Mapping the Central Fold of $tRNA_2^{fMet}$ in the P site of the *Escherichia coli* Ribosome†

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ABSTRACT: 4-Thiouridine (s^4U), a photoreactive analog of uridine, was randomly incorporated into $tRNA_2^{fMet}$ precursor molecules by transcription with T7 RNA polymerase. The s^4U-containing transcripts were trimmed at their 5′-ends with RNase P RNA to yield mature $tRNA_2^{fMet}$. The photoreactive $tRNA_2^{fMet}$ derivatives were aminoacylated and bound to the P site of 70S ribosomes from *Escherichia coli* in the presence of a poly(A,G,U) template. Irradiation of the complexes at 300 nm resulted in the covalent cross-linking of $tRNA_2^{fMet}$ to ribosomal proteins and rRNAs within both the 50S and 30S subunits. The labeled proteins were identified as L1, L27, and S19. 50S-subunit proteins L1 and L27 were attached to nucleotide U17 or U17.1 within the D loop of $tRNA_2^{fMet}$, whereas 30S-subunit protein S19 was cross-linked to nucleotide U47 in the variable loop. Both of these sites occur in or near the central fold of the tRNA. These results permit us to map the D loop of P site-bound tRNA to the region between the central protuberance and the L1 ridge on the 50S ribosomal subunit, while the variable loop can be placed above the cleft on the head of the 30S subunit.

It is now widely accepted that there are three binding sites for tRNA on the *Escherichia coli* ribosome: the A site, which receives aminoacyl-tRNA in the form of a ternary complex with elongation factor Tu and GTP; the P site, which binds peptidyl-tRNA during elongation and fMet-$tRNA^{fMet}$ during initiation; and the E site, through which deacylated tRNA passes before its release (Rheinberger *et al.*, 1981; Grajevskaja *et al.*, 1982; Kirillov *et al.*, 1983; Lill *et al.*, 1984). The locations of these sites have been studied intensively by affinity labeling techniques [for reviews see Cooperman (1980, 1987), Ofengand *et al.* (1986), and Wower *et al.* (1993)], and several models for the arrangement of tRNA molecules on the *E. coli* ribosome have been proposed [see Wower and Zimmermann (1991) and Lim *et al.* (1992)]. From the cross-linking studies, it has been determined that the aminoacyl acceptor end of the P site-bound tRNA contacts the interface side of the 50S ribosomal subunit in the area bounded by proteins L2, L16, and L27, between the central protuberance and the L1 ridge (Wower *et al.*, 1989). This placement of the CCA terminus corresponds well with information about the location of the peptidyl transferase center derived from other studies [see Wower *et al.* (1993)]. The essential interactions of P site-bound tRNA with the 30S ribosomal subunit are confined to the anticodon arm (Kirillov *et al.*, 1983; Rose *et al.*, 1983; Moazed & Noller, 1986). Cross-linking of the 5′-anticodon base of P site-bound *E. coli* $tRNA_1^{Val}$ to nucleotide C1400 of the 16S rRNA was crucial for determining the site at which the anticodon contacts the 30S particle (Prince *et al.*, 1982). Immune electron microscopy studies of covalent $tRNA_1^{Val}$-ribosome complexes have shown that the P site portion of the decoding domain lies in the cleft between the head and the platform of the 30S subunit (Gornicki *et al.*, 1984). One important constituent of the P site on the 30S ribosomal subunit is protein S7, which has been cross-linked to nucleotide 37 in the anticodon loop of yeast $tRNA^{Phe}$ (Sylvers *et al.*, 1992). Cross-linking of the anticodon arm of yeast $tRNA^{Phe}$ containing 2- or 8-azidoadenosine at position 43 to proteins S13 and S19 provided direct evidence that the anticodon stem of P site-bound tRNA is positioned on the head of the 30S subunit, just above the cleft (Wower *et al.*, 1990).

