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tRNA-Guanine Transglycosylase from *Escherichia coli*

Overexpression, Purification and Quaternary Structure

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tRNA-guanine transglycosylase (TGT) is the enzyme responsible for the post-transcriptional modification of specific tRNAs (Asn, Asp, His and Tyr) with queuine. In *E. coli* this modification occurs *via* a two-step reaction: (1) TGT-catalyzed base exchange of guanosine-34 with $preQ_1$ (7-aminomethyl-7-deazaguanine) and (2) addition of a cyclopentenediol moiety to the $preQ_1$-34 tRNA. *E. coli* TGT is normally expressed at very low levels ($\approx$1 mg from 500 g cells). The sequence of the queuine operon of *E. coli* has recently been reported by Reuter *et al.* (1991). We have cloned the *tgt* gene into an overexpressing vector in order to provide a more efficient preparation of TGT. A simple, four-step purification scheme yields 78 mg of homogeneous TGT per liter of cell culture ($A_{600} = 5$ to 6). Amino-terminal protein sequencing confirms the identity of the recombinant protein and indicates that the initiator methionine is retained in the mature form. Native-PAGE of TGT and SDS-PAGE of cross-linked TGT are most consistent with a hexameric quaternary structure for the enzyme. The cross-linking data also suggests that the enzyme exists as a dimer of trimers of identical 42·5 kDa subunits (total M_r = 255 kDa. The enzyme is inactivated by cross-linking with the bisimidoester, dimethylsuberimidate. Substrate (tRNA) protects the enzyme against cross-linking and inactivation by dimethylsuberimidate and against inactivation by modification with ethylacetimidate, a monofunctional, imidoester. This indicates that the enzymic residues (presumably lysines) that are involved in cross-linking and the inactivation are in the active site of the enzyme.

Keywords: queuine; transglycosylase; protein oligomer; tRNA, modified nucleotide; protein cross-linking

1. Introduction

Over 50 modified nucleosides have been discovered in tRNA. In many cases their structures, positions in specific tRNAs, and biosynthetic pathways have been elucidated (Kersten, 1984; Adamiak & Gornicki, 1985). Relatively little is known about their biological roles and the molecular mechanisms by which those roles are performed. However, postranscriptional modifications of tRNA in the anticodon loop have been shown to have varied effects upon the tRNA amino acid identity (Muramatsu *et al.*, 1988), codon–anticodon recognition (Ericson & Björk, 1991), translational fidelity and translational frame-shifting (Hagervall *et al.*, 1990). One of the hypermodified nucleoside bases found in tRNA is the guanine derivative queuine (7-(4,5-*cis*-dihydroxy-1-cyclopenten-3-ylaminomethyl)-7-deazaguanine, Figure 1). While the exact biological role(s) of the queuine modification has not been determined, there is a significant body of evidence that queuine is involved in differentiation, proliferation, and perhaps cellular signaling (for a recent review, see Kersten & Kersten, 1990).

Queuine is present in the wobble position (no. 34) of the anticodon for tRNAs with anticodon sequence GUN (tRNAs Asn, Asp, His and Tyr). In eukaryotes, the queuine modification arises *via* a post-transcriptional base exchange of queuine with the genetically encoded guanine in the intact tRNA:

$$G(34)\text{-tRNA} + \text{queuine} \rightleftharpoons Q(34)\text{-tRNA} + \text{guanine}. \quad (1)$$

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Table 1
Oligonucleotides used in pT7EX and pTGT1

Oligo	Sequence (5′ to 3′)
T7EX1 (120-mer)	CGG TAC CCG GGT CAC ACT GGC TCA CCT TCG
	GGT GGG CCT TTC TGC GTT GGC ATA AGG AGG
	TAA AAG CTT TAG CAT AAC CCC TTG GGG CCT
	CTA AAC GGG TCT TGA GGG GTT TTT TGC TGA
T7EX2 (21-mer)	CGG TAC CCG GGT CAC ACT GGC
T7EX3 (21-mer)	TCA GCA AAA AAC CCC TCA AGA
TGT1 (24-mer)	GAA AGC TTA TGA AAT TTG AAC TGG
TGT2 (24-mer)	GAA AGC TTA ATC AAC GTT CAA AGG

The enzyme responsible for this base exchange is tRNA-guanine transglycosylase (TGT†, E.C. 2.4.2.29). TGT has been isolated from a number of different sources and exhibits different gross structural features such as molecular weight and subunit composition (Singhal, 1983). There appear to be two functionally different classes of the enzyme. The first class of TGTs, represented by the eukaryotic enzymes, exchange queuine into tRNA. The second class, represented by the enzyme from *Escherichia coli*, does not recognize queuine itself but exchanges a queuine precursor (preQ$_1$, Fig. 1), which lacks the cyclopentene diol moiety, for guanosine-34. Further, the *E. coli* TGT does not recognize tRNA containing queuine-34.

The tRNA-guanine transglycosylase from *E. coli* has been isolated by Okada & Nishimura (1979). From 500 g of *E. coli* B, they isolated 0·93 mg of homogeneous protein. They reported that the enzyme is a monomer of 46 kDa M_r. Reuter *et al.* (1991) recently reported the sequence of the queuine operon from *E. coli*, including the sequence of the *tgt* gene. In order to perform detailed studies of the TGT reaction and to fully characterize the enzyme structurally, we have constructed an overexpressing clone of TGT. A purification scheme resulting in milligram quantities of homogeneous enzyme has been developed. Initial structural studies are most consistent with TGT existing as a dimer of trimers in the native state, not as a monomer as previously reported.

