Serine 90 Is Required for Enzymic Activity by tRNA-Guanine Transglycosylase from *Escherichia coli*†

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ABSTRACT: An *Escherichia coli* mutant described by Noguchi *et al.* [Noguchi, S., *et al.* (1982) *J. Biol. Chem. 275,* 6544–6550] contains tRNA lacking the hypermodified wobble nucleoside queuosine (Q) due to an inactive tRNA-guanine transglycosylase (TGT). TGT catalyzes the posttranscriptional base exchange of the Q precursor $preQ_1$ with the genetically encoded guanine in $tRNA^{Asp,Asn,His,Tyr}$. The mutant *tgt* gene was cloned and sequenced; it contained a single point mutation resulting in the change of serine 90 to phenylalanine. Overexpression of the mutant gene yielded TGT(S90F) that showed a reduced solubility and did not purify in the same fashion as the wild-type enzyme. TGT(S90F) has no detectable enzymic activity. To determine whether serine 90 performs a catalytic role in the TGT reaction or whether the loss of activity was caused solely by a conformational change of the enzyme, we used site-specific mutagenesis to construct serine-to-alanine (S90A) and serine-to-cysteine (S90C) mutants. Both S90A and S90C mutants were purified in a manner identical to that used for the wild-type enzyme. SDS–PAGE of dimethyl suberimidate-cross-linked mutants showed a pattern identical to that of the wild-type TGT, indicative of a trimeric quaternary structure. Native PAGE of wild-type and mutant TGTs in the absence and presence of substrate tRNA exhibited band shifts indicating that both mutants retain the ability to bind tRNA. Determination of Michaelis–Menten parameters showed that the mutation of S90 to C yielded a 40-fold reduction in V_{max}/K_M for tRNA and a 5-fold reduction in V_{max}/K_M for guanine, while V_{max} was relatively unchanged (ca. 25% lower). Most of the change in V_{max}/K_M for tRNA came from a 30-fold increase in K_M. The enzymic activity of the S90A mutant was too low to determine V_{max} and K_M; however, the V_{max}/K_M for each substrate was reduced by 4 orders of magnitude compared to the wild type. These results indicate that serine 90 is performing a critical role in the TGT reaction.

The *Escherichia coli* tRNA-guanine transglycosylase (TGT;[1] EC 2.4.2.29) is involved in the biosynthesis of the hypermodified tRNA nucleoside queuosine [Q: 7-(((4,5-*cis*-dihydroxy-2-cyclopenten-1-yl)amino)methyl)-7-deazaguanosine]. Queuosine is present in position 34 (the "wobble position") of $tRNA^{Asp,Asn,His,Tyr}$. Its biosynthesis involves a series of unusual and unique steps. It starts at the nucleotide level, presumably with GTP, which is converted to $preQ_1$ [7-(aminomethyl)-7-deazaguanine] (Figure 1) by a series of uncharacterized reactions. $preQ_1$ is inserted into tRNA by TGT, replacing the genetically encoded guanine. A substituted cyclopentyl group is then transferred to the N-atom of the $preQ_1$-tRNA aminomethyl group in an *S*-adenosylmethionine-dependent reaction by the QueA enzyme, resulting in the formation of epoxyqueuosine [oQ: 7-((*N*-(2,3-epoxy-4,5-*cis*-dihydroxycyclopent-1-yl)amino)methyl)-7-deazaguanosine] (Slany *et al.*, 1993). Finally oQ is converted to Q in a vitamin B12-dependent step (Frey *et al.*, 1988) (Figure 1).

In vitro, the *E. coli* TGT recognizes free guanine and will exchange it into tRNA. This ability serves as the basis for a convenient TGT assay, in which the incorporation of commercially available radiolabeled guanine into tRNA is monitored. The tRNA-guanine transglycosylase from *E. coli* has been isolated (Okada & Nishimura, 1979), and the *E. coli tgt* gene has been sequenced and shown to be organized in an operon with a second Q biosynthesis gene, *queA* (Reuter *et al.*, 1991). An overexpression vector for TGT has been constructed that yields ca. 70 mg of homogeneous TGT per liter of cell culture (Garcia *et al.*, 1993). The quaternary structure of TGT is not precisely known, but our previous results (Garcia *et al.*, 1993) suggested that TGT exists as a dimer of trimers. Subsequently, we have shown that TGT is a trimer and that, in the presence of substrate tRNA, this trimer reversibly dissociates into a TGT monomer·tRNA complex that is detectable as a band shift on native PAGE (S. Chong, A. W. Curnow, and G. A. Garcia, unpublished). This dissociation is specific for substrate tRNA, as nonsubstrate tRNA does not cause a band shift.

