Mutagenesis and Crystallographic Studies of *Zymomonas mobilis* tRNA-Guanine Transglycosylase Reveal Aspartate 102 as the Active Site Nucleophile[†,‡]

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Received August 12, 1996; Revised Manuscript Received October 3, 1996[⊗]

ABSTRACT: Procaryotic tRNA-guanine transglycosylase (TGT) catalyzes the posttranscriptional base exchange of the queuine precursor 7-aminomethyl-7-deazaguanine ($preQ_1$) with the genetically encoded guanine at the wobble position of tRNAs specific for Asn, Asp, His, and Tyr. The X-ray structures of *Zymomonas mobilis* TGT and of its complex with $preQ_1$ [Romier, C., Reuter, K., Suck, D., & Ficner, R. (1996) *EMBO J. 15*, 2850−2857] have revealed a specific $preQ_1$ binding pocket and allowed a proposal for tRNA binding and recognition. We have used band-shift experiments in denaturing conditions to study the enzymatic reaction performed by TGT. The presence of shifted protein bands after incubation with tRNA followed by protein denaturation indicates a reaction mechanism involving a covalent intermediate. Inspection of the X-ray structures and comparison of the different procaryotic TGT sequences highlighted the conserved aspartate 102 as the most likely nucleophile. Mutation of this residue into alanine by site-directed mutagenesis leads to an inactive mutant unable to form a covalent intermediate with tRNA, proving that aspartate 102 is the active site nucleophile in TGT. To investigate the recognition of the wobble guanine in the $preQ_1$ binding pocket, we mutated aspartate 156, the major recognition element for $preQ_1$, into alanine and tyrosine. Both mutants are inactive in producing the final product, but the mutant D156A is able to form the covalent intermediate with tRNA in the first step of the reaction mechanism in comparable amounts to wild-type protein. Therefore, the binding of the wobble guanine in the $preQ_1$ binding pocket is required for the cleavage of the glycosidic bond. The three mutants were crystallized and their X-ray structures determined. The mutants display only subtle changes to the wild-type protein, confirming that the observed biochemical results are due to the chemical substitutions rather than structural rearrangements.

The hypermodified base queuine [Q: 7-[[(4,5-*cis*-dihydroxy-2-cyclopentene-1-yl)amino]methyl]-7-deazaguanine][1] is found in the first (or wobble) position of the anticodon of tRNAs specific for Asn, Asp, His, and Tyr, in most procaryotes and eubacteria. Contrary to the other modified bases which are synthesized on the tRNA level, queuine biosynthesis involves several steps beginning outside the tRNA and requires a base exchange on the tRNA. In eucaryotes, where it is a nutrient, queuine is exchanged directly with the genetically encoded guanine, found at the wobble position of the specific tRNAs, in a single enzymatic step (Katze *et al.*, 1984).

In procaryotes, queuine is synthesized *de novo* [for a review, see Slany and Kersten (1994)], presumably starting from a GTP molecule which is transformed into the queuine precursor $preQ_1$ (7-aminomethyl-7-deazaguanine) (Figure 1).