2. Materials and Methods

(a) *Reagents*

Chemical reagents were purchased from either Sigma or Aldrich, except for dimethylmalonimidate, dimethylsuccimidate and dimethylsebacimidate which were from United States Biochemical. Restriction and DNA-modifying enzymes were from Boehringer-Mannheim. The GeneAmp kit was from Perkin-Elmer/Cetus. The Sequenase kit was from United States Biochemical. *E. coli* strain BL21(DE3)/pLysS was from Novagen. The phage M13K07 and the plasmid pTZ18U were from Pharmacia. Oligonucleotides were synthesized at the University of Michigan, Biomedical Research Resources Core Facility.

(b) *Construction of the expression vector pT7EX*

The forward and reverse PCR primers T7EX2 and T7EX3 (Table 1), respectively, were designed such that T7EX2 has a *Kpn*I restriction site for cloning into a corresponding site in pTZ18U. The reverse primer T7EX3 leaves a blunt end, which will ligate with an S_1 nuclease-treated *Hin*dIII site in pTZ18U. Amplification of the oligo T7EX1 was accomplished following the protocols in the GeneAmp kit. The annealing temperature used was 50°C. After 30 cycles, the amplified DNA was purified through phenol/chloroform extraction and ethanol precipitation. The purified PCR product was then resuspended in 20 μl of sterile water. Ten μl of the amplified insert was digested using 2 units of *Kpn*I in a 20 μl reaction, at 37°C for 2 h. The T7EX/*Kpn*I DNA was purified using the method described above, and resuspended in 20 μl of sterile water.

Ten μl pTZ18U DNA was digested with 2 units of *Hin*dIII in a 20 μl reaction, at 37°C for 2 h. The restriction sample was then heated to 90°C for 15 min and ethanol precipitated. The resulting DNA was resuspended in 10 μl sterile water, and the 3′ protruding ends were removed in a 20 μl nuclease digestion containing 1 unit of S_1 nuclease. The linearized pTZ18U DNA, containing blunt ends, was purified *via* phenol/chloroform extraction and ethanol precipitation. The DNA from the above digestion was resuspended in 10 μl of sterile water. The pTZ18U/*Hin*dIII/S_1 product was digested using 2 units of *Kpn*I in a 20 μl reaction, at 37°C for 2 h. As before, the DNA was purified using phenol/chloroform extraction and ethanol precipitation. The final pTZ18U/*Hin*dIII/S_1/*Kpn*I sample was resuspended in 20 μl sterile water.

Eight μl of T7EX/*Kpn*I and 2 μl of pTZ18U/*Hin*dIII/S_1/*Kpn*I was ligated in a 20 μl reaction containing 2 units of T4 DNA ligase. The ligation reaction was performed at 16°C for 18 h. Five μl of the ligation product was used to

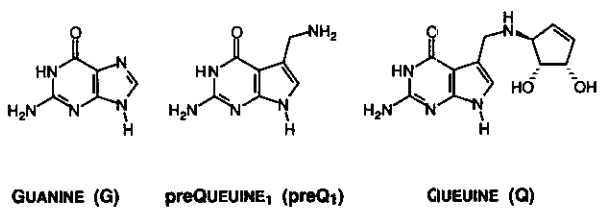

Figure 1. Structures of guanine, preQ$_1$, and queuine.

† Abbreviations used: TGT, tRNA-guanine transglycosylase; PCR, polymerase chain reaction; IPTG, isopropyl-β-thiogalactoside, X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Hepes, hydroxyethylpiperazine-ethylsulfonate; PMSF, phenylmethylsulfonyl fluoride; Amp, ampicillin; Cam, chloramphenicol; CAMP, chloramphenicol/ampicillin.

transform 200 μl of competent *E. coli* TG2. The transformation mixture was placed on ice for 40 min and then the cells were heat shocked at 45°C for 2 min. The transformed cells were then spread on an L-agar plate containing 50 μg ampicillin/ml (L-Amp plate), 10 μg IPTG/ml, and 20 μg X-Gal/ml for blue/white selection. A total of 24 white colonies were picked and template DNA was generated from each following the Pharmacia protocol using M13K07 helper phage. Primary screening for possible constructs was done by checking the size of each template by agarose gel electrophoresis. From the primary screening, five templates appeared to be the correct size of the pT7EX construct. Sequence analysis (using the Sequenase kit and protocols) of the 5 templates revealed that 1 was the pT7EX construct. It should be noted, however, that the results of the 2 independent sequencing trials of pT7EX indicate a 3 base deletion of ACG at positions 243 to 245.

A 500 ml culture of pT7EX/TG2 was started in 2×TY media (5 g/l NaCl, 10 g/l yeast extract, 16 g/l bactotryptone) containing 50 μg ampicillin/ml (2×TY+Amp) and incubated overnight at 37°C with shaking at 300 revs/min. The pT7EX plasmid DNA was isolated using a standard NaOH/SDS lysis protocol. The plasmid was purified by anion exchange chromatography (Mono Q column, Pharmacia) with a linear gradient of 0·25 to 0·45 M-NaCl in 10 mM-Tris (pH 8) with 5 mM-EDTA (TE8). Several runs were required to purify all of the pT7EX. Plasmid containing fractions from each run were pooled and ethanol precipitated. Pure pT7EX DNA was then resuspended in 1 ml sterile water.