While the positions of the acceptor end and the anticodon arm of P site-bound tRNA on the ribosome are now reasonably well-defined, the location of other portions of the tRNA, especially those in the vicinity of the "elbow" or the "central fold" formed by the D and T loops, is still poorly characterized. The available cross-linking data suggest that the elbow of the P site tRNA is located between the areas bounded by proteins L1, L5, L27, and L33 on the 50S ribosomal subunit (Podkowinski & Gornicki, 1989, 1991) and by proteins S5, S7, and S9 on the 30S ribosomal subunit (Abdurashidova *et al.*, 1990; Podkowinski & Gornicki, 1991). However, information about the D and T loops comes either from direct photolysis of tRNA–ribosome complexes with UV light of 254 nm or from cross-linking of tRNAs derivatized with long, photochemically reactive probes. As both of these approaches have certain shortcomings (Wower & Zimmermann, 1991), we decided to reinvestigate the topography of the P site-bound tRNA on the ribosome using tRNA derivatives containing photoreactive nucleotides incorporated throughout the polynucleotide chain and, specifically, within the elbow or central fold. Unlike our previous studies, in which $2N_3A$ and $8N_3A$[1] were inserted into yeast or *E. coli* $tRNA^{Phe}$ by a "cut-and-paste" technique (Wower *et al.*, 1988, 1989, 1993; Sylvers *et al.*, 1992), the present work makes use of *in vitro* tRNA transcripts in which uridines were randomly substituted by the photochemically active uridine analog, 4-thiouridine. The

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[1] Abbreviations: $2N_3A$, 2-azidoadenosine; $8N_3A$, 8-azidoadenosine; s^4U, 4-thiouridine; [s^4U]$tRNA_2^{fMet}$, $tRNA_2^{fMet}$ in which U residues are randomly substituted with s^4U; pre-[s^4U]$tRNA_2^{fMet}$, precursor [s^4U]-$tRNA_2^{fMet}$; AcMet, *N*-acetylmethionyl; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; PMSF, phenylmethane sulfonyl fluoride; BSA, bovine serum albumin.

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transcripts were derived from the gene for a mutant form of *E. coli* $tRNA_2^{fMet}$, in which the C residue at position 17 of the D loop was replaced by U. Photolysis of P site complexes containing s^4U-substituted $tRNA_2^{fMet}$ resulted in the formation of cross-links between U17 or U17.1 and proteins L1 and L27 of the 50S ribosomal subunit and between U47 and protein S19 of the 30S ribosomal subunit. As photoactivated s^4U is expected to form very short cross-links, the labeled proteins are presumed to be in close contact with the central fold of P site-bound tRNA.

EXPERIMENTAL PROCEDURES

Materials. E. coli $tRNA^{fMet}$ (specific amino acid acceptance, 1300 pmol/A_{260}), T4 polynucleotide kinase, calf intestine alkaline phosphatase, puromycin, and mussel glycogen were purchased from Boehringer-Mannheim. Poly(A,G,U) was from Miles Laboratories. Ribonuclease T1 was obtained from Worthington. Restriction enzymes *Bst*NI and *Dra*I were purchased from New England Biolabs. [^{3}H]-Methionine (200 mCi/mmol), [α-^{32}P]CTP and [α-^{32}P]GTP (3000 Ci/mmol), and [γ-^{32}P]ATP (6000 Ci/mmol) were from New England Nuclear. The plasmid kit was from Qiagen Inc. Plasmid pRC3, used for the transcription of precursor $tRNA_2^{fMet}$, was kindly provided by L. H. Schulman (Albert Einstein College of Medicine). Plasmid pDW25, which encodes RNase P RNA from *Bacillus subtilis*, was the gift of N. R. Pace (Indiana University). T7 RNA polymerase was donated by C. T. Martin (University of Massachusetts at Amherst). Published procedures were used for the preparation of s^4UTP (Stade *et al.*, 1989), crude *E. coli* aminoacyl-tRNA synthetases (Wakao *et al.*, 1989), and tight-couple 70S ribosomes from *E. coli* K12 (Robertson & Wintermeyer, 1981). Oligodeoxyribonucleotides were synthesized by the DNA Synthesis Facility at the University of Massachusetts (Amherst, MA). All other chemicals were of reagent grade.

