A Conserved Aspartate of tRNA Pseudouridine Synthase Is Essential for Activity and a Probable Nucleophilic Catalyst†

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ABSTRACT: tRNA pseudouridine synthase I catalyzes the conversion of uridine to pseudouridine at positions 38, 39, and/or 40 in the anticodon loop of many tRNAs. Pseudouridine synthase I was cloned behind a T7 promoter and expressed in *Escherichia coli* to about 20% of total soluble proteins. Fluorouracil-substituted tRNA caused a time-dependent inactivation of pseudouridine synthase I and formed a covalent complex with the enzyme that involved the FUMP at position 39. Asp60, conserved in all known and putative pseudouridine synthases, was mutated to amino acids with diverse side chains. All Asp60 mutants bound tRNA but were catalytically inactive and failed to form covalent complexes with fluorouracil-substituted tRNA. We conclude that the conserved Asp60 is essential for pseudouridine synthase activity and propose mechanisms which involve this residue in important catalytic roles.

Pseudouridine synthases (ΨS)[1] catalyze the conversion of specific uridine residues in RNA to pseudouridine (Ψ), an unusual nucleoside with a carbon−carbon glycosidic linkage (*1*−*3*). The minimal mechanism for the reaction involves cleavage of the *N*-glycosidic bond of the target residue, rotation of the cleaved uracil to juxtapose C_5 of the pyrimidine and C_1' of the ribosyl moiety of RNA, and formation of the $C_1'-C_5$ carbon−carbon bond.

An elaboration of this mechanism based on chemical considerations and analogies with enzymes such as thymidylate synthase involves nucleophilic attack of a Cys residue of the enzyme at C_6 of the pyrimidine to form an essential 5,6-dihydropyrimidine intermediate (*4*) (Scheme 1). The formation of a 5,6-dihydropyrimidine adduct would facilitate all of the reactions necessary for the subsequent conversion to products. It would (i) enhance the lability of the *N*-glycosidic bond (*5*); (ii) provide an axis for a 180° rotation of the pyrimidine ring to juxtapose the C_1' and C_5; and (iii) activate the 5-carbon toward electrophilic attack by the $C_{1'}$. In an attempt to obtain evidence for this mechanism, Kammen et al. showed that tRNA pseudouridine synthase I (ΨSI) activity was inhibited by sulfhydryl reagents (*6*). Further, FUra-RNA, which can form stable 5,6-dihydropyrimidine adducts with enzymes involving such intermediates (*7*) was a potent inhibitor of ΨS (*8*). Conclusive evidence for covalent adduct formation has not been obtained. Thus, although the pathway in Scheme I is consistent with available data, direct evidence for the mechanism is lacking.

In the present work, we describe studies of ΨSI, the enzyme which converts uridine to Ψ at positions 38, 39, and/or 40 in the anticodon loop of many *Escherichia coli* tRNAs. Contrary to previous reports (*8*), we found that the enzyme forms a covalent adduct with FUra-tRNA, thus rekindling our interest in the mechanism involving covalent catalysis by a Cys residue. However, while this work was in progress, sequences of several ΨSs became available, which lacked a conserved Cys residue which might serve the role of such a catalyst (*9*−*13*). Moreover, recent mutagenesis studies unequivocally showed that Cys is not required for catalytic activity of ΨSI (*14*). In searching for alternative potential catalysts, we identified a completely conserved aspartate residue (Asp60 in ΨSI) based on sequence alignments of known and putative ΨSs. Here, we demonstrate that mutagenesis of Asp60 in ΨSI results in the loss of both the catalytic activity and the ability to form a covalent complex with FUra-tRNA. Mechanisms are proposed which involve covalent catalysis by Asp and are in accord with all available data.

MATERIALS AND METHODS

Plasmid Ψ300, which contains the *E. coli* ΨSI gene (*15*) was a gift from M. Winkler (Department of Microbiology and Molecular Genetics, University of Texas at Houston). Plasmid p67YF0, which was used for *in vitro* transcription of yeast $tRNA^{Phe}$ was a gift from O. C. Uhlenbeck (Department of Chemistry and Biochemistry, University of Colorado). [5-^{3}H]UTP (20.1 Ci/mmol) was purchased from Moravek Biochemicals. FUTP was from Sierra Bioresearch. [5′-^{32}P]pCp (3000 Ci/mmol) and [α-^{32}P]ATP (3000 Ci/mmol) were from Amersham. Oligonucleotide synthesis and DNA sequencing were performed by the UCSF Biomolecular Resource Center.

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[1] Abbreviations: Ψ, pseudouridine; ΨS, pseudouridine synthase; ΨSI, tRNA pseudouridine synthase I; [5-^{3}H]Ura-tRNA, [5-^{3}H]uridine containing-$tRNA^{Phe}$; FUTP, 5-fluorouridine triphosphate; FUra-tRNA, 5-fluorouridine-containing $tRNA^{Phe}$; Amp^r, ampicillin resistant; PAGE, polyacrylamide gel electrophoresis.

