Substrate Specificity for 4-Thiouridine Modification in Escherichia coli*

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The biosynthesis of 4-thiouridine (s4U) in Escherichia coli tRNA requires the action of both the thiamin pathway enzyme Thii and the cysteine desulforase IscS. IscS catalyzes sulfur transfer from l-cysteine to Thii, which utilizes Mg-ATP to activate uridine 8 in tRNA and transfers sulfur to give s4U. In this work, we show through deletion analysis of unmodified E. coli tRNAphe that the minimum substrate for s4U modification is a mini-helix comprising the stacked acceptor and T stems containing an internal bulged region. The size of the bulged loop must be at least 4 nucleotides and contain the target uridine as the first nucleotide. Replacement of the T loop sequence with a tetraloop in the deletion substrate increases activity and shows that the TWC primary sequence is not a recognition element. An unmodified tRNAphe transcript in which the 3' sequence is not a recognition element. An unmodified loop sequence with a tetraloop in the deletion substrate uridine as the first nucleotide. Replacement of the T loop sequence with a tetraloop in the deletion substrate increases activity and shows that the TWC primary sequence is not a recognition element. An unmodified tRNAphe transcript in which the 3' terminal ACCA sequence is removed to give a blunt terminus has <0.1% activity, although the addition of a single overhanging base essentially restores activity. In addition, reducing the distance of the 3' terminus relative to U8 by as little as 1 bp severely impairs activity. By dissecting a minimal RNA substrate in the T loop region, a two-piece system consisting of a substrate RNA and a "guide" RNA is efficiently modified. Our results indicate that outside of the modified U8, there is no primary sequence requirement for substrate recognition. However, the secondary and tertiary structure restrictions appear sufficient to explain why s4U modification is limited in the cell to tRNA.

All organisms chemically modify the bases of their tRNA (1, 2). The purpose of these modified nucleosides is varied. Many modifications stabilize codon-anticodon interactions and affect codon specificity while maintaining the correct reading frame (3). Others are required for tRNA recognition by their cognate aminoacyl synthetase (4, 5) and for ribosome function (6). There is also evidence that some modifications are involved in metabolic responses to the environment of the cell (7). Because each modification is enzymatically introduced at the post-transcriptional level, the modification enzymes must recognize a specific base within a highly structured tRNA molecule. These systems thus provide another context for the study of protein-RNA interactions.

The thionucleoside 4-thiouridine (s4U)† is found at position 8 in the tRNA of bacteria and archaea. Its biological role appears to be the result of its photochemical reactivity. Irradiation of bacteria can cause a photocross-link in specific tRNAs between s4U at position 8 and cytidine at position 13 (8). Some of the cross-linked tRNAs are poor substrates for aminoacylation (9). The physiological result of near UV exposure in Escherichia coli (10) and Salmonella typhimurium is that an s4U-dependent growth delay and induction of the stringent response to amino acid starvation. Thus, s4U in tRNA appears to be important in vivo as a physiological sensor for near UV radiation exposure. In vitro, s4U has found use as an effectively zero-length photocross-linking agent (12–14) as well as a chemoselective site for the attachment of biophysical probes (15, 16).

Initial work on s4U biosynthesis in E. coli found a requirement for two enzymes (Fig. 1) (17). The thiI gene was shown to be required for s4U synthesis in E. coli by Mueller et al. (18) and for thiazole biosynthesis in Salmonella typhimurium by Webb et al. (19). We later found that the cysteine desulforase IscS was required for s4U synthesis both in vitro (20) and in vivo (21) and demonstrated in vitro s4U synthesis with an unmodified tRNA substrate. We have shown through gel mobility shift assays that Thii and not IscS binds to tRNA and that Thii binds ATP, most probably for activation of the uridine via adenyllylation (22). The mechanism involves mobilization of sulfur from cysteine by IscS in the form of an enzyme-bound persulfide (23, 24), which is then transferred to a cysteine residue on Thii before final insertion into U8 of tRNA (25–27). The nature of the final sulfur transfer step is unclear, but it has been recently shown that Thii is oxidized to a disulfide under single turnover conditions (28). Thus, Thii has the multiple roles of binding tRNA, activating O4 of uridine 8 using Mg-ATP while accepting a sulfur atom from IscS and transferring it to the 4-position to give the final product, s4U.

