.

The structure of *Zymomonas mobilis* TGT has been solved at 1.85 Å resolution and revealed a non-canonical $(\beta/\alpha)_8$-barrel fold with a zinc binding subdomain. Soaking of TGT crystals with preQ$_1$ and inspection of the structure at 2.2 Å resolution indicated a specific preQ$_1$ binding pocket at the C-terminal face of the barrel [11]. Mutagenesis and crystallographic studies revealed Asp^{102} to be the active site nucleophile of *Z. mobilis* TGT in the base exchange reaction. The proposed mechanism involves two consecutive S_N2 reactions and the formation of a covalent TGT/tRNA intermediate [12]. An *Escherichia coli* mutant has been described that contains tRNAs lacking queuine due to a single point mutation in the *tgt* gene changing the Ser^{90} codon (*E. coli* numbering) to a Phe codon [13]. Mutation of Ser^{90} to cysteine leads to a reduced activity of *E. coli* TGT, mainly due to an increased K_M for the substrate tRNA. The mutation of Ser^{90} to alanine leads to a dramatic decrease of catalytic efficiency, such that V_{max}/K_M is reduced by four orders of magnitude compared to the wild-type [14]. The *E. coli* and *Z. mobilis* TGT show 53% sequence identity and have similar kinetic parameters. Inspection of the crystal structure of the *Z. mobilis* TGT in complex with preQ$_1$ suggested that Ser^{103} (which corresponds to Ser^{90} in *E. coli*) is involved in substrate orientation. In the preQ$_1$ complex, this residue forms a weak hydrogen bond (3.5 Å) to the 2-NH$_2$ group of preQ$_1$. Assuming that G34 of the tRNA occupies the preQ$_1$ site during the first step of catalysis, Ser^{103} cannot act as the catalytic nucleophile due to the long distance of 7 Å to nitrogen N9 in preQ$_1$ [11] and by implication to the 1′ carbon of the G34 ribose. Although it does not appear to be directly involved in catalysis, the importance of this residue is emphasized by the fact that it seems to be invariant among all suggested and characterized TGT enzymes. This fact is true even for TGT enzymes from archaebacteria and eukaryotes, which exhibit deviating substrate specificities from eubacterial TGTs [15].

To further elucidate the function of this residue in the catalytic center of TGT, we have mutated Ser^{103} of *Z. mobilis* TGT to alanine in order to determine the crystal structure of the mutated protein. Here we report the biochemical and structural characterization of the *Z. mobilis* TGT-(S103A).

2. Materials and methods

Reagents and buffers were purchased from Sigma, Aldrich and Gibco-BRL unless stated otherwise. [8-^{3}H]Guanine (50 mCi/mmol) was from Sigma. Oligonucleotides were synthesized at MWG-Biotech.

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0014-5793/99/$20.00
PII: S0014-5793(99)00793-0

2.1. TGT and E. coli tRNA^{Tyr} preparation

Z mobilis TGT, both wild-type and S103A mutant, were prepared as described previously [16,20] and stored at 4°C as microcrystalline suspension. Prior to use, a small sample of the microcrystals was redissolved in a high-salt buffer composed of 10 mM HEPES (pH 7.5), 10 mM $MgCl_2$ and an appropriate concentration of NaCl. The NaCl concentration was adjusted to the lowest possible value to obtain a total dissolution of the microcrystals (e.g. 400 mM NaCl lead to a TGT concentration of 1 mg/ml, 2 M NaCl lead to a maximum TGT concentration of 12 mg/ml). *E. coli* tRNA^{Tyr}-(G34) was prepared as described previously [14] and stored at a concentration of 10 mg/ml in a buffer composed of 5 mM cacodylate (pH 6.5), 2 mM $MgCl_2$ and 0.2 mM EDTA.

2.2. Construction of the TGT mutant S103A

The construction of the mutant was performed via a polymerase chain reaction (PCR) based on a protocol already described [17,18]. The PCR-primers were partially overlapping with both of them containing the mutated codon (Table 1). Successful mutagenesis was verified by sequence analysis of the complete mutated *tgt* gene using an ABI-PRISM 310 automated sequencer, allowing the identification of clones with no additional undesired mutations.

2.3. Activity assays

The determinations of wild-type TGT and TGT(S103A) activities were performed as described previously [16].

