tRNA-Guanine Transglycosylase from *Escherichia coli* Is a Zinc Metalloprotein. Site-Directed Mutagenesis Studies To Identify the Zinc Ligands†

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ABSTRACT: tRNA-guanine transglycosylase (TGT) from *Escherichia coli* catalyzes the exchange of the queuine precursor, $preQ_1$, into tRNA as part of the biosynthetic pathway for the posttranscriptionally modified base, queuine. No significant sequence homologies exist between TGT and any of the proteins in the GenBank database. However, an unusual arrangement of cysteine residues was observed upon manual examination of the TGT sequence. Comparison of this sequence (residues 302−321) revealed similarities to structural zinc-binding motifs in proteins of known structure [Jaffe (1993) *Comments Inorg. Chem. 15*, 67−93]. Within this region of the TGT sequence, there are six residues (four cysteines and two histidines), any four of which could serve as the ligands to the zinc. We report here that wild-type TGT contains ca. 0.8 mol of zinc/mol of subunit, determined by atomic emission spectrometry. In order to determine which enzyme residues are serving as the ligands to the zinc, site-directed mutagenesis studies have been performed. Gross structural probes (native PAGE and CD spectra), enzyme activity assays, and tRNA-binding assays indicate that cysteines 302, 304, and 307 and histidine 317 are the ligands to the zinc. These results also suggest that the zinc site is necessary for TGT homotrimer formation and for tRNA binding.

A key step in the posttranscriptional modification of tRNA with queuine in *Escherichia coli* is the exchange of the queuine precursor, $preQ_1$, into tRNA, replacing the genetically encoded guanosine 34 in the anticodon (Kersten & Kersten, 1990). This reaction is catalyzed by tRNA-guanine transglycosylase (TGT,[1] EC 2.4.2.29) (Okada & Nishimura, 1979; Singhal, 1983). Early in our studies of recombinant-generated TGT we found that the addition of DTT was necessary to protect against loss of enzymic activity (Garcia *et al.*, 1993). This suggested to us that one or more cysteine residues were likely to be important for either catalysis or for maintenance of the enzyme structural integrity.

The *E. coli tgt* gene has been sequenced and was found to code for a 42.5 kDa protein that contains eight cysteine residues (Reuter *et al.*, 1991). No significant sequence homologies were found between *E. coli* TGT and any of the proteins in the GenBank database (Reuter *et al.*, 1991). However, close examination of the TGT sequence reveals an unusual arrangement of cysteine residues (residues 302−321, Figure 1). Compared with the zinc-binding sites of proteins with known structures (Table 1), the cysteine-clustered region in TGT resembles a structural zinc-binding site rather than a catalytic zinc site. Within this region of TGT there are six residues (Cys 302, Cys 304, Cys 307, His 316, His 317, and Cys 321) that could serve as zinc ligands. On the basis of homology alone, any four of these six TGT residues could constitute a zinc-binding motif. The *tgt* gene from the Gram-negative bacterium *Zymomonas mobilis* has recently been sequenced and was found to have ca. 50% identity at the amino acid level with the *tgt* gene from *E. coli* (K. Reuter and R. Ficner, unpublished). The *Z. mobilis* TGT gene contains only five cysteines, compared to the eight cysteines in the *E. coli* gene. All five of the *Z. mobilis* cysteines are conserved with the *E. coli* TGT (Figure 1), including three of the cysteines in the putative zinc-binding region, C145 and C265. The two histidines (H316 and H317) in the putative zinc-binding region are also conserved.

We report here that TGT isolated from an overexpressing clone (Chong & Garcia, 1994b; Garcia *et al.*, 1993) does contain ca. 0.8 mol of zinc/mol of enzyme monomer. In order to determine which enzymic residues are liganded to the zinc, site-directed mutagenesis experiments have been performed. Gross structural probes (native PAGE and CD spectra), enzyme activity assays, and tRNA-binding assays indicate that cysteines 302, 304, and 307 and histidine 317 constitute the four ligands to the zinc. These results also suggest that the zinc site is necessary for TGT homotrimer formation and for tRNA binding.

MATERIALS AND METHODS

Buffers, 5,5′-dithiobis(2-nitrobenzoic acid), *o*-phenanthroline, and *p*-(hydroxymercuri)benzenesulfonic acid were purchased from Sigma. Chelex membrane filters and Chelex resin were purchased from Bio-Rad. [8-^{14}C]Guanine was from Moravek Biochemicals. Wild-type TGT was prepared from overexpressing clones (pTGT5 and pTGT1) as de-

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[1] Abbreviations: TGT, tRNA-guanine transglycosylase; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; PMBS, *p*-(hydroxymercuri)-benzenesulfonic acid; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; ICP-AES, inductively coupled plasma atomic emission spectrometry.