The targeted uridine 8 for s4U biosynthesis is essentially invariant in cytosolic tRNAs. Fig. 2 shows the position of U8 in different representations of the canonical l-shaped tRNA structure. In E. coli, all of the tRNAs contain s4U to some extent, although the levels of s4U in particular tRNAs are found to vary with growth rate (29). Because of the prevalence of the modification, it is reasonable to propose that the tertiary structure and not primary sequence is the major structural determinant for substrate activity. In the structure of most cytosolic tRNAs, U8 is involved in a reverse Hoogsteen base pair with A14, whereas the 2'-hydroxyl of U8 is hydrogen-bonded to A21 (30). The U8-A14 interaction is particularly important for the formation of the core of the tRNA structure by bringing the D loop in closer proximity for base pairing with residues in the T loop (see Fig. 2). An examination of a space-filling model of yeast tRNAphe suggests that although O4 of U8 is not involved in direct tertiary interactions, access to O4 is restricted in the folded structure. Thus, Thii to some extent must invade the
tRNA structure or trap a transient partially unfolded intermo-de to catalyze the chemistry at O4. To gain insight on how Thi recognizes its tRNA substrate, we report on the substrate specificity for s4U modification using variants of unmodified E. coli tRNAs.

MATERIALS AND METHODS

General—ThiI and IscS were overproduced from expression plasmids in E. coli BL21(DE3) and purified as described previously (20). T7 RNA polymerase was purified from strain BL21(pAR219), which was a generous gift of F. Studier. Acrylamido acrylamide was from Integrated DNA Technologies and were purified by denaturing PAGE before use. Deoxysequencing of cloned DNA templates was performed by the University of Wisconsin Biotechnology Center.

Preparation of RNA Substrates—RNA substrates were obtained by runoff transcription using T7 RNA polymerase as described previously (22). Templates for the preparation of short RNAs (<50 nt) were single-stranded DNA oligomers comprising the antisense of the desired RNA sequence and a 5'-17 nt region that is the antisense of the T7 promoter. For example, the template for TPHE39A is 5'-TGG CAC GGT GTC TCC CAA GGA CAC TTX CCG GCC TAT GAT GAC TCG TAT TGG TGC CCG GAC TTG

For longer RNA substrates, double-stranded templates containing the RNA sequence preceded by the T7 promoter sequence were prepared by the method of Peterson et al. (33) using two overlapping oligodeoxynucleotides with a 12-bp region of complementarity. For tRNAs, the sense strand was 57 nt and contained the T7 promoter followed by the work of Ighi (31). This technique incorporates APM into the polyacrylamide gel. The mercury binds to thiouridine groups on the RNA with a Kd of 0.2 μM (35) and acts to retard selectively the mobility of RNAs modified with s4U (31). Stock solutions of sodium sulfide were prepared shortly before performing the assays by dissolving solid Na2S nonahydrate in water and adjusting the pH by sodium bicarbonate. Reaction mixtures (50 μl) were prepared as described above with the exception of sulfide substituted for IscS and [35S]cysteine. Timed aliquots (25 μl) of each reaction mixture were removed and quenched by the addition of 25 μl of phenol/chloroform/isoamyl alcohol (25:24:1). After vortexing and centrifugation for 3 min in a microcentrifuge, the top aqueous layer was passed through a 0.5-ml G-50 spin column. To the eluate was added 25 μl of 8 M urea with 0.05% bromphenol blue and 0.05% xylene cyanol. An aliquot (25 μl) of this mixture was loaded per gel lane. For tRNA's and minor variants, 8% polyacrylamide gels (0.75 mm) containing 100 μM AMP in Tris borate EDTA buffer were run on a MiniProtean II (Bio-Rad) apparatus at 170 V for 1 h at room temperature. The bands were visualized by ethidium bromide staining in Tris acetate EDTA buffer (10 min) and quantitated using a densitometer. Alternatively, [32P]-labeled RNA was utilized and quantitated achieved by PhosphoImager analysis of the dried gel using ImageQuant software from Amersham Biosciences.

