The Role of Cysteine Residues in the Rearrangement of Uridine to Pseudouridine Catalyzed by Pseudouridine Synthase I*  

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Escherichia coli tRNA pseudouridine synthase I (PSUI) catalyzes the conversion of uridine residues to pseudouridine in positions 38, 39, and 40 of various tRNA molecules. In previous biochemical studies with this enzyme (Kammen, H. O., Marvel, C. C., Hardy, L., and Penhoet, E. E. (1988) J. Biol. Chem. 263, 2255–2263) it was reported that cysteine residues are important in maintaining the active structure of the enzyme and are possibly involved in the catalytic reaction mechanism via a covalent cysteine intermediate. In order to further investigate the biochemical properties of PSUI, a high level expression and purification system for the enzyme and its corresponding mutants was developed. PSUI has three cysteine residues among 270 amino acids. In the present investigation, each cysteine residue was individually changed to serine and alanine. In addition, a triple mutant was prepared wherein all three cysteine residues were replaced by alanine. Surprisingly, while two of the three cysteine to serine mutants were inactive, all alanine mutants exhibited near wild-type levels of activity, including the triple mutant. These results provide the first direct and unambiguous chemical evidence against a covalent cysteine intermediate in the rearrangement mechanism of uridine to pseudouridine.

RNA is unique among nucleic acids for its numerous modified nucleotide bases. To date, there have been approximately 93 chemically distinct modified bases identified in various RNAs (1). Among them, pseudouridine (Ψ) is the most common modified nucleotide present in terms of distribution and frequency of occurrence (Fig. 1). It is present in all organisms ranging from prokaryotes to mammals and is found in transfer RNA (tRNA), ribosomal RNA (rRNA), and small nuclear RNA (snRNA) (2–4). Pseudouridine and its N1- and/or N3-derivatized analogs comprise the only carbon nucleotides (carbon–carbon glycosyl bond) so far identified at the polynucleotide level.

The formation of the widely distributed group of pseudouridines is due to a collection of RNA pseudouridine synthases that have a high degree of site specificity. *Escherichia coli* tRNA pseudouridine synthase I (PSUI)1 is one of the few synthases whose gene (truaA) has been identified (5).2 It catalyzes the formation of Ψ in positions 38, 39, and 40 in tRNA molecules (6). The biosynthesis of Ψ takes place at the polynucleotide level and involves an intramolecular rearrangement of U (7–10). Mechanistically, not much is known about the rearrangement, but neither ATP nor cofactors are required (8). Examination of the structure of Ψ suggests that it is chemically formed from U by first cleavage of the carbon–nitrogen glycosyl bond, followed by a 180° flip (or 120° rotation) of the uracil base and then reattachment at C6 to yield Ψ. The overall process can be viewed as a combination of enzymatic events having mechanistic similarities to uracil glycosylases and DNA/RNA methyltransferases. In Ψ formation, the cleavage of uracil from ribose bears analogy to the action of uracil glycosylases, while the reattachment step represents an alklylation event similar to that seen for methyltransferases. A well known mechanistic step in certain enzymatic processing of nucleic acids involves a general Michael addition type mechanism to either uracil or cytosine (11). This is observed for thymidylate synthase (12), dUMP, and dCMP hydroxymethylases (13), DNA (cytosine-5)-methyltransferases (14, 15), and tRNA (m5U54) methyltransferase (16, 17). In addition, reversible Michael adducts have been implicated in the interaction between aminoacyl tRNA synthetases and cognate tRNAs (18, 19). In all cases, the nucleophile in the Michael addition step is the thiol from a cysteine residue of the enzyme. Attack at C6 of the pyrimidine ring forms the covalent cysteine intermediate, which, in the case of methyltransferases, results in activation at C5 for electrophilic attack. Since the late 1970s, an analogous Michael addition type mechanism has been postulated to be also involved in Ψ formation (10, 11, 17–20). The reaction is similar to that of methyltransferases, since both involve an alklylation reaction at C6 of U. In one case the alklylation is intermolecular, while in the other, it is intramolecular. In addition to these reaction similarities, further indication for the possible involvement of a Michael type process was provided by the result that the acid-catalyzed hydrolysis of Ψ proceeds through the addition of water across the 5,6-double bond to form a 5,6-dihydrouridinediastic (21).