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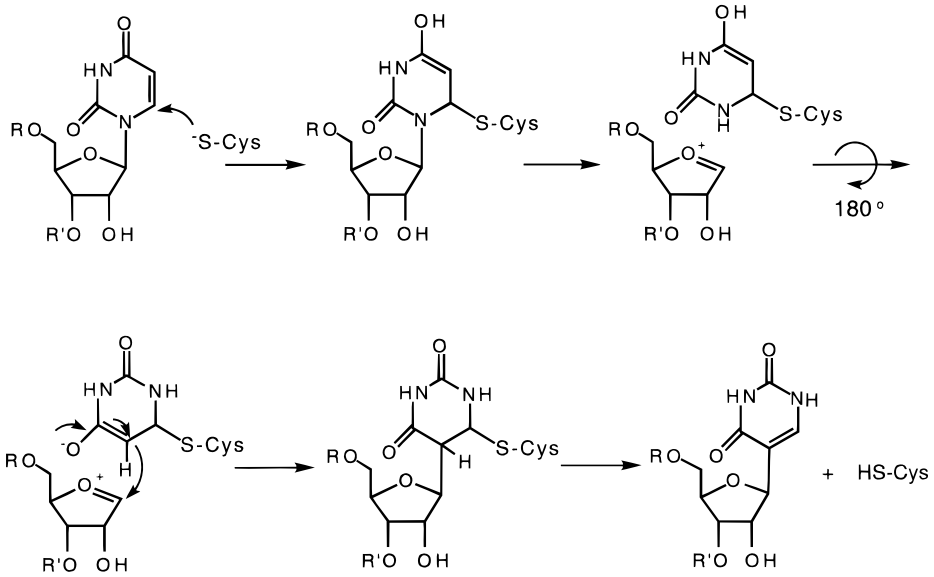
Scheme 1: Proposed Mechanisms for Ψ Conversion in RNA



Construction of the ΨSI Expression Vector pLX1. A PCR reaction was performed with plasmid Ψ300 as the template and two primers: 5′-CTGGCGAAGACTCCATGTCCGACCAGCAACAAC-3′, which hybridizes bp 1 to bp 19 of ΨSI gene (*Bbs*I site is underlined), and 5′-GACGGATCCTCGAGTTAGTCCGCCAGAAATAG-3′, which hybridizes bp 796 to bp 813 (*Xho*I site is underlined). The 0.8 kb PCR product was digested with *Bbs*I and *Xho*I, ligated with *Nco*I/*Xho*I-digested vector pET-15b, and transformed into *E. coli* DH5α. DNA from Amp^r transformants was analyzed by restriction digestion and DNA sequencing.

Mutagenesis. Plasmid encoding ΨSI-D60 mutants were constructed by PCR mutagenesis. PCR reactions were performed using plasmid pLX1 DNA as template and two primers: T7 primer (5′-AATACGACTCACTATAG-3′), which hybridizes upstream from the ΨSI gene within the T7 promoter, and one of the following mutagenic primers, D60A (5′-CCGGTACCGTGTACACCTGC**TGC**AGTACGCCC-3′), D60K/N (5′-CTGCCCGGTACC**A**TG**G**ACCCCTGC**(AT)TT**AGTACGCCC-3′), D60E (5′-CTGCCCGGTACCATG**G**ACCCCTGC**TTC**AGTACGCCC-3′), and D60S (5′-CTGCCCGGTACC**A**TG**G**ACCCCTGC**GGA**AGTACGCCC-3′). The sequence of each of the mutagenic primers spans a natural, unique *Kpn*I site and is complementary to gene sequence beginning at nucleotide 169 except at codon 60 (mutagenized bases in bold) and at positions where new restriction sites (underlined) were introduced for the purpose of restriction analysis. A *Pst*I restriction site was introduced into the D60A primer, and an *Nco*I site was introduced into each of the other primers. For each mutagenesis reaction, the 250 bp PCR product was digested with *Xba*I (38 bp upstream from ΨSI gene) and *Kpn*I and ligated with *Xba*I and *Kpn*I digested plasmid pLX1. Each ligation reaction was transformed into DH5α, and DNA from Amp^r transformants was screened for the presence of the new *Pst*I or *Nco*I sites. DNA from the restriction positive clones was subjected to DNA sequencing.

Plasmid encoding the tRNA^{Phe} (A31G and U39C) mutant under the control of T7 promoter (p67YF0-G31C39) was also constructed by PCR mutagenesis. A PCR reaction was performed using plasmid p67YF0 as template and two primers: 5′-GCCGCCGCAAGGAATGGT-3′, which hybridizes to the sequence preceding a *Sph*I site 245 bp upstream from the T7 promoter, and 5′-GCGAATTCTGTGGATCGAACACAGGACCTCCAG**G**TCTTCAG**C**CTGGCGCT-3′, which hybridizes bp 22 to bp 71 of the tRNA gene (mutagenized bases in bold, the *Eco*RI site is underlined). The 540 bp PCR product was digested with *Sph*I and *Eco*RI and ligated with *Sph*I and *Eco*RI digested plasmid p67YF0. The ligation product was transformed into DH5α, and DNA from Amp^r transformants was screened for the loss of a *Bgl*II site generated by the T39 to C mutation, and subjected to DNA sequencing analysis.

Expression and Purification of ΨSI. Plasmid pLX1 was transformed into BL21(DE3) cells. An overnight culture of pLX1-BL21(DE3) was used to inoculate 6 L of LB (50 mg/L ampicillin). The culture was grown until the cell density reached $A_{600} \approx 0.8$ when IPTG was added to 1 mM final concentration. The induced culture was grown for 3 h at 37 °C, and cells were harvested by centrifugation at 36 000*g* for 15 min at 4 °C.

