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METHOD

Chemical and enzymatic synthesis of tRNAs for high-throughput crystallization

LUKE D. SHERLIN,[1] TIMOTHY L. BULLOCK,[1] T. AMAR NISSAN,[1] JOHN J. PERONA,[1] FREDERICK J. LARIVIERE,[2] OLKE C. UHLENBECK,[2] and STEPHEN A. SCARINGE[3]

[1]Department of Chemistry and Biochemistry, and Interdepartmental Program in Biomolecular Science & Engineering, University of California at Santa Barbara, Santa Barbara, California 93106-9510, USA
[2]Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215, USA
[3]Dharmacon Research, Incorporated, Boulder, Colorado 80301, USA

ABSTRACT

Preparation of large quantities of RNA molecules of a defined sequence is a prerequisite for biophysical analysis, and is particularly important to the determination of high-resolution structure by X-ray crystallography. We describe improved methods for the production of multimilligram quantities of homogeneous tRNAs, using a combination of chemical synthesis and enzymatic approaches. Transfer RNA half-molecules with a break in the anticodon loop were chemically synthesized on a preparative scale, ligated enzymatically, and cocrystallized with an aminoacyl-tRNA synthetase, yielding crystals diffracting to 2.4 Å resolution. Multimilligram quantities of tRNAs with greatly reduced 3′ heterogeneity were also produced via transcription by T7 RNA polymerase, utilizing chemically modified DNA half-molecule templates. This latter approach eliminates the need for large-scale plasmid preparations, and yields synthetase cocrystals diffracting to 2.3 Å resolution at much lower RNA:protein stoichiometries than previously required. These two approaches developed for a tRNA–synthetase complex permit the detailed structural study of "atomic-group" mutants.

Keywords: aminoacyl-tRNA synthetase; RNA ligase; T7 RNA polymerase; X-ray crystallography

INTRODUCTION

Recent years have seen substantial advances in our understanding of the process by which transfer RNAs are specifically aminoacylated by aminoacyl-tRNA synthetases (reviewed in Ibba & Soll, 2000). Ten cocrystal structures of synthetases bound to their cognate tRNAs are now available, and these have stimulated the design and functional assay of modified tRNAs directed at uncovering the structural mechanisms of catalysis and recognition (Beuning & Musier-Forsyth, 1999). However, proper interpretation of these data also requires cocrystal structures of modified tRNA-synthetase complexes, because limiting mutational analysis to enzymology alone leaves unanswered the important question of how a modification exerts its functional effect. Further, the tRNA–synthetase recognition process is quite complex, involving induced-fit conformational changes in both partners as well as indirect readout of nucleotide identities. It is thus expected that deeper insights into specificity will require the comprehensive structure–function analysis of many modifications, highlighting the need for synthetic methods suitable for high throughput.

Current methods for the preparative scale synthesis of multimilligram quantities of tRNAs involve either in vivo overproduction (Perona et al., 1988), or in vitro runoff transcription by T7 RNA polymerase from linearized plasmid templates (Milligan et al., 1987). Of these, the T7 method has been preferred because in vivo processing of mutated transcripts is not always faithful, and because of the necessity for purification of engineered tRNAs from the pool of endogenous molecules. However, experimental difficulties are present with the T7 approach as well. Beyond the absence of naturally occurring nucleotide modifications, in vitro-transcribed tRNAs also often possess 3′ and/or 5′ heterogeneities (Milligan et al., 1987; Pleiss et al., 1998; Helm et al., 1999), requiring either tedious purification at reduced yields or the use of ribozyme sequences to excise the

Reprint requests to: John J. Perona, Department of Chemistry and Biochemistry, University of California at Santa Barbara, Santa Barbara, California 93106-9510, USA; e-mail: perona@chem.ucsb.edu.

full-length tRNA from flanking regions (Price et al., 1995; Ferre D'Amare & Doudna, 1996). Further, the ribozyme approach to addressing length heterogeneity itself can suffer from alternate secondary structure formation, from the need for further purification of the desired RNA, and from generation of a 2′-3′ cyclic phosphate at the 3′ terminus. An alternate method for elimination of 3′ heterogeneity is to employ synthetic DNA templates rather than linearized plasmids. The synthetic DNA is modified at the penultimate or the last 2 nt at the 5′ terminus, using either DNA base analogs (Moran et al., 1996) or 2′-*O*-methyl substitutions (Kao et al., 1999). In analytical scale transcriptions, the 2′-*O*-methyl substitution significantly reduces the proportion of runover transcripts and in some cases also increases the yield.