(c) *Subcloning the* tgt *gene*

The PCR primers TGT1 and TGT2 (Table 1) were designed to amplify the *tgt* gene's open reading frame. Both primers are 24-mers that incorporate 5′ *Hin*dIII restriction sites. Two bases were added 5′ to each restriction site to allow for *Hin*dIII recognition and digestion. (We subsequently learned that more than 3 extra base-pairs are optimal for *Hin*dIII restriction.) The *Hin*dIII sites were added to facilitate cloning into the expression vector pT7EX. Amplification of the *tgt* gene was done using the protocols of the GeneAmp kit (Perkin-Elmer/Cetus). The annealing temperature used was 40°C, and 30 cycles were run. The PCR product was isolated using phenol/chloroform extraction and ethanol precipitation. The ethanol precipitate was resuspended in 50 μl sterile water.

Nine pmol of the PCR-amplified *tgt* gene was digested with 2 units of *Hin*dIII at 37°C for 2 h. The restriction sample was purified as before, and resuspended in 10 μl of sterile water. Half of the restricted *tgt* gene was ligated with pT7EX/*Hin*dIII in a 20 μl reaction using 3 units of T4 DNA ligase. The reaction was done at 15°C overnight. The ligation product was isolated as before, and the DNA was ethanol precipitated. The final pellet was resuspended in 10 μl sterile water. Competent *E. coli* TG2 were transformed with 5 μl of the ligation product, spread on an L-Amp plate, and incubated at 37°C overnight.

Twelve colonies were picked from the L-Amp plate, and 3 ml template cultures of each colony were incubated overnight at 37°C in 2×TY+Amp. Template DNA was prepared as described before and analyzed by agarose gel electrophoresis. Of the 12 colonies, 2 contained the insert. Preliminary sequencing determined that clone no. 11 contained the insert in the correct orientation. The entire insert in clone no. 11 (pTGT1) was sequenced at the University of Michigan, Biomedical Research Resources Core Facility.

(d) *Induction studies of the clone pTGT1*

The cells from the template culture of no. 11 were spread on an L-Amp agar plate. A colony from the incubated plate was picked and a 10 ml culture was started in 2×TY+Amp. Plasmid DNA was purified from this culture, as described before. The final volume of the pTGT1 DNA sample was 100 μl. 200 μl of competent *E. coli* BL21(DE3)/pLysS (Novagen) was transformed with 20 μl of the above pTGT1 sample, spread on an L-agar plate with ampicillin and 30 μg chloramphenicol/ml (L-CAMP plates), and incubated at 37°C overnight. The plasmid pLysS contains the gene for T7 lysozyme, and confers chloramphenicol resistance. An overnight culture of the transformed cells (1 ml) was mixed with 500 μl of 50% glycerol and stored in liquid nitrogen.

Two 150 ml (2×TY+Amp+Cam) cultures of BL21(DE3)/pLysS harboring pTGT1 were incubated at 37°C. When the cultures reached A_{600} of 0·05, one culture was induced by adding IPTG to 1 mM final concentration. The cultures were incubated at 37°C for an additional 6 h. The cells were harvested *via* centrifugation (4500 ***g***, 15 min), and resuspended in 10 ml HEDT buffer (100 mM-Hepes (pH 7·5), 5 mM dithiothreitol and 100 μM-PMSF). The solution was sonicated (4×30 s sonication with 30 s pauses at microtip setting no.4·5, Heat Systems-Sonicator XL2020) for 2 min and stirred at 4°C for 30 min. The cell debris was pelleted *via* ultracentrifugation 100,000 ***g*** for 2 h. The supernatant was assayed tor TGT activity and subjected to SDS-PAGE analysis. A 150 ml (2×TY+Amp) culture of *E. coli* TG2 cells harboring the pTGT1 plasmid was treated in an identical fashion except that hen egg white lysozyme (25 mg) was added to the solution after the sonication.

(e) *mRNA primer extension analysis*

Primer extension analysis was performed as described in section 4·8 of Ausubel *et al.* (1987). A ^{32}P-labeled 21 base oligonucleotide primer (5′[^{32}P]GGT GTC CAG TTC AAA TTT CAT-3′) was designed to anneal to the beginning of the TGT open reading frame in the mRNA. mRNA preparations were performed on 10 ml cultures of *E. coli* TG2, TG2/pTGT1, and BL21(DE3)/pLysS/pTGT1 as described in section 4.4.4 of Ausubel *et al.* (1987). Extension products were separated on 6% (w/v) denaturing PAGE and visualized by autoradiography.

(f) *Preparation and purification of recombinant TGT*

(i) *Cell culture*

E. coli BL21(DE3)/pLysS harboring pTGT1 were grown to the stationary phase (A_{600} = 6·0) in 2×TY media containing 50 μg ampicillin/ml and 30 μg chloramphenicol/ml. The media was inoculated with 100 μl of an overnight culture per 600 ml of media. The cultures were incubated at 37°C with vigorous shaking (300 revs/min) in 2 l baffled flasks (600 ml/flask) overnight.

(ii) *Preparation of crude extract*

Cells from the above culture were harvested by centrifugation (4500 ***g***, 15 min) and the total wet weight of the cells was measured. The cell pellets were resuspended in 60 ml of degassed HEDT buffer. This step and all the subsequent purification steps were performed at 4°C. The

cells were disrupted by 2 min sonication (4 × 30 s pulses as above), slowly stirred for 30 min and then centrifuged at 100 000 *g* for 1·5 h.