Transcription of the $tRNA_2^{fMet}$ Precursor and the RNA Component of RNase P. Plasmid pRC3, which contains the complete precursor $tRNA_2^{fMet}$ sequence flanked by a bacteriophage T7 promoter and a *Bst*NI restriction site, was prepared using Qiagen columns according to the manufacturer's instructions and linearized with *Bst*NI. Transcription of pre-$tRNA_2^{fMet}$ was typically carried out in 240-μL reaction mixtures containing 0.05 μg/μL *Bst*NI-digested plasmid, 1 mM of each of the usual NTPs, 1 μCi of either [α-^{32}P]CTP or [α-^{32}P]GTP, 4 mM s^4UTP, and 2 μM T7 RNA polymerase in 40 mM Tris-HCl (pH 8.1), 20 mM $MgCl_2$, 20 mM DTT, 50 μg/mL BSA, and 1 mM spermidine. When the transcripts were to be used for the analysis of cross-linked ribosomal components, the amount of [α-^{32}P]CTP or [α-^{32}P]GTP was increased to 0.5 mCi and the concentration of nonradioactive CTP or GTP was lowered to 0.2 mM. Reaction mixtures were incubated for 1 h at 37 °C, after which they were extracted with phenol/$CHCl_3$/isoamyl alcohol (25:24:1 by volume) and then with $CHCl_3$/isoamyl alcohol (24:1 by volume). The catalytic RNA component of *B. subtilis* RNase P was transcribed from *Dra*I-cleaved plasmid pDW25. The transcription reaction was carried out in 320 μL under conditions identical to those described for $tRNA_2^{fMet}$, except that the $MgCl_2$ concentration was decreased to 6 mM and both s^4UTP and the radioactive nucleotide were omitted.

Processing of the $tRNA_2^{fMet}$ Precursor with RNase P RNA. Immediately following extraction with $CHCl_3$/isoamyl alcohol, the aqueous phases containing transcripts of $tRNA_2^{fMet}$ precursor and RNase P RNA were pooled and precipitated with ethanol. The transcripts were resuspended in 90 μL of buffer containing 50 mM Tris-HCl (pH 7.5), 250 mM $MgCl_2$, and 2 M NH_4Cl and incubated for 6 h at 37 °C. The reaction mixture was then diluted 6-fold with double-distilled water and precipitated with ethanol. The products were purified by electrophoresis on a denaturing 10% polyacrylamide gel in 100 mM Tris–borate (pH 8.3), and 8 M urea. The mature $tRNA_2^{fMet}$ transcripts were located by autoradiography, eluted from the gel in 0.5 M NH_4OAc, 1 mM EDTA, and 0.5% (w/v) SDS for 12 h at room temperature, extracted with phenol, and precipitated with ethanol.

Aminoacylation of $tRNA_2^{fMet}$. Both the $tRNA_2^{fMet}$ transcript and *E. coli* $tRNA^{fMet}$ were charged with [^{3}H]methionine essentially as described by Wakao *et al.* (1989). Prior to aminoacylation, the $tRNA_2^{fMet}$ transcripts were dialyzed against buffer containing 25 mM Hepes–KOH (pH 7.5), 5 mM magnesium acetate, and 16 mM DTT for 1 h at 4 °C. VS-type Millipore filters were used as dialysis membranes. The extent of aminoacylation was assessed by the measurement of TCA-precipitable ^{3}H radioactivity. Acetylation of aminoacylated tRNAs was carried out according to Haenni and Chapeville (1966).

Formation and Irradiation of P Site Complexes. Noncovalent binding of aminoacylated tRNA to 70S ribosomes was carried out in 10 mM Tris-HCl (pH 7.2), 10 mM $MgCl_2$, 100 mM NH_4Cl, and 1 mM DTT. The molar tRNA/ribosome input ratio was 1:4, and the final concentrations of ribosomes and poly(A,G,U) were 40 A_{260} units/mL and 250 μg/mL, respectively. All reactions were incubated for 15 min at 37 °C. The amount of tRNA noncovalently bound to the ribosomes was estimated by the nitrocellulose filter assay of Schwartz and Ofengand (1974). Binding of aminoacyl-tRNA to the P site was verified by monitoring its ability to react with puromycin as described by Wower *et al.* (1988). Noncovalent tRNA–ribosome complexes were diluted 4-fold with cold binding buffer and irradiated for 20 min at 4 °C in a Rayonet photochemical reactor (Model RPR-100) equipped with six RPR-3000-Å lamps. The percentage of cross-linking was estimated from the distribution of ^{32}P-labeled material on sucrose gradients and on SDS–urea gels (see text).