The first biochemical evidence implicating the importance of cysteine residues in the catalysis of Ψ formation was reported by Kammen et al. (10) using recombinant PSUI. In that study, enzymatic activity was dramatically impaired in the presence of thiol-specific modification reagents such as p-chloromercuribenzoic acid (pCMB), iodoacetate, or 5,5'-dithiobis-(2-nitrobenzoic acid). A similar reduction in activity was observed when reducing thiols were omitted from the reaction buffer. Furthermore, PSUI activity was inhibited by tRNAs containing 5-fluorouridine (5-FU). A mechanism consistent with the above observations was advanced that invoked the use of a nucleophilic cysteine residue (10). Attack of the uridine C5-C6 double bond by SH of cysteine results in a covalent intermediate at C6 (Fig. 2). This would be followed by carbon–nitrogen glycosyl bond cleavage, base rotation, and formation of the carbon–carbon bond between C5 of uracil and C1 of ribose. Elimination

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1 The abbreviations used are: PSUI, pseudouridine synthase I; PCR, polymerase chain reaction; DTT, dithiothreitol; 5-FU, 5-fluorouridine; pCMB, p-chloromercuribenzoic acid; MES, 4-morpholinethanesulfonic acid.

2 Originally identified as hisT but recently renamed truaA (see Ref. 26).
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Michael addition of a thiol group from a cysteine residue in the rearrangement of $U$ to $\Psi$ catalyzed by PSUI is shown. The mechanism is based on previous data supporting the importance of Cys residues in PSUI activity (10) and reaction similarities with known enzymes that modify position C5 of $U$ (20).

Purification of PSUI—The induced cell pellet from a 1-liter culture was resuspended in 10 ml of TEM buffer (10 mM Tris-HCl, pH 8.0, 10 mM MgCl$_2$, 0.1 mM EDTA), and lysed in a French pressure cell. The lysate was centrifuged at 65,000 $\times$ g for 1 h at 4°C. The supernatant was loaded onto a DEAE-cellulose column (28 $\times$ 200 mm) preequilibrated with TESG buffer (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 10% glycerol). The column was eluted with 200 ml of a 0–0.8 M NaCl gradient in TESG buffer. Peak fractions (30 ml) were collected between 0.2 and 0.4 M NaCl and dialyzed against MESG buffer (20 mM MES, pH 6.5, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 10% glycerol) and then loaded onto a phosphocellulose column (25 $\times$ 100 mm) preequilibrated with MESG buffer. The column was eluted with 200 ml of a 0–0.9 M NaCl gradient in MESG buffer. Peak fractions (30 ml) were collected between 0.6–0.7 M NaCl. Solid ammonium sulfate was added to give a final concentration of 60% (w/v). The mixture was centrifuged at 40,000 $\times$ g for 30 min at 4°C. The pellet was dissolved in TESG buffer and dialyzed against TESG buffer followed by 1:1 TESG buffer:glycerol. The final yield of purified PSUI was approximately 20 mg.

Mutagenesis—In vitro mutagenesis was performed by overlap extension site-directed mutagenesis using the polymerase chain reaction as described previously (24). PCR primers used for the generation of mutants were as follows: M(C55S), 5'-CCGGTCTCTTCGCGCGCGGCTA-3' and 5'-TACGCCCGGCGGAGAAGACCGG-3'; M(C154S), 5'-GCGGTGCACTGCTCGAGCCGCGT-3' and 5'-TCCGGGACTGGAGGACTGCGACC-3'. The two flanking PCR primers were the same as those used for subcloning. The conditions for PCR amplification and the subcloning into the pET3d vector were the same as described above for construction of the wild-type expression vector. Overexpression and purification of mutant proteins were analogous to wild-type enzyme. Yields ranged from 2 to 20 mg of purified protein/liter of induced cells.