(iii) *Ammonium sulfate fractionation*

An appropriate amount (47 ml) of saturated ammonium sulfate solution (4·1 M) was added to the 100,000 *g* supernatant (70 ml) to give a final saturaton of 40%. The solution was stirred for 30 min and then centrifuged 16,000 *g*, 1 h). The precipitate was discarded and more saturated ammonium sulfate solution (140 ml) was added to the supernatant (100 ml) to give a final saturation of 75%. The solution was stirred for 30 min and centrifuged (45,000 *g*, 1 h). The precipitate was dissolved in 30 ml of degassed HEDT buffer and dialyzed against 1 liter of the same buffer overnight.

(iv) *Anion exchange chromatography*

The chromatography was performed on a Pharmacia FPLC system. The dialyzed enzyme fraction (47 ml) was filtered through a 0·22 μm sterile filter. Four ml samples of the filtered enzyme were applied to an anion exchange column (Mono Q HR10/10, Pharmacia) equilibrated with degassed HEDT buffer. The column was first washed with 10 ml HEDT buffer and then eluted with 50 ml of a linear gradient of 0·1 to 0·5 M-NaCl in HEDT buffer. The flow rate was kept at 1 ml/min. Fractions (1 ml) containing TGT, determined by guanine exchange assay and SDS-PAGE, were pooled. A portion (8 ml) of the pooled fractions was exchanged into degassed *bis*-Tris buffer (50 mM-*bis*-Tris (pH 6·0), 5 mM-DTT) using microconcentrators (Centricon-10, Amicon). This was then rechromatographed on the Mono Q column under the same conditions as above except *bis*-Tris buffer (pH 6·0) replaced the HEDT buffer. The peak fractions containing TGT were collected and used for the enzyme characterization.

(g) *Polyacrylamide gel electrophoresis*

All electrophoretic analyses were performed on a PhastSystem (Pharmacia). For denaturing gels, 5 μl of protein sample or protein standards (from Pharmacia), mixed with 5 μl of denaturing gel loading buffer (10 mM-Tris·HCl, 1 mM-EDTA, 5% (w/v) SDS, 10% (v/v) β-mercaptoethanol and 0·02% (w/v) bromophenol blue), was heated in boiling water for 5 min. Samples were then subjected to electrophoresis on an 8% to 25% gradient gel (Pharmacia PhastGel) using PhastGel denaturing buffer strips (0·2 M-Tricine, 0·2 M-Tris (pH 8·1), 0·55% SDS, 2% (w/v) agarose). For native gels, samples and protein standards (high molecular weight standards from Pharmacia) were directly applied on an 8% to 25% gradient gel and were electrophoresed using PhastGel native buffer strips (0·88 M-L-alanine, 0·25 M-Tris (pH 8·8), 2% agarose). Electrophoresis was performed following the PhastSystem protocols. Gels were stained using the Coomassie blue staining protocol in the PhastSystem manual.

(h) *Protein assays*

The protein concentrations of enzyme fractions were determined by measuring the absorption at 280 nm and using the absorption coefficient for TGT estimated from the primary sequence of the enzyme using the method described by Cantor & Schimmel (1980):

$$E_{280}^{1\ \mathrm{mg/ml}} = \left(\frac{(5700 \times n_{\mathrm{Trp}}) + (1400 \times n_{\mathrm{Trp}})}{m_{\mathrm{r}}}\right)$$
$$= \left(\frac{(5700 \times 4) + (1400 \times 14)}{42{,}566}\right)$$
$$= 0{\cdot}96. \qquad (2)$$

To confirm the expression yield, the protein concentration of one fraction was analyzed using the Bradford protein assay (Biorad) with bovine serum albumin as standard. This assay determined the protein concentration to be 1·7 mg/ml compared to 1·1 mg/ml calculated using the E_{280}.

(i) *Guanine exchange assay*

A sample (5 μl) of the enzyme preparation was added to a reaction mixture (100 μl total volume) containing 100 mM-Hepes (pH 7·5), 20 mM-$MgCl_2$, 84 nCi (10 μM) of [8-^{14}C]guanine (spec. act.: 56 mCi/mmol) and 2·5 mg/ml (~100 μM) of unfractionated yeast tRNA (Sigma). The reaction mixture was incubated at 37 °C for 1 h. A portion (75 μl) of the reaction mixture was quenched in 3 ml 5% trichloroacetic acid, and the precipitated tRNA was collected on a glass microfiber filter (GF/C, Whatman). Optiphase Biosafe II liquid scintillant (4 ml) was added to the filter in mini scintillation vials and the radioactivity was counted in a liquid scintillation counter (Beckmann LS 5000TD). One unit of the enzyme activity was defined to be the amount catalyzing the incorporation of 1 μmol of guanine into yeast tRNA/min under the above conditions.

(j) *Amino-terminal sequence analysis*

The 9 amino-terminal amino acids were sequenced by the University of Michigan, Biomedical Research Resources Core Facility.

(k) *Chemical cross-linking of TGT*

Bisimidoester cross-linking was performed essentially as outlined for dimethylsubermidate by Davies & Stark (1970). Purified TGT in 0·2 M-triethanolamine (pH 8·0) was mixed with fresh preparations of bisimidoester (in triethanolamine) to give final protein concentrations of 0·48 mg/ml and a reagent concentration of 20 mM in volumes of 50 μl. The reaction mixtures were left at room temperature for 2 h. The cross-linking products were analyzed on SDS-PAGE. Guanine exchange assays were performed on the enzyme stored in triethanolamine buffer to insure that the enzyme was active. Guanine exchange assays were also performed on dimethylsuberimidate-cross-linked protein as follows. Five μl of a 10-fold dilution of preincubated cross-link reaction mixture was used to determine TGT activity *via* the guanine exchange assay described above. After a 1 h incubation at 37 °C, 75 μl samples were quenched in 3 ml of 5% trichloroacetic acid, filtered and counted as above. As controls, identical concentrations of TGT were mixed with the appropriate concentrations of dimethylsuberimidate in triethanolamine buffer and immediately assayed in the same manner as the preincubated TGT samples.

