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Cysteine 265 Is in the Active Site of, But Is Not Essential for Catalysis by tRNA-Guanine Transglycosylase (TGT) from *Escherichia coli*

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Site-directed mutagenesis and X-ray absorption spectroscopy studies have previously shown that the tRNA-guanine transglycosylase (TGT) from *Escherichia coli* is a zinc metalloprotein and identified the enzymic ligands to the zinc [Chong *et al.* (1995), *Biochemistry* **34,** 3694–3701; Garcia *et al.* (1966), *Biochemistry* **35,** 3133–3139]. During these studies one mutant, TGT (C265A), was found to exhibit a significantly lower specific activity, but was not found to be involved in the zinc site. The present report demonstrates that TGT is inactivated by treatment with thiol reagents (*e.g.*, DTNB, MMTS, and N-ethylmaleimide). Further, this inactivation is shown to be due to modification of cysteine 265. The kinetic parameters for the mutants TGT (C265A) and TGT (C265S), however, suggest that this residue is not performing a critical role in the TGT reaction. We conclude that cysteine 265 is in the active site of TGT, but is not performing a critical catalytic function. This conclusion is supported by the recent determination of the X-ray crystal structure of the TGT from *Zymomonas mobilis* [Romier *et al.* (1966), *EMBO J.* **15,** 2850–2857], which reveals that the residue corresponding to cysteine 265 is distant from the putative catalytic site, but is in the middle of a region of the enzyme surface proposed to bind tRNA.

KEY WORDS: Queuine; tRNA modification; chemical modification; cysteine; mutagenesis.

1. INTRODUCTION

tRNA-guanine transglycosylase (TGT)[4] is a key enzyme involved in the post transcriptional modification of tRNA with the hypermodified base queuine (7-(4,5-*cis*-dihydroxy-1-cyclopenten-3-yl aminomethyl)-7-deazaguanine). In *Escherichia coli* the enzyme catalyzes the exchange of a queuine precursor ($preQ_1$, 7-aminomethyl-7-deazaguanine) into tRNA (Okada *et al.*, 1979). This precursor is further modified to queuine by two subsequent enzymatic steps (Nishimura, 1983).

An *E. coli* mutant described by Noguchi *et al.* (1982) contains tRNA lacking the hypermodified wobble nucleoside queosine (Q) due to an inactive tRNA-guanine transglycosylase (TGT). The mutant *tgt* gene was cloned and sequenced; it contained a single point mutation resulting in the change of serine 90 to phenylalanine (Reuter *et al.*, 1994). Studies of TGT (S90A) and TGT (S90C) mutants indicate that serine 90 is performing a critical role in the TGT reaction (Reuter *et al.*, 1994). Homology of the serine 90 site (DSGG) to the active-site serine of trypsin suggests that TGT may

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[4] Abbreviations: TGT, tRNA-guanine transglycosylase; DTNB, dithio-bis-nitrobenzoic acid; DTT, dithiothreitol; MMTS, methylmethanethiosulfonate, NEM, N-ethylmaleimide.

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utilize a serine protease-type nucleophilic catalysis mechanism.

Our early, unpublished observations that the *E. coli* TGT was susceptible to thiol reagents led us to the discovery that TGT is a zinc metalloenzyme. Site-directed mutagenesis studies (Chong *et al.*, 1995) established that cysteines 302, 304, and 307 constitute three of the four TGT ligands to the zinc. These studies in addition to X-ray absorption studies (Garcia *et al.*, 1996) suggested that histidine 317 served as the fourth ligand to the zinc. The very recent determination of the X-ray crystal structure of the TGT from *Zymonomas mobilis* (Romier *et al.*, 1996) confirmed the identity of the three cysteine zinc ligands. However, the residue corresponding to histidine 333 was found to be the fourth ligand to the zinc. Histidine 317 was proposed to be involved in the subunit interface in the *E. coli* TGT, accounting for our observations. Other than the three zinc-ligand cysteines, only one of the eight cysteine-to-alanine mutations yielded a TGT exhibiting a significant reduction in activity. However, this mutant, C265A, was identical to wild-type TGT in quaternary structure and zinc content, and retained the ability to bind tRNA.

