Role of Cysteine Residues in Pseudouridine Synthases of Different Families†

Vidhyashankar Ramamurthy, Steven L. Swann, Christopher J. Spedaliere, and Eugene G. Mueller*

Department of Chemistry & Biochemistry, University of Delaware, Newark, Delaware 19716

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ABSTRACT: The pseudouridine synthases catalyze the isomerization of uridine to pseudouridine in RNA molecules. An attractive mechanism was proposed based on that of thymidylate synthase, in which the thiol(ate) group of a cysteine side chain serves as the nucleophile in a Michael addition to C6 of the isomerized uridine. Such a role for cysteine in the pseudouridine synthase TruA (also named Ψ synthase I) has been discredited by site-directed mutagenesis, but sequence alignments have led to the conclusion that there are four distinct "families" of pseudouridine synthases that share no statistically significant global sequence similarity. It was, therefore, necessary to probe the role of cysteine residues in pseudouridine synthases of the families that do not include TruA. We examined the enzymes RluA and TruB, which are members of different families than TruA and each other. Substitution of cysteine for amino acids with nonnucleophilic side chains did not significantly alter the catalytic activity of either pseudouridine synthase. We conclude, therefore, that neither TruB nor RluA require thiol(ate) groups to effect catalysis, excluding their participation in a Michael addition to C6 of uridine, although not eliminating that mechanism (with an alternate nucleophile) from future consideration.

The pseudouridine synthases catalyze the most prevalent posttranscriptional modification of RNA, the conversion of uridine (U)[1] in RNA to its C-glycoside isomer, pseudouridine (Ψ), as shown in Figure 1. All organisms contain Ψ in RNA, including transfer RNA (tRNA), ribosomal RNA, small nuclear RNA, and small nucleolar RNA (*1*), and Ψ synthases range from mild promiscuity (*2*) to specificity for a single position in one particular RNA molecule (*3*). After the cloning of four genes encoding Ψ synthases (all from *Escherichia coli*) (*3*−*6*), sequence comparisons (*7, 8*) revealed no statistically significant global sequence similarity between the four enzymes, but each had clear homologues and orthologues in the database, leading to the grouping of the Ψ synthases into four "families" (*7*). Two short (<14 amino acids) motifs were found in all four families, and two of the families shared a third short motif (*7, 8*). Furthermore, the similarity in these motifs was not overwhelming (<33% identity). While the sequence information has allowed rapid cloning (nine genes in 4 years) of Ψ synthase genes from *E. coli* (*9*−*11*), yeast (*12*−*14*), mouse (*15*), and human (*15*), the mechanistic details of the isomerization carried out by these enzymes remain unelucidated. Given the limited stretches of similarity (the two shared motifs), findings from an enzyme of one family cannot be confidently extrapolated to members of other families, but instead families must be investigated separately. In fact, the possibility remains open that different families of Ψ synthases catalyze the isomerization of uridine in RNA by mechanisms that differ to a significant degree.

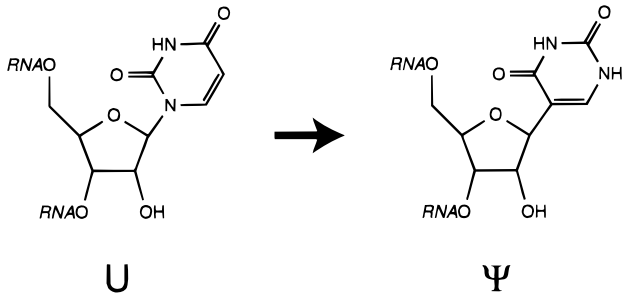

FIGURE 1: Isomerization of uridine (U) to pseudouridine (Ψ) in RNA.

Two attractive mechanisms that do indeed differ significantly have been proposed for the Ψ synthase reaction. The first proposal (Figure 2) was based on the mechanism of thymidylate synthase (*16*). An enzymic nucleophile attacks the substrate U at C6 to form a Michael adduct, and the glycosidic bond then breaks. The rotation of the uracil portion of the Michael adduct about the C6-nucleophile bond repositions C5 near C1′, and they form a new carbon−carbon bond. Expulsion of the enzymic nucleophile and deprotonation of C6 affords Ψ. On the basis of the thymidylate synthase precedent (*17*), the enzymic nucleophile was postulated to be the thiol(ate) of a cysteine side chain, which was supported by the inhibition of TruA (also called Ψ synthase I) upon exposure to thiol-specific reagents (*16*). Later, however, all three cysteine residues in TruA were converted to alanine (singly and together) by site-directed mutagenesis, and the mutant TruA possessed essentially full activity (*18*).