An important limitation of both in vivo overproduction and in vitro transcription is the inability to introduce natural or unnatural modified nucleotides at defined sites. The alternative of direct RNA chemical synthesis has not so far been applied to crystallography of tRNAs, in part because low coupling efficiencies have limited the size of the molecules that can be produced on a multimilligram scale (Wincott et al., 1995). Other synthetic approaches have succeeded in producing full-length tRNAs that could function as substrates for aminoacyl-tRNA synthetases (Lyttle et al., 1991; Goodwin et al., 1994, 1996; Ohtsuki et al., 1996; Persson et al., 2001). However, although synthetic yields up to the near-milligram range had been achieved for these tRNA-sized molecules, the methods were not robust and did not consistently produce high-quality RNA (Goodwin et al., 1994, 1996). RNA crystals derived from these chemical synthesis approaches had thus been confined to short duplexes (Wedekind & McKay, 2000), and to constructs of the hammerhead ribozyme incorporating individual strands up to 32 nt in size (Scott et al., 1995a, 1995b). The recent advances in RNA synthesis utilizing novel 2′-OH protecting groups have now substantially improved coupling efficiency, allowing for much more reproducible syntheses of longer molecules in larger, preparative quantities (Scaringe et al., 1998; Scaringe, 2000). With this methodology, 1.5–2 mg of purified RNAs up to 50 nt in size can be obtained from an 0.4 μmol synthesis. This represents a tripling of the yield even while working at a significantly smaller and more cost-effective scale than the 1 μmol syntheses used previously (Goodwin et al., 1994). These improved, highly reproducible methods set the stage for detailed biophysical analysis of both tRNAs and other RNA species.

Here we present two methods that exploit these recent advances to improve the efficiency and quality of large-scale tRNA preparations suitable for X-ray crystallographic investigations. As an application of the use of synthetic DNA templates incorporating 2′-*O*-methyl modifications, we describe the preparative-scale transcription and cocrystallization of $tRNA^{Gln}$ bound to GlnRS. This approach utilizes partially overlapping single-stranded DNAs that are extended enzymatically to create a duplex template. Reduction in the proportion of 3′-runover transcripts allowed cocrystallization at significantly reduced tRNA:synthetase mole ratios. Second, we generated milligram quantities of $tRNA^{Gln}$ half-molecules by chemical synthesis utilizing the newly developed 2′-ACE methodology (Scaringe et al., 1998; Scaringe, 2000), beginning from 0.4 μmol scale syntheses. Enzymatic ligation with T4 RNA ligase yielded the full-length 75-nt tRNA in sufficient quantities for cocrystallization with the cognate GlnRS enzyme. This approach extends applications of chemical synthesis to crystallization of much larger RNAs, and will permit detailed structural studies of chemically modified species.

RESULTS AND DISCUSSION

Preparative scale RNA synthesis by T7 RNA polymerase

In vitro transcription of *Escherichia coli* U1G $tRNA_2^{Gln}$ by T7 RNA polymerase, from *Bst*NI-linearized plasmid templates, yields preparations in which only 15–20% of the molecules are functional. This is due principally to a large fraction of products containing one or more additional nucleotides on the 3′ terminus (Nissan et al., 1999). The use of this tRNA in cocrystallizations requires a molar excess of 6:1 to 8:1 of tRNA over enzyme, thus necessitating the preparation of large quantities of material (Arnez & Steitz, 1994; Sherlin et al., 2000). The large proportion of nonfunctional tRNA also greatly complicates analysis of aminoacylation kinetics. In an effort to improve the yield of functional transcripts, we developed a methodology for large scale T7 RNA polymerase transcription using a site-specifically modified DNA template derived from combined chemical and enzymatic synthesis in vitro (Kao et al., 1999; Fig. 1). This template incorporates 2′-*O*-methyl sugars at the two 5′ nucleotides in the noncoding DNA strand.

The DNA template was constructed from two strands of DNA 57 and 56 nt in length, which were synthesized at the 0.2 μmol scale and purified by polyacrylamide gel electrophoresis. One strand contains the T7 RNA polymerase promoter and the 5′ portion of the tRNA, and the second encodes the complement of the 3′ portion of the tRNA. The two strands are designed to contain a 10-bp overlap to permit annealing. Full-length duplex DNA is then synthesized with the Klenow fragment of *E. coli* DNA polymerase I (Holowachuk & Ruhoff, 1995; Fig. 1). Because the 75-nt tRNA requires a template of 103 nt, the use of an enzyme for partial synthesis from shorter overlapped oligonucleotides substantially increases the yield of DNA over complete chemical synthesis of the duplex. The 0.2-μmol syn-