Measurements of ThiI Binding to RNA Substrates—For binding studies, mixtures of 50 mM Tris, pH 7.5, 5 mM MgOAc2, 50 mM KCl, 1 mM RNA, and variable concentrations of ThiI were incubated for 10 min at room temperature. Aliquots of 20 μl were then applied via an 8-channel pipette to a modified 96-well plate dot-blot filtration apparatus (Schleicher & Schuell). The apparatus contained both nitrocellulose (Protran BA85, Schleicher & Schuell) and DEAE filters (DE51, Whatman) essentially as described by Wong and Lohman (36). Wells were washed with 100 μl of TE buffer both before and after application of binding solution. Filters were then blotted to remove excess liquid and wrapped in plastic wrap prior to phosphorimaging analysis. The fraction bound to protein was measured directly by comparing the amount of [32P]-labeled RNA bound to nitrocellulose relative to the amount bound to DE51 anion-exchange filter paper. Measurements were performed in triplicate, and data corrected for binding to the filter without RNA. The specific binding of the nitrocellulose filter. The corrected data were plotted as the fraction of total RNA bound to nitrocellulose versus ThiI concentration, and the resulting plot was fitted to a sigmoidal binding curve using Kaleidograph (Synergy).

Assay for Two-piece Trna-s4U Reaction with 45UT and 45UTB RNA—Reaction mixtures (50 μl of total volume) contained 50 mM Tris, pH 7.5, 5 mM MgCl2, 50 mM KC1, 4 mM ATP, 20 μM t-cysteine, 5 μM each of 45UTA and 45UTB RNAs, and 2 μg each of ThiI and IscS. A positive control substrate used was the one-piece substrate TPHE45A at 5 μM, whereas negative control was a reaction with 45UTB RNA omitted.
RNAs were first heated to 85 °C for 2 min and slow-cooled for 1 h in the presence of Tris, MgCl₂, and KCl before the remaining ingredients were added to initiate reaction. After 30 min at 37 °C, an equal volume of phenol:chloroform:isoamyl alcohol (50:48:2) was added and the mixture was vortexed for 20 s. The layers were separated by centrifugation in a microcentrifuge for 4 min, and 25 μl of the top aqueous layer was passed through a G-50 spin column and added to 25 ml of 8 M urea containing 0.1% bromphenol blue and 0.1% xylene cyanol FF. This mixture was mixed by vortexing and heated to 90 °C for 3 min and loaded onto two duplicate 8% denaturing polyacrylamide gels. One of the gels contained 100 μM APM, whereas the other had APM omitted. The gels were run for 45 min at 170 watts in 1/100 Tris borate EDTA buffer. The gels were then soaked in a solution of ethidium bromide in 1/100 TAE buffer for 10 min, and RNA was visualized on a UV transilluminator (Fotodyne).

RESULTS

Deletion Analysis of tRNA³⁵° Transcripts—We used a DEAE filter disc assay described previously (34) to obtain the specific activity for s⁴U modification of RNA variants. The assay was measured for the incorporation of ³⁵S from L-[³⁵S]cysteine into tRNA that was specifically bound on the discs, and the rates were assessed in the linear range of the progress curve at 10% of the product formation. Under these conditions, unmodified tRNA³⁵° gave an initial rate constant (kₐ₀) of 1.0 min⁻¹ at 20 μM (Table I). We found that 20 μM RNA concentration is saturating for all of the RNA variants tested thus far that are substrates. We were not able to obtain accurate Kₘ values because of the limitations of the assay and the apparent low values for Kₘ (<0.1 μM). Using a filter binding assay (36), we were able to measure a Kₐ value for tRNA³⁵° of 1.9 μM (Fig. 3), which is considerably higher than the Kₘ estimate. Values for Kₐ were measured in the absence of other substrates, and this may explain the discrepancy with the Kₘ. In addition, Kₐ may reflect rate constants that indicate a large commitment for catalysis.