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* Corresponding author. Phone: (302) 831-2739. Fax: (302) 831-6335. E-mail: emueller@udel.edu.

[1] Abbreviations: U, uridine; Ψ, pseudouridine; Ψ synthase, pseudouridine synthase; TruA, the Ψ synthase encoded by the gene *truA* (formerly *hisT*), which handles U at positions 37−39 in *E. coli* tRNA (formerly known as Ψ synthase I); TruB, the Ψ synthase encoded by the gene *truB*, which handles U at position 55 in *E. coli* tRNA; RluA, the Ψ synthase encoded by the gene *rluA*, which handles U at position 32 in *E. coli* tRNA and at position 746 in *E. coli* 23S rRNA; [^{3}H]-tRNA, the in vitro transcript of *E. coli* tRNA^{Phe} containing [5-^{3}H]uridine.

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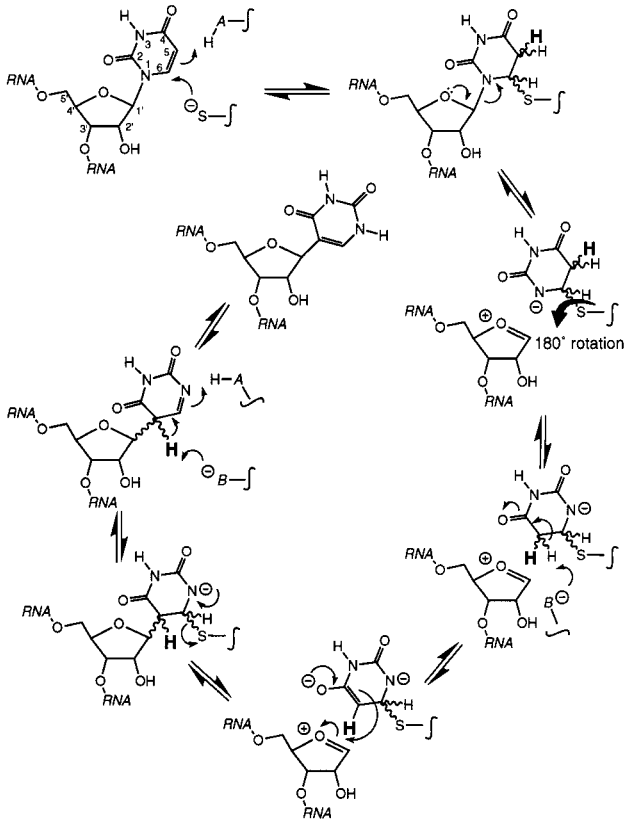

FIGURE 2: Mechanism for Ψ synthases proposed by Kammen et al. (*16*). The thiolate of a cysteine residue is the nucleophile in the Michael addition. Protonation is likely to occur at O^4, but protonation at C5 is depicted to emphasize the similarity of this mechanism to thymidylate synthase, where a methyl donor substitutes for the acid shown here, leading to methylation of C5 (*17*). The oxocarbenium is likely stabilized by ion pairing, nucleophilic trapping, or some other means. For simplicity, the uracil anion is drawn as a bare anion but is likely stabilized by protonation (at O^2 or N1), hydrogen bonding, or metal chelation. *A*-H denotes a general acid; *B* denotes a general base; **H** denotes the tritium in [^{3}H]RNA that is released in the assay. The squiggles denote attachment to the enzyme.