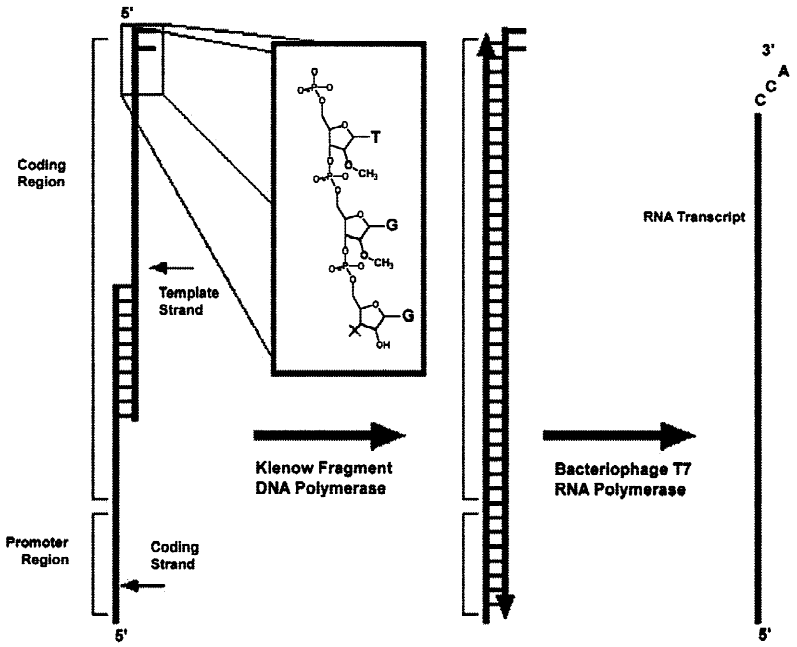

FIGURE 1. Schematic of synthetic template transcription. Overlapping synthetic nucleotides are used as a template for Klenow fragment extension by annealing and extension cycles (see Materials and Methods). The template strand contains 2′-*O*-methyl modifications on the two terminal 5′ residues (inset). The double-stranded fragment then acts as a template for T7 RNA polymerase. The DNA modifications are thought to cause the polymerase to dissociate from the template before adding nontemplated residues, thereby increasing product homogeneity (Kao et al., 1999).

theses produced 4–12 nmol of duplex template after PAGE purification, annealing, enzymatic extension, and purification. This is sufficient material for two to six 1-mL transcription reactions, each of which yield approximately 3 mg of tRNA.

Gel electrophoresis of the transcription products shown in Figure 2 confirms the observation of Kao et al. (1999) that templates with two 5′ *O*-methyl residues yield tRNAs with much less 3′ heterogeneity than the corresponding unmodified templates. More importantly, the proportion of functional transcripts that could be aminoacylated was increased by threefold (Table 1). It was then possible to cocrystallize this tRNA with GlnRS to yield large diffraction-quality crystals at tRNA: enzyme mole ratios as low as 2.5:1 (Table 2). This represents a considerable improvement over the 6:1 to 8:1 ratios required with previous preparations of $tRNA^{Gln}$ derived from in vitro transcription (Arnez & Steitz, 1994; Sherlin et al., 2000). The molar excess of tRNA still required may in part be due to 5′ heterogeneity arising from transcription through the five consecutive guanosines at the 5′ terminus of U1G $tRNA_2^{Gln}$ (Pleiss et al., 1998).

These results indicate that preparative scale synthesis of RNAs up to tRNA size, and likely somewhat larger, can be achieved from chemically synthesized DNA templates incorporating ribose modifications to enhance polymerase fidelity. Further, the strategy of template synthesis by annealing overlapping single-stranded DNAs, coupled to second strand synthesis by Klenow fragment, substantially increases the size of RNA molecules that may be synthesized on a multimilligram scale without the need for preparation of plasmid templates. This application for chemically synthesized DNA templates thus offers a rapid avenue to prepare mutant tRNAs without the need for site-directed mutagenesis.