These observations led to the present study, which establishes that the *E. coli* TGT can be inactivated by treatment with thiol reagents. The results suggest that cysteine 265 is the residue that is responsible for the inactivation and therefore must be in the TGT active site. However, the activities of the cysteine 265 mutants suggest that this residue is not performing a critical role in catalysis. These conclusions are supported by TGT·tRNA complex modeling experiments that suggest that cysteine 265 is on a region of the surface of TGT where tRNA binds (Romier *et al.*, 1996).

2. MATERIALS AND METHODS

Buffers were from Sigma. The tRNA used (*E. coli* $tRNA^{Tyr}$) was prepared from an *in vitro* expression system as described previously (Curnow *et al.*, 1993).

2.1. TGT (wt) and TGT (C265A) Preparation

TGT (wt) was purified as described previously (Chong and Garcia, 1994*b*; Garcia *et al.*, 1993). The TGT (C265A) mutant was prepared as described previously (Chong *et al.*, 1995).

2.2. Construction, Overexpression, and Purification of TGT (C265S)

TGT (C265S) was generated by the "preferential PCR" method (Chong and Garcia, 1994*a*). The mutagenic primer was

5′-GGTTGGCATTACGGAGTCAAACATATC-3′

(mutated codon underlined). The wild-type and mutant TGTs were overexpressed in a *tgt*$^-$ strain of *E. coli, E. coli* K12 (Δ*tgt*)/pLysS, provided by Prof. H. Kersten (University of Erlangen-Nürnberg). The overexpression and purification were performed following the protocols described previously (Chong *et al.*, 1995).

2.3. Kinetic Assays

Assays to determine the kinetic parameters for the tRNA substrate were carried out in a 300-μl reaction mixture containing 100 mM HEPES, *p*H 7·5, 20 mM $MgCl_2$, 5 mM DTT, 40 μM [8-^{14}C]guanine, and from 0.24 to 26 μM tRNA. The enzyme concentration was 173 nM for TGT (C265A), 85 nM for TGT (C265S), or 62 nM for TGT (wt). The assays for the determination of the kinetic parameters for guanine were carried out in the same buffer system in the presence of 20 μM tRNA (for C265A and wild-type) or 26 μM (for C265S). The concentration of [8-^{14}C]guanine was varied from 0.05 to 20 μM. The reaction mixtures were incubated at 37°C and aliquots (70 μl) were taken at various time points. Initial velocities were determined by linear regression of dpm versus time plots. Michaelis–Menten parameters were determined from the initial velocities by nonlinear regression analysis.

2.4. Physical Characterization of TGT Mutants

The purified TGT (C265A) and TGT (C265S) mutants were characterized by SDS-PAGE, native PAGE [including the tRNA "bandshift" assay on native-PAGE (Curnow and Garcia, 1994)], zinc content, and CD spectra following the procedures described previously (Chong *et al.*, 1995). Enzyme concentrations were determined spectrophotometrically using an absorption coefficient of

1.14 mg/mL/OD_{280} previously corrected by total amino acid analysis (Chong *et al.*, 1995). For the native-PAGE bandshift assay 10 μM TGT (wt), 1.5 μM TGT (C265A), and 2 μM TGT (C265S) were incubated either in buffer (25 mM HEPES, *p*H 7.5, 5 mM DTT) alone (odd lanes) or with 50 μM tRNA (even-numbered lanes) for 15 min at 37°C prior to applying 5 μl of each sample to the gel. The native-PAGE was run on an 8–25% gradient gel following the vendor's protocols (Pharmacia Phastsystem).