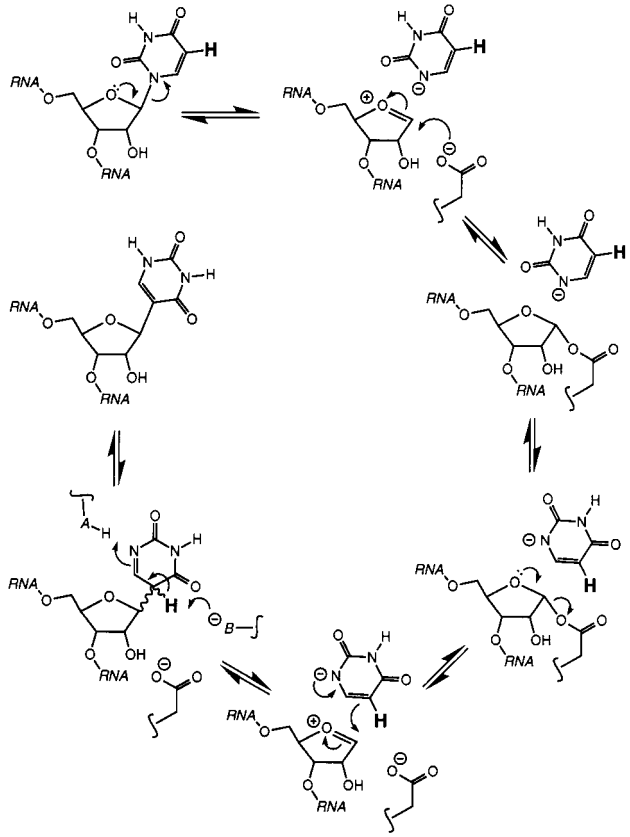

FIGURE 3: Mechanism for Ψ synthases proposed by Santi and coworkers (*19*). Stabilization of the oxocarbenium ion is postulated to occur through formation of an acylal intermediate with an aspartic acid residue shown to be critical for catalysis (*19*, *20*); the original proposal (*19*) invoked S_N2 rather than S_N1 chemistry at C1′. For simplicity, the uracil anion is drawn as a bare anion, but it will likely be stabilized by protonation (at O^4 or O^2 or N1), hydrogen bonding, or metal chelation. *A*-H denotes a general acid; *B* denotes a general base; **H** denotes the tritium in [^{3}H]RNA that is released in the assay. The squiggles denote attachment to the enzyme.

The second proposed mechanism for the Ψ synthase reaction arose from the observation that only a single aspartic acid residue was aligned in all of the known Ψ synthases and their homologues (*19*). Santi and co-workers mutated this aspartic acid residue in TruA, and conversion to alanine, asparagine, glutamate, serine, or lysine resulted in catalytically inactive enzymes that were still able to bind tRNA substrate (*19*). This result and the finding that cysteine residues were *not* critical for TruA activity led to the proposal of a mechanism (Figure 3) in which a Michael adduct does *not* form and the catalytically critical aspartic acid residue forms an acylal intermediate with the ribose ring (*19*). No direct evidence to support this mechanism however has been offered, so it remains an attractive hypothesis rather than an established fact.

These first steps toward elucidating the mechanism by identifying critical amino acids were performed with TruA. In light of the limited sequence similarity to the other families of Ψ synthases, it remains imperative to establish mechanistic facts for Ψ synthases of other families. We have recently reported that the aligned aspartic acid residue is also critical in members of two different families (*20*). The two Ψ synthases examined were TruB, which isomerizes U55 of tRNA (*3*), and RluA, which isomerizes both U746 of 23S rRNA and U32 of tRNA (*5*). The present work examines whether these same two enzymes require cysteine residues for catalytic activity as postulated in the mechanism shown in Figure 2 (*16*).

MATERIALS AND METHODS

General. Activated charcoal (Norit SA-3) was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). Competent JM109(DE3) *E. coli* cells were purchased from Promega Corporation (Madison, WI), and competent BLR-(DE3) pLysS *E. coli* cells were purchased from Novagen (Madison, WI). Isopropyl thio-β-D-galactopyranoside (IPTG), HEPES, and Tris were purchased from Boehringer Mannheim Corporation (now Roche Molecular Biochemicals, Indianapolis, IN). Oligonucleotides ("OPC-purified" grade) were purchased from the Great American Gene Co. (Ramona, CA). QuikChange site-directed mutagenesis kits were purchased from Stratagene (La Jolla, CA). Prime RNase inhibitor was purchased from 5′→3′ Prime, Inc. (Boulder, CO), and Ni−NTA superflow resin was obtained from Qiagen (Chatsworth, CA). Spectra/Por dialysis tubing (12 000−14 000 NMWCO) and all other chemicals were purchased from Fisher Scientific (Philadelphia, PA) or its Acros Organics division (Pittsburgh, PA). A Robocylcer

Gradient 96 thermal cycler (Stratagene) was used for the PCR component of the site-directed mutagenesis. DNA sequencing was performed in the University of Delaware Cell Biology Core Facility using a Long Readir 4200 DNA sequencer (Li-Cor, Inc., Lincoln, NE).

Site-Directed Mutagenesis of TruB and RluA. Plasmids containing the genes *truB* and *rluA* placed in pET15b vectors (Novagen) to fuse a His_6·Tag at N-terminus of overexpressed proteins were generously provided by J. Ofengand. Because the original reports (*3, 5*) do not name these plasmids, they will be referred to as pΨ55 (encoding *truB*) and pΨ746 (encoding *rluA*). Site-directed mutagenesis was performed using the QuikChange protocol (Stratagene) according to the manufacturer's instructions except that the following thermal cycling program was substituted: 95 °C, 1 min; 16× (95 °C, 1 min; 55 °C, 1.5 min; 68 °C, 15 min); 4 °C, hold.