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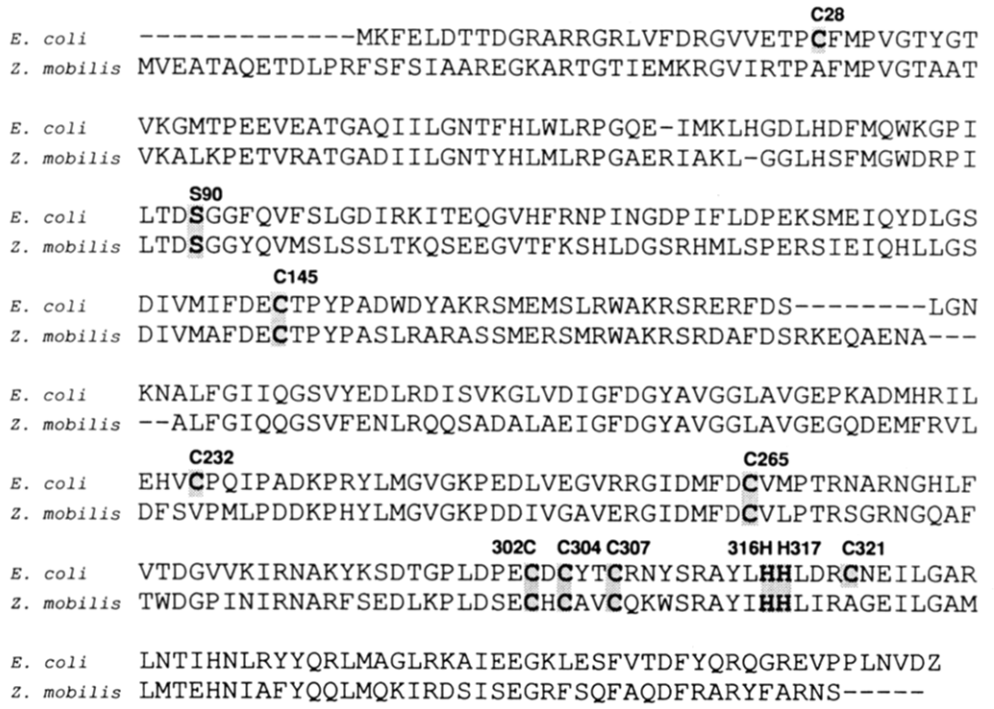

FIGURE 1: Amino acid sequences of TGT from *E. coli* and *Z. mobilis*. The *Z. mobilis* sequence was a personal communication from K. Reuter and R. Ficner.

Table 1: Comparison of Zinc-Binding Sites in Proteins of Known Structure with a Proposed Zinc-Binding Site in TGT[a]

type of zinc	enzyme	ligand	no. of intervening amino acids	ligand	no. of intervening amino acids	ligand	no. of intervening amino acids	ligand
catalytic Zn	alcohol dehydrogenase	C	20	H	106	C	n/a	H_2O
	carboxypeptidase A and B	H	2	E	123	H	n/a	H_2O
	thermolysin	H	3	H	19	E	n/a	H_2O
	β-lactamase	H	1	H	121	H	n/a	H_2O
	phospholipase C	H	3	E	13	H	n/a	H_2O
	alkaline phosphatase	D	3	H	80	H	n/a	H_2O
	adenosine deaminase	H	196	H	80	D	n/a	substrate
structural Zn	aspartate transcarbamoylase	C	4	C	22	C	2	C
	alcohol dehydrogenase	C	2	C	2	C	7	C
	Zif268	C	2−4	C	12	H	3−4	H
	glucocorticoid receptor	C	5	C	13	C	2	C
	GAL4 transcription factor	C	6	C	6	C	9	C
	retroviral gag polyprotein	C	4	C	4	H	4	C
	methionyl-tRNA synthetase[b]	C	2	C	13	C	2	H
	HIV nucleocapsid[c]	C	2	C	4	H	4	C
	PBG synthase	C	2	C	1	C	7	C
	TGT		• • • C D C Y T C R N Y S R A Y L H H L D R C N E • • •					
			302 304 307 316 317 321					

[a] The sequences were taken from the review by Jaffe (1993) except where noted. [b] Taken from Nureki et al. (1993). [c] Taken from Khan and Giedroc (1992). The protein residues that serve as the ligands to the zinc are noted by their one-letter code. The number of intervening amino acids between ligands in the primary sequence of the protein is given.

scribed previously (Chong & Garcia, 1994b; Garcia *et al.*, 1993).

Site-Specific Mutagenesis. The TGT mutants were generated by three different site-specific mutagenesis methods. The mutagenic primers used in these methods are shown in Table 2. For the C304A, C307A, and C321A mutants, two mutagenic primers (1 and 2) were used for each mutant as they were created by the overlap extension method (Ho *et al.*, 1989). The C145A mutant was generated using a combined polymerase chain reaction−ligase chain reaction (PCR−LCR) method, essentially as described by Michael (1994). The remaining mutants were generated using a preferential PCR method (Chong & Garcia, 1994a). All TGT mutant genes were sequenced to confirm that they contained only the desired mutations using the Sequenase (Version 2.0) DNA sequencing kit (United States Biochemicals, Cleveland, OH) and the Long Ranger sequencing gel (J. T. Baker, Inc., Phillipsburg, NJ). The sequencing apparatus was an IBI Model STS-45 standard thermoplate sequencer (International Biotechnologies, Inc., New Haven, CT). The TGT mutant genes were all constructed in the pTGT5 expression plasmid (Chong & Garcia, 1994b).

Overexpression and Purification of the TGT Mutants. All TGT mutants were overexpressed in the TGT deletion strain *E. coli* K12(Δ*tgt*) (provided by Prof. H. Kersten, University of Erlangen, Nürnberg) to eliminate the possibility of

Table 2: Primers Used in Site-Specific Mutagenesis

Mutagenic Primer	Sequence (5' to 3')[a]
C28A	AGG CAT AAA AGC AGG CGT TTC
C145A	ATA CGG CGT AGC CTC ATC AAA
C232A	AAT TTG CGG GGC TAC ATG CTC
C265A	TGG CAT TAC GGC GTC AAA CAT
C302A	GTA GCA ATC AGC CTC AGG ATC
C304A-1	ACA GGT GTA AGC ATC ACA CTC AGG ATC GAG
C304A-2	GAG TGT GAT GCT TAC ACC TGT CGC AAT TAT
C307A-1	ATA ATT GCG CGC GGT GTA GCA ATC ACA CTC
C307A-2	TGC TAC ACC GCG CGC AAT TAT TCA CGC GCT
H316A	ACG GTC AAG ATG AGC CAA GTA AGC GCG
H317A	ACG GTC AAG AGC ATG CAA GTA AGC GCG
H316/317A	GTT GCA ACG GTC AAG CGC TGC CAA GTA AGC
C321A-1	TAT TTC GTT AGC ACG GTC AAG ATG ATG CAA
C321A-2	CTT GAC CGT GCT AAC GAA ATA TTA GGC GCG