In Vitro Transcription—Plasmid pETRP13 containing the partial coding sequence of PSUI with flanking NcoI and BamHI restriction sites were used in the polymerase chain reaction (PCR) to replicate the coding sequence of PSUI from pNU61. A 100-ml reaction mixture contained 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 3.75 mM MgCl$_2$, 0.2 mM each of the deoxyribonucleotide triphosphates (dATP, dCTP, dGTP, and dTTP), 25 pmol each of the primers P1 and P2, 3 ng/ml T7 RNA polymerase, and used without further purification. T7 RNA polymerase was isolated from the E. coli strain BL21/pAR1219 and purified by the method of Wyatt et al. (22). Plasmid pNU61, which carries the coding sequence of the PSUI gene, was a kind gift from Professor Malcolm Winkler. Expression vector pET3d and BL21(DE3) plasmid strain were obtained from Novagen. DNA manipulation and transformation were performed using methods described by Maniatis et al. (23). Restriction digestions and ligations were performed using conditions recommended by the enzyme suppliers. Oligonucleotides were made on an Applied Biosystems 391 DNA synthesizer and purified by 15% denaturing polyacrylamide gel electrophoresis. DNA sequencing was performed using the Sequenase kit from U.S. Biochemical Corp.

Construction of Overexpression System for PSUI—Primers containing the partial coding sequence of PSUI with flanking NcoI and BamHI restriction sites were used in the polymerase chain reaction (PCR) to replicate the coding sequence of PSUI from pNU61. A 100-ml reaction mixture contained 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 3.75 mM MgCl$_2$, 0.2 mM each of the deoxyribonucleotide triphosphates (dATP, dCTP, dGTP, and dTTP), 25 pmol each of the primers P1 and P2, 3 ng/ml pNU61, and 2.5 units of T7 DNA polymerase. Primer was 5’-CTAGCCCATGTTAATGTCCGACCAGCAACAGC-3’ and P2 was 5’-ATCTGGAATCCTTAGTGGCCAGAGAATAACG-3’. The underlined sequences are PSUI sequences, and restriction sites are italicized. The annealing temperature used was 55°C. After amplification, the resulting DNA was digested with NcoI and BamHI, and the fragment was subcloned into the NcoI and BamHI sites of pET3d vector. Both strands of the resulting clone were sequenced. The construct, plasmid pDH101, was transformed into the E. coli BL21(DE3) pLysS strain and grown in NZYM medium containing ampicillin and chloramphenicol. At midlog phase, the cells were induced with 0.2 mM isopropyl-β-D-thiogalactoside for 3.5–4 h and were collected by centrifugation.

EXPERIMENTAL PROCEDURES

Materials—General biochemical reagents and buffers were purchased from common commercial vendors. Radioactive triphosphates were obtained from Amersham. [5-3H]UTP was purchased from Anodtek (Irvine, CA). Mature yeast tRNA$^*$ was purchased from Sigma. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs. T7g DNA polymerase was purchased from Perkin-Elmer.Ribonucleotide5'-triphosphates were purchased from Pharmacia Biotech. Aniline (99.5%) and pCMB were purchased from Aldrich, and used without further purification. T7 RNA polymerase was isolated from the E. coli strain BL21/pAR1219 and purified by the method of Wyatt et al. (22). Plasmid pNU61, which carries the coding sequence of the PSUI gene, was a kind gift from Professor Malcolm Winkler. Expression vector pET3d and BL21(DE3) plasmid strain were obtained from Novagen. DNA manipulation and transformation were performed using methods described by Maniatis et al. (23). Restriction digestions and ligations were performed using conditions recommended by the enzyme suppliers. Oligonucleotides were made on an Applied Biosystems 391 DNA synthesizer and purified by 15% denaturing polyacrylamide gel electrophoresis. DNA sequencing was performed using the Sequenase kit from U.S. Biochemical Corp.

Construction of Overexpression System for PSUI—Primers containing the partial coding sequence of PSUI with flanking NcoI and BamHI restriction sites were used in the polymerase chain reaction (PCR) to replicate the coding sequence of PSUI from pNU61. A 100-ml reaction mixture contained 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 3.75 mM MgCl$_2$, 0.2 mM each of the deoxyribonucleotide triphosphates (dATP, dCTP, dGTP, and dTTP), 25 pmol each of the primers P1 and P2, 3 ng/ml pNU61, and 2.5 units of T7 DNA polymerase. Primer was 5’-CTAGCCCATGTTAATGTCCGACCAGCAACAGC-3’ and P2 was 5’-ATCTGGAATCCTTAGTGGCCAGAGAATAACG-3’. The underlined sequences are PSUI sequences, and restriction sites are italicized. The annealing temperature used was 55°C. After amplification, the resulting DNA was digested with NcoI and BamHI, and the fragment was subcloned into the NcoI and BamHI sites of pET3d vector. Both strands of the resulting clone were sequenced. The construct, plasmid pDH101, was transformed into the E. coli BL21(DE3) pLysS strain and grown in NZYM medium containing ampicillin and chloramphenicol. At midlog phase, the cells were induced with 0.2 mM isopropyl-β-D-thiogalactoside for 3.5–4 h and were collected by centrifugation.
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RESULTS