An *E. coli* mutant described by Noguchi *et al.* (1982) was found to contain tRNAs lacking queuosine due to an inactive tRNA-guanine transglycosylase. In this study we have sequenced the *tgt1* allele from this mutant and show that the loss of activity is due to a single point mutation changing serine 90 to phenylalanine. TGT(S90F) exhibits altered physical properties compared to the wild-type enzyme, suggesting that the introduction of the phenylalanine residue had caused a change in the enzymic conformation. In order

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[1] Abbreviations: TGT, tRNA-guanine transglycosylase; oQ, epoxyqueuosine; Q, queuosine; $preQ_1$, 7-(aminomethyl)-7-deazaguanine; PCR, polymerase chain reaction; DMS, dimethyl suberimidate; SDS, sodium dodecyl sulfate; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; PAGE, polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; DTT, dithiothreitol. Mutant enzymes are referred to according to the pattern TGT(S90F), that is, serine 90 mutated to phenylalanine. Abbreviations for oligonucleotides are defined in Table 1.

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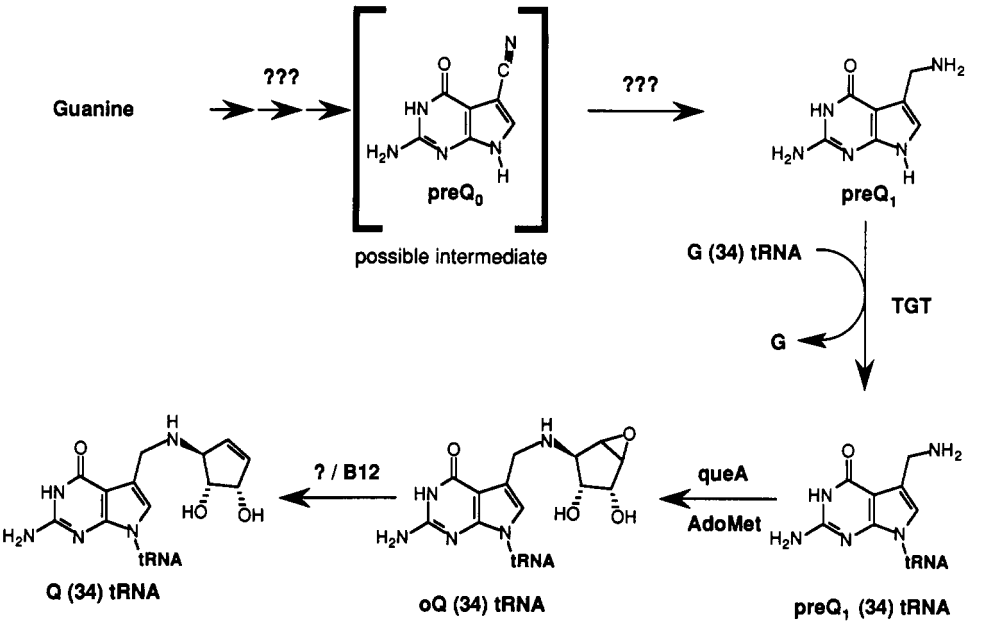

FIGURE 1: Biosynthesis of queuosine–tRNA in *E. coli*.

Table 1: Oligonucleotides Used in Mutagenesis

oligo	sequence (5′ to 3′)[a]
KR1A	G GGG CCG ATC CTC ACC GAC GCC GGC GGC TTC
KR1C	G GGG CCG ATC CTC ACC GAC TGC GGC GGC TTC
KR2	CAG AGA CAT CTC CAT GGA GCG TTT TGC GT
KR3A	T GAA GAC CTG GAA GCC GCC GGC GTC GGT GA
KR3C	T GAA GAC CTG GAA GCC GCC GCA GTC GGT GA
KR4	ACT CAC TAT AGG GAA TTC GAG CTC GGT AC

[a] Mutated codons are underlined.

to further define the role of serine 90 in the TGT reaction, we have used site-specific mutagenesis to replace it by alanine and cysteine. Here we report the characterization of the TGT-(S90A) and TGT(S90C) mutants.

MATERIALS AND METHODS

Reagents. Buffers were purchased from Sigma. Nucleoside triphosphates and helper phage M13KO7 were from Pharmacia. The GeneAmp kit was from Perkin-Elmer. The Sequenase version 2.0 kit was from United States Biochemicals. Restriction endonucleases were from Boehringer Mannheim. [α-^{32}P]dATP and [8-^{3}H]guanine were from Amersham Corp. Centricon-30 concentrators were from Amicon. Oligonucleotides (Table 1) were synthesized at the University of Michigan Biomedical Research Resources Core Facility.