[a] Mutated codons are underlined.

contamination of the mutant protein with chromosomally encoded, wild-type TGT. The plasmid pLysS was cotransformed into K12(Δ*tgt*) to facilitate the cell lysis. The procedures for cell growth, induction of protein overexpression, and cell harvesting were as described previously (Chong & Garcia, 1994b). The culture media and cell resuspension buffer were supplemented with $ZnSO_4$ (100 μM). The proteins were purified by a modification of the previously described method (Garcia *et al.*, 1993). After the first anion-exchange chromatography step (Pharmacia MonoQ, pH 7.5), the TGT fractions were ammonium sulfate precipitated (75% saturation) and resuspended in 1 M ammonium sulfate, 100 mM HEPES, pH 7.5, and 5 mM DTT. The sample was then applied to a Pharmacia HiLoad Phenyl Sepharose column and eluted with a gradient from 1 to 0 M ammonium sulfate in the same buffer. In order to maximize the yields of C302A, C304A, C307A, H317A, and H316−317A mutants, the 100000*g* supernatants were directly brought to an ammonium sulfate saturation of 75% without the 40% saturation of the ammonium sulfate fractionation step.

Protein Concentration. The protein concentrations were determined by measuring the absorption at 280 nm and using a corrected absorption coefficient for TGT. Total amino acid analysis was performed by the University of Michigan Biomedical Resources Core Facility. Duplicate determinations at two different (by a factor of 2) TGT concentrations were used to determine a correction factor for a theoretical absorption coefficient for TGT calculated from the primary sequence. The corrected absorption coefficient for TGT is given by eq 1.

$$\epsilon_{280}^{1\text{mg/mL}}\ (\text{corrected}) = 1.14 \qquad (1)$$

Metal Ion Quantification. Wild-type and mutant TGTs were desalted (Pharmacia Fast Desalting column) into metal ion free buffer (Chelex-treated 50 mM HEPES, pH 8). The metal concentrations were determined by atomic emission on solutions containing 0.2−1 mg of protein/mL using a Leeman Labs Plasma-Spec III inductively coupled plasma atomic emission spectrometer (ICP-AES). No interferences were observed from the matrix solution. The method detection limits (3σ basis) for the analyses were 0.2 μM Zn, 4 μM Hg, 0.6 μM Fe, Ca, Mg, V, Cu, and Co, 0.1 μM Mn and Sr, and 1 μM Ni. The reported precision is based on replicate and gravimetric standard analyses.

Enzyme Assays. An aliquot of the enzyme preparation (for the mutant enzymes, 100 ng was used) was added to a reaction mixture (400-μL total volume) containing 100 mM HEPES (pH 7.5), 20 mM $MgCl_2$, 10 μM [8-^{14}C]guanine (specific activity 56 mCi/mmol), and 10 μM [ca. 5 times its K_M (Curnow *et al.*, 1993)] unmodified, *E. coli* $tRNA^{Tyr}$ (ECY2). tRNA (ECY2) was prepared via *in vitro* transcription as described previously (Curnow *et al.*, 1993). The reaction mixtures were incubated at 37 °C, and aliquots (75 μL) were taken at various time points. Upon removal from the reaction mixture, the aliquots from ECY2 were precipitated by adding 2 mL of 5% trichloroacetic acid (TCA) solution. The precipitates were then collected on glass fiber filters (Whatman GF/C). The filters were then rinsed, dried, and quantitated via liquid scintillation counting. Initial velocities were determined by linear regression of DPM versus time plots and along with the protein concentrations were used to calculate specific activities.

Dialysis against o-Phenanthroline. Samples of wild-type TGT (20 μM in 500 μL of 100 mM HEPES, pH 7.5, 10 mM DTT), prepared from Zn-supplemented media, were dialyzed against 100 mM phosphate, pH 6.0, and 1 mM DTT in the presence and absence of 10 mM *o*-phenanthroline for 14 days at 4 °C under argon. The TGT samples were desalted into 25 mM HEPES, pH 7.5, and 10 mM DTT buffer prior to native PAGE, activity, and zinc content determinations. UV spectrophotometry indicated that no detectable *o*-phenanthroline remained bound to the enzyme.

Physical Characterization of the TGT Mutants. The purified TGT mutants were characterized by SDS−PAGE and native PAGE (including the tRNA-binding assay on native PAGE) as previously described (Curnow & Garcia, 1994).

Circular Dichroism (CD) Spectra of Wild-Type and Mutant TGTs. Wild-type and mutant TGTs were dialyzed into 2 mM phosphate buffer (pH 7.5). The samples were diluted in the same phosphate buffer so that their final concentrations were 0.1 mg/mL before the measurement. The CD spectra were measured on a Jasco J-710 spectropolarimeter (Jasco Inc., Easton, MD, University of Michigan, Biomedical Research Core Facility). 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.).

RESULTS

TGT Metal Ion Content. Wild-type TGT was analyzed by inductively coupled plasma atomic emission spectrometry (ICP-AES) to determine if zinc was present in the protein. As shown in Table 3, zinc was found to be present at 0.83 (±0.04) mol/mol of enzyme along with a minor amount of iron (0.07 mol/mol of enzyme). Subsequent preparations of wild-type TGT were found to have similar zinc contents (average zinc content over four different preparations = 0.79 ± 0.05). A number of other metals were examined and no detectable amounts were found. We prepared TGT from cells grown in 2× TY media (which we have determined

Table 3: Metal Content of Purified TGT[a]

metal	metal/enzyme
Zn	0.83 (±0.04)
Fe	0.07 (±0.03)
Mn, Sr	<0.003
Mg, Ca, V, Co, Cu	<0.02
Ni	<0.03

[a] TGT was desalted into metal-free buffer as described in Materials and Methods. Two 1.5-mL samples of TGT from different desalting runs were each analyzed for all of the metals above. The values reported are an average of the two determinations. TGT concentrations were determined using the corrected UV absorption coefficient.