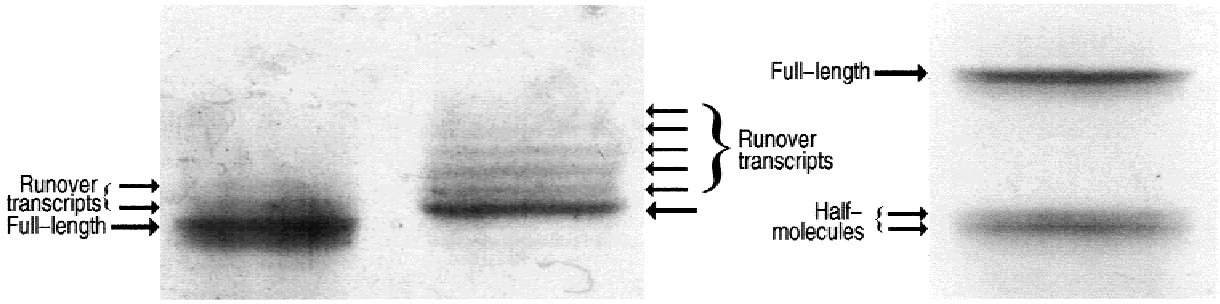

FIGURE 2. Polyacrylamide gel electrophoresis of tRNAs. Left: tRNA synthesized enzymatically by T7 RNA polymerase using a duplex oligodeoxynucleotide template containing 2′-*O*-methyl modifications. Center: tRNA synthesized enzymatically by T7 RNA polymerase using a plasmid template lacking modifications. Right: tRNA synthesized chemically and subjected to ligation by T4 RNA ligase.

TABLE 1. Aminoacylation efficiency of $tRNA^{Gln}$ constructs.

Source of tRNA	Aminoacylation (pmol/40 μg)
In vivo overexpression	1,600[a]
T7 in vitro–plasmid template	300 ± 47
T7 in vitro–2′-*O*-methyl template	900 ± 115
Chemical synthesis	1,100 ± 95

[a]Taken from Perona et al. (1988).

Enzymatic ligation and crystallization of chemically synthesized tRNA

The T7 transcription approach does not permit the site-specific introduction of chemically modified ribonucleotides, many of which are very useful in structure–function analysis. In addition, many tRNAs (including *E. coli* $tRNA_2^{Gln}$) begin with nucleotides unfavorable for efficient transcription initiation by T7 RNA polymerase. To address these limitations, we exploited recent advances in the synthesis of RNA using 2′-ACE technology (Scaringe et al., 1998; Scaringe, 2000) to generate milligram quantities of a full-length intact 75-nt $tRNA^{Gln}$ species derived entirely from chemical synthesis. Unmodified 36 and 39-mer *E. coli* $tRNA_2^{Gln}$ half-molecules corresponding to nt U1-A37 and U38-A76 were each synthesized at the 0.4-μmol scale, and annealed together to generate the nicked molecule (Fig. 3). The nick was then sealed using bacteriophage T4 RNA ligase (Kaufman & Littauer, 1974; Bruce & Uhlenbeck, 1982; Satoh et al., 2000), and the efficacy of the procedure assessed by denaturing polyacrylamide gel electrophoresis. This analysis shows that the half-molecules were enzymatically ligated at approximately 70% efficiency (Fig. 2). A comparable efficiency of ligation was obtained for half-molecules joined between U38 and U39 (data not shown). T4 RNA ligase was chosen for this step rather than T4 DNA ligase (Moore & Sharp, 1992; Han & Dervan, 1994), because of the lower efficiency of the latter enzyme and its requirement for a bridging oligodeoxynucleotide. The high coupling efficiency of the chemical synthesis provides a typical yield of 2.5–3 mg of purified full-length tRNA after ligation, beginning with the 0.4-μmol scale synthesis of each half-molecule.

$tRNA^{Gln}$ generated in this manner was further purified by gel electrophoresis and then tested for substrate activity in glutaminylation assays with *E. coli* GlnRS. Under conditions of enzyme excess that allow maximal aminoacylation, glutamine could be attached to approximately 70% of the substrate molecules, a somewhat better efficiency than achieved with enzymatic transcription from 2′-*O*-methyl modified templates (Table 1). Aminoacylation levels of the synthetic $tRNA^{Gln}$ are only slightly lower than those measured for purified $tRNA_2^{Gln}$ derived from in vivo overexpression (Perona et al., 1988).

To assess the suitability of the chemically synthesized tRNA for biophysical studies, the molecule was cocrystallized with GlnRS and ATP under conditions similar to those previously developed for the native com-

TABLE 2. X-ray data collection and refinement statistics.