Cloning Procedures. DNA manipulations and restriction fragment isolations were performed according to well-established methods (Sambrook *et al.*, 1989).

DNA Sequence Determination. Sequence analysis was performed by the dideoxy chain termination method using the modified T7 DNA polymerase (Sequenase, USB) following the vendor's protocols. ssDNA served as a template. The *tgt1* allele-containing *Sal*I/*Bam*HI fragment was cloned into M13mp19 for sequencing purposes. pTGT1 is a phagemid derived from pTZ18U (Garcia *et al.*, 1993). ssDNA was produced upon infection of pTGT1-transformed cells with helper phage M13KO7.

Expression and Purification of the tgt1 Gene Product. A 0.8-kb *Bss*HII fragment containing the codon for S90 was replaced in the TGT overexpression plasmid pTGT1 (Garcia *et al.*, 1993) by the corresponding fragment of *tgt1* containing the F90 codon and transformed into SJ1505/pLysS. The transformed *E. coli* strain constitutively expressed the mutant *tgt* gene in high amounts. The mutated TGT was purified according to the protocol of Garcia *et al.* (1993). Because the mutant protein lacks detectable enzymic activity, its enrichment was followed by SDS–PAGE. TGT(S90F) purified differently from wild-type TGT. After ultracentrifugation of the cell lysate at 100000*g*, a significant portion of the mutant TGT was found in the pellet. Therefore the ultracentrifugation step was replaced by a 10000*g* centrifugation leaving TGT-(S90F) in the supernatant. The subsequent 45% $(NH_4)_2SO_4$ saturation of the supernatant resulted not only in the precipitation of contaminating protein, as is the case for wild-type protein, but also in the precipitation of TGT(S90F). Therefore a 40% precipitation was performed during which the majority of TGT(S90F) remained in the supernatant. In the subsequent MonoQ anion-exchange chromatography of the redissolved and dialyzed pellet of the second 70% $(NH_4)_2SO_4$ precipitation, TGT(S90F) eluted at a slightly lower salt concentration than wild-type TGT (0.27 instead of 0.30 M NaCl).

Construction of S90A and S90C Mutants. The construction of the S90A and S90C mutants was performed by a PCR method (Ho *et al.*, 1989). In this protocol two sections of the template overlapping in the region to be mutagenized are amplified (Figure 2). The "inside" primers of both reactions carry the mutation. The respective remaining primer of each reaction is called the "outside" primer. The products of both reactions are mixed, denatured, and allowed to reanneal. Some of the DNA molecules form heteroduplexes overlapping at their 3′ ends. These heteroduplexes are extended and amplified with the outside primers only. The resulting fragment carries suitable restriction sites on each side of the mutation to allow the fragment to be subcloned into the desired plasmid, replacing the corresponding original fragment. In the case of pTGT1, a 560-bp fragment carrying the S90 codon was mutagenized by amplification. This section of DNA could be cloned as an *Eco*RI/*Nco*I fragment. The PCR amplifications were performed with about 100 ng of template DNA according to the vendor's protocol, using 30 cycles. The annealing temperature was 35 °C in all cases. The sequences of the primers used are