Overexpression of PSUI and Mutants—The previously known plasmids, pNU61 and λpho containing the trua (hisT) gene sequence of PSUI were formerly the best available source for recombinant PSUI (6, 10). In this system, PSUI is under the control of its natural promoter and is produced in less than 0.2% of total soluble protein. In order to further study the biochemical properties of this enzyme, initial efforts were directed at creating a high level PSUI expression system. The PSUI coding sequence was amplified by PCR using pNU61 as template. Two extra amino acids (methionine and valine) were added in front of PSUI in order to create the correct reading frame. The PCR product was digested with NcoI and BamHI and subcloned into the corresponding sites of pET3d to give pDH101. Here, PSUI is under control of the T7 promoter. Upon induction with 0.2 mM isopropyl β-D-thiogalactoside, the major component of the soluble cell extracts was overexpressed PSUI. The expression levels and purification results are shown in Fig. 3. Approximately 20 mg of pure PSUI can be obtained from 1 liter of induced cells. All mutants were expressed and purified analogously to the wild-type protein, affording 2–20 mg/liter.

Activities of Mutant Enzymes—Based on prior biochemical evidence implicating the participation of cysteine residues in the catalysis of U to Ψ (10), the role of these cysteines was specifically examined by site-directed mutagenesis. E. coli PSUI has three cysteine residues located at positions 55, 154, and 169. Each cysteine was individually changed to a serine to generate mutant proteins, M(C55S), M(C154S), and M(C169S). The activities of these mutants were tested, and the results are presented in Table I. While M(C55S) maintained activity levels similar to those of wild-type enzyme, M(C154S) and M(C169S) possessed little to no enzymatic activity. To further examine the function of these residues, a similar series of mutants was prepared in which cysteine was replaced by the nonpolar residue alanine. Three mutants were made wherein Ala replaced Cys in positions 154 (M(C154A)), 169 (M(C169A)), and in all three locations 55, 154, and 169, which afforded the triple mutant, M(AAA). The activities of these mutants were tested, and the results are presented in Table I. In contrast to the Ser mutants, all Ala mutants showed high levels of activity, even the triple mutant, which has all three Cys replaced by Ala.

Sulfhydryl Requirement—Kammen and co-workers (10) previously investigated the thiol dependence of PSUI on catalysis. It was reported that the thiol-specific reagent, pCMB, irreversibly inactivated the enzyme. Table II shows the inactivation of PSUI in the presence of 0.25 mM pCMB, but contrary to the previous report, the inhibition was reversible upon the addition of 10 mM DTT. Further, the activities of Ala mutants, M(C154A), M(C169A), and M(AAA) were also examined in the presence of 0.25 mM pCMB (Table II). All proteins showed a dramatic impairment of activity in the presence of pCMB (minus DTT) except the triple mutant, M(AAA), which lacks Cys residues.

Determination of Vmax and Km—Ψ activity of wild-type and triple mutant M(AAA) enzymes were measured as a function of increasing substrate concentration (Fig. 4). Initial velocities were plotted against substrate concentration, from which Vmax and Km were determined. Vmax values for wild-type and M(AAA) enzymes are 380 ± 24 and 240 ± 28 pmol s−1, respectively. Km values for wild-type and M(AAA) enzymes are 86 ± 14 and 110 ± 27, respectively. Replacement of all three Cys residues with Ala in M(AAA) resulted in only a small change in both Km and Vmax parameters as compared with wild-type enzyme. These mutations caused a 1.2-fold increase in Km and a 1.6-fold decrease in Vmax. The overall Vmax/Km ratio was lowered by a factor of 2 for the triple mutant, M(AAA).

| Reaction system | % of control | 3H release |
|----------------|-------------|-----------|
| PSUI           | 100         | 100       |
| M(C55S)        | 100         |           |
| M(C154S)       | 1           |           |
| M(C169S)       | 95          |           |
| M(C154A)       | 97          |           |
| M(C169A)       | 97          |           |
| M(AAA)         | 92          |           |