SDS−PAGE analysis was used to detect the 31 kD ΨSI in the column fractions during the purification. All of the following procedures were performed at 4 °C. Cells from 6 L of induced pLX1-BL21(DE3) were resuspended in 50 mL of buffer A (10 mM KH_2PO_4, pH 7.0, 0.5 mM EDTA, 10% glycerol, 0.5 mM DTT) containing 0.5 M $(NH_4)_2SO_4$. The cell suspension was lysed by two passes through a French pressure cell at 16 000 psi, and centrifuged at 36 000*g* for 25 min. The supernatant was adjusted to 1 M $(NH_4)_2SO_4$ by addition of 25 mL of buffer A containing 2 M $(NH_4)_2SO_4$ with stirring. The lysate was cleared by centrifugation, and the supernatant (75 mL) was loaded onto a 50 mL phenyl-Sepharose column equilibrated with buffer A containing 1 M $(NH_4)_2SO_4$. The column was washed with 500 mL of the same buffer and eluted with a 500 mL linear gradient from 1 to 0 M $(NH_4)_2SO_4$. ΨSI eluted as a broad peak between 0.8 and 0 M $(NH_4)_2SO_4$. Fractions containing the ΨSI were pooled, dialyzed against buffer A and loaded onto a DEAE-Sepharose column (15 mL) equilibrated with buffer A. ΨSI did not bind to the DEAE column, and the flow through was loaded directly onto a S-Sepharose column

(20 mL) equilibrated with buffer A. The S-Sepharose column was washed with 200 mL of buffer A, and the enzyme was eluted with a 200 mL linear gradient of 0 to 250 mM KCl in buffer A. ΨSI eluted at about 130 mM KCl. The fractions containing ΨSI were pooled and concentrated using a Centriprep-10 concentrator. The ΨSI-D60A, D60S, D60E, D60N, and D60K mutant enzymes were purified by similar procedures.

tRNA Synthesis. tRNA was synthesized by *in vitro* transcription catalyzed by T7 RNA polymerase using *Bst*NI linearized p67YF0 as template. The 500 μL reaction contained 4 mM of each NTP, 20 mM $MgCl_2$ and 0.1 mg/mL T7 RNA polymerase (*16*). [5-^{3}H]Ura-tRNA, FUra-tRNA, and [α-^{32}P]FUra-tRNA were prepared in similar reaction mixtures containing 0.1 mM [5-^{3}H]UTP (1.0 Ci/mmol) instead of UTP, 1.6 mM FUTP instead of UTP, 1.6 mM FUTP and 0.1 mM [α-^{32}P]ATP (4.0 Ci/mmol) instead of UTP and ATP, respectively. The synthetic tRNAs were purified using Qiagen columns according to manufacturer's instructions (Qiagen Inc.).

3′-End labeling of tRNA. The purified *in vitro* transcribed tRNA was labeled at the 3′ end with [5′-^{32}P]pCp using T4 RNA ligase (*17*). The 3′-end-labeled tRNA was purified by electrophoresis on 7 M urea−12% PAGE.

Tritium Release Assay. The tritium release assay was a modification of a reported procedure (*18*). A typical 100 μL reaction contained 2 nM ΨSI and 1.5 μM [5-^{3}H]Ura-tRNA (2.0×10^4 dpm/pmol) in TNE buffer (20 mM Tris·Cl, pH 8.0, 0.1 M NH_4Cl, and 2 mM DTT). Reactions were incubated at 15 or 22 °C. Aliquots of 18 μL were removed at time intervals up to 20 min and quenched with 1 mL of 5% Norit A in 0.1 N HCl. The mixtures were centrifuged for 5 min, and the supernatants were again treated with 0.5 mL of 5% Norit A in 0.1 N HCl. Mixtures were centrifuged for 10 min, the supernatants were filtered through glass wool, and 1.0 mL of each filtrate was counted in 5 mL of Aquasol-2 (New England Nuclear).

Nitrocellulose Filter Binding Assay. Reaction mixtures (200 μL) containing various amounts of ΨSI (1 nM to 5 μM) and 0.1 nM [3′-^{32}P]FUra-tRNA (5.4×10^6 cpm/pmol) were incubated in TNE buffer at 15 °C for 15 min. Two 95 μL aliquots of each reaction mixture were applied to duplicate nitrocellulose membranes wetted with wash buffer (25 mM potassium phosphate, pH 7.4), and the filters were washed with six 1 mL aliquots of the wash buffer, dried briefly in air, and counted in 5 mL of Aquasol II (*19*).

SDS−PAGE and Urea−PAGE Gel Shift Assays. Gel shift assays of the ΨSI−FUra-tRNA complex were performed using 12% SDS−PAGE as described for tRNA (m^5U54) methyltransferase−FUra-tRNA complex (19). Reaction mixtures containing 12.5 μM ΨSI and 0.5 μM [3′-^{32}P]FUra-tRNA (6.6×10^3 cpm/pmol) in 100 μL TNE buffer were incubated at 15 or 22 °C. Ten microliter aliquots of each reaction mixture were removed at various times between 2 and 300 min and added to equal volumes of 2× SDS loading buffer (125 mM Tris·Cl, pH 6.8, 4% SDS, 1.5 M 2-mercaptoethanol, 20% glycerol, and 0.005% bromophenol blue). The samples were loaded onto gels without heating. Gels were either stained with Coomassie Blue R250 or subjected to autoradiography. For quantitation of radioactivity, the gels were exposed to a phosphor screen, and scanned using a phosphorimage scanner.

An identical reaction mixture was incubated overnight and denatured in 7 M urea for 2 h at room temperature. The sample was diluted 1:1 in formamide loading buffer (0.005% bromophenol blue in formamide) and loaded onto 7 M urea−12% PAGE without heating.

Stability of ΨSI−FUra-tRNA Complex. [3′-^{32}P]FUra-tRNA (0.2 μM, 1.0×10^4 cpm/pmol) was incubated with 2 μM ΨSI in 50 μL TNE buffer at 15 °C overnight. Ten microliters of the reaction mixture was diluted to a final volume of 40 μL containing 10 μM unlabeled FUra-tRNA and incubated at 15 °C. Aliquots (8 μL) were removed at 1.5, 3, 6 , and 9.5 h, added to an equal volume of 2× SDS loading buffer, and analyzed using SDS−PAGE and autoradiography.

RNase A Digestion of the ΨSI-FUra-tRNA Complex. The ΨSI−[α-^{32}P]FUra-tRNA complex (labeled at 5′-P of A residues) was formed by incubating 10 μM ΨSI and 10 μM [α-^{32}P]FUra-tRNA in a 20 μL reaction at 22 °C for 6 h. RNase A (0.1 μg) was added to the reaction mixture and digestion was carried out for 1 h at 37 °C. The digested complex was subjected to SDS−PAGE and autoradiography showed a radioactive band migrating as a 33 kDa protein.