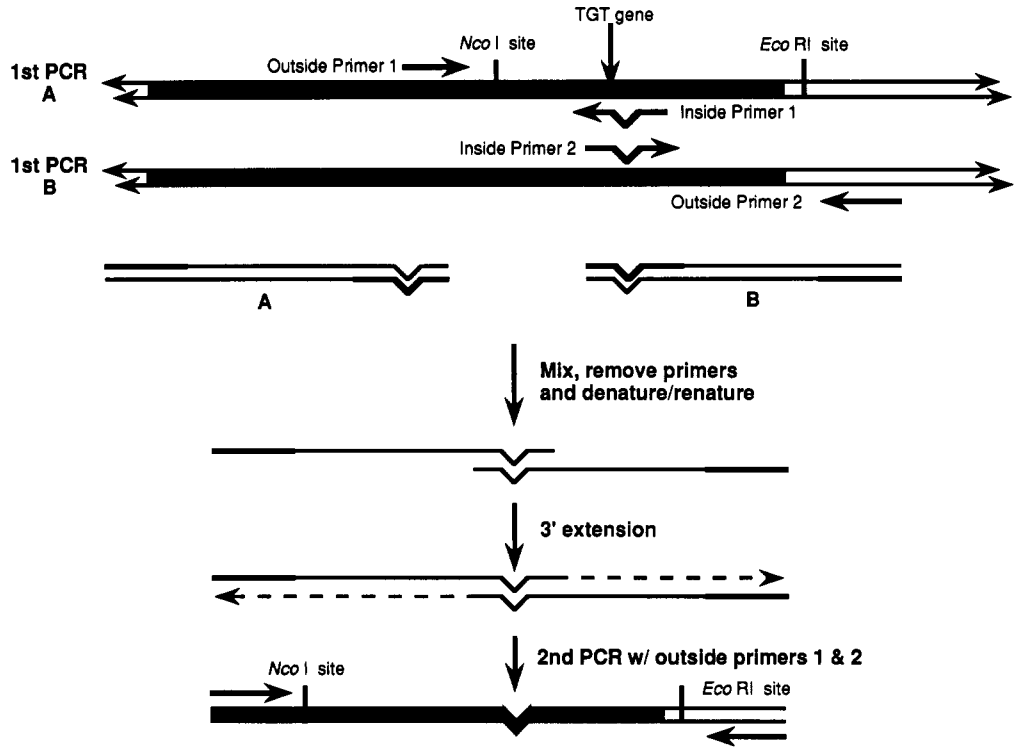

FIGURE 2: Mutagenesis scheme.

given in Table 1. For the first and second PCR reactions pTGT1 served as a template. For the first PCR reaction, inside primer KR1A (containing the A mutation) or KR1C (containing the C mutation) and outside primer KR2 carrying the *Nco*I restriction site were used. For the second, simultaneously performed reaction, inside primer KR3A (A mutation) or KR3C (C mutation) and outside primer KR4 carrying the *Eco*RI site were used. The amplified fragments were phenol extracted and ethanol/ammonium acetate precipitated, leaving the primers and dNTPs in the supernatant. The dried and redissolved PCR products were mixed, denatured, and reannealed. They served as a template for a third PCR amplification with outside primers KR2 and KR4 only. The resulting 580-bp fragments were cut with *Nco*I and *Eco*RI and ligated into *Eco*RI/*Nco*I-digested pTGT1. The ligation mixture was transformed into *E. coli* strain TG2 according to Sambrook *et al.* (1989). Successful mutagenesis was verified by sequence analysis of the complete mutated *tgt* genes allowing the identification of clones with no additional unwanted mutations.

TGT Preparation. Wild-type TGT and mutated forms of TGT were isolated from BL21(DE3)/pLysS or SJ1505/pLysS transformed with the TGT overexpression plasmid pTGT1 or mutated derivatives of pTGT1, with the exception of TGT-(S90F) (see above), as described previously (Garcia *et al.*, 1993). Transformation of cells with pTGT1 leads to a constitutive overproduction of TGT.

Isolation of E. coli tRNA^{Tyr}. G(34)-containing tRNA was extracted from the *tgt*$^-$ mutant *E. coli* strain SJ1505 by anion-exchange chromatography on DEAE cellulose according to standard methods. Specific G(34)-containing tRNA^{Tyr} was prepared by hydrophobic-interaction chromatography on a phenyl-Superose column (Pharmacia) followed by chromatography on benzoylated DEAE-cellulose essentially according to Gillam and Tener (1971).

Chemical Cross-Linking of TGT. Protein samples (0.6 mg/mL) were incubated with freshly prepared 40 mM dimethyl suberimidate (DMS) in 100 mM triethanolamine buffer (pH 8.5) for 2 h at room temperature (Davies & Stark, 1970).

Polyacrylamide Gel Electrophoresis. For denaturing polyacrylamide gel electrophoresis (SDS–PAGE), 2-μL aliquots of the protein samples were added to 2 μL of SDS loading buffer, boiled, applied to an 8–25% gradient Phastgel (Pharmacia), and electrophoresed using SDS–PAGE buffer strips. For native polyacrylamide gel electrophoresis, 4-μL aliquots of enzyme (0.6 mg/mL) and enzyme (0.6 mg/mL) + tRNA (10 μM) were applied to an 8–25% gradient Phastgel and electrophoresed using native buffer strips (Pharmacia). The electrophoresis and Coumassie blue staining were performed following the vendor's protocols.