FIG. 3. SDS-polyacrylamide gel electrophoresis analysis of overexpression and purification of PSUI. Analysis using 10% SDS-polyacrylamide gel electrophoresis. Lane 1, protein molecular weight markers; lane 2, pNU61 plasmid expression; lanes 3 and 4, before and after induction with isopropyl β-D-thiogalactoside of expression plasmid pDH101; lane 5, pooled fractions after phosphocellulose column; lane 6, homogeneous PSUI after (NH₄)₂SO₄ precipitation and resuspension.
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5 mM tRNA and 5 mM enzyme were used in each reaction. Wild-type enzyme (PSUI) was used as control, representing approximately 80% conversion of U to Ψ. The enzyme was pretreated with or without PCMB at 30 °C for 15 min prior to the start of the reaction. All reactions contained 0.25 μM β-MCE from enzyme solution. Reactions were performed as described under “Experimental Procedures.”

| Reaction system | % of control |
|-----------------|--------------|
| Complete system (no pCMB) | 100 95 97 92 |
| Omit DTT (no pCMB) | 98 95 95 92 |
| Omit DTT (with 0.25 mM pCMB) | 1 3 5 79 |
| Omit DTT (with 0.25 mM pCMB) and then add 10 mM DTT | 100 94 97 91 |

a Complete system consists of enzyme, [3H]tRNA, 50 mM Tris-HCl, 10 mM MgCl₂, and 10 mM DTT.
b After 20 min, a final concentration of 10 mM DTT was added to the reaction, which was then allowed to proceed for an additional 20 min.

DISCUSSION

A compilation of E. coli tRNA sequences reveals Ψ residues at seven different positions, namely 13, 32, 38, 39, 40, 55, and 65 (1–4). There are nine identifiable positions in which Ψ is found in E. coli tRNA (4). E. coli PSUI specifically catalyzes the conversion of U to Ψ in positions 38, 39, and 40 in tRNA (6). This particular modification is highly dependent on the tertiary structure of the tRNA. During the course of the present study, Ofengand and co-workers cloned the genes for several Ψ synthases from E. coli. Ψ55 synthase (truB) specifically modifies U to Ψ at position 55 in tRNA (26). Ψ746 synthase (rtaU) modifies both positions 32 in tRNA and 746 in 23 S rRNA (27). And finally, Ψ516 synthase (rsuA) modifies position 516 in 16 S rRNA (28). Surprisingly, although all Ψ synthases catalyze the same type of reaction in RNA, wherein U is rearranged to Ψ, there is no obvious sequence homology between these enzymes (27). All of these synthases contain two or three cysteine residues, but there is no conserved alignment of these residues in their primary sequence.

Previous studies on Ψ formation suggested that the mechanism of this reaction involves cleavage of the uracil base followed by reattachment of the base to the ribose at C₅ of uracil (7–10). A well-established mechanistic pathway of enzymes that effect reaction at C₅ of pyrimidines (i.e., methyltransferases) utilizes covalent cysteine intermediates via a Michael type addition (11–17, 20). Similarly, cysteine residues have been implicated to play an important role in the catalyzed rearrangement of U to Ψ by PSUI (10). Synthase activity was shown to be irreversibly inactivated by the addition of pCMB (10). This inactivation has been verified in the present study, but in contrast to the previous report, the inactivation was found to be reversible. A similar reversibility has been reported for tRNA (m⁵U54) methyltransferase (16). On the other hand, a previous study showed that while various Ψ synthase activities were inhibited in the presence of 5-FU tRNAs, no covalent intermediates could be detected (29). This result, however, may very well be a consequence of the underlying mechanism of the transformation and not the absence of a covalent intermediate. Fig. 5 illustrates this point. It is conceivable that while 5-FU residues have been used with great success in trapping covalent cysteine intermediates in methyltransferases (12, 16), they may not be applicable for studying Ψ formation in the same manner, due to the difference in their reaction pathways. Since the rearrangement of U to Ψ results in a free N₃-H imino proton, β-elimination of a covalent intermediate at C⁵ could conceivably take place. This would release the enzyme from its substrate, and thus prevent trapping of a covalent adduct. Such an elimination pathway is not available in the mechanism of U or C methylation by methyltransferases.