(l) *Substrate protection against cross-linking and inactivation*

TGT was incubated with various concentrations of dimethylsuberimidate as above in the presence or absence

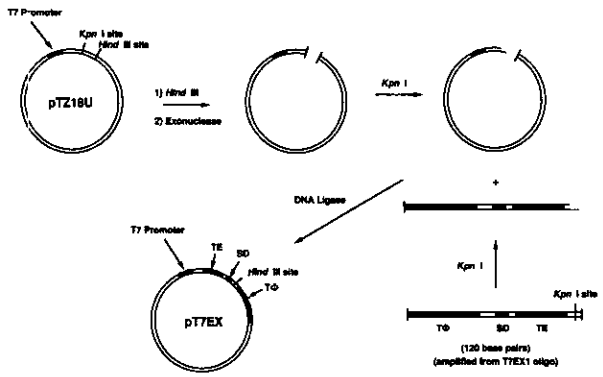

Figure 2. Construction of pT7EX. TE: early transcription terminator (*E. coli* RNA polymerase); SD: Shine-Delgarno sequence; and TΦ: strong transcription terminator (T7 RNA polymerase).

of 10 μM tRNA†. Guanine exchange assays were performed as above and relative activities were calculated with an incubation of the enzyme without dimethylsuberimidate as reference. Inactivation of TGT by incubation with ethylacetimidate was performed in an identical manner. In this case a number of time points were taken in the guanine exchange assay and initial velocities were used to determine relative activities.

3. Results

(a) *Construction of the expression vector pT7EX*

The expression vector pT7EX was designed as a derivative of phagemid pTZ18U (see Fig. 2 for a construction outline). A 120 base oligonucleotide was designed (T7EX1) such that it contains, 5′ to 3′, the early transcription terminator (TE) from T7 DNA, ribosome binding site, *Hind*III cloning site, and a strong transcription terminator (TΦ) for T7 RNA polymerase (the terminator sequences are described in Dunn & Studier, 1983). After PCR amplification of the oligo T7EX1, it was ligated into the multiple cloning site of pTZ18U. DNA sequence analysis (data not shown) revealed that a three-base deletion had been introduced into the insert. This is not an uncommon phenomenon when performing DNA amplification with *Taq* DNA polymerase. The CG portion of the deletion constitutes the last two bases in the TE sequence.

(b) *Subcloning the* tgt *gene*

Agarose gel analysis of the 12 recombinant clones revealed that two of the 12 samples were of the expected size. The results of the preliminary sequence analysis indicated that one of the clones contained the *tgt* gene in the corrected orientation (no. 11, pTGT1), and the other contained the *tgt* gene in the opposite orientation (no. 5). The entire insert of clone no. 11 was sequenced (data not shown) and was found to exactly match the *tgt* gene sequence reported by Reuter *et al.* (1991). There was, however, a one-base deletion of a G in the TE sequence of the vector, with the result that the TE sequence in pTGT1 has three bases deleted. It was not thought that this would impair the usefulness of pTGT1, therefore the deletions were not corrected.

† Unmodified *E. coli* $tRNA^{tyr}$ was prepared by *in vitro* transcription after the method of Milligan & Uhlenbeck (1989). This will be the subject of a subsequent publication.

Table 2
Enzyme activities of TGT expression samples

Culture	Total protein (mg)	Specific activity (U†/mg × 10^5)
TG2/pTGT1	111	25·9
BL21(DE3)/pLysS/ pTGT1(−IPTG)	138	12·5
BL21(DE3)/pLysS/ pTGT1(+IPTG)	63	65·0

† U ≡ 1 μmol [8-^{14}C]guanine incorporated into tRNA per min at 100 μM-yeast tRNA, 10 μM-[8-^{14}C]guanine, 37 °C.

(c) *Induction studies of the clone pTGT1*

T7 lysozyme serves a dual purpose in the BL21(DE3)/pLysS strain of bacteria. The lysozyme helps to reduce the basal levels (non-induced) of T7 RNA polymerase activity by binding and inhibiting the polymerase. Another function of the T7 lysozyme, is that it facilitates the purification of a cloned gene product. Gentle sonication disrupts the inner cell membrane, releasing the T7 lysozyme which then lyses the cells. Unexpectedly, a crude preparation of TGT from non-induced cells exhibited a high level of TGT activity. Denaturing gel electrophoresis (SDS-PAGE) showed a similar profile for induced and non-induced cells. A 100,000 ***g*** supernatant of an extract of *E. coli* TG2 harboring pTGT1 also showed a high level of expression of TGT when analyzed by both TGT activity assay and SDS-PAGE. The activities of these preparations are listed (see Table 2) and an SDS-PAGE is shown in Figure 3.