Kinetic Assays. The K_M determinations were carried out in a reaction mixture containing 100 mM Tris·HCl (pH 7.5), 20 mM $MgCl_2$, 2 mM DTT, and various concentrations of [8-^{3}H]guanine and *E. coli* tRNA^{Tyr} in a total volume of 50 μL. The enzyme concentration was 2 μg/mL ($\sim$50 nM monomer) for wild-type TGT, 4 μg/mL ($\sim$100 nM monomer) for S90C, and 20 μg/mL ($\sim$500 nM monomer) for S90A. The reaction mixtures were incubated at 37 °C, and 10-μL aliquots were taken at various time points. Upon removal from the reaction mixture the aliquots were transferred to filter disks (Schleicher and Schuell) and precipitated with 10% TCA. The filters were washed with 5% TCA and ethanol, dried, and quantitated via liquid scintillation counting. Initial velocities were determined by linear regression of dpm versus time plots. Michaelis–Menten parameters were determined from the average of three replicate determinations of initial velocity data (converted to μM s^{-1} mg^{-1}) by nonlinear regression analysis.

RESULTS

Cloning and Sequence Analysis of the Mutant tgt1 Gene. The *tgt1* gene was cloned from chromosomal DNA of the *E. coli tgt*$^-$ strain SJ1505 as a *Sal*I/*Bam*HI fragment identical to the corresponding fragment of the wild-type chromosome

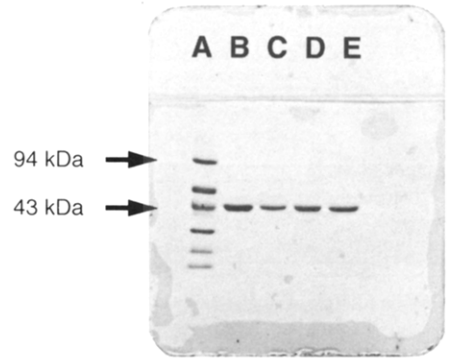

FIGURE 3: SDS–PAGE of TGT preparations. Lane A, MW standards; lane B, TGT(wt); lane C, TGT(S90F); lane D, TGT(S90C); lane E, TGT(S90A).

(Reuter *et al.*, 1991). SJ1505 had been created by P1 transduction of the *tgt1* allele from strain JE7337 (Noguchi *et al.*, 1982) into strain SJ1502 (Frey *et al.*, 1988). *In vitro* expression of the resulting *tgt1*-containing plasmid and subsequent SDS–PAGE showed that the *tgt1* gene product was of the same size as the wild-type tRNA-guanine transglycosylase (data not shown). Sequence analysis revealed that *tgt1* contained a single point mutation resulting in the change of serine 90 to phenylalanine (TCC → TTC).

Purification of the tgt1 Gene Product. The modified purification procedure for TGT(S90F) described in Materials and Methods yielded a mutant enzyme preparation approximately 90% pure as estimated by SDS–PAGE (Figure 3). No significant enzymic activity of TGT(S90F) was detectable. The solubility of TGT(S90F) in TGT reaction buffer was determined to be 14 mg mL^{-1} by concentration via ultrafiltration with Centricon-30 concentrators. The solubility of wild-type TGT amounted to >50 mg mL^{-1}. The altered physical properties of the S90F enzyme compared to those of the wild-type enzyme suggest that the phenylalanine residue not only had inactivated TGT but also had caused some alteration in the conformation of the enzyme. On native PAGE, TGT(S90F) migrates to an apparent M_r of ca. 45 and does not exhibit a band shift when incubated in the presence of tRNA (data not shown).

Construction and Overexpression of TGT(S90A) and TGT(S90C). In order to determine whether S90 is critical for the TGT reaction or whether the loss of activity in S90F was due solely to the alteration of the enzymic conformation, S90A and S90C mutants were constructed. The mutations were introduced into the TGT overexpression plasmid pTGT1 using a PCR-dependent technique described by Ho *et al.* (1989). The TGT genes of the mutated pTGT1 derivatives were completely sequenced to verify the successful mutagenesis and to exclude potential additional mutations. In order to exclude that the characterization of the mutated proteins was impaired by copurified chromosomally encoded wild-type TGT, the mutated pTGT1 derivatives were transformed to the *E. coli tgt*⁻ strain SJ1505/pLysS, which expresses the inactive mutant TGT(S90F). Very small amounts of contaminating chromosomally encoded TGT(S90F) were not thought to interfere with the characterization because of its distinct purification behavior and its lack of activity. A TGT deletion mutant, K12 (Δ*tgt*)/pLysS (F. Ullrich and H. Kersten, unpublished results), transformed with pTGT1 was found not to express TGT in sufficient amounts due to the rapid loss of the expression plasmid (data not shown). To exclude potential host strain-specific artifacts, wild-type pTGT1 was transformed into SJ1505/pLysS also. Although TGT prepared from this host strain did not differ from TGT isolated from BL21(DE3)/pLysS in any of its properties, these transformants served as a source for all wild-type TGT preparations used for the subsequent experiments. By SDS–PAGE of total cell lysates it was verified that all enzyme variants were overproduced in similar amounts (data not shown).