	Synthetic tRNA	In vitro-transcribed tRNA
Space group	$C222_1$	$C222_1$
Cell constants		
a,b,c (Å)	239.18, 94.47, 115.82	236.45, 92.89, 114.71
α,β,γ (°)	90.0, 90.0, 90.0	90.0, 90.0, 90.0
Resolution (Å)	40.0–2.4	60.0–2.26
Number of reflections	457,637	402,134
Unique reflections	51,582	48,063
R_{merge}[a]	6.9% (44.3%)[b]	4.4% (35.6%)[b]
I/σ[c]	16.7 (2.9)[b]	13.3 (2.1)[b]
Completeness	99.8 (99.6)[b]	77.3 (51.6)[b]
Multiplicity	4.8 (4.6)[b]	2.9 (2.0)[b]
R_{cryst}[d]	24.3%	22.1%
R_{free}[e]	29.6%	27.4%
RMSD		
Bonds (Å)	0.009	0.008
Angles (°)	1.576	1.577

[a] $R_{merge} = (\sum_h \sum_i |\langle F_h \rangle - F_{hi}|)/(\sum_h F_h)$ where $\langle F_h \rangle$ is the mean structure factor magnitude of *i* observations of symmetry-related reflections with Bragg index *h*.

[b] Numbers in parentheses refer to statistics for the highest 0.1 Å resolution shell.

[c] I/σ, average intensity over sigma.

[d] $R_{cryst} = (\sum_h \sum_i ||F_{obs}| - |F_{calc}||)/(\sum |F_{obs}|)$ where F_{obs} and F_{calc} are the observed and calculated structure factor magnitudes.

[e] R_{free} is calculated with a test set containing 10% of the data removed at the start of refinement.

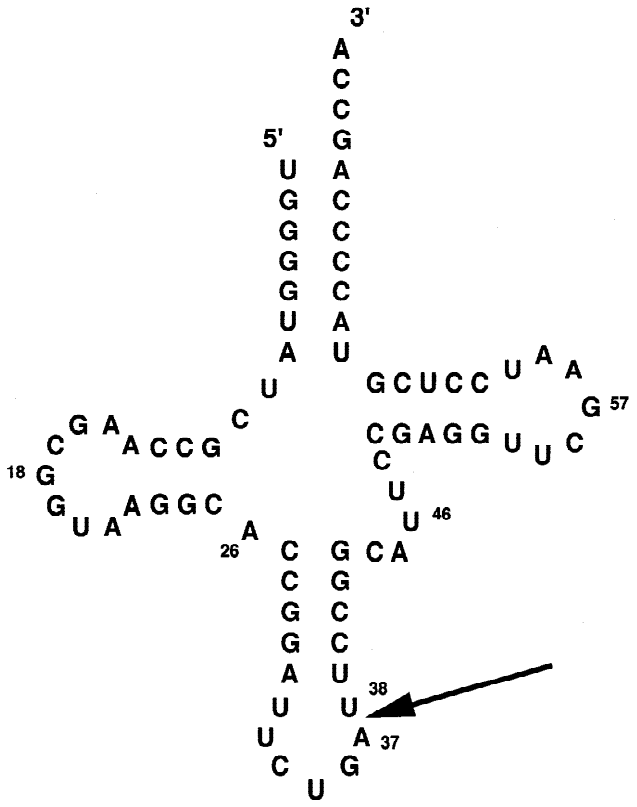

FIGURE 3. Cloverleaf secondary structure of *E. coli* $tRNA_2^{Gln}$. The arrow indicates the position of ligation of half-molecules, to form the complete tRNA. A U1G mutation was introduced into the sequence for the bacteriophage T7 transcription of plasmid-derived and synthetic template tRNAs to promote transcription.

plex (Perona et al., 1988; Rould et al., 1989). Large orthorhombic crystals were obtained whether or not the tRNA was subjected to the additional gel electrophoresis purification step. Further, the crystals grow from solutions containing equivalent or only slight molar excess of tRNA with respect to enzyme. This is in contrast to the high 6:1 to 8:1 tRNA:GlnRS mole ratios required for cocrystallization of $tRNA^{Gln}$ obtained from in vitro transcription reactions (Arnez & Steitz, 1994; Bullock et al., 2000; Sherlin et al., 2000), and is also improved compared to the 2.5:1 ratio required using tRNA derived from 2′-*O*-methyl modified templates (see above and Materials and Methods). The structure of the GlnRS-$tRNA^{Gln}$ complex was determined at 2.4 Å resolution from crystals containing tRNA that was not further purified after enzymatic ligation (Table 2). Electron density maps calculated in the region of the anticodon loop show an intact sugar–phosphate backbone at the ligation junction between A37 and U38 (Fig. 4). This shows that the tRNA half-molecules present in the crystallization mixture do not inhibit crystal formation, and indicates that modified tRNAs produced in this manner will be suitable for detailed structural analysis.