Analysis of tRNA–Protein Complexes. Irradiated ribosomes were dissociated into subunits by centrifugation through 10–30% sucrose gradients in 10 mM Tris-HCl (pH 7.6), 50 mM KCl, 0.25 mM $MgCl_2$, and 0.05% (v/v) 2-mercaptoethanol in a Beckman VTi50 rotor at 40 000 rpm for 2 h. Proteins cross-linked to the $tRNA_2^{fMet}$ transcripts were analyzed by one-dimensional polyacrylamide gel electrophoresis (Laemmli & Favre, 1973), two-dimensional polyacrylamide gel electrophoresis (Metz & Bogorad, 1974), reverse-phase HPLC (Kerlavage *et al.*, 1983; Kerlavage & Cooperman, 1986), and the "agarose" immunological method (Gulle *et al.*, 1988). Prior to electrophoretic analysis, tRNA–protein complexes were digested with RNase T1 (4000 units/mL) in 10 mM Tris-HCl (pH 8.1), 0.5 mM EDTA, 8 M urea, and 0.06 mg/mL PMSF at 4 °C overnight. In the morning, the concentrations of RNase T1 and PMSF were brought to 8000 units/mL and 0.12 mg/mL, respectively, and incubation was continued for 2 h at 37 °C. For HPLC, RNase T1-treated tRNA–subunit complexes were mixed with unlabeled subunits, extracted with acetic acid, and precipitated with acetone as described (Kerlavage & Cooperman, 1986). Separation of total protein complements from each subunit was achieved by reverse-phase HPLC on a Synchropak RP-P C_{18} column using a convex 15–45% acetonitrile gradient containing 0.1%

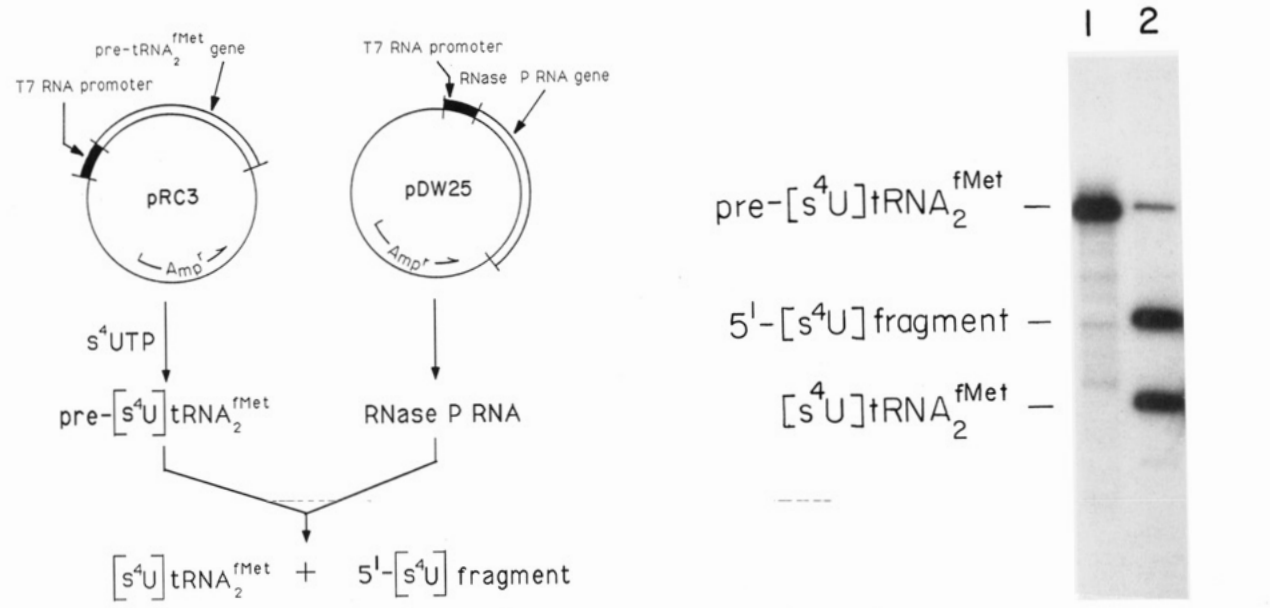

FIGURE 1: Preparation of *E. coli* tRNA$_2^{fMet}$ randomly substituted with s^{4}U. (left) Scheme depicting the *in vitro* synthesis of pre-[s^{4}U]-tRNA$_2^{fMet}$ and RNase P RNA and the subsequent processing of the pre-[s^{4}U]tRNA$_2^{fMet}$ transcript. (right) Analysis of the pre-[s^{4}U]-tRNA$_2^{fMet}$ by denaturing PAGE before (lane 1) and after (lane 2) treatment with RNase P RNA.

trifluoroacetic acid (Kerlavage *et al.*, 1983). Fractions were collected at 1-min intervals, and the radioactivity of each was determined by scintillation counting. Immunological tests were carried out on individual tRNA–protein complexes that were isolated by electrophoresis of [s^{4}U]tRNA-labeled ribosomal subunits through a 4% polyacrylamide gel containing SDS and urea, as described by Brimacombe *et al.* (1988).