As shown in Table I, all of the full-length E. coli tRNA transcripts we have studied are good substrates at 20 μM. This is consistent with the study of Emilsson et al. (29) who found that all tRNAs contain s⁴U in vivo, although some are modified more efficiently than others at high growth rates. They reported that tRNA₂³⁵°, now known as the active form of the single tRNA³⁵° in E. coli (37), was poorly modified at all of the growth rates studied. We measured the in vitro activity of
unmodified tRNA\textsubscript{Glu} as a substrate and found that it has a lower \( k_{\text{obs}} \) by over 2-fold. Thus, although the magnitude of the reduced activity is small, it is consistent with their results. Modifications normally present in this tRNA \textit{in vivo} may modify the activity further. The unmodified transcripts of tRNALys, tRNASer-2, and tRNAArg were also found to be good substrates, consistent with \textit{in vivo} results (26).

Initial efforts to study the structure activity relationship of the tRNA were to introduce subtle disruptions of the tertiary fold in the vicinity of U8. We first mutated the target uridine 8 to make sure our \textit{in vitro} system was specific for this base alone. An unmodified tRNA\textsubscript{Phe} transcript containing either a U8C or U8A mutation was inactive as a substrate. Binding studies of WT tRNA\textsubscript{Phe} and the U8C mutant surprisingly showed no difference in the value of \( K_d \) within experimental error (Table I). This finding suggests that the U8C mutant can adopt a structure that is bound by ThiI, but because this structure lacks the target, uridine it is not modified.

Because U8 is involved in tertiary interactions with A14, we proceeded to make an analogous change in A14. An A14U mutation would be expected to disrupt the interaction and loosen the tertiary fold and reduce the effective concentration of properly folded tRNA. We found that this mutant had the same activity as the WT tRNA. Thus, relaxation of the tertiary fold has no effect on s4U modification, which may be expected if a nonnative structure is bound by the enzyme.

Defining the Minimal RNA Substrate—We subsequently introduced more drastic alterations of the tRNA structure. We sequentially replaced the D, anticodon (AC), and T stem loops with a two-nucleotide spacer and found that these RNAs were good substrates (data not shown). A tRNA mutant lacking both the D and AC stem loops was still processed at 50% of the rate of the full-length tRNA under saturating conditions. Replacement of the T\textsubscript{WC} loop of this RNA with a GAAA tetraloop resulted in a 39-nt RNA (Fig. 4, TPHE39A) that was an efficient substrate at saturation with activity nearly two-thirds that of the full-length tRNA\textsubscript{Phe} transcript (Table II). Binding measurements give a \( K_d \) of \( 6 \pm 2 \mu\text{M} \), indicating that some binding interactions are lost in the ground state but have little effect during catalysis. This was confirmed by our estimate of the \( K_m \) for TPHE39A to also be \(< 0.1 \mu\text{M} \). A UUCG tetraloop gave similar results. Thus, the conserved T\[GRAPHIC] sequence of the T loop is not a recognition element for s4U synthesis \textit{in vitro}.

Other deletion constructs that retained the larger T loop of tRNA\textsubscript{Phe} had lower specific activity as substrates (data not shown). The tetraloop sequence may act to stabilize TPHE39A and prevent alternative folded structures. Mutation of the target uridine 8 in the minimal substrates abolished s4U activity (for example, see TPHE39C in Table II). This shows that the small substrates are still binding in broadly the same orientation in the active site as the full-length tRNA. Reduction in the length of either stem in TPHE39A led to a significant decrease in activity.