Table 4: TGT Specific Activity and Zinc Content plus or minus Zn Supplementation and after Dialysis against *o*-Phenanthroline[a]

sample	[TGT] (μM)	[Zn] (μM)	Zn/TGT	relative specific activity
A	45.0 (±1.8)	35.6 (±1.8)	0.79	100 (±2)
B	43.0 (±1.8)	35.2 (±1.8)	0.82	103 (±2)
C	1.58 (±0.08)	1.24 (±0.06)	0.78	28 (±3)
D	1.53 (±0.08)	<0.14	<0.1	2.4 (±0.5)

[a] TGT samples were prepared from clones grown in 2× TY media (A) and 2× TY media supplemented with 100 μM $ZnCl_2$ (B). The third and fourth TGT samples (C and D) were generated from a 14-day dialysis of TGT against pH 6.0 buffer and buffer + 5 mM *o*-phenanthroline as described in the text. TGT activities, zinc content, and protein concentrations were performed as described in Materials and Methods. Relative specific activities are averages of replicate determinations.

by ICP-AES to contain approximately 25 μM Zn) and 2× TY media supplemented with 100 μM $ZnCl_2$. The TGT preparations are, within experimental error, identical and contain approximately 0.8 mol of Zn/mol of TGT (Table 3). Mutant TGTs were analyzed for zinc content in an identical manner.

Dialysis of TGT against o-Phenanthroline. TGT dialyzed against buffer containing *o*-phenanthroline for 14 days (4 °C, under argon) has a significantly reduced content of zinc (<0.1 mol of zinc/mol of enzyme versus 0.78 mol of zinc/mol of enzyme dialyzed against buffer alone; Table 4). The "*o*-phenanthroline-dialyzed" enzyme exhibits a 90% reduction in specific activity compared to enzyme dialyzed against buffer alone (Table 4). The relatively low enzyme recoveries for TGT dialyzed against buffer alone and buffer containing *o*-phenanthroline (30% and 40%, respectively) are due to denaturation of the enzyme under the dialysis conditions. A precipitate was observed after dialysis and was removed by centrifugation and filtration prior to analysis. SDS−PAGE of the precipitate reveals a single protein band at ca. 43 kDa, consistent with the precipitate being TGT (data not shown).

Zinc Contents of Site-Specific Mutants. The mutants C28A, C232A, C265A, and H316A have zinc contents, within experimental error, identical to that of wild-type TGT (Table 5). The C145A and C321A mutants exhibited slightly reduced zinc contents compared to wild type. Mutation of three of the four proposed cysteine ligands (C302, C304, and C307) to alanine resulted in essentially complete loss of zinc. Of the two conserved histidine residues in this region, His 317 appears to be the fourth ligand to zinc since the H317A mutant lost more than 50% of the zinc. This is supported by the observation that the double mutant, H316/317A, has only 2% zinc.

Enzymic Activity of Site-Specific Mutants. The TGT mutants were also subjected to enzymic activity determinations. The C145A, H316A, and C321A mutants retained activities comparable to that of wild-type TGT. The C28A, C232A, and H316A mutants had activities that were slightly different from wild type, most likely reflecting a subtle change in enzyme structure. The C265A mutant had a substantially reduced activity (ca. 7%) compared to wild type. All the putative cysteine ligand mutants (C302A, C304A, and C307A) had no detectable activity. The putative histidine ligand mutant H317A had only 1.4% of the wild-type activity. The H316/317A double mutant had very low activity (0.9%).

Table 5: Specific Activity and Zinc Contents of Wild-Type and Mutant TGTs[a]

TGT	cysteine homology[b]	specific activity (nmol min^{-1} mg^{-1})	relative activity (%)	μM Zn/μM enzyme	substrate tRNA binding[c]
wt		7.4 (0.1)	100	0.73 (0.03)	+
C28A	NC	5.6 (0.2)	76	0.80 (0.04)	+
C145A	C	7.5 (1.3)	101	0.66 (0.03)	+
C232A	NC	9.2 (0.4)	124	0.77 (0.04)	+
C265A	C	0.55 (0.06)	7	0.82 (0.04)	+
C302A	C	<0.01	<0.1	0.04 (0.01)	−
C304A	C	<0.01	<0.1	0.06 (0.02)	−
C307A	C	<0.01	<0.1	0.05 (0.01)	−
H316A	C	5.8 (0.3)	78	0.76 (0.03)	+
H317A	C	0.10 (0.01)	1.4	0.35 (0.01)	−
H316/317A	C	0.07 (0.04)	0.9	0.02 (0.01)	−
C321A	NC	7.6 (1.1)	103	0.69 (0.03)	+

[a] Enzymes were prepared from media (2× TY) which was supplemented with 100 μM $ZnSO_4$, as described in Materials and Methods. [b] C indicates residues absolutely conserved between the *E. coli* and *Z. mobilis* TGT sequences; NC indicates residues that are not conserved. [c] A plus (+) indicates that the mutant TGT retains the ability to bind tRNA; a minus (−) indicates no detectable tRNA binding based upon the native PAGE band-shift assay as described in Materials and Methods.