(d) *mRNA transcription start site*

Autoradiography of primer extension products showed only a band corresponding to labeled primer in the TG2 lane (data not shown). Both the

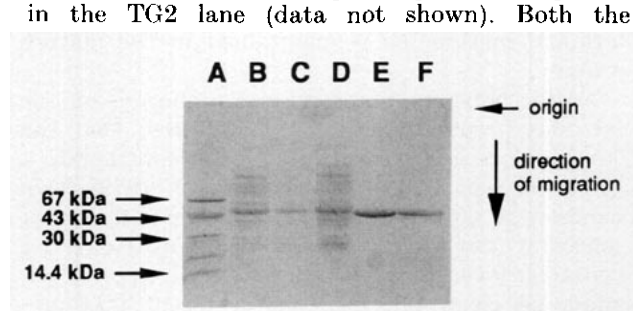

Figure 3. SDS-PAGE of TGT expression samples. Lane A, M_r standards; lane B, TG2/pTGT1; lane C, BL21(DE3)/pTGT1 + IPTG; lane D, BL21(DE3)/pLysS/ pTGT1-IPTG; lanes E and F, TGT standard.

Table 3
TGT purification from BL21(DE3)/pLysS/pTGT1

Fraction	Total protein (mg)	Volume (ml)	Total activity (DPM/min × 10^{-8})	Specific activity (U/mg × 10^5)	Yield (%)	Fold purification
B 100,000 *g* supernatant	7973	70	1·99	15·4	100	1 ×
C 40–75% $(NH_4)_2SO_4$	3934	47	2·32	36·2	116	2·4 ×
D FPLC Mono Q (pH 7·5)	313	66	1·34	264	67	17 ×
E FPLC Mono Q (pH 6·0)†	4·8	1	0·06	804	n/a	52 ×

† Only 8 ml of D were rechromatographed at pH 6·0. This represents only one fraction of the pH 6·0 peak. Guanine exchange assays were performed as described in Materials and Methods with the exception that 70 mM-Hepes was used in a total volume of 150 μl. There is no detectable difference in TGT activity between assays performed in 70 mM-Hepes and 100 mM-Hepes.

TG2/pTGT1 and BL21(DE3)/pLysS/pTGT1 lanes appeared identical with a number of faint bands and the major band comigrating with a 171(±1) base long oligonucleotide standard (data not shown). This indicates that transcription is starting 150 base-pairs upstream of the ATG start codon of TGT.

(e) *Preparation and purification of recombinant TGT*

A 3·6 l culture was prepared and the cells were allowed to grow under the conditions described in Materials and Methods. The culture reached an A_{600} of six after 18 hours. A total wet weight of 38 g of cells were obtained after harvesting. Previous experiments showed that most of the TGT was precipitated at 50% to 70% saturation range (data not shown). We chose to use a 40% to 75% range to maximize yield at this early step in the purification scheme. During anion exchange chromatography, the enzyme eluted as a single peak at about 0·3 M-NaCl. A total of 313 mg of protein was obtained after the first Mono Q chromatography step with an apparent 67% yield and 17-fold purification. The enzyme reached ~99% purity after rechromatography at pH 6·0. The purification procedure is summarized in Table 3 and Figure 4.

(f) *Physical characterization of recombinant TGT*

The nine amino-terminal residues corresponded to those predicted from the gene sequence (data not shown). This analysis also indicated that the amino terminal methionine is maintained in the mature protein.

Native polyacrylamide gel electrophoresis on our previous preparations of TGT indicated that the enzyme does not exist as a monomer contrary to a literature report (Okada & Nishimura, 1979). Two bands were observed, with a significant amount of smearing, that corresponded to apparent trimer and pentamer forms (data not shown). Native polyacrylamide gel electrophoresis on our present TGT preparation (Figure 5A) shows predominantly the higher molecular weight band, also with some smearing. The quaternary structure of the native enzyme based upon the apparent molecular weight of the band is difficult to judge, but is most consistent with either a pentameric or hexameric state.

Denaturing gel electrophoresis (SDS-PAGE) of TGT treated with bisimidoesters of varying chain length is shown in Figure 5B. The dimethylsuberimidate-treated TGT exhibits a heavy band in the SDS-PAGE corresponding to monomer and a lighter band corresponding to an apparent trimer (Fig. 5B, lane D). At higher concentrations of reagent, dimethylsuberimidate-treated TGT shows faint bands at larger M_r (data not shown). SDS-PAGE of dimethylsebacimidate-treated TGT (Fig. 5B, lane E) shows bands corresponding to monomer, trimer, tetramer, pentamer, and a faint band at larger M_r that may correspond to hexamer. No dimer band is observed in any of these experiments. Additionally, the monomer band in each of the bisimidoester-treated TGT lanes migrates to a slightly higher M_r than untreated TGT. Incubation samples of various dilutions of TGT with dimethylsuberimidate show no change in trimer to monomer band intensities, indicating that the cross-linking of TGT is most probably not due to an intermolecular reaction (data not shown).

Treatment of TGT with dimethylsuberimidate also resulted in the inactivation of the enzyme (Fig. 6A). This inactivation and cross-linking were both prevented by the presence of 10 μM-tRNA

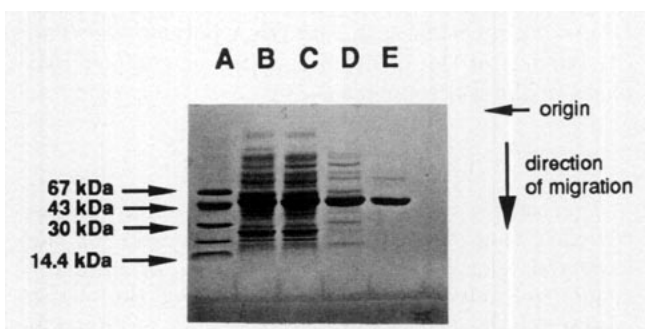

Figure 4. SDS-PAGE of TGT preparation. Lane A, M_r standards; lane B, 100,000 *g* supernatant; lane C, 40% to 75% ammonium sulfate precipitate; lane D, Mono Q (pH 7·5) eluate; and lane E, Mono Q (pH 6·0) eluate. Lanes B, C and E contain 2 μl of a 1 : 10 dilution of sample. Lane D contains 2 μl of a 1 : 7 dilution of sample in order to load a similar mount of protein to that in lane E.