In order to clarify these mechanistic ambiguities, the participation of cysteine residues in maintaining Ψ synthase activity was directly investigated by site-directed mutagenesis. Initially, serine was chosen to replace cysteine, since structurally and functionally serine is comparable with cysteine. The three cysteine residues of PSUI located in positions 55, 154, and 169 were each individually mutated to serine. An E. coli tRNA^[htRNA] transcript containing uridine residues labeled with ³H at C₅ was used as the substrate. This tRNA contains a modifiable U at position 39, which is transformed to Ψ when exposed to PSUI (3). The extent of the U to Ψ rearrangement was monitored by the tritium release assay (8). This assay takes advantage of proton release from C₅ during the course of the U to Ψ transformation. Monitoring ³H release from a tritium-labeled substrate provides a relatively easy method for measuring enzyme activity. While mutant M(C55S) had little effect on activity in comparison with wild-type PSUI, mutants M(C154S) and M(C169S) showed virtually no activity, thus indicating that these mutations severely impaired catalytic function (Table 1). Both mutants M(C55S) and M(C154S) were capable of binding tRNA^[htRNA] as assayed by electrophoretic mobility shift assays.

### Table II

| Reaction system | % of control |
|-----------------|--------------|
| Complete system | 100 95 97 92 |
| Omit DTT | 98 95 95 92 |
| Omit DTT and DTT | 1 3 5 79 |
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a Complete system consists of enzyme, [³H]tRNA, 50 mM Tris-HCl, 10 mM MgCl₂, and 10 mM DTT.
b After 20 min, a final concentration of 10 mM DTT was added to the reaction, which was then allowed to proceed for an additional 20 min.

### Fig. 4

Kinetic analysis of wild-type and mutant enzymes. Determination of kinetic parameters, \( k_m \) and \( V_{max} \), for wild-type (WT, ○) and mutant (M(µA), ●) enzymes. Reactions were performed as described under “Experimental Procedures.” Initial velocities were determined by linear regression of DPM versus time plots. Kinetic parameters were the average of three separate determinations.

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In order to clarify these mechanistic ambiguities, the participation of cysteine residues in maintaining Ψ synthase activity was directly investigated by site-directed mutagenesis. Initially, serine was chosen to replace cysteine, since structurally and functionally serine is comparable with cysteine. The three cysteine residues of PSUI located in positions 55, 154, and 169 were each individually mutated to serine. An E. coli tRNA^[htRNA] transcript containing uridine residues labeled with ³H at C₅ was used as the substrate. This tRNA contains a modifiable U at position 39, which is transformed to Ψ when exposed to PSUI (3). The extent of the U to Ψ rearrangement was monitored by the tritium release assay (8). This assay takes advantage of proton release from C₅ during the course of the U to Ψ transformation. Monitoring ³H release from a tritium-labeled substrate provides a relatively easy method for measuring enzyme activity. While mutant M(C55S) had little effect on activity in comparison with wild-type PSUI, mutants M(C154S) and M(C169S) showed virtually no activity, thus indicating that these mutations severely impaired catalytic function (Table 1). Both mutants M(C55S) and M(C154S) were capable of binding tRNA^[htRNA] as assayed by electrophoretic mobility shift assays.
whereas mutant M(C169S) did not (data not shown). These results tentatively suggested that Cys$^{154}$ and Cys$^{169}$ were important in maintaining activity, while Cys$^{99}$ was relatively benign. Furthermore, the catalytic involvement by Cys$^{154}$ remained a possibility, since mutant M(C154S) maintained the ability to bind but not to modify. Although Cys and Ser are structurally similar in the sense that they are both nucleophilic, they possess significant differences in polarity. The hydroxyl group on Ser is highly polar, while the sulphydryl on Cys is relatively nonpolar. Assuming Cys occupies a position in a hydrophobic environment, the introduction of a polar residue like Ser could significantly perturb the active enzyme structure. In order to address this possibility, a second set of mutants was generated that replaced Cys by the nonpolar residue Ala. Since Ser substitution at positions 154 and 169 had the greatest effect on activity, these positions were individually changed to Ala. In contrast to the Ser mutants, however, both Ala mutants, M(C154A) and M(C169A), showed similar activity to wild-type enzyme (Table I). This result suggests that the loss of activity in the serine mutants M(C154S) and M(C169S) is not mechanistically based but is more likely due to a perturbation of the active enzyme structure by serine substitution. To further confirm these results, a triple mutant was generated in which all three cysteine residues in positions 55, 154, and 169 were changed to alanine. Again, similar findings were observed (Table I). The triple mutant, M(AAA), possessed high levels of activity, and its kinetic parameters were compared with those of wild-type enzyme (Fig. 4). Both wild-type and triple mutant enzymes exhibited comparable kinetic parameters in $K_m$ and $V_{max}$. A 1.2-fold increase in $K_m$ and a 1.6-fold decrease in $V_{max}$ were observed for the mutant enzyme as compared with wild-type enzyme. This resulted in an overall 2-fold decrease in the parameter, $V_{max}/K_m$. Since this parameter is a measure of enzyme efficiency, the Cys to Ala mutations resulted in the generation of a relatively active but somewhat less efficient enzyme in both binding and $V_{max}$. More importantly, however, is the magnitude of the overall change in $V_{max}/K_m$, which is relatively small. This suggests that the wild-type and mutant enzymes proceed by a common mechanism that does not involve a covalent cysteine intermediate. To further substantiate the thiol independence on catalysis, $\Psi$ activity was also examined in the presence of the thiol modification reagent, pCMB (Table II). Analogous to wild-type PSUI, the enzymatic activities of M(C154A) and M(C169A) were lost in the presence of pCMB, but upon subsequent addition of DTT the activities were restored. The triple mutant, M(AAA), however, which lacks cysteine residues, was not significantly affected by the addition of pCMB. Only a slight diminution in activity was observed. This result verifies that the activity measured for mutant M(DDD) was solely due to mutant enzyme and not the presence of endogenous wild-type enzyme, since wild-type protein is virtually inactive under these conditions. From these experiments, it is clear that cysteine residues are not necessary for maintaining catalysis during $\Psi$ formation. Additionally, Cys$^{154}$ and Cys$^{169}$ in the wild-type enzyme may occupy a hydrophobic position within the protein fold that is sensitive to substitution by a polar substitute and/or chemical modification.