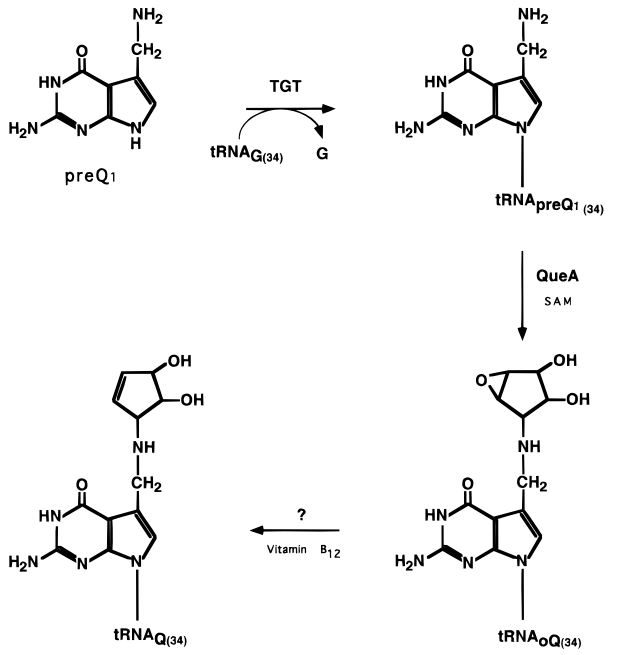

FIGURE 1: Last steps of the queuine biosynthetic pathway. $preQ_1$, 7-aminomethyl-7-deazaguanine; oQ, epoxyqueuine; Q, queuine; SAM, *S*-adenosylmethionine.

This precursor is exchanged with the guanine at the wobble position by the tRNA-guanine transglycosylase (TGT; EC 2.4.2.29) enzyme (Okada *et al.*, 1979). The tRNA-bound $preQ_1$ is further modified into epoxyqueuine [oQ: 7-[[*N*-

[†] This work was supported in part by the Deutsche Forchungsgemeinschaft.

[‡] The coordinates have been deposited within the Brookhaven Protein Data Bank under the names 1WKD, 1WKE, and 1WKF.

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[⊗] Abstract published in *Advance ACS Abstracts,* November 15, 1996.

[1] Abbreviations: TGT, tRNA-guanine transglycosylase; $preQ_1$, 7-aminomethyl-7-deazaguanine; oQ, epoxyqueuine; Q, queuine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MW, molecular weight(s); PCR, polymerase chain reaction; HEPES, *N*-2-(hydroxyethylpiperazine)-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

S0006-2960(96)02003-X CCC: $12.00

Table 1: Oligonucleotides Used in Mutagenesis

oligo	sequence (5′ to 3′)[a]
T7	AATACGACTCACTATAGGGAG
T7reverse	CTAGTTATTGCTCAGCGGTG
D102A_NM[b]	GTCAAAATAGGCCGATCCCA
D102A_M[c]	TGGGATCGGCCTATTTTGACGGCTAGCGGCGGGTATCAGG
D156A_NM	AAGGCCATTACGATATCACTG
D156A_M	CAGTGATATCGTAATGGCCTTTGCCGAATGCACGCCTTATCCAG
D156Y_NM	AAGGCCATTACGATATCACTG
D156Y_M	CAGTGATATCGTAATGGCCTTTUACGAATGCACGCCTTATCCAG

[a] The mutated codon is underlined. [b] NM, nonmutated. [c] M, mutated.

(2,3-epoxy-4,5-*cis*-dihydroxy-2-cyclopent-1-yl)amino]methyl]-7-deazaguanine] by the *S*-adenosylmethionine-dependent enzyme QueA (Slany *et al.*, 1993). Final conversion of epoxyqueuine to queuine is carried out by an unknown enzyme in a vitamin B_{12}-dependent reaction (Frey *et al.*, 1988) (Figure 1).

TGT was first isolated from *Escherichia coli* extracts (Okada & Nishimura, 1979) and the corresponding *tgt* gene cloned and sequenced (Reuter *et al.*, 1991). The *E. coli* protein was overexpressed and purified (Garcia *et al.*, 1993), and the anticodon stem−loop together with the $U_{33}G_{34}U_{35}$ sequence was shown to be the only requirement, besides magnesium, for recognition (Curnow *et al.*, 1993; Nakanashi *et al.*, 1994; Curnow & Garcia, 1995). Two residues, serine 90 and cysteine 265, have been shown to be potentially involved in the *E. coli* TGT catalytic mechanism (Reuter *et al.*, 1994; Chong *et al.*, 1995). Recently, the *tgt* gene from *Zymomonas mobilis* was cloned and its product overexpressed, purified, and characterized (Reuter & Ficner, 1995). Both *E. coli* and *Z. mobilis* enzymes have a molecular mass around 43 kDa, show 53% sequence identity, and have similar kinetic parameters. *Z. mobilis* TGT has been crystallized and its structure solved at 1.85 Å resolution (Romier *et al.*, 1996a,b). The enzyme has an unexpected $(\beta/\alpha)_8$-barrel fold with a zinc binding domain insertion. Soaking of the TGT crystals with the substrate $preQ_1$ revealed a specific $preQ_1$ binding pocket at the C-terminal face of the barrel. These results together with the electrostatic potential at the surface of the enzyme enabled the modeling of tRNA binding and recognition. Inspection of the experimental and modeled structures lead us to propose a catalytic mechanism composed of two consecutive S_N2 reactions involving the formation of a covalent intermediate. Only a few residues which might act as nucleophiles were found in the vicinity of the $preQ_1$ pocket, with aspartate 102 being the most properly placed to play this role.