The QuikChange protocol amplifies the entire plasmid by PCR with primers containing mismatches to alter a codon. For mutant TruB with one cysteine residue replaced, pΨ55 was used; for mutant RluA with one cysteine residue replaced, the plasmid template was pΨ746. All references to amino acid positions are in terms of the primary sequence of the native protein (not the His_6·Tagged protein; this particular His_6·Tag adds 20 amino acids to the N-terminus). The primers used for each mutation are presented below with the upper primer broken into codons and the altered codon (and its complement on the other primer) denoted in bold type:

TruB C58A

CC GGC ATG TTG CCG ATT **GCC** CTC GGG GAA GCG AC

GT CGC TTC CCC GAG **GGC** AAT CGG CAA CAT GCC GG

TruB C174A

G CTG GAG CTG GAA ATT CAC **GCC** TCA AAA GGC ACT TAT ATC CGC

GCG GAT ATA AGT GCC TTT TGA **GGC** GTG AAT TTC CAG CTC CAG C

TruB C193A

GGT GAA AAA CTC GGC **GCT** GGC GCG CAT GTT ATT TAC C

G GTA AAT AAC ATG CGC GCC **AGC** GCC GAG TTT TTC ACC

Rlu A C117A

GAA CTG CCG CTG ATT **GCC** GAC TGG CCA AAC CGC CCG

CGG GCG GTT TGG CCA GTC **GGC** AAT CAG CGG CAG TTC

RluA C128A

GC CCG AAA CAG AAA GTC **GCT** TAC GAA ACG GGT AAA CCT G

C AGG TTT ACC CGT TTC GTA **AGC** GAC TTT CTG TTT CGG GC

The success of the mutagenesis protocol was confirmed by sequencing the entire gene. Generally, two of three plasmids isolated from independent colonies after the QuikChange protocol were sequenced and had only the desired mutation. In the initial mutagenesis of Cys-193 of TruB however the targeted C193A was not obtained, rather the C193V enzyme resulted, which served our purposes equally well. The generation of RluA and TruB with either two or three cysteine residues mutated to alanine (the double and triple mutants) were made by subjecting plasmids encoding appropriate single or double mutants to the QuikChange protocol. The names of the plasmids encoding the mutant Ψ synthases are presented in Table 1. The sequences of all plasmids described in this paper can be found at http://www.udel.edu/chem/mueller. The nucleotide sequence of *truB* is in the GenBank Database (AE000397, the complement of nucleotides 8456−9400), and TruB is in the Swissprot Database (file P09171). The nucleotide sequence of *rluA* is in the GenBank Database (AE000116, the complement of nucleotides 2481−3140), and RluA is in the Swissprot Database (file P39219).

Table 1: Plasmids Described in This Paper and the Mutations Present in the Encoded Ψ Synthases[a]

name	gene	mutation	parent plasmid
pΨ746	*rluA*	wild-type	pET15b
pBH201	*rluA*	C128A	pΨ746
pBH203	*rluA*	C117A	pΨ746
pBH204	*rluA*	C117A/C128A	pBH201
pΨ55	*truB*	wild-type	pET15b
pBH302	*truB*	C58A	pΨ55
pBH303	*truB*	C174A	pΨ55
pBH304	*truB*	C193V	pΨ55
pBH306	*truB*	C58A/C174A	pBH302
pBH307	*truB*	C58A/C193A	pBH302
pBH308	*truB*	C174A/C193A	pBH303
pBH309	*truB*	C58A/C174A/C193A	pBH307

[a] Plasmids encoding the wild-type RluA (pΨ746) and TruB (pΨ55) were constructed by Ofengand and co-workers (*3, 5*). All proteins expressed from these plasmids have an N-terminal His_6·Tag. The parent plasmid was used as the template in the Quick Change protocol to generate the mutation denoted. The sequences of all plasmids can be found at http://www.udel.edu/chem/mueller.

Overexpression and Purification of the Ψ Synthases. Overexpression of wild-type and mutant TruB and RluA was achieved by induction of expression by addition of IPTG to a culture of λDE3-lysogenized *E. coli* harboring the appropriate plasmid (Table 1). The cells were harvested 3 h after induction, and the Ψ synthase was purified by chromatography over Ni−NTA resin, eluting with a step of imidazole. Detailed procedures have been published elsewhere (*20*). Protein concentrations were determined by the Biuret method with bovine serum albumin as the standard (*21*); along with $A_{280\ nm}$, the determined concentrations have been used to calculate the extrinction coefficients for both RluA ($\epsilon_{280\ nm} = 6.6 \times 10^3\ M^{-1}\ cm^{-1}$) and TruB ($\epsilon_{280\ nm} = 2.1 \times 10^4\ M^{-1}\ cm^{-1}$). We have determined that our previously reported (*20*) storage conditions for RluA result in loss of activity (though not precipitation), so we now store RluA as described by Wrzesinski et al. (*5*): 30 μg/mL in 1:1 glycerol:20 mM Tris·HCl, pH 7.9, containing NaCl (0.35 M); −20 °C.