This methodological approach involving enzymatic ligation of chemically synthesized tRNA half-molecules will be suitable as a basis for producing equivalent high yields of selectively modified tRNAs. Although the incorporation of modified nucleosides has previously been found to reduce coupling efficiencies, the new synthetic approaches exploited here are not sensitive to their presence. Equivalent high yields of RNAs, including tRNA half-molecules, have been obtained with species incorporating 2′-deoxy, T, Ψ, Gm, Um, or s^4U modifications. Experiments to crystallize $tRNA^{Gln}$ variants possessing site-specific modified groups are in progress.

The two procedures described here should be applicable to the crystallization and structural analysis of other tRNAs in unliganded and protein-bound states, as well as to RNAs possessing different biological functions. The chemical synthesis and ligation method has the advantage that both base and sugar–phosphate modifications can be incorporated at defined positions, making readily possible the crystallographic study of precise single and multiple “atomic group” mutants for RNAs up to approximately 100 nt in size. Particularly in the case of tRNAs, the precise 3′-end synthesis ensures a high proportion of functional transcripts and thus more accurate kinetic analyses as well. In experimental systems where only natural base substitutions are required, the first approach utilizing chemically modified oligodeoxynucleotide templates may be preferred because of the somewhat higher cost of RNA synthesis. Other crystallized RNA and RNA–protein systems that should be immediately amenable to either of these approaches include small catalytic RNAs from natural sources (Pley et al., 1994; Scott et al., 1995a; Ferre D’Amare et al., 1998), aptamers arising from in vitro selection schemes (Wedekind & McKay, 1999; Sussman et al., 2000), and ribosomal RNA domains cocrystallized with ribosomal proteins (Agalarov & Williamson, 2000).

MATERIALS AND METHODS

Enzyme purifications

T7 RNA polymerase was purified from *E. coli* BL21 cells containing the plasmid *p*AR1219 (Grodberg & Dunn, 1988). Purified enzyme was stored at −20 °C in 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 50% glycerol, 20 mM sodium phosphate, pH 7.8. Glutaminyl-tRNA synthetase was purified by a two-column procedure (Hoben & Soll, 1985) from *E. coli* K12Δ*H1*Δ*trp* cells containing the overexpression vector *p*MN20 (Perona et al., 1988). The enzyme was stored at −20 °C at 20 mg/mL in a buffer containing 50% glycerol, 50 mM KCl, 20 mM KH_2PO_4, pH 7.2, 20 mM β-mercaptoethanol.

Enzymatic synthesis of RNA

All oligodeoxynucleotides used as templates for enzymatic transcription were obtained from Integrated DNA Technolo-