2.5. Inhibition of TGT Activity by Thiol Reagents

TGT (wt) was depleted of exogenous thiols (*i.e.*, DTT) by desalting into degassed 25 mM HEPES buffer (*p*H 7.8) using a Fast Desalting column (HR 10/10, Pharmacia) or by dialyzing against the degassed 25 mM HEPES buffer (*p*H 7.5). TGT (wt), 7.6 μM in a final volume of 50 μl, was incubated at room temperature with increasing amounts of thiol reagents (from 1 to 17.5 μM) for 10 min (in the case of DTNB and MMTS) or 90 min (in the case of N-ethylmaleimide). The reaction was stopped by diluting the reaction mixture 100-fold. The specific activity of the diluted enzyme was determined using standard buffer conditions (see above) and saturating concentrations of guanine and tRNA. These values were plotted against the mole ratio of reagent versus enzyme (Fig. 3A). As a reference, TGT specific activity was determined under the same conditions in the absence of thiol reagent and was set to 100%.

2.6. DTT Reversal of Thiol Reagent Inhibition of TGT Activity

The experiment was performed as above except that only one concentration (15 μM) of the thiol reagent was used and 10 mM DTT was present during the dilution of the reaction mixture and in the activity assay (Fig. 3B).

2.7. Substrate Protection of TGT from the Inhibition by Thiol Reagents

TGT (15.2 μM) was first incubated with 40 μM tRNA at 37°C for 20 min. The substrate-protected TGT was then incubated with 15 μM thiol reagent (7.6 μM enzyme) for 60 min. The reaction was stopped by diluting the reaction mixture 100-fold. The TGT activity was then determined as above (Fig. 3B).

2.8. DTNB Titration of TGT (wt) and TGT (C265S)

The method used was according to Habeeb (1971) with some modifications. TGT (wt) (6 μM) and TGT (C265S) (4.5 μM, depleted of exogenous thiols as described above for wt), prepared as described above, were individually mixed with DTNB (1 mM in 25 mM HEPES, *p*H 7.8) and the absorbance at 412 nm was immediately followed as a function of time in a Gilford Response® UV/VIS spectrophotometer. The net absorbance was converted into concentration using the molar extinction coefficient for the nitrobenzoate thiolate chromophore of 13,600 OD M^{-1} cm^{-1} at 412 nm. The number of the titrated sulfhydryl groups per monomer TGT was plotted as a function of titration time (Fig. 4).

The specific activities of DTNB-modified wild-type and mutant enzymes were also determined. TGT (wt) (7.5 μM) or TGT (C265S) (9 μM), in a final volume of 20 μl, were incubated at room temperature with increasing amounts of DTNB [3.7, 7.5, and 15 μM for wild-type TGT, 4.5, 9, 18 μM for TGT (C265S)] for 10 min. Three replicate determinations were performed at each DTNB concentration. The averages of these determinations were then plotted versus mole ratio DTNB (Fig. 5).

3. RESULTS

3.1. Overexpression and Purification of TGT (C265S)

The TGT (C265S) mutant was generated by a "preferential PCR" method (Chong and Garcia, 1994*a*). The mutated TGT gene insert was sequenced to verify successful mutagenesis and the absence of adventitious mutations. TGT (C265S) was overexpressed in the *E. coli* K12 (Δ*tgt*)/pLysS strain to exclude wild-type TGT contamination. The mutant protein expression level was similar to that of wild-type TGT (data not shown). TGT (C265A) and TGT (C265S) mutants were purified to homogeneity as previously described (Chong *et al.*, 1995).

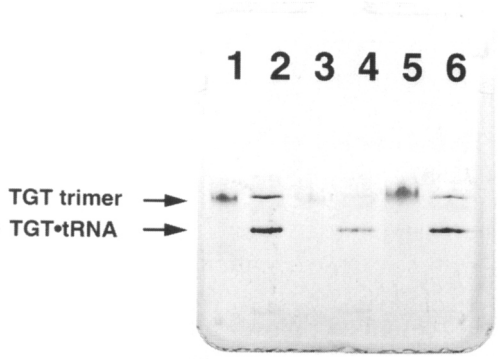

Fig. 1. Native-PAGE bandshift assay. Lanes 1 and 2; TGT (wt); 3 and 4; TGT (C265A); 5 and 6, TGT (C265A), in the absence (odd-numbered lanes) and presence (even-numbered lanes) of tRNA.