Ψ Synthase Activity Assay. The assay for Ψ synthase activity measures the liberation of tritium from C5 when labeled U in RNA is isomerized to Ψ and is a slight modification of the tritium release assay reported by Nurse et al. (*3*). The substrate was the in vitro transcript of *E. coli* $tRNA^{Phe}$ containing [5-^{3}H]uridine ([5-^{3}H]tRNA), which was prepared as described earlier (*20*). The concentration of [5-^{3}H]tRNA was measured in two ways. First, $A_{260\ nm}$ was determined, and the concentration was calculated using $\epsilon_{260\ nm} = 5 \times 10^5\ M^{-1}\ cm^{-1}$ (*22*). Second, the tritium activity was determined by liquid scintillation counting, and the specific radioactivity of the initial [5-^{3}H]UTP was used along with the sequence of *E. coli* $tRNA^{Phe}$ to determine the concentration of [5-^{3}H]tRNA. Substrate preparations were not deemed pure until the two methods (spectrophotometric and radiochemical) returned concentrations that agreed within 10%.

Table 2: Kinetic Parameters for Wild-Type and Mutant RluA and TruB

enzyme	k_{cat} (s^{-1})	K_m (nM)	k_{cat}/K_m (M^{-1} s^{-1})
	TruB		
wild-type	0.12 ± 0.01	146 ± 14	7.5×10^5
C58A	0.26 ± 0.01	177 ± 22	1.5×10^6
C174A	0.16 ± 0.01	127 ± 80	1.3×10^6
C193V	0.18 ± 0.01	218 ± 4	8.3×10^5
triple (C58A/C174A/C193A)	0.11 ± 0.01	124 ± 10	8.9×10^5
	RluA		
wild-type	0.099 ± 0.003	108 ± 20	9.2×10^5
C117A	0.070 ± 0.012	420 ± 60	1.7×10^5
C128A	0.15 ± 0.02	160 ± 55	9.4×10^5
double (C117A/C128A)	0.074 ± 0.012	157 ± 25	4.7×10^5

Unlabeled tRNA transcript was not judged to be pure unless the ratio of $A_{260\ nm}$ to $A_{280\ nm}$ was greater than 1.9 and >90% of the $A_{260\ nm}$ units were retained in a Microcon-3 ultrafiltration device. In all cases when purity was initially deemed insufficient, the initially measured $A_{260\ nm}$ overestimated the actual concentration of tRNA.

A typical assay mixture (250 μL) was 50 mM HEPES buffer, pH 7.5, containing ammonium chloride (100 mM), DTT (5 mM), EDTA (1 mM), Prime RNase inhibitor (30 units), and [5-^{3}H]tRNA (112−1125 nM, 1.216 μCi/nmol tRNA). After 5 min at 37 °C, a small volume (<5 μL) of a solution of Ψ synthase (final concentration = 20 nM) was added to initiate reaction. Aliquots (47.5 μL) were removed periodically (30 s−10 min) and quenched by dilution into 0.1 M HCl (0.5 mL) containing Norit-SA3 (12% w/v). Mixtures were spun in a microcentrifuge (5 min, maximum speed), and the supernatant was filtered through a plug of glass wool. The pellet was resuspended twice in 0.1 M HCl (0.5 mL) and again centrifuged; the supernatants from these washes were separately passed through the plug of glass wool. An aliquot (1 mL) of the combined filtrates (∼1.4 mL) was mixed with Scintisafe Econo 2 scintillation fluid (10 mL) and subjected to scintillation counting. Our routine assays for activity contain 500 nM [5-^{3}H]tRNA. The variation in the rate of the reaction as a function of substrate concentration was determined in triplicate for all enzymes except RluA C117A and RluA C117A/C128A, with which duplicate determinations were performed. The variation between replicate runs ranged between 2% and 7%. Each set of initial velocities was fit to the Briggs−Haldane equation using DeltaGraph 4.5 (SPSS, Inc., Chicago) to determine V_{max} (and hence k_{cat}) and K_m. The uncertainties (Table 2) are the standard deviations in the independent fits of each set of initial velocities.