Identification of the Nucleotide Attachment Site of the ΨSI-FUra-tRNA complex . The radioactive 33 kDa band from SDS−PAGE of the above RNase A digest was extracted with water and incubated at 90 °C for 10 min to disrupt the complex. After precipitating the radioactive oligonucleotide with cold ethanol, the oligonucleotide was purified on 7 M urea−PAGE. The ^{32}P-oligonucleotide migrating slightly faster than the bromophenol blue marker was extracted with 0.5 M NH4OAc, 20 mM EDTA, and 0.1% SDS and digested with RNase T2 for nearest neighbor analysis (*20*). The [^{32}P]-3′-NMPs in the digest were separated on 2d-cellulose TLC and analyzed by autoradiography.

Inhibition and Inactivation of ΨSI by FUra-tRNA. Twenty nanomolar ΨSI (final concentration) was added to reaction mixtures (final volume of 80 μL) containing 3.3 μM [5-^{3}H]-Ura-tRNA (5.3×10^4 dpm/pmol) and varying amounts of FUra-tRNA (0, 100, 250, and 500 nM). Incubation was performed at 15 °C, and 18 μL aliquots were removed at 1′ and 2′ and mixed with 1 mL of 5% Norit A in 0.1 N HCl. Initial rates were analyzed by the tritium release assay as described above. The K_i value was determined as described (*21*).

The time-dependent inactivation of ΨSI by FUra-tRNA was analyzed by preincubating ΨSI with FUra-tRNA and assaying for enzyme activity. ΨSI (100 nM) was incubated with FUra-tRNA (1 μM) in TNE buffer. Eighteen microliter aliquots of the mixture were removed at 0, 10, 20, and 40 min and diluted 10-fold into the assay buffer containing 3.65 μM [5-^{3}H]Ura-tRNA. Initial rates of tritium release were measured.

Effect of Uracil on the Covalent Complex Formation. ΨSI (12.5 μM) and [3′-^{32}P]FUra-tRNA (0.5 μM, 6.6×10^3 cpm/pmol) were incubated in 100 μL TNE buffer at 15 °C in the presence or absence of 5 mM uracil. Ten microliter aliquots of the reaction were removed at 2, 5, 10, 20, 40, 80, 240, and 390 min and analyzed by SDS−PAGE. Gels were subjected to autoradiography and phosphorimage scanning for quantitation of the ΨSI−[3′-^{32}P]FUra-tRNA complex.

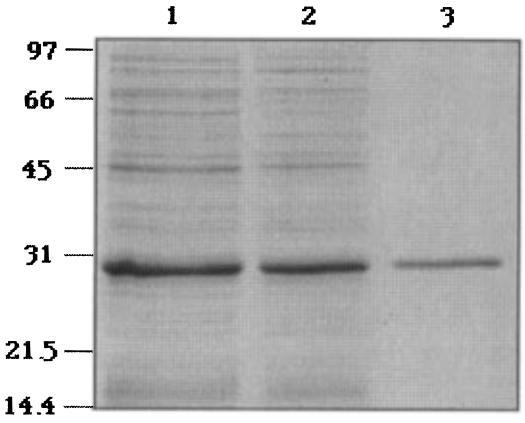


FIGURE 1: SDS−PAGE of the overexpression and purification of ΨSI. Lane 1, crude extract; lane 2, phenyl-Sepharose chromatography pool; lane 3, pool after DEAE-Sepharose chromatography and S-Sepharose chromatography.

RESULTS

Cloning and Overexpression of ΨSI and ΨSI-D60 Mutants. The gene encoding ΨSI was subcloned from plasmid Ψ300 using PCR to add convenient restriction sites. The 5′ primer contained a *Bbs*I site which generates a *Nco*I-compatible overhang at the ATG start codon and the 3′ primer contained a *Xho*I site. The *Bbs*I-*Xho*I restricted PCR fragment containing the ΨSI gene was ligated into the *Nco*I−*Xho*I restricted vector pET-15b to yield pLX1, which contains the ΨSI gene under the control of the T7 promoter. DNA sequence analysis of the entire gene confirmed the correct sequence. IPTG induction of pLX1/BL21(DE3) cells yielded expression of ΨSI at about 20% of the total soluble proteins (lane 1, Figure 1).

PCR mutagenesis of pLX1 was used to isolate plasmids carrying D60A, D60S, D60E, D60N, and D60K mutants of ΨSI. The 5′ PCR primer spanned the T7 promoter, and the 3′ mutagenic PCR primer contained codon 60, a *Kpn*I site located 13 base pairs downstream, and new restriction sites for restriction screening. PCR-generated mutagenic fragments were exchanged into pLX1 utilizing the *Kpn*I site and an *Xba*I site located between the T7 promoter and the ΨSI gene. Restriction analysis of plasmid DNA identified the presence of the mutations which were then confirmed by sequence analysis. In the case of D60K/N where a degenerate primer was used, three isolates were sequenced to resolve the individual mutants.

Purification of Wild-Type and Mutant ΨSIs. Cell extracts were prepared from IPTG-induced cultures of pLX1/BL21-(DE3). ΨSI was purified by sequential chromatography on phenyl-Sepharose, DEAE-Sepharose, and S-Sepharose (Table 1). The final enzyme preparation was homogeneous as judged by SDS−PAGE (lane 3, Figure 1). The yield of the purified enzyme was approximately 12 mg/L of culture. Each of the D60 mutant enzymes (D60A, D60S, D60E, D60N, and D60K) was purified by a procedure similar to that for wild-type ΨSI, with yields ranging from 2.5 to 10 mg/L of culture.