Analysis of Internal Loop Containing the Target Uridine—Fig. 5 shows the relationship between internal loop size and activity with TPHE39A (7-nt loop) as reference substrate. The data show that a 6-nt loop with a base deleted from two different points in the loop (TPHE38B and TPHE38C) gives a slightly better substrate. The specific activity drops significantly with a 5-nt loop and diminishes rapidly as the loop is further contracted. The extreme case where a single uridine is bulged out of a long stem loop is inactive (not shown). Alternative structures predicted by MFold (38) were probed by sub-

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Substrate & \( X \) & \( V_{\text{max}} \) & \( K_{s} \) \\
\hline
TPHE39A & 5'-AGUGUC & 100 & 0.39 \\
TPHE38B & AGUGUC & 109 & 0.39 \\
TPHE38C & GUGUC & 105 & 0.39 \\
TPHE37B & AGUG & 54 & 0.39 \\
TPHE36A & AGU & 3.5 & 0.39 \\
TPHE35B & GU & 0.5 & 0.39 \\
\hline
\end{tabular}
\caption{Kinetic data for small RNA substrates (relative to tRNA\textsuperscript{Phe}) shown in Fig. 5.}
\end{table}
Substituting different bases in the 6- and 7-nt loops. Substitutions that decreased the possibility of base pairing within the internal loop gave modest increases in activity (data not shown). Because the structure of tRNA is complex in this region, these small substrates do not provide direct insight to the contouring of the active site but do show that multiple bases are required and that primary sequence is probably of little importance.

Effects of Changes at the 5' and 3' Termini—Because the modified U8 is relatively close to the acceptor terminus of the tRNA, this may be a site for recognition by ThiI. Extension of the small substrates in the 5' direction as shown in substrate TPHE45A (Fig. 4) leads to little reduction in activity (Table II). Extension of the full-length tRNA in the 3' direction is also well tolerated. However, when the acceptor stem in either full-length tRNA^Phe or the small substrates are extended significantly as a duplex (TPHE-ZIP or TPHE47A), activity is drastically reduced.

The 3' Extension Is a Positive Determinant for s^4U Activity—The only invariant primary structural element that remains in common between TPHE39A and natural tRNA^Phe is the 3'-terminal NCCA. We first prepared a blunt-ended derivative of TPHE39A. This RNA was not a substrate for s^4U synthesis at concentrations as high as 40 μM. This indicated that in the context of a minimal substrate, the 3'-terminal ACCA was absolutely required for activity. We then deleted the 3'-ACCA from the full-length tRNA^Phe transcript (Fig. 6, TPHEBLUNT). The initially purified transcript contained mostly N-1-length RNA and was a good substrate for the enzyme. However, when we purify the T7 transcript to single nucleotide homogeneity, we find that the blunt-ended tRNA is also no longer an observable substrate (<0.1%). Fig. 7 shows APM affinity gel analysis of s^4U reactions with full-length tRNA^Phe compared with the truncated substrate TPHEBLUNT. In both initial rate studies (Fig. 7, panel A) and during extended incubations (panel B), we see no s^4U formation in the purified blunt tRNA.

We also prepared a series of 3'-truncated substrates lacking A, CA, and CCA from the 3' end of the tRNA and carefully purified the transcripts. As shown in Table III, the addition of a single nt to the blunt tRNA (TPHE-CCA) restores activity. The range in activity of these truncated derivatives at 20 μM

Fig. 6. Structures of unmodified E. coli tRNA^Phe variants that probe the 5' and 3' terminus. Data for these variants are shown in Table III. For each structure, the remainder of the sequence is identical to tRNA^Phe.
concentration was 0.36–0.5 min⁻¹. The primary sequence recognition of this single-stranded region is not stringent. A variant of tRNA⁺₅₃ with a 3'-GUUG (Fig. 6, TPHE-GUUG) that replaces the ACCA is only reduced in activity by ~2-fold. Thus, Thl appears to have a binding site for the 3’-overhanging discriminator base N73 but does not appear to interact with the conserved 3’-terminal CCA in a sequence-specific manner. All of the effects at the terminus are more pronounced in the small substrates, which may amplify defects in binding interactions with Thl during catalysis.