RESULTS

Site-Directed Mutagenesis. Wild-type TruB has three cysteine residues (Cys-58, Cys-174, and Cys-193), and wild-type RluA has two (Cys-117 and Cys-128). The QuikChange site-directed mutagenesis protocol is suited to alter one codon (or perhaps two contiguous codons) at a time, so three rounds of mutagenesis were required to achieve TruB with all three cysteine residues altered (the C58A/C174A/C193A triple mutant). The three mutant TruB that retain only one of the cysteine residues of the wild-type enzyme (the double mutants) were therefore constructed en route to the triple mutant, for the time was identical and the effort required was negligibly greater than producing a single double mutant because the plasmid bearing each single mutant could be used in parallel mutagenesis reactions. The generation of the C193V rather than the C193A TruB was unintended, but this substitution of valine for cysteine was deemed suitable for our purposes because valine has only slightly greater steric bulk than cysteine (essentially an "extra" β-methyl group). Table 1 lists all of the generated mutants and the names of the plasmids encoding them.

Overexpression and Purification. The genes *truB* and *rluA* were cloned into pET15b, so that they are postioned behind a T7 promoter. Expressed TruB and RluA have an N-terminal

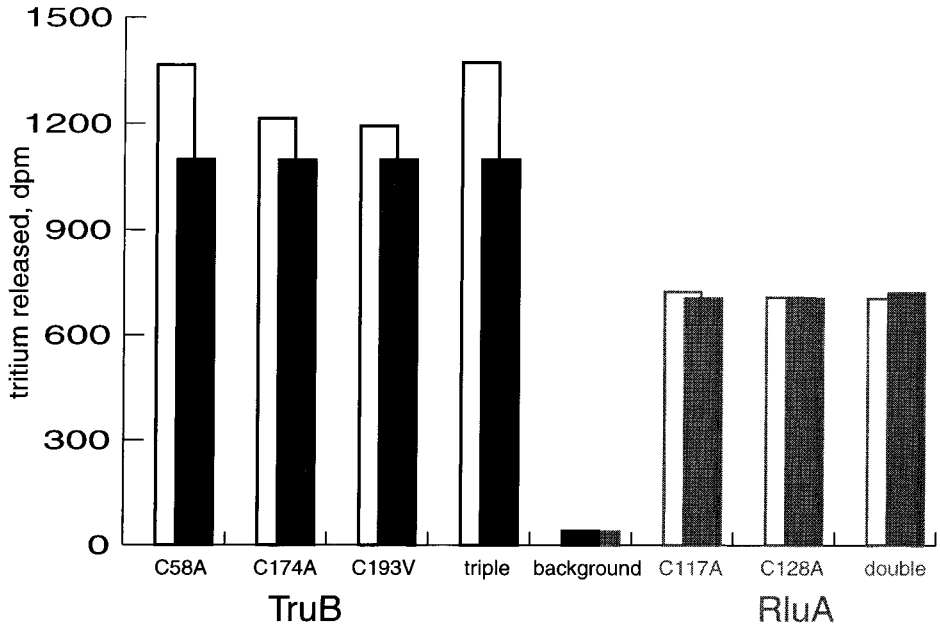

FIGURE 4: Activity assays of Ψ synthases. The solid bars denote the activity of the wild-type enzyme (TruB in black, RluA in gray), and the open bars denote the activity of the mutant enzymes as denoted. Tritium release is proportional to the amount of U isomerized (see text for details). "Triple" denotes the C58A/C174A/C193A TruB, and "double" denotes the C117A/C128A RluA. "Background" shows the counts returned when [5-^{3}H]tRNA is put through the assay workup without exposure to a Ψ synthase.

His_6•Tag (*3, 5*). These plasmids were used to overexpress wild-type TruB and RluA and to generate all of the mutant enzymes described in this paper. All Ψ synthases were overexpressed by induction of T7 RNA polymerase production from the λDE3 lysogenized in the host cells and were purified to very near homogeneity by chromatography over a "nickel column". The yields (15−40 mg from a 500-mL culture) and storage properties of mutant and wild-type enzymes were indistinguishable.

Activity Assays and Determination of Kinetic Parameters. Assay of Ψ synthase activity monitors the appearance in solvent of tritium from the transcript of *E. coli* $tRNA^{Phe}$ containing [5-^{3}H]uridine ([5-^{3}H]tRNA), which reflects Ψ formation because isomerization of U necessarily results in loss of the proton at C5 to solution. Our standard assay for activity contains 500 nM [5-^{3}H]tRNA and 20 nM enzyme; the end point (2−10 min) was in each case confirmed to be in the linear regime by monitoring tritium release as a function of time (data not shown). The mutant RluA and TruB were fully active (Figure 4).