Identification of Cross-Linked Residues in tRNA. The residues of s^{4}U-labeled tRNA$_2^{fMet}$ transcripts that were cross-linked to ribosomal proteins were identified either by the "partial hydrolysis" technique of Abdurashidova *et al.* (1990) or by the oligodeoxynucleotide protection method described below. Both analyses were carried out using tRNA that had been dephosphorylated with calf intestine alkaline phosphatase and labeled with ^{32}P at the 5'-end in the presence of [γ-^{32}P]-ATP and T4 polynucleotide kinase prior to cross-linking (D'Alessio, 1982).

In the partial hydrolysis method, [5'-^{32}P]tRNA–protein complexes were isolated by PAGE according to Brimacombe *et al.* (1988) and incubated in 50 mM Na_2CO_3 (pH 9.4) for 3 min at 90 °C to generate a tRNA ladder. This mixture was then extracted with phenol to remove oligonucleotides covalently attached to the protein. Protein-free oligonucleotides, which partitioned into the aqueous phase, were precipitated with ethanol in the presence of 0.1 μg/mL glycogen and separated by electrophoresis on a denaturing 20% polyacrylamide gel along with the sequence ladders derived from 5'-^{32}P-labeled *E. coli* tRNA^{fMet} and tRNA$_2^{fMet}$ transcripts. RNA sequencing reactions were performed by the enzymatic method of Donis-Keller (1979).

For oligodeoxynucleotide protection analysis, individual [5'-^{32}P]tRNA–protein complexes were incubated with 0.5 mg/mL proteinase K in 100 mM Tris-HCl (pH 7.7), 25 mM EDTA, 150 mM NaCl, and 1% (w/v) SDS for 30 min at 37 °C. Proteinase K, as well as the peptides released from the cross-linked proteins, was removed from the digestion mixture by phenol extraction. Proteinase K-treated complexes were precipitated with ethanol and annealed to oligodeoxyribonucleotides complementary to residues 1–12 or 1–18 of the tRNA at final concentrations of 60 or 40 μM, respectively, by incubation in a buffer containing 40 mM Tris-HCl (pH 7.7), 5 mM NaCl, 1 mM DTT, 30 μg/mL BSA, 4% (v/v) glycerol, and 50 μg/mL *E. coli* tRNA^{fMet} for 10 min at 65 °C. After the reaction mixture was cooled slowly to 4 °C, RNase T1 (6000–9000 units/mL) was added and the mixture was incubated at 4 °C overnight. RNase T1 was inactivated by phenol extraction, and the digestion products were separated by electrophoresis on a denaturing 12% polyacrylamide gel. In control experiments, 5'-^{32}P-labeled tRNA$_2^{fMet}$ transcripts were substituted for covalent tRNA–protein complexes.

RESULTS

Preparation and Aminoacylation of [s^{4}U]tRNA$_2^{fMet}$. Photoreactive tRNA$_2^{fMet}$ derivatives in which uridine residues were randomly substituted with s^{4}U were prepared by a two-step procedure (Figure 1). Precursor [s^{4}U]tRNA$_2^{fMet}$ was first synthesized by *in vitro* transcription of *Bst*NI-cleaved plasmid pRC3 with T7 RNA polymerase. The pre-[s^{4}U]-tRNA$_2^{fMet}$ molecules were then processed by *B. subtilis* RNase P RNA transcribed *in vitro* from *Dra*I-digested plasmid pDW25. From 70 to 90% of the precursors were converted to mature [s^{4}U]tRNA$_2^{fMet}$ in the latter reaction. Enzymatic sequencing of mature, 5'-^{32}P-labeled [s^{4}U]tRNA$_2^{fMet}$ showed that the primary structure of the transcripts corresponded to what was expected (see Figure 2).