Relative Orientation of 3’ End and Modified U8—Because the acceptor stem is helical and there is evidence of an interaction at both of its ends, the relative orientation between U8 and the 3’ end of the tRNA may be an important recognition element for Thl. Thus, we varied the distance between the two by adding or subtracting a single base pair in the acceptor stem. As shown in Table III, the addition of one base pair in the substrate TPHE + 1BP causes a relatively modest effect. This is not surprising because E. coli tRNA⁺₅₃, which contains an extra base pair in the acceptor stem by base pairing with the 3’-CCA (TPHE-ZIP) significantly reduces activity. Deletion of a single base pair in the acceptor stem causes a decrease in rate of nearly an order of magnitude. As in the case of the U8C mutant, Kᵣ is only slightly affected (not shown). In the context of the minimal substrate, deletion of a single base pair in the stem (TPHE37A) abolishes activity. Because it is unlikely that decreasing the acceptor stem by one bp affects the stability of the helix, this is evidence that orientation between U8 and the terminus is important for activity and may involve direct interaction between Thl and position 73.

Sulfide Is Able to Replace lscS/Cysteine as a Substrate for s⁴U Synthesis—We have reported previously that sulfide is able to act as an alternative source of sulfur for s⁴U synthesis in vivo, but the level of s⁴U is only 1–2% of that typically found in E. coli when an lscS mutant is grown in the presence of up to 10 mM sulfide (40). In vitro, we find that sulfide is an excellent source of sulfur for s⁴U synthesis but only at millimolar concentrations. We used [³²S]labeled tRNA⁺₅₃ as substrate and measured the fraction of modified tRNA using affinity electrophoresis (31) and PhosphorImager analysis. Reaction times were 2 min in the linear range of the progress curve. As shown in Fig. 8, the optimal sulfide concentration for s⁴U modification of tRNA⁺₅₃ is ~50 mM. Higher sulfide concentrations are inhibitory. At 50 mM sulfide and 20 μM tRNA, the initial rate constant for Thl is ~1.4 min⁻¹, which is similar to the kᵣ of 1.0 for the reaction with lscS/cysteine. This result is in agreement with the lower levels of s⁴U produced in vivo at 10 mM concentration, although the free sulfide concentration inside the cells was not known.

A Two-piece Trans-system Is an Efficient Substrate—The simple structural requirements for s⁴U activity as demonstrated in the minimal substrate TPHE39A suggested that a two-piece RNA system may combine to act as a substrate. For practical reasons related to transcription yields, we used the extended substrate TPHE45A as a model substrate (Fig. 9, top) for the trans-system. Dissection of the phosphodiester backbone in the tetraloop of TPHE45A gives two RNAs that are expected to anneal to form the proper structure for recognition by Thl. Indeed, when we prepared the “substrate” RNA 45cutA and the “guide” RNA 45cutB and tested them for s⁴U activity, we found that 45cutA was modified at nearly the same efficiency as the one-piece TPHE45A substrate. Fig. 9 (bottom) shows APM affinity gel analysis of the reactions of TPHE45A (lane 1) compared with the trans-system (lane 2). Panel A shows the migration of the RNAs in the absence of APM, whereas panel B shows migration of s⁴U-modified RNA in the affinity gel. Modification of substrate RNA 45cutA is dependent on the presence of the guide RNA 45cutB (lane 3). Analysis of the reactions by [³⁵S]transfer disc assay gave similar results. Initial rates for s⁴U formation in TPHE45A and the two-piece system were 1.11 and 0.92 pmol/s, respectively. In addition, use of either a DNA guide or a RNA guide lacking the 3’-CCA terminus results in no s⁴U modification (data not shown). A 25-fold excess of the DNA form of the guide (45cutB-DNA) shows little or no inhibition (lane 4). This may indicate that the shape of the acceptor helix is important for activity or that specific 2’-hydroxyl groups are sites of recognition.

DISCUSSION

There are now a number of examples of structure activity relationship studies for tRNA modification enzymes. Grosjean et al. (41) have recently separated these enzymes into two major groups. Group I enzymes do not require an intact tRNA for substrate recognition and instead recognize structural domains. This group includes the tRNA A37 N²-dimethylallyltransferase (MiaA) (42), G37 guanine trans-glycosylase in E. coli tRNA⁵⁷ (43), the U54 methyltransferase RUMT (44), and 2-thiouridine modification of 5-methyl-U54 to give s⁴T54 (45). Each of these enzymes recognizes a small stem loop structure with a varying degree of primary sequence recognition. Group II modification enzymes require a properly folded tRNA for substrate recognition. These include the m⁵G37 methyltransferase, which modifies tRNA⁵⁷ in E. coli (46, 47) and Salmonella (48), the m⁴G26 dimethyltransferase (49), and the Y35 synthase in Arabidopsis (50). Using tRNA⁴⁰ variants injected into Xenopus oocytes, Grosjean et al. (41) have found that Y13, Y40, and m⁴G37 are extremely sensitive to changes in tRNA tertiary structure, whereas m²G26 was less so and T54, Y55, m³A58, m⁵C49, and m⁶G6 were largely insensitive (40).