The determination of k_{cat} and K_m was carried out for wild-type and mutant enzymes (Table 2). The instability under assay conditions of both RluA and TruB at concentrations less than 20 nM and the low K_m values for these enzymes combine to hamper measurement of initial velocities at substrate concentrations below K_m. Figure 5 is representative of the kinetic data used to generate the values in Table 2. Initial velocities as a function of substrate concentration were determined in duplicate for RluA C117A and RluA C117A/C128A and in triplicate in all other cases. The uncertainty in K_m reflects the difficulty in obtaining data at [tRNA] < K_m, but the reproducibility in determining k_{cat} values is gratifying.

DISCUSSION

The thiol(ate) groups of cysteine side chains are frequently found in enzyme active sites where they play crucial roles in catalysis, and sometimes these roles are uniquely suited to a thiol(ate) among the functional groups of the 20 common amino acids. In the case of the Ψ synthases, a role for a cysteine side chain as the nucleophile for a Michael addition to C6 of the isomerized U (Figure 2) was proposed based on the mechanism of thymidylate synthase (*16*). The cysteine side chains in the Ψ synthase TruA have been shown not to be essential (*18*), but the lack of statistically significant global sequence similarity between four families of Ψ synthases (*7, 8*) mandates that members of each family be investigated. We chose RluA and TruB as representatives of different Ψ synthase families, performing site-directed mutagenesis to probe the catalytic role of cysteine residues.

Assays for Ψ synthase activity (Figure 4) show that all of the examined mutant RluA and TruB are active. The differences in measured activity between wild-type and mutant TruB hover on the edge of statistical significance, and in all cases the mutant TruB appear more active. As shown in Table 2, the k_{cat} values for the mutant RluA and TruB do not differ much from those of the wild-type enzymes, and the K_m values are also largely unperturbed by mutation of the cysteine residues in these two Ψ synthases. The greatest difference between a mutant and wild-type enzyme is for C117A RluA, whose k_{cat} is 70% that of wild-type RluA and whose K_m is increased roughly 4-fold relative to the wild-type RluA, a very mild variation which mutation of the second cysteine in RluA apparently compensates (Table 2). We stress that these are fine variations in kinetic parameters whose accurate determination is hampered by the inability to obtain initial velocity data at [tRNA] < K_m. Even if determined to statistical certainty, the interpretation of such