3.2. Physical Characterization of TGT (C265A) and TGT (C265S)

Native-PAGE (Fig. 1) showed that both mutants retained the trimeric gross structure identical to the wild-type enzyme and exhibited a bandshift after incubation with the substrate tRNA, indicating that they retained the ability to bind tRNA. The zinc contents of these two mutants were determined to be 0.82 ± 0.04 per monomer enzyme for C265A and 0.85 ± 0.03 per monomer enzyme for C265S. Wild-type TGT contains from 0.76 to 0.85 zinc atoms per monomer, varying with the preparation (Chong *et al.*, 1995).

The CD spectra of TGT (C265A) and TGT (C265S) were very similar to that of wild-type TGT (Fig. 2). The secondary structural elements were predicted following the method of Johnson (1990) from the CD data using Young's Protein Secondary Structure Program (Jasco Inc.). The TGT (C265A) mutant was predicted to have 65% α-helix, no β-sheet, 21% β-turn, and 14% random coil, whereas TGT (C265S) was predicted to have 79% α-helix, no β-sheet, 16% β-turn, and 6% random coil. These predicted values are very similar to those for wild-type TGT, which were found previously to be $70 \pm 3\%$ α-helix, no β-sheet, $19 \pm 1\%$ β-turn, and $12 \pm 1\%$ random coil (Chong *et al.*, 1995).

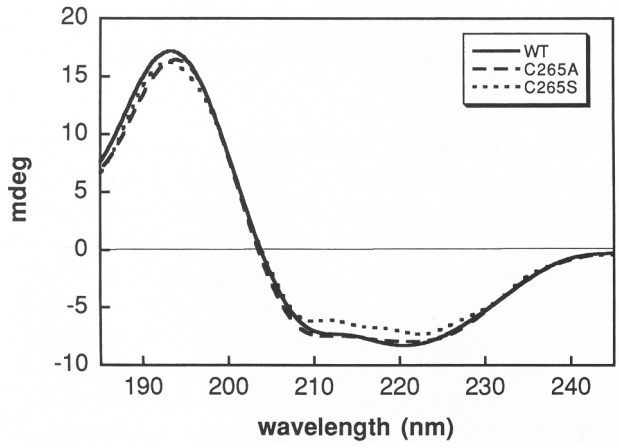

Fig. 2. CD spectra of TGT (wt), TGT (C265S), and TGT (C265A).

3.3. DTNB Titration of TGT (wt) and TGT (C265S)

Modification of wild-type TGT with ca. 1.5 equivalents of DTNB completely inactivates the enzyme (Fig. 3A). Similar results are achieved when methylmethanethiosulfonate (MMTS) or N-ethylmaleimide (NEM) are used (Fig. 3A). Preincubation of enzyme with substrate tRNA protects TGT from inactivation by treatment with thiol reagents (Fig. 3B). In the cases of DTNB and MMTS treatment, inactivation can be reversed by the addition of dithiothreitol (Fig. 3B).

Treatment of TGT (wt) with excess DTNB under native conditions titrates two thiols immediately and a third thiol is titrated after ca. 15 min (Fig. 4). Under denaturing conditions (3% SDS), two to three thiols are titrated immediately and a total of ca. eight thiols are titrated within 5 min (data not shown). This is consistent with the number of cysteines (eight per monomer) predicted from the *tgt* gene sequence. Treatment of TGT (C265S) with excess DTNB under native conditions yields one less thiol titrated than does TGT (wt) treated in an identical fashion (Fig. 4).

3.4. DTNB Inactivation of TGT (wt) and TGT (C265S)

DTNB titrations of TGT (wt) and TGT (C265S) were conducted and the specific activities of the treated enzymes were determined at various concentrations of DTNB. The results (Fig. 5) indicate that TGT (C265S) retained more than 75% of its activity when the enzyme was treated with two equivalents of DTNB. Under the same conditions wild-type enzyme retained only 2% of its original activity.