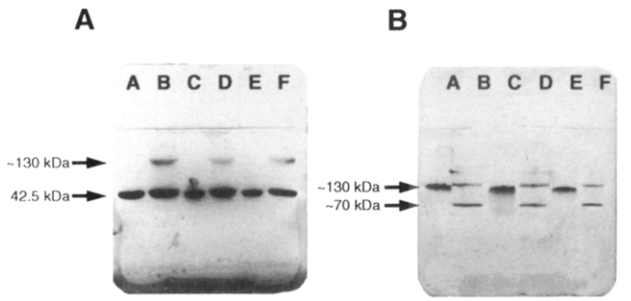

FIGURE 4: SDS–PAGE of DMS-treated TGTs and native PAGE of tRNA binding to TGTs. (A) SDS–PAGE of DMS-treated TGTs. Lane A, TGT(wt); lane B, TGT(wt) + DMS; lane C, TGT(S90C); lane D, TGT(S90C) + DMS; lane E, TGT(S90A); lane F, TGT(S90A) + DMS. TGT samples (0.6 mg/mL) were incubated with 40 mM DMS at room temperature for 2 h. Aliquots (2 μL) were added to 2 μL of SDS sample buffer and applied to an 8–25% gradient Phastgel (Pharmacia). Electrophoresis (SDS buffer strips) and coumassie blue staining were carried out by following the vendor's protocols. (B) Native PAGE of TGT binding to tRNA. Lane A, TGT(wt); lane B, TGT(wt) + tRNA; lane C, TGT(S90C); lane D, TGT(S90C) + tRNA; lane E, TGT(S90A); lane F, TGT(S90A) + tRNA. TGT samples (0.6 mg/mL) were incubated with 100 μM tRNA at 37 °C for 5 min prior to loading. Aliquots (4 μL) were were applied to an 8–25% gradient Phastgel (Pharmacia). Electrophoresis (native buffer strips) and Coumassie blue staining were carried out by following the vendor's protocols.

Purification and Characterization of TGT(S90A) and TGT(S90C). Wild-type enzyme and both mutant TGT enzymes were prepared according to the method of Garcia *et al.* (1993) and were shown to purify in an identical fashion. The solubility of S90A and S90C was >50 mg mL^{-1}, as was the case for wild-type TGT. Although the three-dimensional structure of TGT is not known, we have observed that TGT exists as a trimer (S. Chong, A. W. Curnow, and G. A. Garcia, unpublished). SDS–PAGE of TGT treated with the cross-linking reagent dimethyl suberimidate (DMS) exhibits a band corresponding to a monomer ($M_r \approx 42.5$) and a second band corresponding to a trimer ($M_r \approx 125$). DMS-treated TGT(S90A) and TGT(S90C) showed patterns identical to that of the wild-type (Figure 4).

tRNA Binding to Wild-Type and Mutant TGT. On native PAGE, wild-type TGT migrates to an apparent M_r (~130) corresponding to a homotrimer. When incubated with saturating levels (100 μM) of substrate tRNA, the TGT band shifts to an apparent M_r (~70) consistent with a TGT monomer·tRNA complex (A. W. Curnow and G. A. Garcia, unpublished). Both TGT(S90A) and TGT(S90C) exhibit behavior identical to that of the wild-type TGT on native PAGE (Figure 4B), indicating that both mutants retain the ability to bind tRNA.

Determination of Kinetic Parameters. Kinetic studies were performed with the wild-type, S90C, and S90A enzymes. Plots of initial velocity versus $tRNA^{Tyr}$ and guanine concentrations showed that wild-type TGT and the S90C mutant both followed Michaelis–Menten kinetics (Figure 5). The mutation of S90 to C caused a 30-fold increase in $K_M(tRNA^{Tyr})$. K_M(guanine) was increased by a factor of 4. The mutation barely affected V_{max}, decreasing it by only ca. 25%. The catalytic efficiency represented by V_{max}/K_M was therefore decreased by a factor of ca. 40 for $tRNA^{Tyr}$ and by a factor