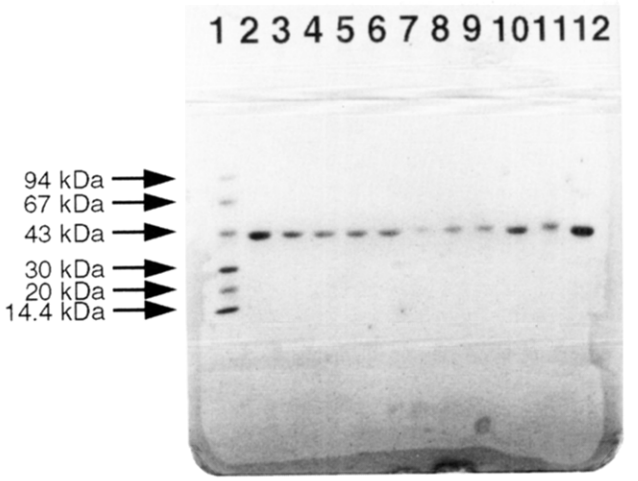

FIGURE 2: SDS−PAGE of wild-type (wt) and mutant TGTs. Approximately equal amounts of TGT protein were applied to each lane. Proteins were run on a Pharmacia Phastsystem and visualized using Coomassie blue staining following vendors' protocols. Lanes: 1, MW standards; 2, wt; 3, C28A; 4, C145A; 5, C232A; 6, C265A; 7, C302A; 8, C304A; 9, C307A; 10, H316A; 11, H317A; 12, C321A.

PAGE Analyses of Site-Specific Mutants. SDS−PAGE (Figure 2) indicates that all of the desired mutants were expressed and purified essentially to homogeneity. All of the mutants migrate to the same M_r (corresponding to 42 500), indicating that there was no proteolysis of any of the mutants.

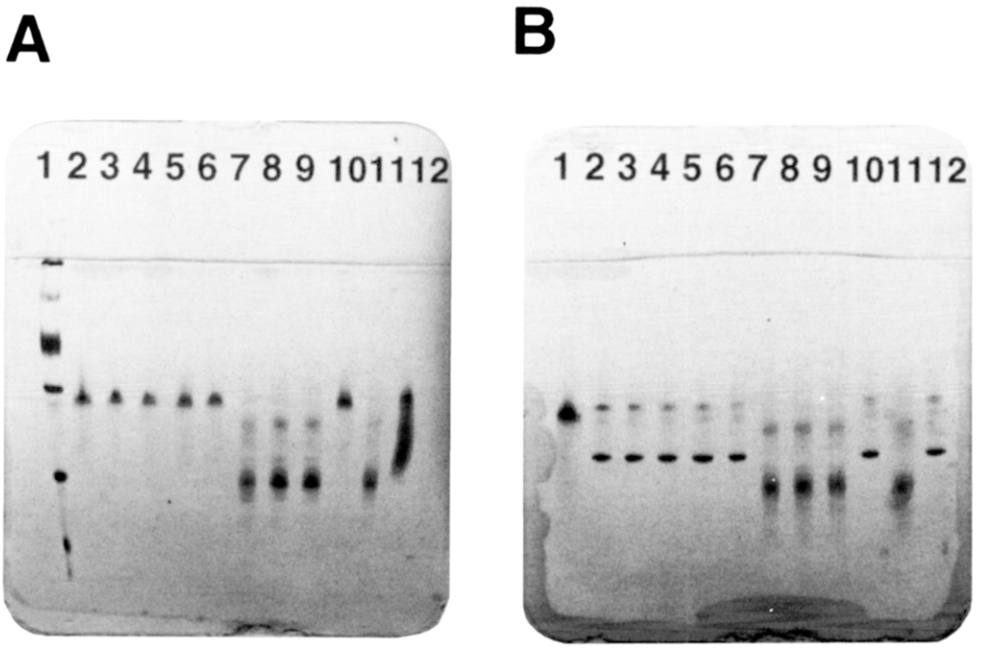

FIGURE 3: Native PAGE of wt and mutant TGTs in the absence (A) and presence (B) of tRNA. Approximately equal amounts of TGT protein were applied to each lane. (A) Lanes: 1, MW standards; 2, wt; 3, C28A; 4, C145A; 5, C232A; 6, C265A; 7, C302A; 8, C304A; 9, C307A; 10, H316A; 11, H317A; 12, C321A. (B) Lanes: 1, wt; 2, wt + ECY2; 3, C28A + ECY2; 4, C145A + ECY2; 5, C232A + ECY2; 6, C265A + ECY2; 7, C302A + ECY2; 8, C304 + ECY2A; 9, C307A + ECY2; 10, H316A + ECY2; 11, H317A + ECY2; 12, C321A + ECY2.

The C302A, C304A, C307A, and H317A mutants migrate to a much smaller apparent M_r (ca. 42 000) than wild-type TGT on native PAGE (Figure 3A, lanes 7, 8, 9, and 11), indicating that these mutants do not form the TGT homotrimer. The C321A mutant exhibits an unusual smearing on native PAGE (Figure 3A, lane 12). Upon incubation with tRNA (ECY2) in the absence of guanine or preQ$_1$, wild-type TGT dissociates into a TGT (monomer)·tRNA complex (Curnow & Garcia, 1994). Figure 3B shows the native PAGE of mutant TGTs incubated with tRNA (ECY2). The mutants C302A, C304A, C307A, and H317A exhibit no change in migration in the presence of tRNA (Figure 3B, lanes 7, 8, 9, and 11), indicating that they do not bind tRNA. The C321A mutant does exhibit the same band shift in the presence of tRNA as wild-type TGT and all of the other mutants, again indicating that, under these conditions, these mutants bind tRNA identically to wild-type TGT.