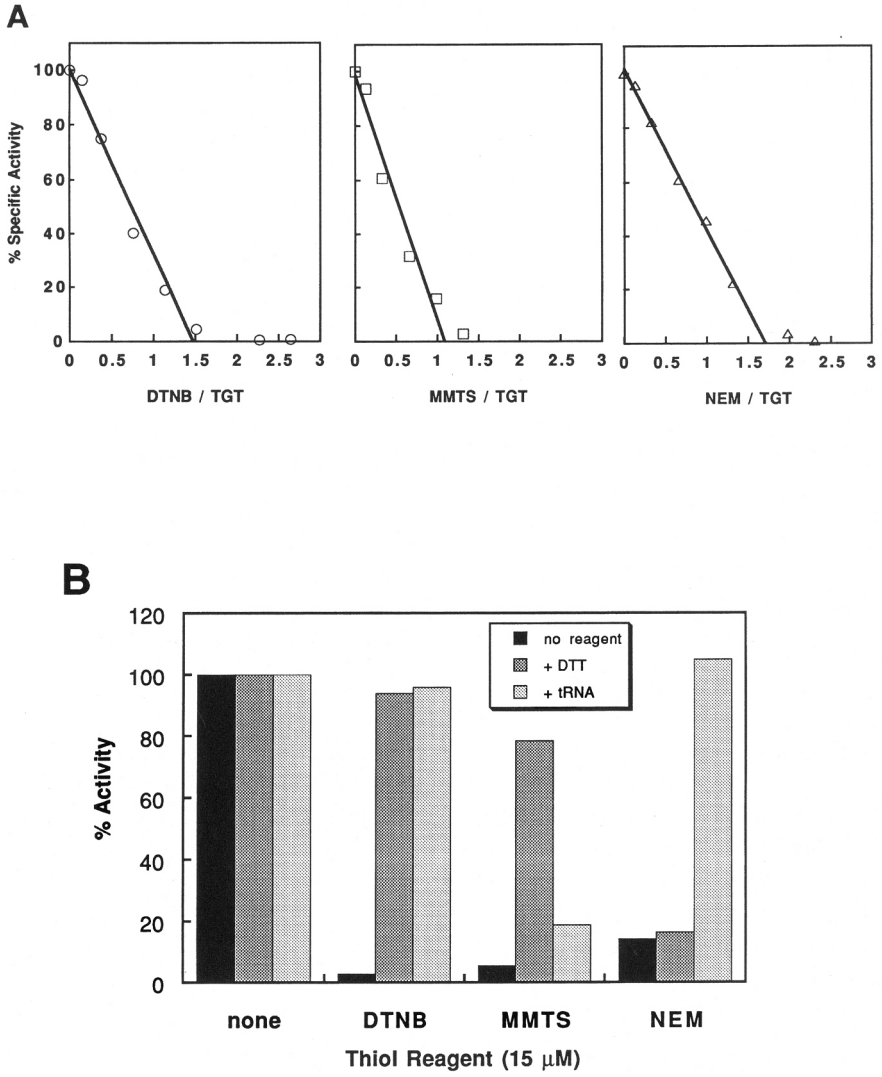

Fig. 3. Inactivation of TGT (wt%) by thiol reagents. (A) Inhibition of TGT activity by thiol reagents. The lines are linear fits of the initial portions (the first five or six points) of the titrations. (B) Effect of adding DTT and the substrate tRNA on the inhibition by thiol reagents. The data plotted are averages of at least two replicate experiments.

3.5. Determination of Kinetic Parameters of TGT Mutants

The kinetic parameters of TGT (C265A) and TGT (C265S) were determined. Compared to wild-type TGT, the mutation of cysteine 265 to alanine did not cause a large change in K_M for either substrate (Table I). The mutation decreased V_{max} to ca. 20% of the value for the wild-type. The catalytic efficiency represented by V_{max}/K_M was decreased by ca. 90% for $tRNA^{Tyr}$ and 70% for guanine compared to the wild-type. The mutation of cysteine 265 to serine resulted in only small changes in K_M and V_{max}. Compared to the result for the wild-type, K_M for $tRNA^{Tyr}$ increased 1.5-fold and K_M for guanine twofold. The mutant retained approximately 70% of the wild-type V_{max}.