2.4. Band-shift assay

Wild-type or mutated TGT (3 μM) was first incubated with a 20-fold excess of *E. coli* tRNA^{Tyr} at 37°C for 1 h in a reaction mixture containing 10 mM HEPES (pH 7.4) and 20 mM $MgCl_2$. The reaction mixtures were then analyzed by native polyacrylamide gel electrophoresis (PAGE) as described previously [12]. Gels were stained with Coomassie brilliant blue R-250 (Merck) followed by silver staining (Sigma).

2.5. Crystallization and preQ$_1$ soaking

PreQ$_1$ was synthesized as described previously [19]. Wild-type TGT and TGT(S103A) were crystallized as described previously [20]. Crystals were soaked for 3 days at 22°C in a buffer composed of 5% (w/v) PEG 8000, 10% (v/v) DMSO, 100 mM Tris (pH 8.5), 1 mM DTT and 50 mM preQ$_1$.

2.6. Structure determination

X-ray data were collected on an RAXIS-IV image plate system mounted on a Rigaku RU300 rotating anode generator operating at 50 kV and 100 mA, using focusing mirrors (MSC, USA). The crystal to detector distance was 120 mm and images of 1° oscillation with a 5 min exposure time were collected at −173°C. Diffraction data were processed using the programs DENZO and SCALEPACK [21]. To collect data under cryo-conditions crystals were flash frozen in a solution containing 50 mM HEPES (pH 7.5), 16% (w/v) PEG 8000 and 20% (v/v) glycerol as a cryo-protectant.

The structures were refined by several cycles of energy minimization, using X-PLOR [22] and manual correction using ARP [23] and O [24].

3. Results

3.1. Construction and purification of TGT(S103A)

In order to characterize the structure and function of Ser^{103} in the base exchange reaction, a serine to alanine mutation of *Z. mobilis* TGT was constructed. The mutation S103A was introduced into the *Z. mobilis tgt* overexpression plasmid pETZM4 [16] by PCR using an optimized method for site-directed mutagenesis [17,18]. To verify successful mutagenesis and exclude potential additional mutations, the complete insert of the mutated plasmid was sequenced. The resulting plasmid pETZM4-S103A was transformed into *E. coli* BL21(DE3) for overexpression of the mutated *tgt* gene as described previously [16]. TGT(S103A) could be purified identically to the wild-type TGT, indicating a correct folding of the enzyme.

Table 1
Oligonucleotides used in mutagenesis

Oligo	Sequence (5′ to 3′)[a]
S103A-s	CTATTTTGACGGATGCCGGCGGGTATCAG
S103A-a	CCTGATACCCGCCGGCATCCGTCAAAAAG

[a]The mutated codon is underlined.

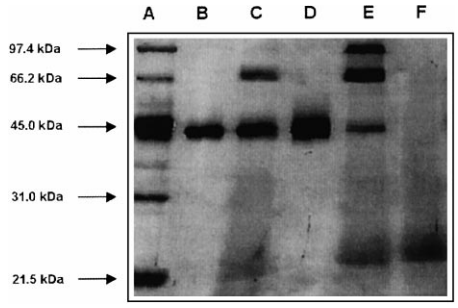

Fig. 1. Silver-stained SDS-PAGE of wild-type TGT and TGT(S103A) in the absence or presence of tRNA^{Tyr}-(G$_{34}$). Lane A, molecular mass standards; lane B, TGT (wt); lane C, TGT (wt)+tRNA; lane D, TGT(S103A); lane E, TGT(S103A)+tRNA; lane F, tRNA.

3.2. Characterization of the TGT(S103A) mutation by activity assay and denaturing gel electrophoresis

The measurement of TGT(S103A) activity revealed a drastic reduction of activity compared with wild-type *Z. mobilis* TGT as positive control. Even at high substrate and enzyme concentrations the initial velocities were too low to determine V_{max} and K_M because of the pronounced errors obtained for the measured values. This finding was consistent with the properties of *E. coli* TGT(S90A) [14]. It has been shown that *E. coli* TGT(S90A) binds cognate tRNA under native conditions [14]. To see if *Z. mobilis* TGT(S103A) retained