Circular Dichroism Spectra of Site-Specific Mutants. The CD spectra for wild-type and mutant TGTs are presented in Figure 4. Panel A shows replicate spectra for different preparations of wild-type TGT for comparison. The spectra have two minima in the 210−220-nm range and a high extinction maximum at ca. 195 nm. Panel B shows the spectra for wild-type TGT and the mutants of "non-zinc ligand" residues. The mutant spectra are qualitatively identical to that for wild-type TGT. The slight variability in the amplitude of the spectra may be due to slight errors in protein concentration determination. The protein concentrations were all determined by the UV assay which has been corrected for wild-type TGT by total amino acid analysis. This correction may, however, be slightly different for the mutant enzymes, resulting in slightly different enzyme concentrations. Panel C shows the spectra for wild-type and five mutants of the proposed "zinc ligand" residues. The spectra of the zinc ligand mutants are qualitatively different from that of wild type. These mutant spectra exhibit a significant attenuation of the maximum at 195 nm, a change in the relative extinction of the 210 and 220 minima, and a shift of these minima to shorter wavelength.

The predicted secondary structure for wild-type TGT (Table 6) indicates that the enzyme is predominantly α-helical (70 ± 3%), with no predicted β-sheet structure, 19 ± 1% β-turn, and 12 ± 1% random coil. The secondary structures for the non-zinc ligand mutants exhibit the same, predominantly α-helical (average α-helix prediction = 68 ± 4%) prediction. On the other hand, the zinc ligand mutants exhibit secondary structure predictions that are significantly different from that of wild-type TGT. These mutants are predicted to have 29 ± 4% α-helix, 28 ± 5% β-sheet, 14 ± 3% β-turn, and 29 ± 1% random coil.

DISCUSSION

Zinc has been known to play an essential role in the structure and catalysis of many enzymes including some DNA- and RNA-binding proteins (Berg, 1990; Vallee & Auld, 1990). The binding sites for catalytic zinc (a zinc that is directly involved in the catalysis) and structural zinc in these proteins have distinctly different sequence homologies (Jaffe, 1993; Vallee & Auld, 1990). Vallee and Auld analyzed the X-ray crystal structures of 11 enzymes containing a catalytic zinc and 2 enzymes containing a structural zinc (Vallee & Auld, 1990). Jaffe later extended the analysis to 24 enzymes (Jaffe, 1993). From their analyses, these authors deduced a number of structural features ("rules") that would help determine the amino acids that are most likely to serve as the ligands to either a catalytic or a structural zinc. They found that structural zinc is tetrahedrally coordinated to at least two, often four, cysteine ligands, two of which are usually separated by fewer than four intervening amino acids. In those cases where less than four cysteines are involved, one or two histidines serve as the remaining zinc ligands. These structural zinc ligands are often derived from a continuous stretch of 15−35 amino acids.

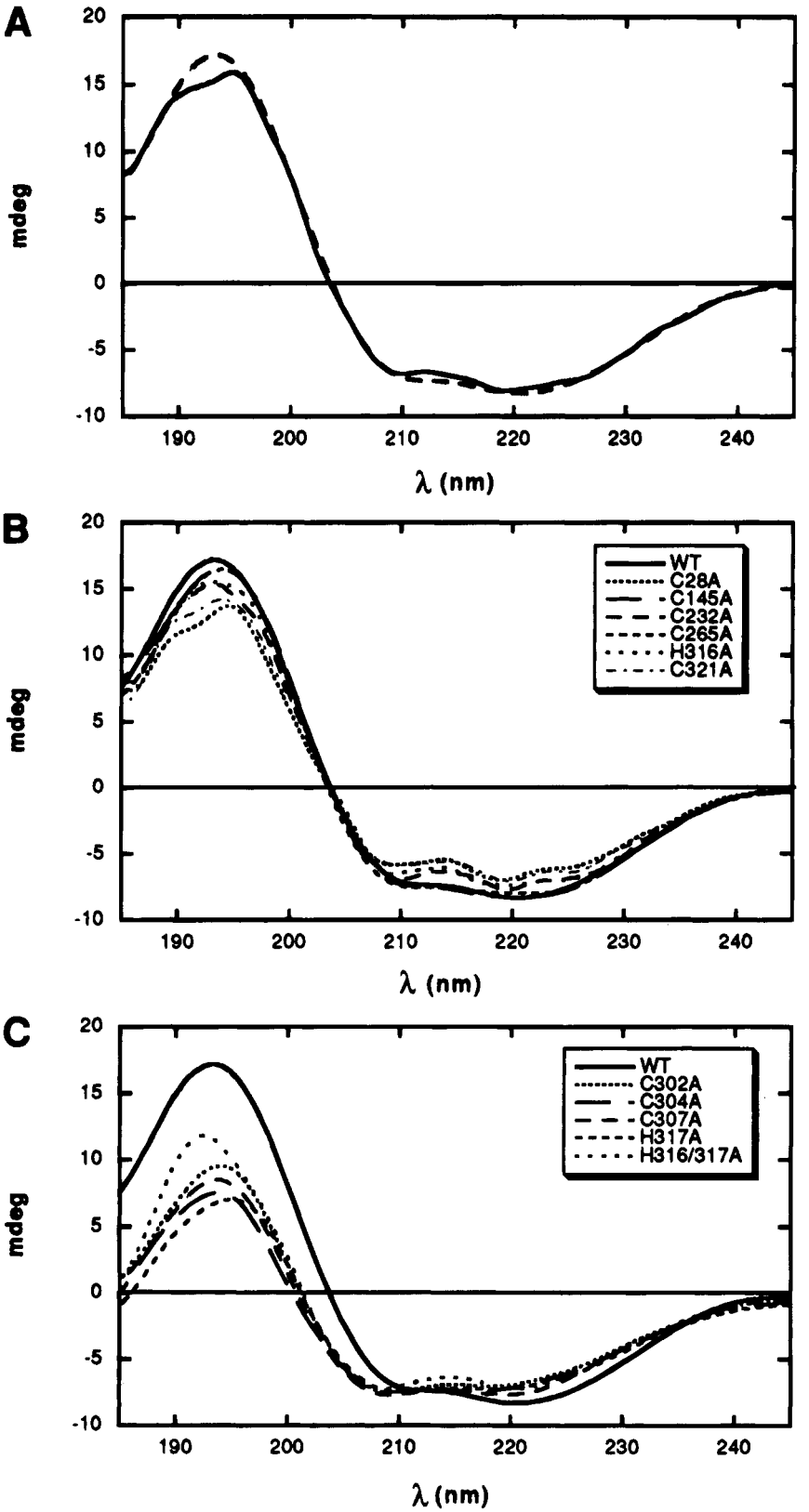

FIGURE 4: CD spectra for wild-type and mutants TGTs: (A) replicate CD spectra for wild-type TGT; (B) CD spectra for wild-type TGT (bold line) and six mutants of non-zinc ligand residues; (C) CD spectra for wild-type TGT (bold line) and four mutants of zinc ligand residues.