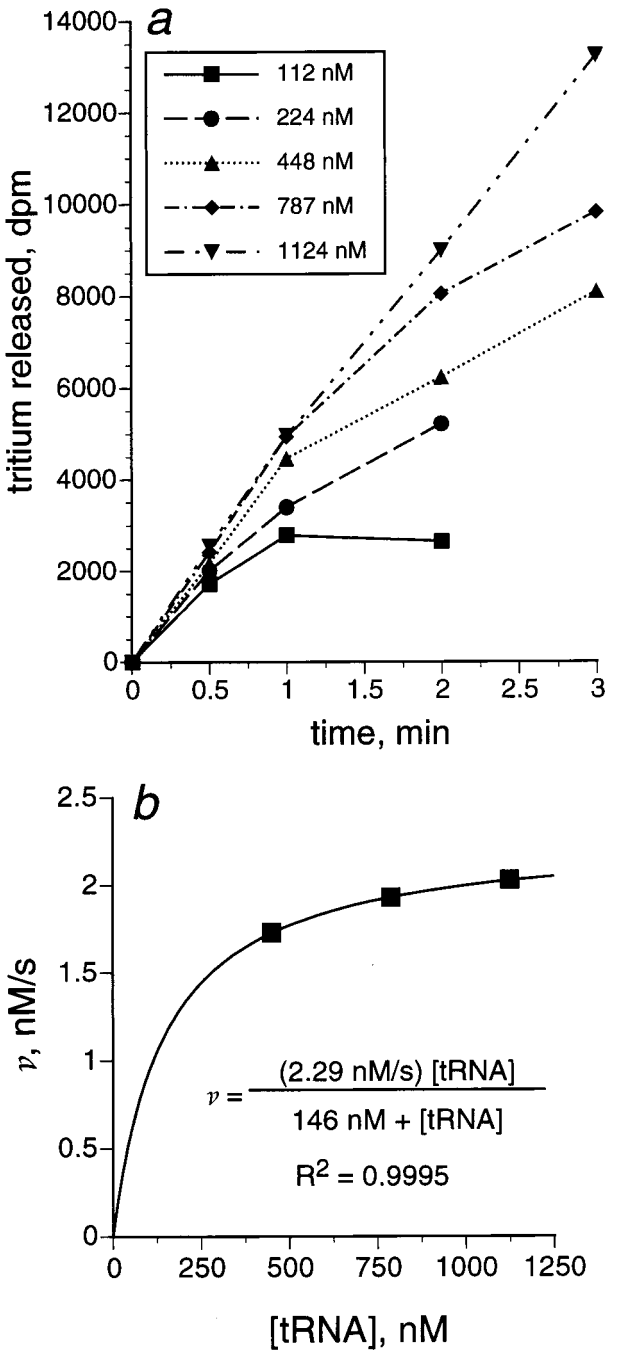

FIGURE 5: Kinetic data for wild-type TruB. (a) The time course of tritium release catayzed by 20 nM TruB. This time course was performed in triplicate; the other two data sets are omitted for graphical clarity but give initial velocities that vary by 3% or less from the data shown. (b) The fit of the initial velocity data (panel a and duplicate runs) to the Briggs−Haldane equation. The size of the points reflects the uncertainty (3%) between independent initial velocity determinations. At [tRNA] = 112 and 224 nM, curvature is obvious by 1 min, so the 30 s time points cannot be confidently used to calculate initial velocities; data for [tRNA] =112 and 224 nM were therefore excluded from the fit.

subtle variations in kinetic parameters would require very detailed structural knowledge, almost certainly the determination of the structure of the complex between tRNA and both wild-type and mutant RluA.

To our knowledge, k_{cat} and K_m values have not previously been published for RluA. Santi and co-workers (*23*) have determined k_{cat} and K_m values for the wild-type TruB to be 0.24 s^{-1} and 780 nM, respectively, which differ somewhat from our determinations (0.12 s^{-1} and 146 nM). The apparent 5.3-fold discrepancy in K_m is slightly understated, for different extinction coefficients were assumed for $tRNA^{Phe}$: we used 5×10^5 $(M\ tRNA)^{-1}\ cm^{-1}$ (*22*); Santi and co-workers used $6.25 \times 10^5\ M^{-1}\ cm^{-1}$ (*23*). When normalized to $5 \times 10^5\ M^{-1}\ cm^{-1}$, the K_m value reported by Santi and co-workers becomes 975 nM, and the discrepancy in K_m values increases to 6.7-fold. At least part of this difference may lie in the conditions used, for Santi and co-workers carried out their assays in 20 mM Tris·HCl, pH 8.0, containing NH_4Cl (100 mM) and EDTA (2 mM), while we assayed in 50 mM HEPES buffer, pH 7.5, containing NH_4Cl (100 mM), DTT (5 mM), and EDTA (1 mM).[2] Regardless of this rather mild discrepancy with the previously reported kinetic parameters for wild-type TruB, the values in Table 2 are internally consistent and allow an accurate comparison between the wild-type and mutant TruB and RluA.

Replacement of each cysteine, singly or together, in both RluA and in TruB did not seriously impair catalytic activity, clearly indicating that *the cysteine residues in these enzymes are not catalytically essential*. We can, therefore, rule out the participation of the cysteine thiol(ate)s at the active site of these two Ψ synthases, just as such participation had been ruled out for TruA (*18*), a member of a third family of these enzymes. In particular, our results exclude the proposal (Figure 2) that a cysteine thiol(ate) serves as the nucleophile for a Michael addition to C6 of the isomerized U (*16*). A Michael addition by another nucleophilic side chain cannot be excluded, although to our knowledge no such reaction has been demonstrated by an enzymic nucleophile other than a cysteine thiolate. One alternate candidate as a Michael nucleophile, however, is the aspartic acid residue implicated by sequence alignments (*7, 8*) and subsequently shown to be essential in all four Ψ synthase families (*19*, *20*, *26*, *27*). This critical amino acid could form a Michael adduct (Figure 2, with a carboxylate in place of the thiolate) or serve as a general acid/base in addition to its proposed (*19*) formation of an acylal intermediate (Figure 3).

While a great deal of work remains to establish the events at Ψ synthase active sites, any mechanism uniquely requiring the chemical properties of a cysteine thiol(ate) group are now excluded. We can, therefore, focus our attention on other amino acids as catalytic residues, initially those implicated by sequence alignments and soon, we hope, those implicated by structural information (*24*, *25*).

[2] It must be noted that we and the Santi group used identically His_6·Tagged TruB (*3*), so any variation in K_m does *not* arise from interaction between the polycationic N-terminal His_6·Tag and the polyanionic $tRNA^{Phe}$ substrate.

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