Kinetic Parameters for Wild-Type and Mutant ΨSIs. For determination of kinetic parameters for Ψ formation, the substrate was unmodified [5-^{3}H]uracil-labeled yeast tRNA^{Phe} generated by run-off *in vitro* transcription using linearized plasmid p67YF0. ΨSI catalyzes release of tritium from [5-^{3}H]Ura-tRNA upon the conversion of uridine to pseudouridine. The initial velocities of tritium release were measured in reactions containing 2 nM ΨSI and variable concentrations of [5-^{3}H]Ura-tRNA (0.1−3 μM). Rates were linear for approximately 10−25% of the reaction. Initial rates were plotted against substrate concentrations, and the k_{cat} and K_m values were obtained by nonlinear least-squares fit of the data to the Michaelis−Menton equation. The kinetic parameters were originally determined at 22 °C. We found that the enzyme lost approximately 50% activity at 22 °C in 1 h, so we also determined kinetic parameters at 15 °C where the enzyme is stable for at least 1 h. At 22 °C, the k_{cat} was 0.73 s^{-1}, and K_m was 0.94 μM; at 15 °C, the k_{cat} was 0.18 s^{-1} and K_m was 1.8 μM.

Incubation of [5-^{3}H]Ura-tRNA with excess enzyme for 1 h released 0.9 mol of tritium/mol of [5-^{3}H]Ura-tRNA; theoretical for the substrate used was 1.0 mol/mol of [5-^{3}H]-Ura-tRNA.

The catalytic activities of ΨSI-D60A, D60S, D60E, D60N, and D60K mutants toward the substrate [5-^{3}H]Ura-tRNA were analyzed by the tritium release assay. D60 mutants (20 nM) were incubated with 6 μM [5-^{3}H]Ura-tRNA at 15 °C for up to 60 min under standard assay condition. No detectable tritium release was observed for any of the D60 mutants (≤0.01% of wild-type ΨSI) (Table 2).

Binding of Wild-Type and Mutant ΨSIs to tRNAs. A nitrocellulose filter binding assay was used to measure the apparent dissociation constants (K_d) of wild-type ΨSI and mutant enzymes with FUra-tRNA (*19*). The K_d values were determined by measurement of bound complexes using a constant amount of [3′-^{32}P]FUra-tRNA (0.1 nM) and varying enzyme concentration (1 nM to 5 μM) (Figure 2). K_d values were obtained by nonlinear least-squares fit of the binding data to an equation that calculates free ligand concentration (*19*). Wild-type ΨSI and each of the D60 mutant enzymes had similar K_d values *vs* FUra-tRNA: 93 nM for wild-type ΨSI and 89−169 nM for the D60 mutants (Table 2). We also measured the K_d value for the ΨSI-D60A mutant

Table 1: Purification Summary of ΨSI

fraction	vol (mL)	protein (mg)	specific activity (units/mg)[a]	units[a] ($\times 10^{-4}$)	yield (%)
crude extract	50	760	246	18.7	100
phenyl-Sepharose pool	130	386	158	6.1	33
S-Sepharose pool	45	72	734	5.2	28

[a] One unit is defined as the amount of enzyme that releases 1 nmol of tritium from [5-^{3}H]Ura-tRNA in 5 min at 22 °C under standard assay conditions.

Table 2: Characterization of Wild-Type and Mutant ΨSIs

	K_d (nM)		
	FUra-tRNA	tRNA	k_{cat} (s^{-1})
WT ΨSI	93	nd[a]	0.18
ΨSI-D60A	108	72	*b*
ΨSI-D60S	169	nd	*b*
ΨSI-D60E	159	nd	*b*
ΨSI-D60N	154	nd	*b*
ΨSI-D60K	89	nd	*b*

[a] Not determined. [b] No detectable tritium release was observed (≤0.01% of wild-type ΨSI).

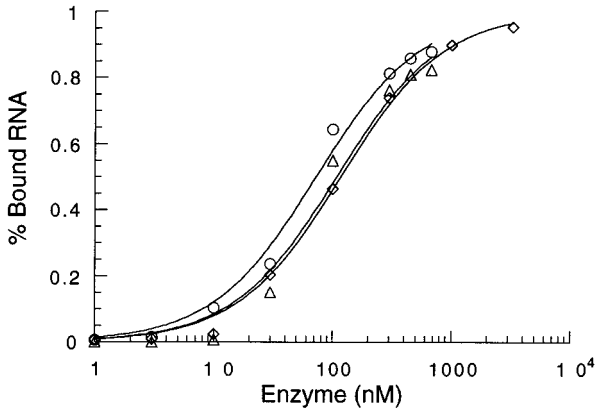


FIGURE 2: Nitrocellulose filter binding assay of ΨSI *vs* [3′-^{32}P]-FUra-tRNA (diamond), D60A mutant *vs* [3′-^{32}P] FUra-tRNA (triangle), and D60A mutant *vs* [3′-^{32}P]tRNA (circle). Reaction mixtures containing various amounts of enzyme and 0.5 nM [3′-^{32}P]FUra-tRNA or [3′-^{32}P]tRNA were incubated in TNE buffer at 15 °C for 15 min. Aliquots of each reaction mixture were applied to the nitrocellulose membranes, and filters were washed and counted in Aquasol II.

enzyme with native, unmodified tRNA. The K_d value for this complex was 72 nM, similar to those of wild-type ΨSI and the D60 mutants for FUra-tRNA. The K_d for the ΨSI−FUra-tRNA(G31C39) complex was 32 nM.

Inhibition of ΨSI by FUra-tRNA. When FUra-tRNA was present in the tritium release assay mix at the time of enzyme addition, an immediate inhibition was observed. The K_i value for this inhibition was determined to be 0.1 μM. Upon incubation of 100 nM ΨSI with 1 μM FUra-tRNA at 15 °C prior to addition of substrate, there was a time-dependent inactivation of the enzyme with a $t_{1/2}$ of 35 min.