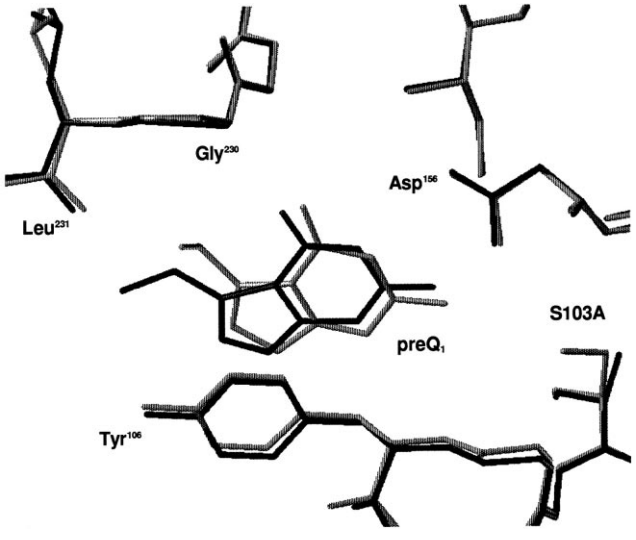

Fig. 2. The alignment of all main chain atoms of wild-type TGT (grey) and TGT(S103A) (black) shows a slight rotation of the preQ$_1$ molecule towards the amide of Gly^{230}. Additional density indicates a rotation of the aminomethyl group of preQ$_1$ by ∼95° in the TGT(S103A) structure, allowing an interaction with the main chain carbonyl oxygen of Leu^{231}.

Table 2
Data collection and refinement statistics

TGT(S103A)	
Space group	C2
Cell constant *a* (Å)	90.60
Cell constant *b* (Å)	65.04
Cell constant *c* (Å)	70.74
Cell constant β (deg)	96.25
Resolution (Å)	71–200
Wavelength (Å)	1.54
Temperature of data collection (K)	100
No. of reflections	27 018
Completeness of all data (%)	97.4
R^{a}_{symm} for all data (%)	5.7
Completeness of outer shell (%)	95.1
R_{symm} in outer shell (%)	24.3
R^{b}_{free} (%)	19.9
R-factor[b] (%)	16.1

[a] $R_{symm} = \Sigma|I-\langle I\rangle|/\Sigma I$, where I is the observed intensity and $\langle I\rangle$ is the average intensity for multiple measurements.
[b] The R_{free} [26] was calculated from a random selection of reflections constituting ~10% of the data; the *R*-factor was calculated with the remaining intensities.

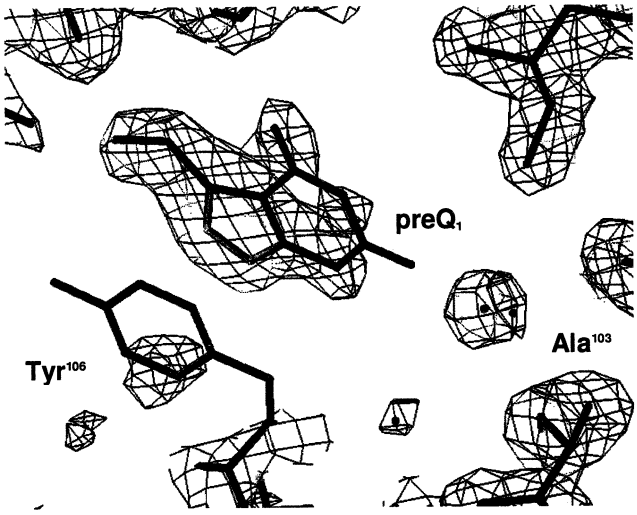

Fig. 3. Representation of the 2.0 Å resolution $2f_o-f_c$ map contoured at 1.0 σ of the TGT(S103A) binding pocket in complex with $preQ_1$. Electron density of the refined structure is partially missing for the C2-NH_2 group of the $preQ_1$ molecule and for the side chain of Tyr^{106}, suggesting a reduced occupancy of the substrate in the binding pocket.

the ability to form the covalent protein/tRNA intermediate shown for wild-type TGT, we performed a band-shift experiment under denaturing conditions as described previously [12] using wild-type TGT as positive control. Both wild-type and mutated TGT were incubated at 37°C for 1 h in the presence and absence of a 20-fold excess of *E. coli* $tRNA^{Tyr}$-(G34). Analysis by SDS-PAGE clearly showed the presence of shifted bands (~66 kDa) consistent with a TGT monomer·tRNA complex (Fig. 1). Surprisingly, an additional band at a higher molecular weight (~97 kDa) was observed in the case of TGT(S103A) seen also for *Z. mobilis* TGT(D156A) [12], for which no explanation can be given. Nevertheless, the results confirm that TGT(S103A) is able to form a covalent intermediate with the tRNA and that Ser^{103} cannot be the active site nucleophile involved in the formation of a covalent bond to tRNA. Consistent with these results, we also find that the