The specific amino acid acceptance of the [s^{4}U]tRNA$_2^{fMet}$ derivative was found to be approximately 1000 pmol of methionine/A_{260} unit of tRNA. This figure compares favorably with the values of 1000 and 1400 pmol/A_{260} unit determined for unsubstituted tRNA$_2^{fMet}$ transcripts and native *E. coli* tRNA^{fMet}, respectively. These results show that the two transcripts are equally competent for aminoacylation and indicate that limited s^{4}U substitutions in tRNA$_2^{fMet}$ transcripts do not significantly affect recognition of the tRNA by methionyl-tRNA synthetase, despite differences in the hydrogen-bonding properties of U and s^{4}U (Katritzky & Waring, 1962; Kyogoku *et al.*, 1967; Iwahashi & Kyogoku, 1977). Met-[s^{4}U]tRNA$_2^{fMet}$ was acetylated by the method of Haenni and Chapeville (1966) prior to the cross-linking experiments.

Cross-Linking of AcMet-[s^{4}U]tRNA$_2^{fMet}$ to E. coli Ribosomes. AcMet-[s^{4}U]tRNA$_2^{fMet}$ was bound to the P site of

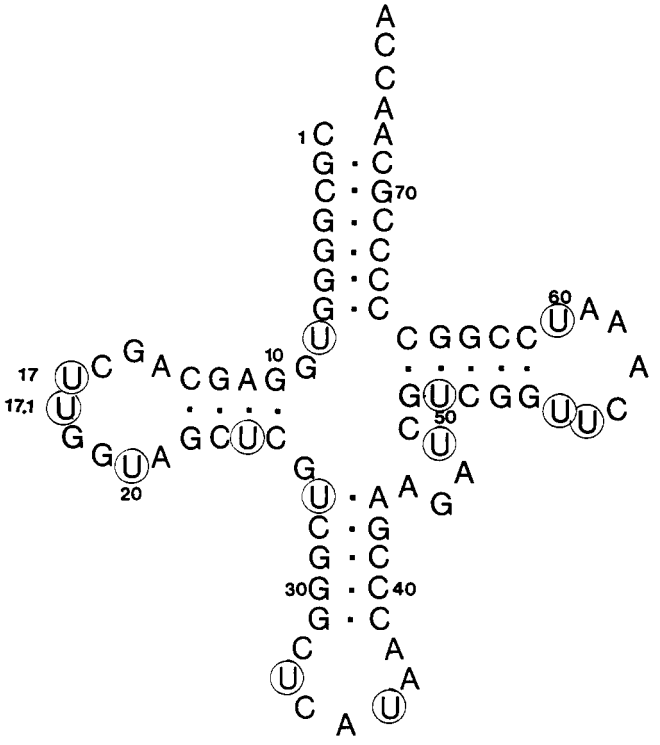

FIGURE 2: Cloverleaf structure of the *E. coli* $tRNA_2^{fMet}$ transcript. Sites of possible s^4U incorporation are encircled.

poly(A,G,U)-programmed 70S tight-couple ribosomes at a molar tRNA/ribosome input ratio of 1:4. Under these conditions, 70–80% of the tRNA associated with the 70S particles. Similar results were obtained for non-aminoacylated $tRNA_2^{fMet}$. In the absence of poly(A,G,U), the extent of binding of AcMet-[s^4U]$tRNA_2^{fMet}$ to 70S ribosomes was about 40%. On average, 90% of the noncovalently bound AcMet-[s^4U]$tRNA_2^{fMet}$ was sensitive to reaction with puromycin, demonstrating that the tRNA derivatives were located in the P site.

Noncovalent complexes containing AcMet-[s^4U]$tRNA_2^{fMet}$, poly(A,G,U), and ribosomes were irradiated with 300-nm lamps to induce cross-linking. The 30S and 50S subunits were then dissociated and fractionated by centrifugation on sucrose gradients at 0.25 mM Mg^{2+} to determine the distribution of the cross-linked tRNA. Of the AcMet-[s^4U]-$tRNA_2^{fMet}$ initially bound to the 70S ribosomes, 4% became covalently attached to each of the two ribosomal subunits (Figure 3a; Table I). Control experiments demonstrated that cross-linking was dependent upon both UV irradiation and the presence of s^4U in the tRNA (Figure 3b,c). The fraction of noncovalently bound AcMet-[s^4U]$tRNA_2^{fMet}$ that became cross-linked to the ribosomes was essentially the same when poly(A,G,U) was omitted or when non-aminoacylated [s^4U]$tRNA_2^{fMet}$ was used (not shown). As illustrated in Figure 4, all of the covalently bound AcMet-[s^4U]$tRNA_2^{fMet}$ was able to react with puromycin, confirming that the cross-linked tRNA remained at the ribosomal P site.