4. DISCUSSION

The primary sequence of TGT from *E. coli* contains eight cysteine residues in the monomer. In the native state two equivalents of cysteine are immediately titrated and a third cysteine is titrated

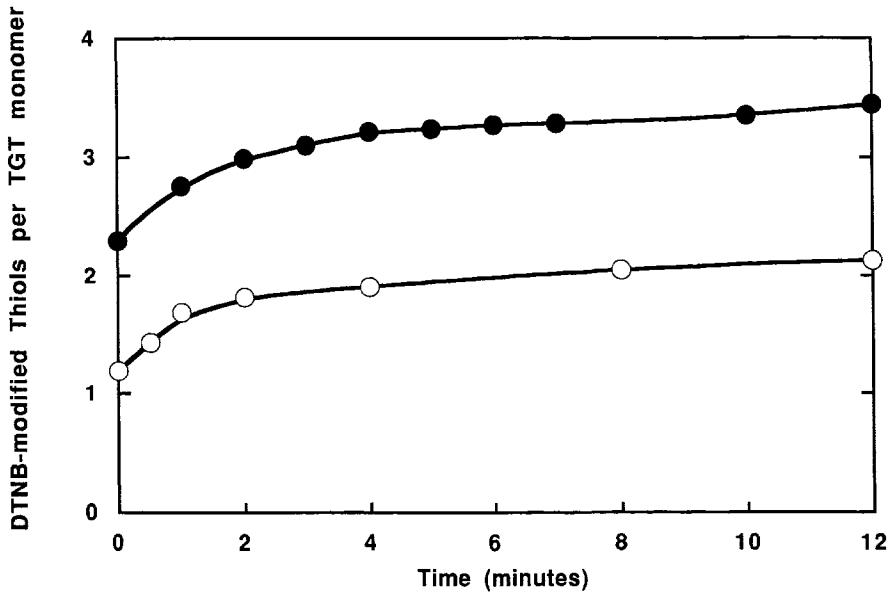

Fig. 4. DTNB titration of TGT (wt) and TGT (C265S). TGT (wt) is represented by the filled circles and TGT (C265S) is represented by the open circles. Samples were titrated under conditions of excess DTNB as described in Materials and Methods.

within 20 min (Fig. 4). All three thiol reagents (DTNB, MMTS, and NEM) were found to inhibit the activity of TGT. In order to determine if there was a linear relationship between the amount of thiol reagents added and their capacity to inhibit TGT, the percentage activity was plotted as a function of the amount of thiol reagent added per mole of the enzyme. As shown in Fig. 3A, TGT is completely inactivated by treatment with between one and two equivalents of thiol reagent. The near-complete reversal of DTNB and MMTS inhibition of the TGT activity by treatment with DTT (Fig. 3B) is consistent with the formation of intermolecular disulfide bonds between TGT and the reagents. In the case of N-ethylmaleimide, treatment with DTT did not reverse the inhibition, consistent with the formation of a thio-ether bond between TGT and this reagent (Fig. 3B). Substrate tRNA fully protected TGT from inhibition by DTNB and N-ethylmaleimide, and 75% protection was achieved in the case of MMTS (Fig. 3B). These results suggest that the cysteine residue titrated by the thiol reagents is located in or very near the active site of TGT and may be involved in the catalysis of the TGT reaction.

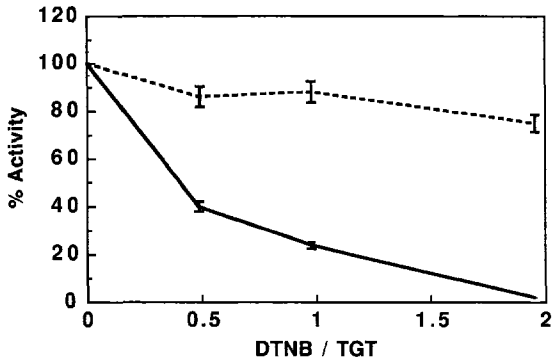

Fig. 5. Effect of DTNB modification on the activities of TGT (wt) and TGT (C265S). The solid line is for wild-type TGT and the dashed line is for TGT (C265S). The data were obtained from the average of three replicate determinations at each DTNB concentration. The standard deviations are represented by error bars (some are too small to be visible). For ease of comparison the activities plotted are relative to the specific activity of the untreated enzyme. Note that the absolute specific activity of TGT (C265S) is ca. 50% of that for the wild type.