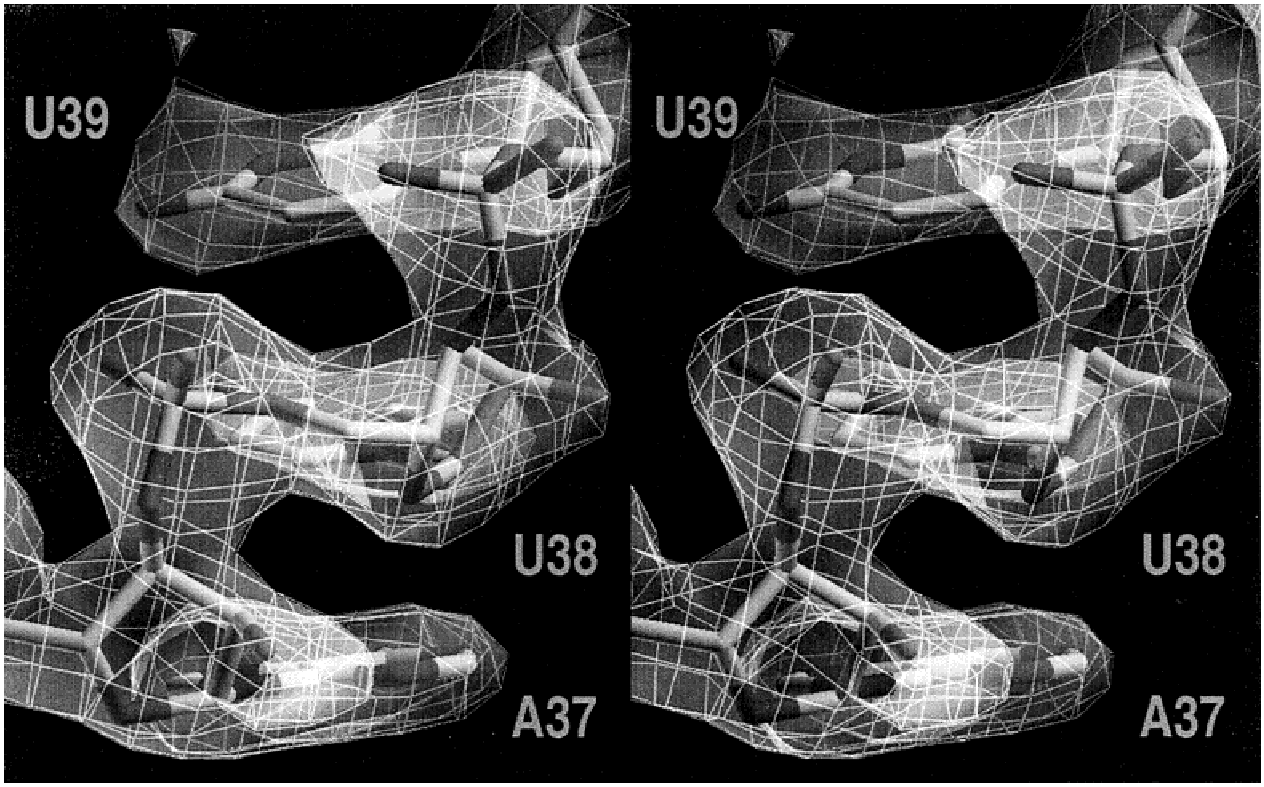

FIGURE 4. Divergent stereo view of the anticodon region of $tRNA^{Gln}$. The overlay of a simulated annealing omit map contoured at 0.8σ indicates ligation between residues 37 and 38 to form the full-length tRNA.

gies in PAGE-purified form. Oligonucleotides were designed such that the sense strand corresponding to the 5′ end of the sequence possesses a 10-bp overlap with the 3′ antisense strand (Fig. 1; Bullock et al., 2000). The oligonucleotides used for construction of the *E. coli* $tRNA_2^{Gln}$ gene are: 5′-AAT TCCTGCAG**TAATACGACTCACTATA**GGGGGTATCGCCA AGCGGTAAGGCACCGG-3′; 5′-mTmGGCTGGGGTACGA GGATTCGAACCTCGGAATGCCGGAATCAGAATCCGGTG CCTT-3′, where mT and mG represent the 2′-*O*-methyl nucleotides and the underlined portions represent the overlapped region. Bold type indicates the T7 RNA polymerase promoter.

Oligonucleotides were mixed to an equimolar concentration of 4 μM in a reaction solution containing 400 μM dNTPs, 10 mM Tris-HCl, pH 7.5, 10 mM $MgSO_4$, 7.5 mM DTT, and 50 U/mL Klenow fragment polymerase (Promega). The mixture was cycled between 283 K and 310 K at 30 s intervals for eight cycles, after which the DNA was precipitated in 65% ethanol/0.3 M sodium acetate, pelleted, and resuspended in 100 μL PMS buffer (5 mM PIPES, pH 7.5/10 mM $MgSO_4$).

Transcription was performed in solutions containing 250 mM HEPES-KOH, pH 7.5, 30 mM $MgCl_2$, 2 mM spermidine, 40 mM DTT, 0.1 mg/mL bovine serum albumin, 5 mM dNTPs, 5 μg inorganic pyrophosphatase (Boeringer Mannheim), 50 U RNasin (Amersham), 40 μg/mL T7 RNA polymerase, and 1 μM DNA template from the Klenow extension reaction (Milligan et al., 1987; Pokrovskaya & Gurevich, 1994; Sherlin et al., 2000). The 2-mL reaction mixture was incubated at 310 K for 8–10 h, at which time RQ1 RNase-free DNase (Promega) was added to 10 U/mL and the incubation continued for a further 2–3 h. The reactions were then loaded on a 5-mL DE-52 (Whatman) column preequilibrated with 100 mM HEPES-KOH, pH 7.5, 12 mM $MgCl_2$, and 200 mM NaCl. The column was washed with 30 mL equilibration buffer and the RNA eluted with a solution of 100 mM HEPES-KOH, pH 7.5, 12 mM $MgCl_2$, 600 mM NaCl. Fractions containing tRNA were dialyzed into PMS buffer and refolded by heating to 343 K followed by slow cooling to room temperature. The tRNA was precipitated with ethanol, resuspended in PMS buffer, and further dialyzed into PMS buffer prior to further use.