Table I: Cross-Linking of AcMet-[s^4U]$tRNA_2^{fMet}$ to Ribosomal Components

component	% cross-linking	% distribution
30S subunit	4	
16S rRNA		55
S19		45
50S subunit	4	
23S rRNA		37
L1		11
L27		42

To determine the relative distribution of the photoaffinity label between proteins and ribosomal RNAs, the 50S- and 30S-subunit fractions were analyzed on a 4% polyacrylamide gel containing both urea and SDS, which effectively separates AcMet-[s^4U]$tRNA_2^{fMet}$–rRNA and AcMet-[s^4U]-$tRNA_2^{fMet}$–protein complexes from one another, as well as from free, photolyzed AcMet-[s^4U]$tRNA_2^{fMet}$ (Figure 5). In the 50S fraction, 37% of the cross-linked AcMet-[s^4U]-$tRNA_2^{fMet}$ became attached to 23S rRNA (Table I). The remainder of the AcMet-[s^4U]$tRNA_2^{fMet}$ cross-linked to the 50S subunit was distributed between two protein complexes, marked I and II on the autoradiogram presented in Figure 5. No cross-links were detected between AcMet-[s^4U]-$tRNA_2^{fMet}$ and 5S rRNA. A similar analysis of the 30S fraction indicated that 55% of the covalently incorporated AcMet-[s^4U]$tRNA_2^{fMet}$ was associated with 16S rRNA, while 45% was bound to ribosomal proteins (complex III, Figure 5; Table I). The results were essentially identical when non-aminoacylated [s^4U]$tRNA_2^{fMet}$ was used in place of AcMet-[s^4U]$tRNA_2^{fMet}$.

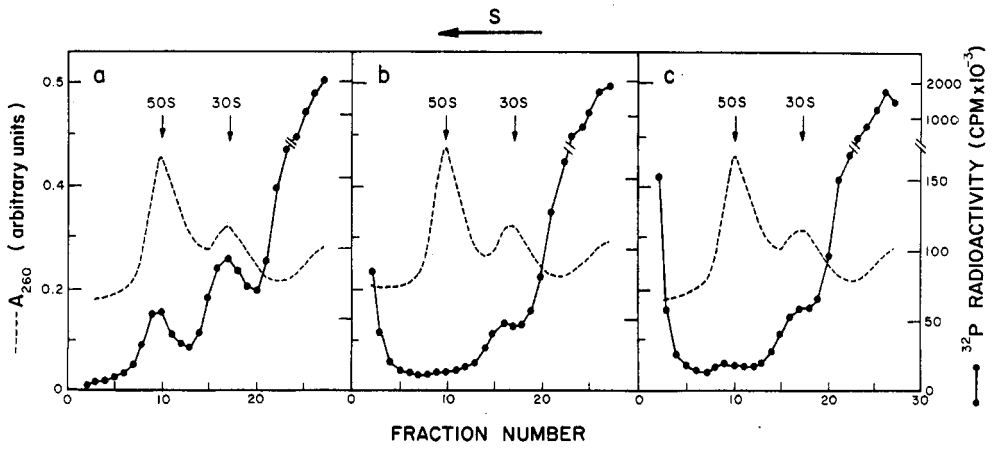

FIGURE 3: 3. Cross-linking of AcMet-[s^4U]$tRNA_2^{fMet}$ to 50S and 30S subunits and dependence of the cross-linking reaction on UV irradiation and the presence of s^4U in $tRNA_2^{fMet}$: (a) UV-irradiated AcMet-[^{32}P][s^4U]$tRNA_2^{fMet}$–poly(A,G,U)–70S ribosome complexes; (b) non-irradiated AcMet-[^{32}P][s^4U]$tRNA_2^{fMet}$–poly(A,G,U)–70S ribosome complexes; and (c) UV-irradiated [^{32}P]$tRNA_2^{fMet}$–poly(A,G,U)–70S ribosome complexes. The complexes were prepared as described in Experimental Procedures and centrifuged through 10–30% sucrose gradients containing 0.25 mM Mg^{2+} to separate ribosomal subunits.