The formation of $\Psi$ is not a simple process; it involves a multistep mechanism. PSUI has to first bind the tRNA, next cleave the uracil base from ribose, and then rotate and ligate it back on the ribose of the tRNA. All of these events occur in a site-specific manner on the tRNA. Chemically, the cleavage step in the formation of $\Psi$ is catalytically similar to the cleavage event seen in uracil-DNA glycosylases that catalyze the cleavage of uracil residues during DNA repair (30). In principle, mutants that lack cysteine residues could facilitate the initial step in the U to $\Psi$ rearrangement, which involves cleavage of the carbon–nitrogen glycosyl bond. If a covalent cysteine intermediate was necessary for retaining uracil after cleavage from ribose, then the absence of a catalytic cysteine residue could result in the site-specific formation of an abasic site. Since the tritium release assay involves the absorption of substrates on charcoal, this assay is able to distinguish between the release of free [5-3H]uracil (resulting from glycosylase activity) and $^3H^+$ (resulting from $\Psi$ formation). This was verified using [5-3H]uracil as a control. In all experiments with Cys to Ala mutant enzymes, no evidence of the formation of abasic sites could be detected. Finally, further evidence was provided using aniline-facilitated chemical cleavage of modified tRNA (25). For tRNAs modified by wild-type and mutant enzymes, no cleavage of the tRNA was observed when incubated under acidic conditions followed by treatment with aniline (data not shown). These results indicate that no abasic sites were generated in tRNA after modification with mutant PSUI enzymes, thus validating the mechanistic interpretation of the Ala mutants.

In conclusion, mechanistic pathways are never proven; rather, the elimination of plausible routes serves to support the hypothesis. The catalytic formation of $\Psi$ residues by PSUI does not involve covalent cysteine-derived intermediates as previously thought. This may be a general feature of $\Psi$ synthases. Furthermore, mutational studies presented here demonstrate that enzyme activity is still maintained even in the absence of Cys residues within the protein. Mechanistically, the formation of $\Psi$ appears to resemble more closely the action of a uracil glycosylase than it does a methyltransferase. The active site of this enzyme is no doubt guarded from releasing uracil after its initial cleavage from ribose to allow for rotation and realignment at C5. Current efforts are underway involving crystallization trials of PSUI bound to its tRNA substrate in order to elucidate the mechanistic details of this intriguing rearrangement.

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