To further characterize the reaction performed by TGT, we have analyzed TGT−tRNA complexes by gel electrophoresis in denaturing conditions. Evidence of a covalent intermediate prompted us to mutate aspartate 102 by site-directed mutagenesis to assay its role in catalysis. Aspartate 156, a major recognition element for $preQ_1$, was also mutated to study the role of the $preQ_1$ binding pocket in the specific recognition of the wobble guanine. Here we report the biochemical and structural characterization of the TGT mutants D102A, D156A, and D156Y.

MATERIALS AND METHODS

Reagents. All chemicals were purchased from Sigma unless stated. Restriction endonucleases were from New England Biolabs and DNA-modifying enzymes from Boehringer Mannheim. [8-^{3}H]Guanine was from Amersham Corp. Oligonucleotides were synthesized by the oligonucleotide service at EMBL.

TGT and E. coli tRNA^{Tyr} Preparation. *Zymomonas mobilis* TGT, either wild-type or mutants, was prepared as described previously (Reuter & Ficner, 1995; Romier *et al.*, 1996a) 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 NaCl, up to the desired protein concentration. The NaCl concentration was adjusted as low as possible to obtain a total dissolution of the microcrystals (i.e., 400 mM for 1 mg/mL and 2 M for 12 mg/mL). *E. coli* tRNA^{Tyr}-(G_{34}) was prepared as described previously (Reuter et al., 1994) 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.

Polyacrylamide Gel Electrophoresis. For polyacrylamide gel electrophoresis (PAGE), 1.5 μL of the TGT solutions (1 mg/mL) was mixed with 10 μL of reaction buffer (10 mM HEPES, pH 7.5, 10 mM $MgCl_2$, and 1 mM EDTA) and 2 μL of either distilled water or tRNA (10 mg/mL; i.e., 20-fold excess of tRNA). The reaction mixtures were then incubated 1 h at 37 °C. Following incubation, 7 μL of a SDS loading buffer [60 mM Tris, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, and 0.01% (w/v) Bromophenol blue] was added to the reaction mixtures which were left for 1 h at 37 °C to allow proper unfolding. The samples were then applied, together with protein markers (Biorad), onto a SDS gel composed of a 4% stacking part (pH 6.8) and a 15% running part (pH 8.9). Electrophoresis was performed at 100 V in the presence of a SDS running buffer composed of 25 mM Tris, pH 8.8, 200 mM glycine, and 0.1% (w/v) SDS. Gels were stained with Coomassie brilliant blue R-250 (Biorad) followed by silver staining (Sigma).

Construction of the TGT Mutants D102A, D156A, and D156Y. DNA manipulations and fragment isolations were performed according to well-established methods (Sambrook *et al.*, 1989). The construction of the mutants was performed by polymerase chain reaction (PCR; Ho *et al.*, 1989) following a protocol already described (Reuter *et al.*, 1994). The external primers used were T7-specific primers. The inside primers were only partially overlapping, with only one of them containing the mutated codon (Table 1). To minimize errors produced by PCR, only the first half of the mutated gene (which contains the mutation) was used for ligation. This fragment was obtained by digestion of the PCR fragments with both *Nco*I and *Eco*RI restriction enzymes (*Z. mobilis tgt* gene has a single *Eco*RI restriction site). The second half of the gene was obtained by digestion of the wild-type gene with both *Eco*RI and *Bam*HI restriction