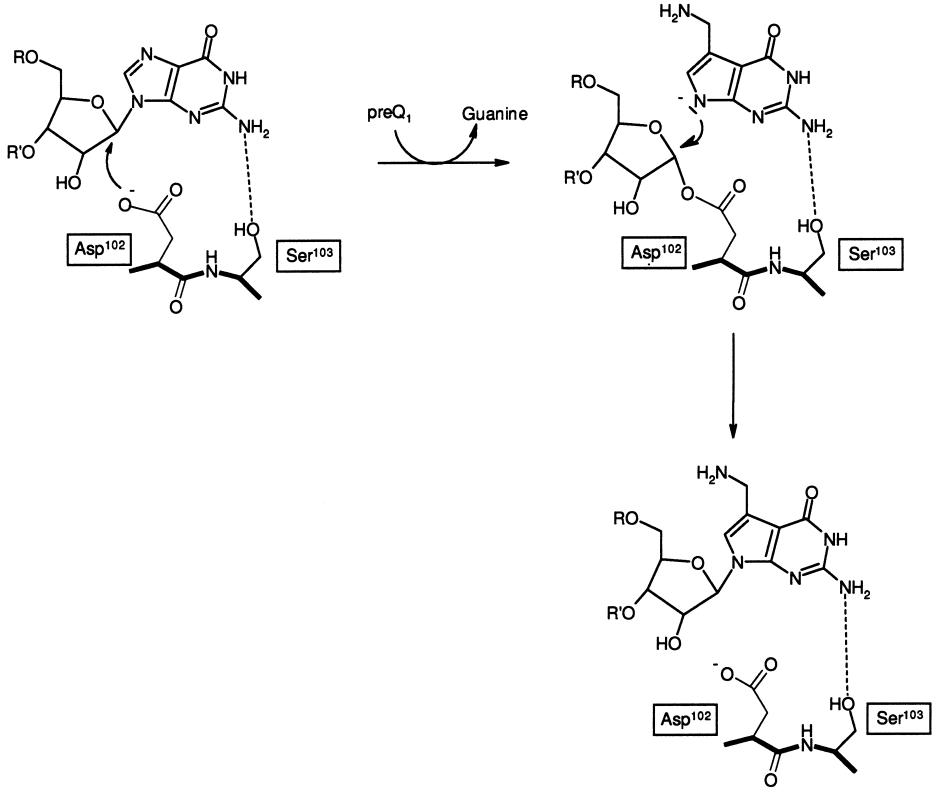

Fig. 4. Proposed catalytic mechanism with Asp^{102} as the active site nucleophile. The nucleophilic attack of Asp^{102} at C1′ atom of the guanosine results in a covalent TGT/tRNA intermediate. Subsequent nucleophilic substitution by the deprotonated $preQ_1$ molecule preserves the β-configuration and leads to the modified $preQ_1$-tRNA. The residue Ser^{103} performs a critical role in substrate orientation.