The *tgt* gene from *Z. mobilis* has ca. 50% identity at the amino acid level with the *E. coli tgt* gene (K. Reuter and R. Ficner, unpublished). Of the eight cysteines in the *E. coli* TGT, five are conserved (Figure 1). The five conserved cysteines include cysteines 302, 304, and 307, but not 321. Additionally, both of the histidines in this region (316 and 317) are also conserved. It is clear that sequence analysis and homology arguments are insufficient to discriminate between the possible zinc-binding residues present in TGT. We therefore sought first to determine if zinc was indeed present in TGT and second, to use site-directed mutagenesis to provide evidence for the identity of the zinc ligands.

Table 6: Secondary Structure Predicted from CD Spectra of Wild-type and Mutant TGTs[a]

TGT	α-helix (%)	β-sheet (%)	β-turn (%)	random coil	zinc content[b]
wt[c]	68, 72	0, 0	20, 18	12, 11	+
C28A	67	0	20	13	+
C145A	65	0	22	13	+
C232A	69	0	19	12	+
C265A	65	0	21	14	+
C302A	29	32	12	27	−
C304A	25	31	14	30	−
C307A	26	31	14	29	−
H316A	74	0	17	9	+
H317A	29	23	20	29	−
H316/317A	37	24	11	28	−
C321A	72	0	18	10	+
wt[d]	70 (±3)	0	19 (±1)	12 (±1)	
Zn plus mutants[d]	68 (±4)	0	20 (±2)	12 (±2)	
Zn minus mutants[d]	29 (±5)	28 (±5)	14 (±3)	29 (±1)	

[a] Secondary structural elements were predicted from the CD spectra following the method of Johnson (1990) using Young's protein secondary structure program (Jasco Inc.). [b] A plus (+) indicates that the mutant TGT contains essentially wild-type levels of zinc; a minus (−) indicates substantially reduced or no zinc bound. [c] Values for two different preparations of TGT are shown. [d] Values are the averages for the two preparations of wild-type TGT and of the indicated mutants with the standard deviations in parentheses.

Wild-type and mutant enzymes were isolated from recombinant clones (pTGT1 and pTGT5) that overexpress TGT to the level of 70 mg/L of cell culture. At this level of protein overexpression it is possible that the cells are not able to fully incorporate zinc into all of the enzyme. This would account for the 0.8 mol of zinc/mol of enzyme that we have measured. The presence of a small amount of iron is consistent with this explanation as the iron may be adventitiously binding in unoccupied zinc sites. However, supplementation of the growth media with 100 μM $ZnCl_2$ or $ZnSO_4$ did not yield any increase in TGT zinc content (Tables 4 and 5). The protein concentration determination method has been calibrated by total amino acid analysis performed in duplicate at two different concentrations of wild-type TGT, so it is unlikely that our results are due to an error in the protein concentration determination. Likewise, the error in the zinc determination method cannot account for the discrepancy between the zinc content and absolute, 1:1 stoichiometry. We have attempted to express TGT in a defined medium lacking zinc and in the same medium supplemented with either cobalt or cadmium. In all three cases, the weakly expressed TGT was found only in the insoluble pellet (data not shown). It would appear that the presence of Zn during TGT translation is necessary for optimal expression and proper folding of the protein. Denaturation of TGT followed by renaturation in zinc-supplemented buffer may be necessary to obtain TGT with 1:1 zinc stoichiometry.

TGT assays were performed in the presence of various concentrations of zinc chloride (data not shown). At concentrations greater than 1 μM, zinc chloride inhibits the reaction. This inhibition is not due to chloride since the assays are routinely run in the presence of 20 mM $MgCl_2$. It has been demonstrated (Mayaux & Blanquet, 1981) that the Phe-tRNA synthetase is inhibited by zinc binding to the protein, not to tRNA. It is therefore unlikely that the zinc is interacting with the tRNA in the TGT reaction, but this possibility cannot be ruled out. Inhibition by zinc has also been noted for the tyrosine-dependent DAHP synthase from *E. coli*, a zinc-containing enzyme (Stephens & Bauerle, 1991). It is likely that the zinc inhibition of TGT activity is due to zinc binding to residues at the active site. Consistent with this hypothesis is the observation that TGT is inhibited by cadmium (Curnow and Garcia, unpublished).

In order to further investigate the relationship between the presence of zinc and TGT activity, we attempted to generate "apo-TGT" by removing the zinc. Treatment of TGT with EDTA alone is not sufficient to remove zinc from TGT preparations. Modification of TGT sulfhydryls by DTNB followed by treatment with EDTA does not release enzyme-bound zinc (data not shown). In their studies of the T4 gene 32 protein, Giedroc *et al.* (1986) were able to remove the protein-bound zinc by treatment with *p*-(hydroxymercuri)-benzenesulfonic acid (PMBS) followed by dialysis to remove the zinc. While PMBS treatment of TGT does yield chemically modified and inactive enzyme, we were unable to generate apo-TGT. It may be that the protein partially denatures as a result of PMBS modification and that the zinc and PMBS may not be accessible to solvent. In a study of the *E. coli* Ala-tRNA synthetase, it was found that *o*-phenanthroline, a zinc chelator, inhibits the activity of this zinc-containing enzyme (Miller *et al.*, 1991). These authors were unable to determine if the *o*-phenanthroline inhibition of Ala-tRNA synthetase was due to removal of zinc or to *o*-phenanthroline binding to zinc on the enzyme and inhibiting its activity. We find that dialysis of TGT against *o*-phenanthroline results in loss of both enzymic activity and zinc with no indication of *o*-phenanthroline remaining bound to the enzyme.