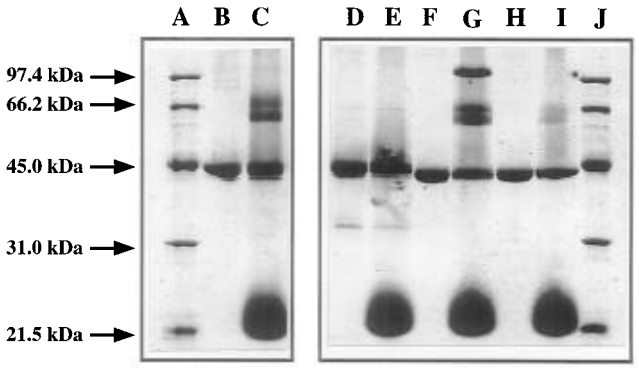

FIGURE 2: Silver-stained SDS−PAGE of wild-type TGT and TGT mutants D102A, D156A, and D156Y, in the absence or presence of $tRNA^{Tyr}(G_{34})$. Lane A, molecular mass standards; lane B, TGT(wt); lane C, TGT(wt) plus tRNA; lane D, TGT(D102A); lane E, TGT(D102A) plus tRNA; lane F, TGT(D156A); lane G, TGT(D156A) plus tRNA; lane H, TGT(D156Y); lane I, TGT(D156Y) plus tRNA; lane J, molecular mass standards.

endonucleases. Both fragments were ligated overnight at 14 °C together with a pET-9d vector previously digested with both *Nco*I and *Bam*HI restriction enzymes. Sequence analysis was performed by the EMBL sequencing service using an automatic sequencer (Voss *et al.*, 1995).

Activity Assays. The determination of the TGT wild-type and mutant activities was performed as described previously (Reuter & Ficner, 1995).

Structure Determination. The three mutants were crystallized and data collection performed under cryogenic conditions as described previously (Romier *et al.*, 1996a). The structures were refined by several cycles of energy minimization, using X-PLOR (Brünger, 1992), and manual corrections, using TURBO-FRODO (Roussel & Cambillau, 1992).

RESULTS

Study of Wild-Type TGT−tRNA Complexes in Denaturing Conditions. In order to assess the possible formation of a covalent intermediate during TGT reaction, wild-type TGT−tRNA complexes were studied in denaturing conditions. Wild-type TGT was first incubated at 37 °C for 1 h in the presence or in the absence of *E. coli* $tRNA^{Tyr}(G_{34})$. Following incubation, a SDS-containing buffer was added to the reaction mixtures which were then left 1 h at 37 °C. The choice of such a temperature was made to prevent hydrolysis of the putative covalent bond between the protein and the tRNA. For similar reasons, no β-mercaptoethanol was added to the SDS denaturing buffer. Analysis of the denatured samples by SDS−PAGE clearly shows the presence of shifted protein (estimated to about 10% of the total protein loaded) after incubation with the tRNA (Figure 2). This result confirms that the TGT reaction involves the formation of a covalent intermediate.

Construction and Purification of the Mutants. The structures of *Z. mobilis* TGT and of its complex with $preQ_1$ have been previously solved by X-ray crystallography (Romier *et al.*, 1996a,b). Assuming the formation of a covalent intermediate during TGT reaction, structural inspection highlighted aspartate 102 as the putative nucleophile (Romier *et al.*, 1996b). To verify this hypothesis, this residue was mutated into alanine. Additionally, aspartate 156, which is found at the bottom of the $preQ_1$ binding pocket hydrogen-bonding the 1-NH and 2-NH_2 groups of $preQ_1$, was mutated into alanine and tyrosine to study the binding of the wobble guanine in the $preQ_1$ binding pocket. The three mutants were constructed by site-directed mutagenesis (Ho *et al.*, 1989) as previously described for *E. coli* TGT mutants (Reuter *et al.*, 1994). The mutated genes were inserted into a pET-9d vector and overexpressed, and their products were purified as reported previously (Reuter & Ficner, 1995; Romier *et al.*, 1996a). The mutants could all be purified by preparative crystallisation, as for the wild-type, indicative of a proper folding of the enzyme.