Chemical synthesis of RNA

RNA oligonucleotides were chemically synthesized using the 5′-*O*-silyl ether protecting group in conjunction with an acid-labile 2′-*O*-orthoester (Scaringe et al., 1998; Scaringe, 2000). Oligonucleotides were deprotected by incubation at 60 °C in 100 mM acetic acid, pH 3.8, lyophilized, and stored at −20 °C until ready for use. RNAs corresponding to tRNA half-molecules (with the break between nucleotides A37 and U38) were resuspended in PMS buffer and annealed by mixing molar equivalents to 50 μM and heating to 363 K in ligase reaction buffer (50 mM Tris-HCl, pH 7.8/25 mM KCl/10 mM $MgCl_2$/10 mM DTT/1 mM ATP) for 5 min, followed by slow cooling to room temperature at a rate of 1 K/min. T4 RNA ligase (New England Biolabs) was added to 0.1 U/mL and the mixture was incubated for 6 h at 310 K. Ligation was confirmed by denaturing polyacrylamide gel analysis, and the full-length RNA purified by repeated phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. The resulting pre-

cipitate was resuspended in PMS buffer to a concentration of approximately 1 mM for crystallization. Samples used for aminoacylation kinetics were gel purified, recovered from the gel slice by extraction with organic solvents, and refolded according to the above protocol.

Aminoacylation assays

Plateau aminoacylation values were determined by the filter-binding method, as described (Hoben & Soll, 1985; Nissan et al., 1999). The fraction of transcripts capable of aminoacylation was determined by reactions carried out under conditions of enzyme excess, and varying the concentration of tRNA. The concentration of ATP was 4 mM and that of [^{3}H]-labeled glutamine was 680 μM. The counting efficiency of [^{3}H]-glutamine was corrected as described (Nissan et al., 1999).

Crystallization and X-ray structure determinations

Crystals of GlnRS bound to tRNA transcribed in vitro from 2′-*O*-methyl-containing templates were grown in the presence of glutamine and the ATP analog α,β-methyleneadenosine 5′-triphosphate (AMPCPP; Sigma Chemical Co.). The crystals were grown by microseeding with crystals of the GlnRS-tRNA^{Gln}-ATP ternary complex. Two microliters of a diluted stock of crushed crystals in 2.25 M ammonium sulfate, 20 mM PIPES, pH 7.0, 10 mM $MgSO_4$, 20 mM β-mercaptoethanol, and 60 mM glutamine were mixed with 2 μL of the GlnRS-tRNA^{Gln} complex containing 5 mg/mL GlnRS, a 2.5:1 mole ratio of tRNA to enzyme, and 2 μM AMPCPP. These crystals grew in 1–2 weeks by vapor diffusion over a reservoir containing 2 M ammonium sulfate, 20 mM PIPES, pH 7.0, 10 mM $MgSO_4$, 20 mM β-mercaptoethanol. After growth, crystals were stabilized for 5 min in a solution containing 2.25 M ammonium sulfate, 20% glycerol, 20 mM PIPES, pH 7.0, 20 mM β-mercaptoethanol, 2 μM AMPCPP, and 60 mM glutamine, and then transferred for 1 min to a second stabilizer containing 2 M ammonium sulfate, 20% sucrose, 15% xylitol, 20% glycerol, 20 mM PIPES, pH 7.0, 20 mM β-mercaptoethanol, 2 μM AMPCPP, and 10 mM glutamine, prior to freezing.

Crystals of GlnRS bound to tRNA derived from chemical synthesis were grown in the presence of 2 mM ATP, from solutions in which the tRNA:enzyme mole ratio varied from 1:1 to 1.5:1. The tRNA in these crystallizations was not gel purified and contains the unligated half-molecules in a proportion of approximately 30%. These crystals were obtained by vapor diffusion at 298 K from solutions containing 2.25 M ammonium sulfate, 20 mM PIPES, pH 7.0, 10 mM $MgSO_4$, 20 mM β-mercaptoethanol (Perona et al., 1988; Rould et al., 1989; Arnez & Steitz, 1994). The final concentration of the complex was kept in the 6–10 mg/mL range. The crystals were prepared for data collection by equilibration for 5 min in a stabilization solution containing 2.3 M ammonium sulfate, 20% glycerol, 80 mM PIPES, pH 7.0, 30 mM $MgSO_4$, 0.02% NaN_3, and 1 mM ATP. Crystals were then transferred to a cryostabilization solution containing 2.6 M ammonium sulfate, 20% glycerol, 20% sucrose, 15% xylitol and 1 mM ATP for 1 min.

For data collection, all crystals were mounted in a cryoloop, dunked in liquid nitrogen, and placed in a cryostream at 100 K. Diffraction amplitudes were collected at the Stanford Synchrotron Research Laboratory on beamline 9-1. Data were reduced with the programs XDISPLAYF and DENZO (Otwinowski, 1993). The structures were refined with the XPLOR package (Brunger et al., 1987) using, in each case, the 100 K structure of Rath et al. (1998) as a starting model.

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