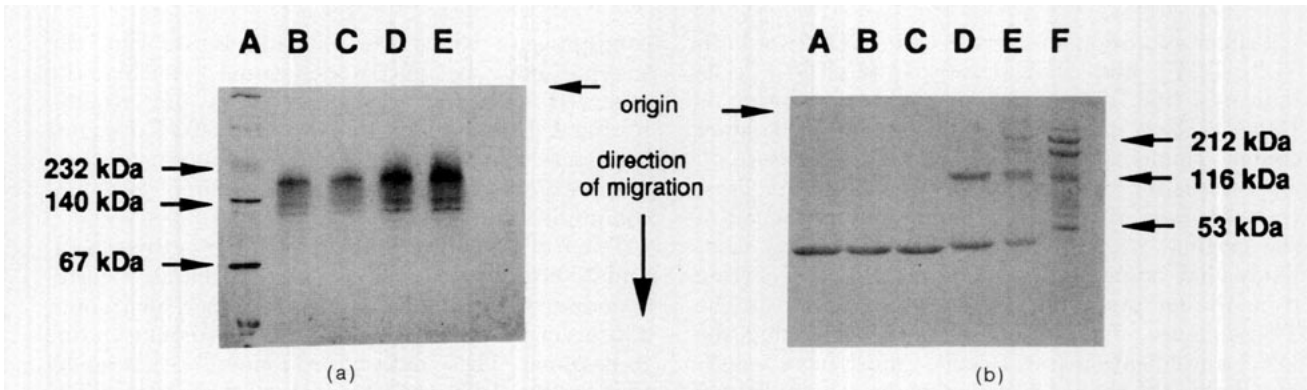

Figure 5. Native-PAGE of TGT and SDS-PAGE of cross-linked TGT. A. Native PAGE of TGT from fraction E (Table 3). Lane A, M_r standards; lanes B and C, 1 : 10 dilution of sample; lanes D and E, 1 : 5 dilution of sample. B, SDS-PAGE of bisimidoester-cross-linked TGT. Lane A, untreated TGT; lane B, TGT treated with dimethylmalonimidate; lane C, TGT treated with dimethylsuccinimidate; lane D, TGT treated with dimethylsuberimidate; lane E, TGT treated with dimethylsebacimidate; lane F, M_r standards. Cross-linking reactions were performed as described in Materials and Methods.

substrate ([Fig. 6A and B). Incubation of TGT with ethylacetimidate, a monofunctional imidoester, resulted in a loss of activity that was also protected by the presence of tRNA (Fig. 6A). As a control, TGT incubated under identical conditions, in the absence of any modifying reagent, showed no loss of activity (data not shown).

4. Discussion

(a) *Construction of the expression vector pT7EX and TGT vector pTGT1*

The pTZ vectors, from which pT7EX is derived, utilize the T7 RNA polymerase to direct transcription of cloned genes (Studier *et al.*, 1990). In the plasmid pT7EX, the ribosome binding site engineered upstream of the *Hin*dIII cloning site should allow for efficient translation initiation of cloned open reading frames that do not contain 5′ flanking regions. The addition of the TΦ terminator downstream of the cloning site reduces the length of RNA transcribed by T7 RNA polymerase upon induction. The yield of mRNA corresponding to the *tgt* gene should then be maximized, with little, or no extraneous RNA being produced. Transcription of T7 DNA by *E. coli* RNA polymerase ends at a site immediately following the early transcription terminator sequence described by Dunn & Studier (1983). The T7 RNA polymerase does not terminate at the TE sequence. This terminator was incorporated into pT7EX so that any extraneous transcription by *E. coli* RNA polymerase would be terminated before the inserted gene thereby minimizing non-induced expression of the protein.

Contrary to our design, this vector expresses TGT at very high levels in the absence of any induction of T7 RNA polymerase. We have investigated this further and have found that the vector overexpresses TGT when incorporated into an *E. coli* host that does not contain the T7 RNA polymerase gene (TG2). In collaboration with R. W. Woodard (University of Michigan), we have cloned the *kdsA* gene into pT7EX (G. D. Dotson, G. A. Garcia, R. Dua & R. W. Woodard, unpublished results). We observe the same lack of induction control in the overexpression of the *kdsA* gene.