Of the eight cysteine-to-alanine TGT mutants studied previously (Chong *et al.*, 1995) TGT (C265A) was the only mutant that had a significant loss in activity while still maintaining the wild-type zinc content and gross structure. Cysteine 265 is conserved in the TGT genes from *E. coli* and *Z. mobilis,* which share only 50% identity at the amino acid level (Reuter and Ficner, 1995). It is also located in the middle of a stretch of 12 conserved amino acids. It was therefore speculated that cysteine 265 might serve a key catalytic role in the TGT reaction mechanism. Changing cysteine 265 into alanine results in only a tenfold decrease in

Table I. Kinetic Parameters for TGT (wt), TGT (C265S), TGT (C265A)[a]

		K_M (μM)	V_{max} (μM sec^{-1} mg^{-1})	V_{max}/K_M (sec^{-1} mg^{-1})	Ratio of V_{max}/K_M to wt value
tRNA (ECY2)	wt	2.0 (0.12)	11.0 (0.2)	5.5 (0.5)	1
	C265S	2.8 (0.3)	8.0 (0.2)	2.9 (0.4)	0.5
	C265A	2.5 (0.4)	1.8 (0.1)	0.7 (0.2)	0.1
Guanine	wt	1.2 (0.13)	10.1 (0.3)	8.4 (1.3)	1
	C265S	2.5 (0.5)	7.2 (0.5)	2.9 (0.8)	0.4
	C265A	0.8 (0.1)	2.0 (0.1)	2.5 (0.5)	0.3

[a] Standard deviations are shown in parentheses.

the catalytic efficiency (V_{max}/K_M), whereas a previously described serine-90-to-alanine mutation causes approximately four orders of magnitude decrease in activity (Reuter *et al.*, 1994). Kinetic studies on TGT (C265S) show that this mutant enzyme retains 70% of the wild-type V_{max}, V_{max}/K_M for tRNA is 50% of the wild-type value, and V_{max}/K_M for guanine is 40% (Table I). This does not support a key catalytic role for cysteine 265 in the TGT reaction.

If cysteine 265 is the enzymic residue that is modified by the thiol reagents leading to inactivation of wild-type TGT, then one would expect that the C265S mutant would no longer be susceptible to inactivation by treatment with thiol reagents. Figure 5 shows that the activity of TGT (C265S) does not change significantly when treated with varying amounts of DTNB. Under the same conditions, wild-type TGT is essentially totally inactivated by the addition of two equivalents of DTNB. These studies indicate that it is DTNB modification of cysteine 265 in wild-type TGT which results in the inactivation of the enzyme. This suggests that cysteine 265 is located in or very near the active site. However, the activities of the C265S and C265A mutants clearly indicate that this residue is not performing a key role in catalysis. The higher activity of the C265S mutant relative to the C265A mutant suggests that polarity or hydrogen bonding ability may be involved in the role of cysteine 265.

These conclusions are supported by the recently published X-ray crystal structure of the TGT from *Z. mobilis* (Romier *et al.*, 1996), which reveals that cysteine 265 is on the surface of the enzyme. A model of tRNA bound to the enzyme (the structure of the TGT·tRNA complex was not determined) suggests that cysteine 265 lies underneath the tRNA and may contribute to tRNA binding. The authors state that cysteine 265 is too far from the preQ$_1$ binding site to have a role in catalysis. The model of the TGT·tRNA complex is entirely consistent with the results presented here, which include substrate protection of the enzyme against thiol reagents. The structural model is also consistent with the conclusion that the enzyme is inactivated by chemical modification of cysteine 265 with thiol reagents because they impair the enzyme's ability to bind tRNA.

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