SDS−PAGE Analysis of the ΨSI−FUra-tRNA Complex. The formation of a stable ΨSI-FUra-tRNA complex was detected using the SDS−PAGE gel shift assay described for tRNA (U54) methyltransferase (*18*, *19*), except that the samples were loaded onto SDS−PAGE gels without prior heating at 100 °C. FUra-tRNA showed a mobility equivalent to a protein of 25 kD, and the free ΨSI showed a mobility equivalent to 31 kD. Incubation of ΨSI with [3′-^{32}P]FUra-tRNA resulted in the appearance of a new Coomassie-stained band with a mobility equivalent to a 55 kD protein on SDS−PAGE. The protein band at 55 kD comigrated with the radioactivity from [3′-^{32}P]FUra-tRNA, indicating that this band represents a protein−RNA complex which is stable to SDS denaturation. The ΨSI−[3′-^{32}P]FUra-tRNA complex was also observed upon denaturation in 7 M urea and electrophoresis in polyacrylamide gel containing 7 M urea. No complex was observed between ΨSI and the substrate tRNA or between ΨSI and the mutant FUra-tRNA(G31C39) under the same conditions.

To determine the rate of formation of the ΨSI−FUra-tRNA complex, ΨSI (12.5 μM) and [3′-^{32}P]FUra-tRNA (0.5 μM) were incubated at 15 or 22 °C and the reaction was terminated at intervals between 2 min and 5 h by denaturation with 2% SDS. A time-dependent increase of ΨSI-FUra-tRNA complex was observed on SDS−PAGE (Figure 3) with apparent first-order rate constants of 0.44 h^{-1} at 22 °C, and 0.24 h^{-1} at 15 °C. With a 25-fold excess of enzyme in the reaction, 75% of the [3′-^{32}P]FUra-tRNA was converted to the ΨSI−[3′-^{32}P]FUra-tRNA complex after 5 h of reaction

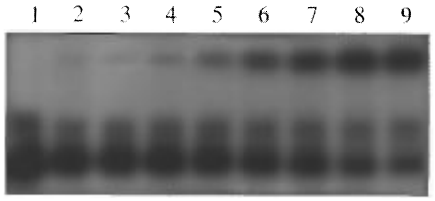


FIGURE 3: Autoradiogram of SDS−PAGE showing the complex formation between ΨSI and FUra-tRNA. ΨSI was incubated with [3′-^{32}P]FUra-tRNA and denatured at 0 min (lane 1), 2 min (lane 2), 5 min (lane 3), 10 min (lane 4), 20 min (lane 5), 40 min (lane 6), 80 min (lane 7), 160 min (lane 8), and 300 min (lane 9). The faster migrating band is free FUra-tRNA and the slower is the ΨSI-FUra-tRNA complex.

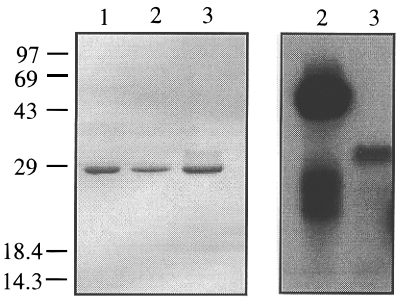


FIGURE 4: SDS−PAGE analysis of RNase A digestion of the ΨSI−[α-^{32}P]FUra-tRNA complex. (Left) Coomassie stained gel. Lane 1, ΨSI; lane 2, 10 μM ΨSI and 10 μM FUra-tRNA were incubated at 22 °C for 6 h and denatured in SDS loading buffer; lane 3, the reaction mixture in lane 2 treated with 0.005 μg/μL of RNase A for 1 h at 37 °C and denatured in SDS loading buffer. (Right) Autoradiography of the same gel. Lane 2, the lower lane is free [α-^{32}P]FUra-tRNA, and the upper lane is the ΨSI−[α-^{32}P]FUra-tRNA complex. Lane 3, complex between ΨSI and a small fragment of [α-^{32}P]FUra-tRNA after the RNase A digestion.

at 22 °C; complete conversion was observed after overnight incubation.

We attempted to "exchange" the FUra-tRNA from the preformed ΨSI−[3′-^{32}P]FUra-tRNA complex (0.05 μM FUra-tRNA and 0.5 μM ΨSI) with excess unlabeled FUra-tRNA (10 μM). Once the complex was formed, no release of [3′-^{32}P]FUra-tRNA was observed for 9.5 h.

To confirm that the complex observed by denaturing gel electrophoresis involved a covalent linkage between ΨSI and FUra-tRNA, we digested the ΨSI−FUra-[α-^{32}P]tRNA complex with RNase A, which specifically attacks single-stranded RNAs 3′ to pyrimidine residues. For tRNA^{Phe}, the smallest RNA fragment that would contain U_{39} after the RNase A digestion would be 5′-GAAGAU$_{39}$-3′. After complete RNase A digestion (37 °C for 1 h), a ^{32}P-labeled complex was detected on SDS−PAGE as a band migrating at 33 kD (Figure 4), indicating that the complex between ΨSI and a small ^{32}P-labeled RNA fragment can be isolated under denaturing conditions.

The ΨSI-D60 mutants do not form observable covalent complexes with FUra-tRNA. After overnight incubation of 5 μM of wild-type ΨSI and each of the D60 mutants (D60A, D60S, D60E, D60N, and D60K) with 0.5 μM [3′-^{32}P]FUra-tRNA, the reaction mixtures were analyzed by SDS−PAGE. Complex formation was observed for the wild-type enzyme, but no complex was detected for any of the mutant enzymes.