Table 2: Data Collection and Refinement Statistics

mutant	D102A	D156A	D156Y
cell constants			
a (Å)	90.7	90.2	90.4
b (Å)	63.7	64.5	64.3
c (Å)	72.5	72.0	72.0
β (deg)	96.4	96.9	97.2
resolution (Å)	2.6	2.2	2.2
completeness (%)	98.8	97.7	93.6
R_{sym}[a] (%)	6.5	3.7	3.8
resolution range (Å)	6.0−2.6	6.0−2.2	6.0−2.2
R-factor[b] (%)	20.4	20.3	19.9
R-free[b] (%)	27.6	24.6	25.0
rms to wild-type[c] (Å)	0.17	0.15	0.19

[a] $R_{sym} = \sum|I - \langle I\rangle|/\sum I$, where I is the observed intensity and $\langle I\rangle$ is the average intensity for multiple measurements. [b] The *R*-free (Brünger, 1992) was calculated from a random selection of reflections constituting ~10% of the data; the *R*-factor was calculated with the remaining intensities. [c] The rms value was calculated with all main-chain atoms including the C_β.

Characterization of the Mutants by Activity Assays and Denaturating Gel Electrophoresis. Activity assays using wild-type TGT as positive control showed a total loss of activity for the three mutants. As is evident from the lack of a band-shift under denaturing conditions, the D102A mutant is not able to form a covalent intermediate with its tRNA substrate whereas the two other mutants still bind covalently tRNA (Figure 2). Surprisingly, in the case of the D156A mutant, an additional band is observed at a higher molecular weight. So far no explanation could be given for this band. However, when incubating the reaction mixtures in the SDS loading buffer at 56 °C rather than at 37 °C, this band was not observed any more whereas the other shifted bands are still observed (data not shown).

Structural Characterization. The X-ray structures of the three mutants have been determined and refined (Table 2). The three structures display only minor changes compared to the wild-type structure. The three mutations are clearly confirmed by the electron density maps (Figure 3). One important change concerns the D156A and D156Y mutants where the side chain of aspartate 102 breaks the hydrogen bond network it formed with the side chains of asparagine 70, histidine 73, and glutamine 107, and flips toward the $preQ_1$ binding pocket. In the case of the D156Y mutant, aspartate 102 is seen forming a hydrogen bond with the hydroxyl group of tyrosine 156 (Figure 3).

DISCUSSION

So far, the catalytic mechanism of procaryotic tRNA-guanine transglycosylases is poorly understood. The determination of the structure of *Z. mobilis* TGT by X-ray crystallography (Romier *et al.*, 1996a,b) revealed that TGT adopts an irregular $(\beta/\alpha)_8$-barrel fold. Soaking of the crystals with the $preQ_1$ substrate showed the presence of a specific $preQ_1$ binding pocket at the C-terminal face of the barrel.