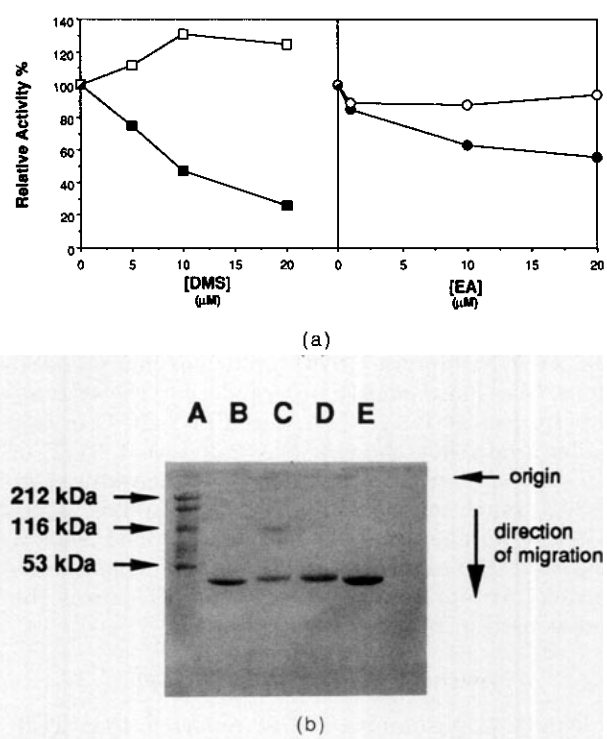

Figure 6. tRNA protection of TGT cross-linking. A, Plot of inactivation of TGT and protection by tRNA. Percent relative activity is plotted against concentration of dimethylsuberimidate (DMS, squares) and ethylacetimidate (EA, circles). Filled symbols correspond to incubations in the absence of tRNA and open symbols in the presence of tRNA, performed as described in Materials and Methods. Values plotted are the average of 2 replicate determinations. B, SDS-PAGE of dimethylsuberimidate-treated TGT. Lane A, M_r standards; lane B, untreated TGT; lane C, TGT treated with 20 mM-dimethylsuberimidate; lane D, TGT treated with 20 mM-dimethylsuberimidate in the presence of 10 μM-tRNA; lane E, untreated TGT with 10 μM-tRNA.

Primer extension analysis of *tgt* mRNA from both TG2/pTGT1 and BL21(DE3)/pLysS/pTGT1 cells indicates that the start point of transcription is 150(±1) base pairs upstream of the TGT start codon. Immediately preceding this transcription start site is the *lac* promoter (−10 and −35 regions) that was part of the blue/white selection portion of the original pTZ18U vector. Therefore it is most likely that transcription of *tgt* mRNA is occurring from the *lac* promoter system. The deletion in the TE sequence of pT7EX that occurred during the DNA amplification (see Results) must have inactivated the TE sequence as a terminator for *E. coli* RNA polymerase. Studier *et al.* (1990) have noted that higher copy vectors which contain the *lac* operator sequence may bind all the cellular *lac* repressor in cell lines which do not overexpress the *lacI* gene. They note this can cause apparently constitutive expression from vectors which rely on DE3 lysogen for the production of T7 RNA polymerase for gene expression. It may be that, in a similar fashion, these high copy pT7EX constructs are titrating out the *lac* repressor and thereby depressing their own *lac* promoter. There is, however, an increase in specific activity when BL21(DE3)/pLysS/pTGT1 cells are induced with IPTG, suggesting that T7 RNA polymerase-mediated transcription is occurring. Once transcribed, the *tgt* mRNA has a sequence around the ribosome binding site that is most favorable for high levels of translation using the criteria of DeBoer & Hui (1990). This may contribute to the relatively high levels of TGT expression from this vector.

(b) *Purification of TGT from pTGT1*

Initially we used the buffer system described by Okada & Nishimura (1979) in our enzyme preparations. We subsequently observed that the enzyme rapidly lost activity when stored at −20°C in this buffer (data not shown). We found that TGT in Tris/mercaptoethanol lost activity when stored at −20°C much more rapidly that TGT in Tris alone. After a number of trials, we determined that a degassed Hepes/DTT (HEDT) buffer system was optimal for our enzyme preparations. This was the buffer used in the present preparation.

(c) *Quaternary structure of TGT*

Okada & Nishimura (1979) reported that TGT was apparently monomeric. They reached this conclusion based upon the protein M_r they determined by SDS-PAGE (~46 kDa) and the enzyme's elution from a sizing gel column relative to standard proteins (~56 kDa). In our preparation, we find that the protein does not appear to be a monomer. Negative gel electrophoresis of TGT shows no bands corresponding to either monomeric or dimeric protein. The native gels show a significant amount of smearing which may be due to the fact that the native gels are run at pH 8·8. In preliminary experiments we find that the enzyme rapidly loses activity above pH 8 (data not shown). Therefore, under the conditions in which the native gels are run, the enzyme may be partly denatured, causing the observed smearing. Consistent with our results, Reuter & Kersten have observed that TGT does not behave as a monomer on sizing gel columns in their preparations (K. Reuter & H. Kersten, personal communication).

Denaturing gel electrophoresis of dimethylsebacimidate-cross-linked TGT shows bands corresponding to monomer, trimer tetramer, pentamer, and a very faint higher M_r band that could be due to hexamer. These data are consistent with a model that native TGT exists in a hexameric state. The absence of any cross-linked dimer band suggests that the hexamer consists of two trimers in which the monomers are tightly bound together in the trimer, with a looser association of the two trimers. The protection by substrate (tRNA) against cross-linking and inactivation suggests that the enzymic residues involved (presumably lysines) are in the active site of the enzyme. Inactivation of the enzyme by the monofunctional imidoester, ethylacetimidate, and the appearance of a relatively intense monomer band in the cross-linked and inactivated TGT suggest that the cross-linking occurs inefficiently compared to simple chemical modification of the enzymic residues. This is also supported by the migration of the monomer band to a slightly higher M_r in the SDS-PAGE of cross-linked TGT. This could be due to dimethylsuberimidate having alkylated a number of protein residues in a monovalent or intrasubunit fashion.

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