Identification of the Nucleotide of [^{32}P]FUra-tRNA attached to ΨSI. The FUra-tRNA-ΨSI complex labeled with 5′-[^{32}P]AMP was completely digested with RNase A to give

ΨSI covalently bound to a ^{32}P oligonucleotide. The complex was isolated, disrupted by heating, and the recovered oligonucleotide digested with RNase T2; this provided the component 3′-NMPs in which nucleotides 3′ to the [^{32}P]AMP of the oligonucleotide contained the ^{32}P label. Analysis by 2d TLC showed only 3′-[^{32}P]AMP and 3′-[^{32}P]GMP in a ratio of 0.8:2.0; this indicated the presence of A^{32}pA and 2G^{32}pA in the oligonucleotide bound to ΨSI. Of the 12 fragments expected from RNase A digested α-[^{32}P]ATP-labeled tRNA^{Phe}, only oligonucleotides G34 to FU39 and A64 to FU68 contain A^{32}pA and G^{32}pA, and only the former, which has the target FU at position 39, contains the dinucleotides in the proportion found. Hence, we conclude that the covalent FUra-tRNA -ΨSI complex involves the FUMP at position 39.

Effects of Uracil on the Covalent Complex Formation. We performed an experiment designed to ascertain whether the target fluorouracil dissociated from the enzyme during formation of the ΨSI−FUra-tRNA covalent complex. Here, we treated ΨSI (12.5 μM) with [3′-^{32}P]FUra-tRNA (0.5 μM) in the presence and absence of excess uracil (5 mM) and measured ΨSI−FUra-tRNA covalent complex formation *vs* time. Complex formation was measured by stopping the reaction at various time points between 2 min and 6 h and analyzing by SDS−PAGE. The initial rates of complex formation (0.22 h^{-1}) were identical with or without uracil in the reaction mixture.

DISCUSSION

We have undertaken studies on the mechanism of Ψ synthases. The enzyme used here is a highly expressed recombinant ΨSI which converts U residues at positions 38, 39, and/or 40 of *E. coli* tRNAs to Ψ. The substrate was *in vitro* synthesized, unmodified yeast tRNA^{Phe}, which contains a single target for ΨSI at U39. The purified recombinant protein catalyzed formation of 0.9 mol of Ψ/mol of tRNA^{Phe}, and steady state kinetic parameters showed a K_m of 1.8 μM and k_{cat} of 0.2 s^{-1} at 15 °C.

We began this work attempting to verify a proposed mechanism of ΨSI which involves formation of a covalent adduct between a Cys thiol of the enzyme and the C_6 of the U39 target of tRNA^{Phe} (Scheme 1). By analogy with enzymes which are known to utilize this mechanistic feature (*4*, *18*), we sought to determine whether tRNA containing fluorouracil would form a stable covalent complex with ΨSI. Previous workers have reported that FUra-tRNA was a potent inhibitor of ΨSI, but did not corroborate covalent bond formation (*3*, *8*). We showed that when initial velocity assays were performed shortly after mixing components, FUra-tRNA was an effective inhibitor of ΨSI with $K_i \approx 0.10$ μM. However, upon incubation of 0.10 μM ΨSI with 1 μM FUra-tRNA, there was a time-dependent loss of enzyme activity. Treatment of ΨSI with [3′-^{32}P]FUra-tRNA gave a protein−[3′-^{32}P]FUra-tRNA complex which, if not heated in loading buffer, was isolable on 1% SDS−PAGE or 7 M Urea−PAGE. The ΨSI−[3′-^{32}P]FUra-tRNA complex formed with rate of 0.24 h^{-1}, and upon addition of unlabeled FUra-tRNA there was no loss of radioactivity after as long as 9.5 h. Further, treatment of the ΨSI−[α-^{32}P]FUra-tRNA complex with RNase A followed by SDS−PAGE gave a small RNA fragment associated with the protein. Thus, contrary to previous reports, we concluded that ΨSI forms a covalent complex with FUra-tRNA.

Incubation of ΨSI with FUra-tRNA(G31C39) mutant, containing C instead of FU at position 39, showed no covalent complex, which demonstrates that fluorouracil in the target 39 position is essential for covalent complex formation. In addition, we showed that FUra-tRNA is attached to ΨSI via a linkage to a hexanucleotide fragment (5′-GAAGAFU) containing a single FUMP from position 39 of tRNA. Taken together, the results indicate that the FUMP at position 39 of FUra-tRNA is involved in covalent bond formation with ΨSI.

In the mechanism of Scheme 1, the target uracil (or fluorouracil) remains covalently bound to the enzyme until product is formed; in mechanisms not involving nucleophile addition to C_6, uracil (or fluorouracil) is transiently bound by noncovalent forces after the *N*-glycoside has been cleaved but before $C_1'-C_5$ formation and could dissociate from the enzyme. We performed an experiment designed to ascertain whether fluorouracil dissociates from the enzyme during formation the ΨSI−FUra-tRNA covalent complex. Here, we treated ΨSI with [3′-^{32}P]FUra-tRNA in the presence and absence of excess uracil and measured ΨSI−[3′-^{32}P]FUra-tRNA covalent complex formation *versus* time. We reasoned that if *N*-glycoside hydrolysis occurred and the cleaved fluorouracil was loosely bound to the enzyme, it could dissociate and be replaced by uracil. C_5 of uracil could then attack C_1' of the sugar to form the normal product Ψ and lead to turn over and dissociation of the complex; thus, formation of the ΨSI−FUra-tRNA covalent complex would be depressed by uracil. The result of the experiment was that the rates of covalent complex formation were the same with and without uracil. Thus, we concluded that, in the ΨSI−FUra-tRNA covalent complex, fluorouracil remained tightly bound to the enzyme.