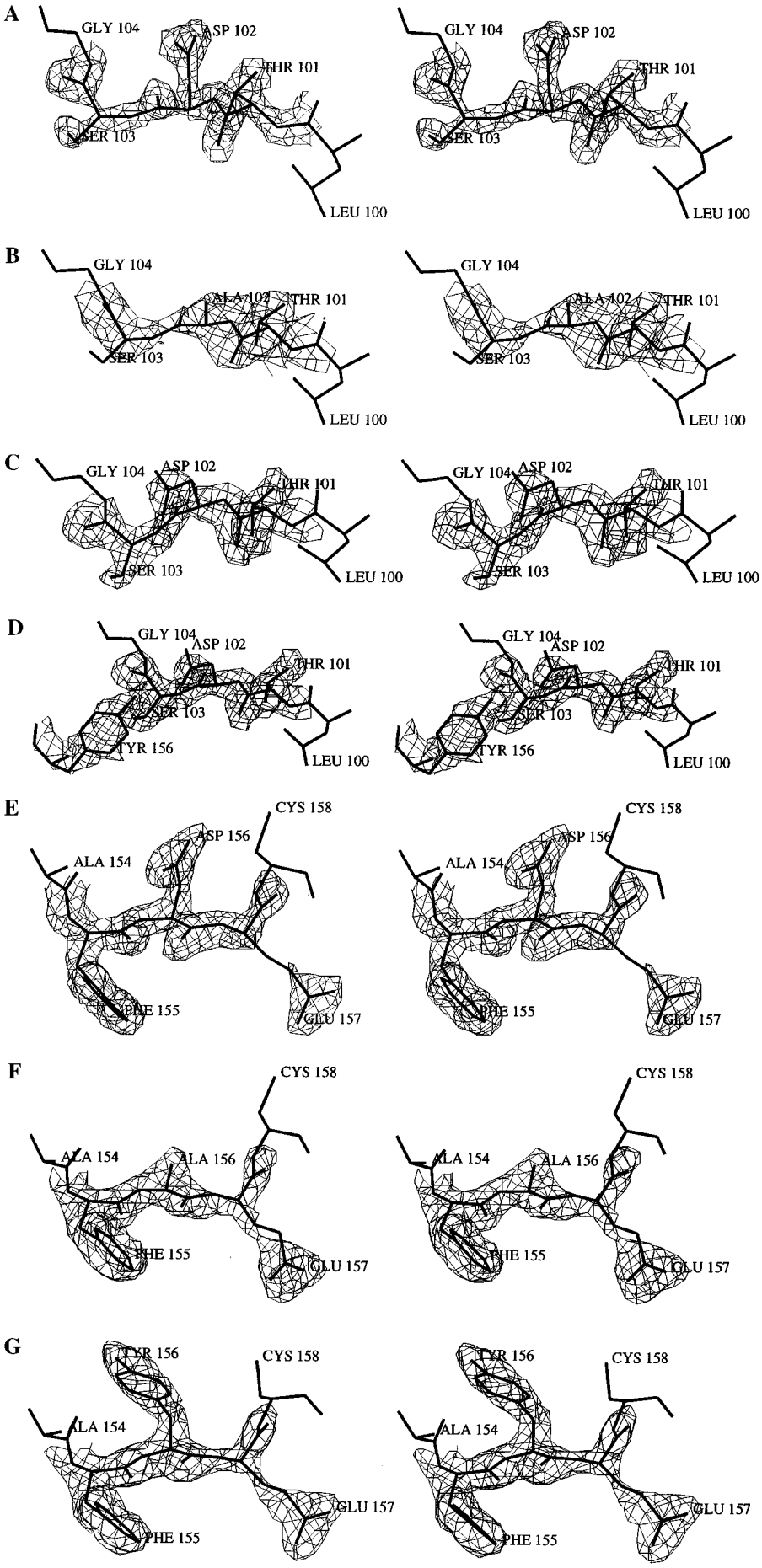

FIGURE 3: Stereoview of the $2F_o - F_c$ electron density maps of TGT wild-type and mutants. All maps are contoured at 1.5 σ. (A) Residue 102, TGT(wt); (B) residue 102, TGT(D102A); (C) residue 102, TGT(D156A); (D) residue 102, TGT(D156Y); (E) residue 156, TGT(wt); (F) residue 156, TGT(D156A); (G) residue 156, TGT(D156Y).

Furthermore, the electrostatic potential at the surface of the protein is characterized by a bipolar charge distribution with a negatively charged region in the center of the barrel and a positively charged region on the zinc subdomain located above the barrel plane. Altogether, these results enabled the modeling of a TGT−tRNA complex where the anticodon stem−loop phosphate backbone is recognized by the positively charged region, whereas the anticodon is recognized by the negatively charged region inside the barrel (Romier *et al.*, 1996b).