The TGT mutants C28A, C145A, C232A, C265A, H316A, and C321A all have zinc contents within the range of zinc contents that we have observed for wild-type TGT (Table 5). These mutants all retain the ability to bind tRNA as evidenced by the native PAGE band-shift assay (Figure 3B). With the exception of C265A, these mutants have specific activities comparable to that of wild-type TGT. (The C28A, C232A, and H316A mutants have real, but very small differences in activity, most probably due to subtle effects of the mutations upon the enzyme that are not detectable by the gross structural probes used in this study.) Preliminary results suggest that cysteine 265 is in the TGT active site and may be directly involved in catalysis. Further studies of this residue are in progress. The CD spectra and secondary structure predictions are consistent with the conclusion that these mutants have no gross structural differences from wild-type TGT. Taken in total, these data strongly suggest that none of these residues are involved in binding zinc.

The mutants C302A, C304A, C307A, and H316/317A have barely detectable levels of zinc, indicating a severe attenuation of the mutant enzymes' abilities to bind zinc. Likewise, they have extremely low, if any, detectable activity. This is mirrored in the native PAGE analyses where they are all found not to form the TGT homotrimer nor to bind tRNA (Figure 3). Interestingly, these mutants do migrate to a relatively defined position on native PAGE, indicating that they do have significant tertiary structure. Both qualitatively and quantitatively, the CD spectra and the secondary structure predictions reveal that these mutants have significant differences in secondary structure from the wild-type TGT. The secondary structure predictions for these

mutants indicate a large decrease in α-helical content and an increase in β-sheet content. We believe that this is not a real increase in β-sheet structure but reflects a loss of α-helix that the prediction program interprets as β-sheet.

The mutant H317A has a significant amount of zinc bound (35%) and a significant, albeit very low, level of activity. One possible explanantion is that the histidine at position 316 may be able to partially fulfill the role of the fourth ligand to the zinc in the absence of His 317. The discrepancy between the activity level (1.4%) and the zinc content (35%) of the H317A mutant could be due to a subtle change in enzyme structure caused by this shifting of the ligand structure around the zinc, resulting in a mutant enzyme with a lower specific activity. The results of the H316/317A double mutant, above, are consistent with this hypothesis.

All of these results point strongly to the conclusion that Cys302, Cys304, Cys307, and His317 are the zinc ligands in the *E. coli* TGT. This conclusion is consistent with the homology comparison between *E. coli* and *Z. mobilis* TGT amino acid sequences (Figure 1). Each of the four residues proposed to bind zinc in the *E. coli* TGT are absolutely conserved with the *Z. mobilis* sequence. At present, it is not known if the *Z. mobilis* TGT contains zinc. We have recently received an overexpression vector for the *Z. mobilis* TGT from Dr. K. Reuter (University of Erlangen, Nürnberg) and intend to investigate this. Preliminary extended X-ray absorption fine structure (EXAFS) analyses (D. Tierney, K. Clark, J. Penner-Hahn, S. Chong, and G. Garcia, unpublished) indicate that the zinc site in wild-type TGT has an S_3N-type ligand structure, consistent with the three cysteine and one histidine ligand structure that the present study proposes.

Jaffe has reviewed the structures of catalytic and structural zinc sites in proteins and has used these data along with the results of chemical modification studies and EXAFS analysis (Dent *et al.*, 1990) to predict the structural zinc site in mammalian and yeast porphobilinogen synthase to be CX_2-$CXCX_7C$ and *E. coli* porphobilinogen synthase to be $CXCX_7$-CX_3C (Jaffe, 1993). This is similar to the zinc site that we have found in TGT in that a CXC sequence appears in these sites as well.

It has recently been demonstrated that the zinc ion in the Met-tRNA synthetase from *E. coli* is liganded by four cysteines in a $CX_2CX_9CX_2C$-type zinc-finger structure (Fourmy *et al.*, 1993a,b; Landro & Schimmel, 1993). The authors propose that alteration of the zinc domain by extensive dialysis against *o*-phenanthroline propagates a structural change in the active site causing a reduction in activity (Fourmy *et al.*, 1993b). The human estrogen receptor uses two four-cysteine zinc-finger domains to bind to DNA (Schwabe *et al.*, 1990). The receptor binds as a dimer with the first zinc finger binding to the DNA and the second zinc finger dimerizing with the second zinc finger of the other subunit. In this way the receptor can recognize a palindromic DNA sequence with an intervening DNA sequence. The dimerized, second zinc fingers span the intervening sequence. The enzyme aspartate carbamoyltransferase contains a four-cysteine structural zinc domain in the regulatory subunits that serves as the interface between the regulatory subunits and the catalytic subunits (Ke *et al.*, 1988). Binding of effector molecules to the allosteric domain of the regulatory subunit propagates a conformational change in the zinc domain of the regulatory subunit. This conformational change in the zinc subunit appears to mediate the T to R transition via the zinc domain's interactions with the catalytic subunits. Our data suggest that the zinc-binding site in TGT is involved in the oligomerization of the enzyme into the homotrimer state, perhaps not unlike that found for the zinc domains in aspartate transcarbamoylase and the estrogen receptor.

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