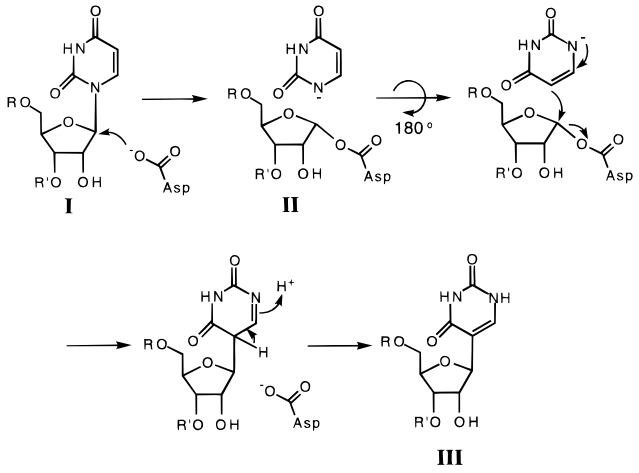
While this work was in progress, the sequences of several proven and numerous putative Ψ synthases became available, and there were no conserved Cys residues that might be assigned the role as the proposed nucleophilic catalyst (*12*, *13*). Further, Zhao, and Horne (*14*) reported that mutation of the Cys residues of ΨSI to Ala did not result in a loss of enzyme activity. Thus, it was apparent that the proposed mechanism involving thiol addition to C_6 of the target uracil was incorrect and was therefore abandoned.

Sequence alignment of three of four recently identified Ψ synthase families (*rlu*A, *rsu*A, and *tru*B) identified two conserved regions, one of which contains a "RLD" motif in which an aspartate residue is completely conserved (*12*, *13*). When we aligned the fourth family (*tru*A) along with these three families, the Asp residue (Asp60 in ΨSI) was retained as the only completely conserved amino acid (Figure 5). On the basis of this finding, we assumed that Asp60 of ΨSI might serve an important role in catalysis and prepared and examined several mutants at that position. In this group, we sampled mutants which presented side chains which were small (Ala), charged (Lys and Glu), and neutral-hydrophilic (Ser and Asn). All of these mutants were inactive in catalyzing Ψ formation; they reversibly bound FUra-tRNA as in wild-type ΨSI but were unable to form covalent adducts with FUra-tRNA. Thus, Asp60 of ΨSI is essential for the enzymatic activity and covalent attachment of FUra-tRNA.

rluA family	---**HRLD**--T	SG----A---	-A---L----	--------Y-	A-V-G-----
rsuA family	---**GRLD**---	-GL-L-T--G	--------P-	----K-Y---	----------
truA family	---**GRTD**-GV	-A--Q-----	----------	----------	----------
truB family	GH-**G-LD**P--	TG-L-IC---	AT--------	--K-Y-----	L---------

FIGURE 5: Alignment of the consensus sequences of the four putative Ψ synthase families. The consensus sequence motif common to all families is in bold letters. An aspartate residue is the only completely conserved amino acid in all four families.

Scheme 2



Taking into account all available data, we can propose two possible minimal mechanisms for ΨSI which involve covalent complex formation of the Asp60 residue of the enzyme with UMP 39 of $tRNA^{Phe}$.

First, the conserved Asp residue may serve the role of nucleophilic catalyst in the mechanism depicted in Scheme 1. However, although possible from chemical considerations, there is no precedent for carboxylate attack on C_6 of pyrimidines.

In considering alternative mechanisms, we recognized that aspartate or glutamate residues may serve as nucleophilic catalysts in glycosidases and form glycosyl-enzyme covalent adducts during the hydrolysis leading to products with retention of anomeric configuration (*22*, *23*). Indeed, tRNA-guanine transglycosylase, which catalyzes the replacement of G34 with preQ1, uses aspartate as a nucleophilic catalyst to attack the C_1' carbon leading to a covalent intermediate which is isolable on SDS−PAGE (*24*, *25*).

Thus, a second possible mechanism for ΨS is proposed in Scheme 2. Here, the carboxylate of the conserved Asp attacks the C_1' carbon of the ribose to displace the uracil moiety and form covalent intermediate II. Still bound to the enzyme, the uracil rotates so the C_5-carbon is juxtaposed to the C_1' and then displaces the Asp to form the $C_1'-C_5$ bond of Ψ. This mechanism is consistent with the retention of anomeric configuration at C_1' of the product Ψ. Also, as shown by early model studies (*26*), the nucleophilicity of the C_5 position of uracil may be enhanced for carbon−carbon bond formation by participation of the uracil N_1 anion. In the experiments described using FUra-tRNA, it can be seen that an intermediate analogous to II containing tightly, but not covalently, bound fluorouracil could account for the covalent isolable intermediate.

In summary, we initially sought to obtain evidence for a proposed mechanism of ΨS, which involved covalent bond formation between a Cys residue of the protein and the target uridine residue of RNA. In accord with this mechanism, we showed that FUra-tRNA formed a covalent complex with ΨSI and that fluorouracil was tightly bound to the enzyme throughout the reaction. We also showed that the target FUMP 39 of FUra-tRNA was directly involved in the covalent adduct with ΨSI. However, sequence analysis of ΨSs and mutagenesis studies reported elsewhere ruled out the proposed involvement of a Cys nucleophile in the mechanism. We then focused attention to the single conserved residue in ΨSs and Asp60 in ΨSI. We showed that mutagenesis of this residue did not affect reversible binding to tRNA but abolished catalysis and covalent bond formation with FUra-tRNA. Finally, we have proposed two possible mechanisms in accord with all available data which involve (i) covalent catalysis by reaction of the carboxylate of Asp60 at C_6 of the target pyrimidine, or (ii) covalent bond formation between the conserved Asp and the 1′ carbon of the sugar at the target U residue. We are currently attempting to verify the mechanism of ΨS by biochemical approaches and X-ray crystallography.

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