The crystal structure of queuosine, the nucleoside of queuine, shows that the β-configuration is preserved at the ribose C1′ (Yokoyama *et al.*, 1979). A direct nucleophilic attack of the glycosidic bond by $preQ_1$ can therefore be excluded. Consequently, a S_N1 or two consecutive S_N2 reactions could be catalyzed by the enzyme. The former mechanism is unlikely due to the presence of many water molecules in the center of the barrel and to the absence of a site in the $preQ_1$ pocket vicinity where the ribose could be hidden from the solvent. Since the TGT reaction does not require additional energy provided by GTP or ATP, the second proposed mechanism most likely involves the formation of a covalent intermediate. Inspection of the structures shows that only four conserved residues may act as nucleophiles: histidine 73, aspartate 102, aspartate 280, and cysteine 281. Although histidines and cysteines are considered better nucleophiles than carboxylates, only aspartate 102 was close enough to the $preQ_1$ binding pocket to act as a nucleophile.

We have presented here evidence that the catalytic mechanism of TGT involves a covalent intermediate. Analysis of the wild-type TGT−tRNA complex by gel electrophoresis in denaturing conditions shows the presence of shifted protein bands (Figure 2). The presence of two shifted bands might be due to partially digested tRNA. To investigate whether aspartate 102 was the TGT active site nucleophile, this residue was mutated into alanine by site-directed mutagenesis, and the D102A mutant was assayed for its ability to form a covalent intermediate. In this case, no shifted protein bands were observed in denaturing conditions (Figure 2), and the mutant was found to be totally inactive. No changes were observed in its crystal structure, which could account for the loss of activity or its inability to form a covalent intermediate. Altogether, these results confirm the role of a nucleophile played by aspartate 102 in the catalytic mechanism of TGT. Even though carboxylates are considered as poor nucleophiles, nucleoside 2-deoxyribosyltransferase, which catalyzes the same kind of reaction, also has a catalytic mechanism involving a covalent intermediate and makes use of a carboxylate (Glu98) as nucleophile (Porter *et al.*, 1995; Porter & Short, 1995; Short *et al.*, 1996). The structure of this enzyme has recently been solved (Armstrong *et al.*, 1996), but its fold and the environment of the nucleophile are not comparable with those of TGT.

In *E. coli* TGT, two residues—serine 90 and cysteine 265—have been shown to affect the catalytic activity of the enzyme (Reuter *et al.*, 1994; Chong *et al.*, 1995). However, both mutants retain some activity when mutated into alanines which excludes their role as active site nucleophiles. The location of the corresponding residues in the structure of *Z. mobilis* TGT suggests a structural role for the side chains of both residues.

The results presented here confirm the catalytic mechanism proposed on the basis of the crystal structure determination of TGT (Romier *et al.*, 1996b) (Figure 4). In this mechanism, the carboxylate group of aspartate 102 first attacks the wobble guanosine at the C1′ atom, leading to guanine release and formation of a covalent bond between the aspartate side chain and the ribose in an α configuration. It has not been shown so far whether this attack is facilitated by some other mechanisms as, for instance, a protonation of the leaving guanine. The second reaction catalyzed by TGT is the substitution of aspartate 102 by $preQ_1$, leading to the formation of the final $preQ_1$-modified tRNA. It has been proposed (Hoops *et al.*, 1995) that deprotonation of $preQ_1$ nitrogen N9 occurs before it attacks the C1′ atom (see Figure 4 for atom numbering). The mechanism of this deprotonation is still to be clarified.