In this study, we show the specificity determinants for substrate activity in s⁴U modification. The minimal substrate requirements are a mini-helix containing an internal bulge of at least 5 nt with a uridine at the 5’ end of the bulge. This mini-helix is similar to the mini-helices shown to be substrates for aminoacyl-tRNA synthetases (51), but in that system, there is no requirement for an unpaired U8 (52). Thl could be characterized as a Group I modification enzyme, although the minimal substrate is more complex than that of stem loop-recognizing enzymes. Other tRNA-modifying enzymes have minimal recognition elements similar to that of Thl. These include Rnase P (53) and the archael G15 guanine trans-glycosylase that initiates archaeosine biosynthesis (54). Both recognize bulged mini-helix portions of the tRNA. For human Rnase P, a mini-helix comprising the acceptor and T stems is inactive but becomes an efficient substrate with the insertion of a single nucleotide at position 8 (53). Recently, the crystal structure of the G15 guanine trans-
glycosylase from *Pyrococcus horikoshii* was solved showing that the enzyme binds the novel A form of tRNA (55). This intriguing structure involves a rearrangement of the tRNA core to expose nucleotides U8-G22 while preserving the coaxially stacked mini-helix comprised of the acceptor and T-stems. It is conceivable as the authors suggest that this alternate form of tRNA is also a preferred substrate for s^4^U modification (55). Binding an alternate tRNA conformation would solve the problem of inaccessibility to U8 in the L-shape tRNA. However, for s^4^U modification, the acceptor and T-stem would be recognized rather than the acceptor-D stem loop region. Sequence homology searches have failed to show similarity between Thil and the archael G37 guanine *trans*-glycosylase, although this does not rule out a similar three-dimensional fold.

The 3'-NCCA overhang is also an important recognition element for s^4^U modification, although the sequence specificity is not stringent. The lack of activity of the blunt tRNA mutant is unlikely to be physiologically relevant because tRNA processing does not involve such a structure. However, the variant TPHE(-CCA) with a single base overhang is an intermediate in tRNA maturation and we show that it is nearly as active as the mature tRNA. Thus, all of the tRNA processing intermediates appear to be good substrates for s^4^U modification at a high concentration. Our results also suggest that the relative orientation of U8 with the tRNA terminus is important for substrate recognition. This discrimination may be a key component that ensures that only tRNA is modified to s^4^U in the cell.

In addition to RNA substrate specificity, we also investigated the specificity of the sulfur donor. We find that millimolar concentrations of inorganic sulfide can replace IscS/L-cysteine in the reaction. The optimal sulfide concentration was 50 mM, and higher concentrations were inhibitory under our assay conditions. This contrasts with our measurement of an apparent $K_m$ for cysteine in the coupled assay of $2.5 \pm 1.2 \mu M$. Thus, sulfide binding by Thil is weak ($K_m > 20 \mu M$) and physiologically irrelevant. However, the fact that sulfide is an efficient substrate at its optimal concentration provides some support for a mechanism of s^4^U synthesis in which nascent sulfide is produced by internal reduction of a Thil persulfide (27). The alternative mechanism involves direct attack of a Thil persulfide on the activated uridine before internal reduction. It is also

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**Fig. 8.** APM gel shift assay showing s^4^U formation by Thil with inorganic sulfide in place of IscS/L-cysteine. Reactions mixtures were carried out as described under “Materials and Methods” using 10 $\mu M$ 32P-labeled *E. coli* tRNA<sub>Phe</sub> transcript as substrate and 180 nM Thil in a final volume of 50 $\mu l$. Reactions were run at 37 °C for 2 min.