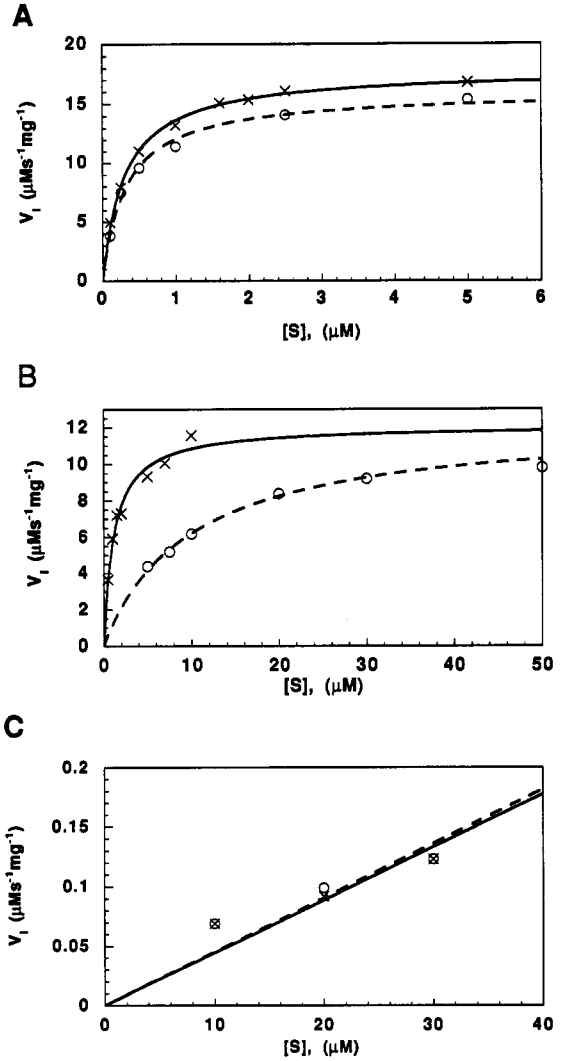

FIGURE 5: Michaelis–Menten plots of wild-type and serine 90 mutant TGTs. The × symbols and solid lines represent the values and fit for varied guanine, and the circle symbols and dashed lines represent the values and fit for varied tRNA. A, TGT(wt); B, TGT(S90C); C, TGT(S90A).

of 5 for guanine compared to the wild type. The enzymic activity of the S90A mutant turned out to be extremely reduced. The very low initial velocities measured even at high substrate and enzyme concentrations had a high error range. This fact made it impossible to determine V_{max} and K_M. However, V_{max}/K_M values for tRNA and guanine were determined from linear regression of initial velocity versus substrate concentration at substrate concentrations below K_M. We estimate that K_M for each substrate must be greater than 30 μM. The kinetic parameters of TGT(wt), TGT(S90C), and TGT(S90A) are shown in Table 2.

DISCUSSION

In the present study it was shown that a single point mutation in the *tgt1* allele of an *E. coli* mutant described by Noguchi *et al.* (1982) caused the mutation of serine 90 to phenylalanine. During the purification of the mutant protein, it became obvious that it not only was inactive but also exhibited physical properties that significantly differed from those of the wild-type protein. Its altered behavior upon ammonium sulfate precipitation and anion-exchange chromatography and its low solubility indicated that the introduction of the phenylalanine residue had caused a conformational change in the enzyme.

Table 2: Kinetic Parameters for TGT (wt), TGT (S90C), and TGT (S90A)[a]

	K_M (μM)	V_{max} (10^{-6} M s^{-1} mg^{-1})	V_{max}/K_M (s^{-1} mg^{-1})
TGT (wt)			
tRNA^{Tyr}	0.32(0.03)	16.0(0.4)	50(3)
guanine	0.30(0.02)	17.8(0.2)	59(3)
TGT (S90C)			
tRNA^{Tyr}	9.7(0.9)	12.3(0.4)	1.3(0.1)
guanine	1.1(0.2)	12.1(0.5)	11(1.5)
TGT (S90A)			
tRNA^{Tyr}	nd	nd	0.0045(0.0005)
guanine	nd	nd	0.0044(0.0007)

[a] The numbers in parentheses are the standard errors of the fits. nd = not determinable. The kinetic parameters were calculated from an average of three sets of initial velocity determinations for each mutant and substrate combination.