E. coli TGT(S90A) mutant forms a covalent complex with both full-length tRNA and minihelix RNA (data not shown).

3.3. Structural characterization

Soaking crystals of TGT(S103A) with $preQ_1$ and subsequent structure analysis was used to determine the binding mode of the substrate. The X-ray structure has been solved at 2.0 Å resolution (Table 2) and shows only minor changes compared to the wild-type structure (Fig. 2). The serine to alanine mutation is clearly confirmed by the electron density map. A $preQ_1$ molecule could easily be fit in the difference electron density. The $2f_o-f_c$ electron density map of the refined TGT(S103A) structure including the substrate clearly indicates a similar orientation of the $preQ_1$ molecule as observed in the wild-type structure [11]. The base is intercalated between the side chains of Met^{260} on one side, and Tyr^{106} and Cys^{158} on the other side. Specific recognition is allowed through hydrogen bonding between the side chain of Asp^{156} and the 1-NH and 2-NH_2 groups, and between the amide of Gly^{230} and oxygen O6. These residues are strictly conserved in all presently known TGT sequences of prokaryotes, archaebacteria and eukaryotes, suggesting a common guanine recognition motif [15]. Interestingly, difference electron density indicates a rotation of the exocyclic methylene-amino group of $preQ_1$ by $\sim 95°$ in the TGT(S103A) complex, allowing an interaction with the main chain carbonyl oxygen of Leu^{231} (Fig. 2). The 2-NH_2 group of $preQ_1$ forms a hydrogen bond (2.8 Å) to an additional water molecule in the vicinity of the serine to alanine mutation. This water molecule is not present in the wild-type structure, and thus formally replaces the OH functional group of Ser^{103}. In the wild-type TGT/$preQ_1$ complex, the 2-NH_2 group is involved in a weak hydrogen bond (3.5 Å) to Ser^{103}, suggesting that this residue assists in the correct orientation of the substrate required for the base exchange reaction [11]. In the present complex, no electron density was observed for the C2-NH_2 group of $preQ_1$ in the $2f_o-f_c$ map at a contour level of 1.0 σ (Fig. 3), nor could electron density be properly assigned to the hydroxyphenyl ring of Tyr^{106}, indicating that this residue is not fully occupied in the position adjacent to the $preQ_1$ molecule (Fig. 3). A superposition based on all main chain atoms between wild-type TGT and TGT(S103A) shows a slight rotation of the $preQ_1$ molecule towards the amide group of Gly^{230} (Fig. 2).

4. Discussion

The results presented here support the catalytic mechanism proposed on the basis of structural and mutagenesis studies described previously [11,12]. The X-ray structure of queuosine monophosphate shows that the β-configuration of the ribose is preserved [25]. Consequently, two consecutive S_N2 reactions are most likely involved in the base exchange reaction catalyzed by TGT as indicated earlier [11,12]. In this mechanism, a covalent TGT/tRNA intermediate is formed as a consequence of the preceding nucleophilic attack by the carboxylate of Asp^{102} at the C1′ atom of the wobble guanosine (Fig. 4). After replacement of guanine by $preQ_1$ at the binding site, the deprotonated $preQ_1$ attacks carbon C1′ of the ribose, resulting in the final $preQ_1$-modified tRNA. In our band-shift experiment, we have demonstrated that TGT(S103A) still binds covalently to tRNA, in agreement with the above postulated mechanism. Therefore, residue Ser^{103} cannot be involved in the nucleophilic attack of the wobble guanosine. In the enzyme assay, we observed a dramatic reduction of activity of *Z. mobilis* TGT(S103A) compared to the wild-type TGT. However, total inactivation upon mutation of the *Z. mobilis* TGT Ser^{103} to alanine could not be determined, consistent with the results obtained for *E. coli* TGT(S90A) [14]. The X-ray structure of *Z. mobilis* TGT(S103A) showed difference density for a $preQ_1$ molecule, suggesting a similar binding mode to that observed for wild-type TGT. This clearly implies that the wobble guanine is still recognized in the $preQ_1$ binding pocket of TGT(S103A). However, the lack of well resolved electron density attributable to the C2-NH_2 group of $preQ_1$ and to the side chain of Tyr^{106} in the refined structure of TGT(S103A) indicates an elevated flexibility of the $preQ_1$ molecule in the binding pocket. In the wild-type TGT, the 2-NH_2 group of $preQ_1$ forms a weak hydrogen bond (3.5 Å) to Ser^{103} [11]. This interaction could be crucial for immobilizing the wobble guanosine in a binding position suitable for the nucleophilic attack and tighter binding of the intermediate transition state with Asp^{102} (Fig. 4). Removal of the Ser^{103} hydroxyl group by an alanine replacement is accompanied by a change to a more hydrophobic environment. In the refined TGT(S103A) structure, an additional water molecule is detected in the vicinity of Ala^{103}, allowing for an extra hydrogen bond (2.8 Å) to the 2-NH_2 group of $preQ_1$. This water molecule could mimic the Ser-OH recognition site, however it obviously does not retain the orientational fixation of the Ser^{103} hydroxyl group binding to the 2-NH_2 group of the wobble guanosine. We therefore conclude that Ser^{103} plays a critical role in the appropriate immobilization and orientation of the substrate for the base exchange reaction.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft (Grant: KL 1204/1-1 and SFB 286).

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