**Fig. 9.** Top, design of a two-piece trans-RNA substrate for s^4^U modification by *E. coli* Thil. Substrate TPHE45A is cleaved at the GAAA tetraloop to give a substrate RNA (45cutA) and a guide RNA (45cutB). The arrow shows the uridine that is modified to s^4^U. Bottom, APM affinity gel analysis of two-piece trans-s^4^U reaction. Panel A is denaturing polyacrylamide gel without APM, whereas panel B shows duplicate gel containing 100 $\mu M$ APM. Lane 1, one-piece substrate TPHE45A (5 $\mu M$); lane 2, two-piece system containing 5 $\mu M$ each of 45cut.A and 45cut.B; lane 3, 5 $\mu M$ 45cut.A only; lane 4, 5 $\mu M$ each of 45cut.A and 45cut.B with 125 $\mu M$ 45cut.B DNA.
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possible that both mechanisms may be operative depending on the source of sulfur.

Finally, we have shown that by dissecting the substrate TPHE45A into two pieces, two RNAs can be annealed to form the correct structure for substrate recognition by ThiI. In this system, the modified RNA strand acts as the substrate, whereas the unmodified strand acts as a guide RNA to direct ThiI binding and catalysis via complementary regions. The efficient modification of the two-piece substrate suggests that the reaction may be engineered to direct modification at any uridine in a separate RNA molecule. We have recently found that the two-piece system can be extended in either direction with little loss in activity and have successfully introduced s4U at other specific sites in tRNA.2 This finding suggests that ThiI may bind the tRNA in a clamp-like manner with both ends of the RNA exposed. Future work will concentrate on mapping the binding of tRNA onto ThiI and optimization of the trans-modification system.

REFERENCES

1. Bjork, G. R. (1995) in tRNA: Structure Biosynthesis and Function (Soll, D., and RajBhandary, U. L., eds) pp. 165–205, ASM Press, Washington, D. C.
2. Limbach, P. A., Crain, P. F., and McCloskey, J. A. (1994) Biochemistry 33, 5302–5311.
3. Urbonavicius, J., Qian, Q., Durand, J. M., Hagervall, T. G., and Bjork, G. R. (1995) in tRNA: Structure Biosynthesis and Function (Soll, D., and RajBhandary, U. L., eds) pp. 165–205, ASM Press, Washington, D. C.
4. Tremblay, T. L., and Lapointe, J. (1986) Biochem. Cell Biol. 64, 313–315.
5. Zuck, M. (1989) Science 244, 48–52.
6. Hrada, F., Sato, S., and Nishimura, S. (1972) FEBS Lett. 19, 352–354.
7. Qin, P. Z., Hideg, K., Feigon, J., and Hubbell, W. L. (2003) Biochemistry 42, 6772–6783.
8. Lipsett, M. N. (1972) J. Biol. Chem. 247, 1458–1461.
9. Mueller, E. G., Buck, C. J., Palenchar, P. M., Barnhart, L. H., and Paulson, J. L. (1998) Nucleic Acids Res. 26, 2060–2061.
10. Webb, E., Class, K., and Downs, D. M. (1997) J. Bacteriol. 179, 4399–4402.
11. Kambamathi, R., and Lauhon, C. T. (1999) Biochemistry 38, 16561–16568.
12. Lauhon, C. T., and Kambamathi, R. (2000) J. Biol. Chem. 275, 20996–21010.
13. Hara, H., Horiuchi, T., Saneyoshi, M., and Nishimura, S. (1970) Biochem. Biophys. Res. Commun. 38, 905–911.
14. Lin, C.-K., and Altman, S. (1995) J. Mol. Biol. 255, 87–95.
15. Szweykowska-Kulinska, Z., and Beier, H. (1992) FEBS Lett. 305, 119–123.
16. Peterson, E. T., and Uhlenbeck, O. C. (1992) Nature 358, 476–478.
17. Webb, E., Class, K., and Downs, D. M. (1997) J. Bacteriol. 179, 4399–4402.
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