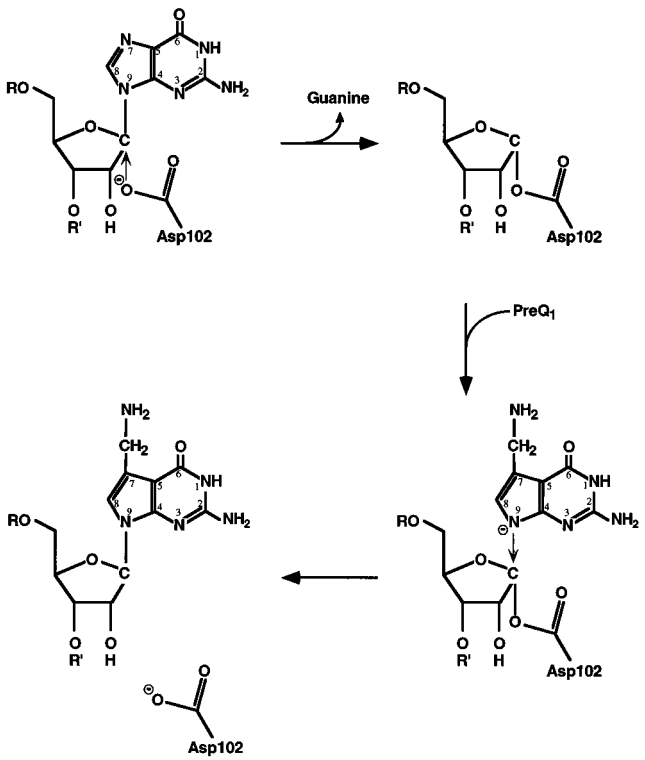

FIGURE 4: Proposed catalytic mechanism with aspartate 102 as the active site nucleophile. The carboxylate group of the aspartate first attacks the C1′ atom, leading to the covalent intermediate with a covalent bond in α configuration. The deprotonated $preQ_1$ molecule attacks then the C1′ atom, leading to the final $preQ_1$-modified tRNA.

In our original proposal for the reaction mechanism, we assumed that the wobble guanine is recognized in the $preQ_1$ binding pocket prior to the cleavage of the glycosidic bond by aspartate 102. To verify this hypothesis, we mutated aspartate 156, the major recognition element for $preQ_1$. A mutation into alanine was designed to prevent proper binding of either guanine or $preQ_1$, and a mutation into tyrosine was made to block the pocket. Both mutants are totally inactive in producing the final product, but they are still able to form a covalent intermediate with tRNA in the first step of the reaction mechanism (Figure 2). However, while the amount of shifted protein for the D156A mutant is similar to the wild-type, it is almost not detectable in the case of the D156Y mutant. This clearly implies that the $preQ_1$ pocket is involved in the binding of the wobble guanine. The presence of some covalent complex, which is however hardly detectable on the silver-stained gel, with the D156Y mutant suggests that alternative conformations of the anticodon loop enable aspartate 102 to come close to the C1′ position. The determination of the crystal structure of TGT in complex

with tRNA should help in clarifying the mode of binding for the anticodon loop.

Finally, another structural and functional aspect concerns the conformation of the aspartate 102 side chain. In the wild-type protein, the carboxylate of this aspartate is involved in a hydrogen-bonding network formed by the side chains of asparagine 70, histidine 73, and glutamine 107. The structures of the D156A and D156Y mutants reveal that the aspartate 102 side chain is rotated by almost 180° toward the $preQ_1$ binding pocket (Figure 3). This conformational change might be due to the missing repulsive charge of aspartate 156. We have previously postulated that, in order to perform its catalytic role, aspartate 102 must break its hydrogen bonding network and flip toward the wobble guanosine (Romier *et al.*, 1996b). We assumed that binding of one of the uracils of the recognized $U_{33}G_{34}U_{35}$ sequence may perturb this network, freeing the carboxylate of the aspartate. However, the structures of the TGT mutants D156A and D156Y reveal that such a flip is possible without prior perturbation of the hydrogen-bonding network. It may therefore be possible that such a flip also occurs spontaneously in presence of the wobble guanosine, even though a complementary action is always possible.

ACKNOWLEDGMENT

We wish to thank Gunter Stier for help with the mutagenesis.

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BI962003N