In order to determine whether the hydroxyl group of serine 90 is of any importance in catalysis, we have constructed two mutants, serine 90 to alanine (S90A) and serine 90 to cysteine (S90C). The purification behavior, solubility, and quaternary structure of TGT(S90A) and TGT(S90C) did not differ from those of wild-type TGT, indicating that the overall, gross conformation has been maintained in these mutants. Both mutants also bind substrate tRNA, as shown in the native PAGE band shift assays (Figure 4B). We have previously determined by Coumassie blue and ethidium bromide staining that the band at $M_r \sim 70$ contains both protein and nucleic acid (A. W. Curnow and G. A. Garcia, unpublished). Transfer RNA migrates to an apparent M_r of ~25 under these conditions. This band shift phenomenon is specific to substrate tRNA and is reversible. Its significance is currently under investigation.

The V_{max} of the wild-type TGT determined in this report is similar to that reported by Curnow *et al.* (1993) (15×10^{-6} Ms^{-1} mg^{-1} after correcting for the different aliquot volumes and converting units). The K_M values determined in this study differ by a factor of 7 for tRNA^{Tyr} and 3 for guanine from the values reported by Curnow *et al.* (1993). This difference may be explained by the different buffer conditions used in the two studies and the different tRNA^{Tyr} preparations.

The S90C mutant exhibits a 40-fold reduction in V_{max}/K_M for tRNA and a 5-fold reduction in V_{max}/K_M for guanine, while V_{max} is relatively unchanged (ca. 25% lower). Most of the change in V_{max}/K_M for tRNA comes from a 30-fold increase in K_M. Due to the very low activity of the S90A mutant, V_{max} and K_M values could not be determined. However, estimates of V_{max}/K_M have been determined from linear regression of initial velocities versus substrate concentration in the range below K_M. The TGT(S90A) V_{max}/K_M values for tRNA and for guanine are very similar and approximately 4 orders of magnitude lower than those for wild-type TGT. These data indicate that serine 90 is performing a critical role in the TGT reaction that can be partially fulfilled by cysteine.

It is intriguing to speculate that a serine protease-type mechanism may obtain for the TGT reaction. In such a mechanism, serine 90 would displace guanine 34, forming a covalent enzyme·tRNA complex. The serine would then be displaced by the incoming base (in this case the radiolabeled guanine, *in vivo* preQ$_1$). The extremely low activity of the S90A mutant and the fact that both mutants are seen to bind tRNA in the band shift assays (which were performed under saturating concentrations of tRNA) are consistent with this interpretation. The active site serine of rat trypsin has been mutated to cysteine (Higaki *et al.*, 1989), resulting in a 10^6-fold loss in k_{cat}. An X-ray crystal structure indicates that the

sulfhydryl of the mutant trypsin is occluding the oxyanion hole that stabilizes the transition state (McGrath *et al.*, 1989). This is postulated to account for the diminution in k_{cat}. For TGT, there is no need for an oxyanion hole because the transition state (with a guanine leaving group) would not be expected to resemble the oxyanion transition state of trypsin. Therefore, one might expect a much smaller effect on k_{cat} (in our case V_{max}) upon mutation of the TGT serine 90 to cysteine since there is no oxyanion hole to occlude. This would also explain the observed insensitivity of TGT to the classical serine protease inhibitor phenylmethanesulfonyl fluoride (PMSF), which requires an oxyanion hole for activity. The fact that K_M(tRNA) is affected to a much larger extent than V_{max} may be due to a steric effect of the much larger sulfur of cysteine versus the oxygen of serine.

Alternatively, the TGT serine 90 may simply bind to and help orient the tRNA substrate in the TGT active site. Mutagenesis studies of aspartate 229 in thymidylate synthase have been reported recently (Liu & Santi, 1993). The authors concluded that aspartate 229 is involved in the proper orientation of the dUMP substrate and facilitates catalysis, although it is not absolutely required for catalysis. Substitution of the TGT serine 90 hydroxyl by a thiol (*e.g.*, the cysteine mutant) may decrease the tRNA binding affinity, but may still allow for proper orientation of the tRNA. This is consistent with a small change in V_{max} and a large increase in K_M (tRNA). Removal of the serine hydroxyl group (*e.g.*, the alanine mutant) would then be expected to have a large effect upon both binding and orientation of tRNA, consistent with the greatly decreased V_{max}/K_M that we have observed. Further experiments to distinguish between these possible roles for serine 90 are in progress.

It seems that serine 90 is located in a functionally significant region of the enzyme. The *tgt* gene of the Gram-negative bacterium *Zymomonas mobilis* shows only about 50% identity to the *E. coli tgt* gene at the amino acid level. However, Serine 90 is conserved in this gene and is found exactly in the middle of a stretch of 11 conserved amino acids (K. Reuter and R. Ficner, unpublished results).

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