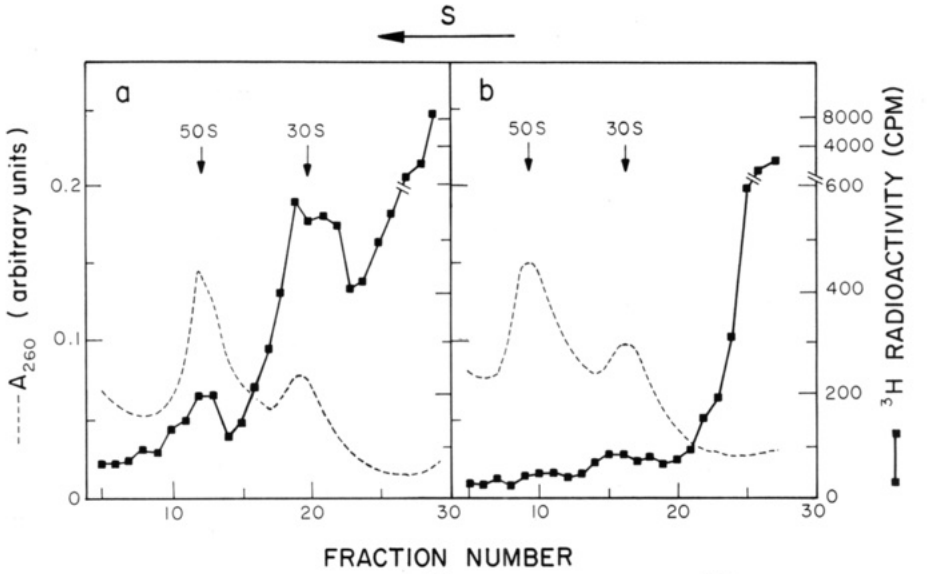

FIGURE 4: Puromycin reactivity of cross-linked AcMet-[s^{4}U]tRNA$_2^{fMet}$. Ac[^{3}H]Met-[s^{4}U]tRNA$_2^{fMet}$–poly(A,G,U)–70S ribosome complexes were irradiated at 300 nm and separated into subunits by sucrose gradient centrifugation (a) before and (b) after treatment with puromycin, as described in the legend to Figure 3.

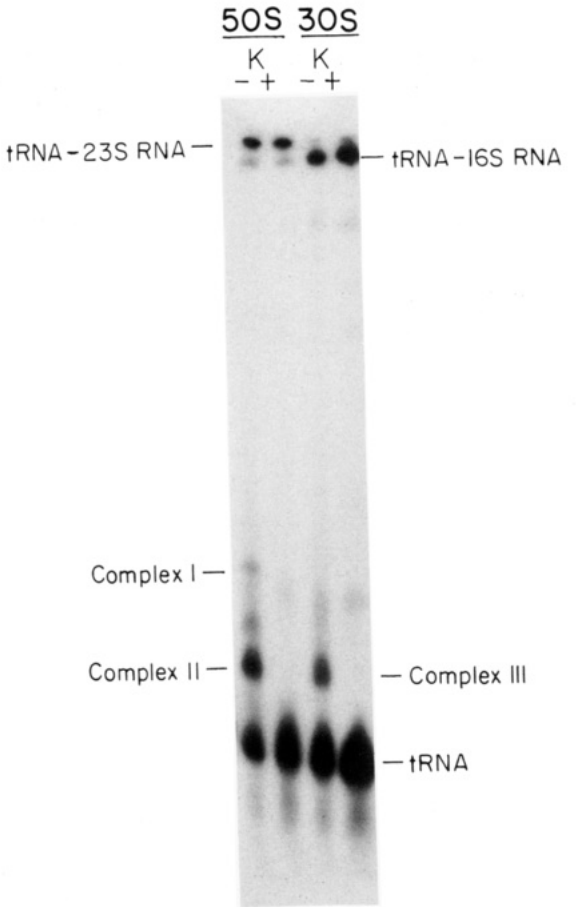

FIGURE 5: Distribution of cross-linked AcMet-[s^{4}U]tRNA$_2^{fMet}$ among 16S rRNA, 23S rRNA, and ribosomal proteins. Covalent complexes of AcMet-[s^{4}U]tRNA$_2^{fMet}$ with 50S and 30S subunits were isolated as in Figure 3a and analyzed by PAGE in the presence of SDS and urea according to Brimacombe *et al.* (1988). Complexes I, II, and III were identified as tRNA–protein complexes by their susceptibility to proteinase K: untreated samples, –K; samples treated with proteinase K, +K.

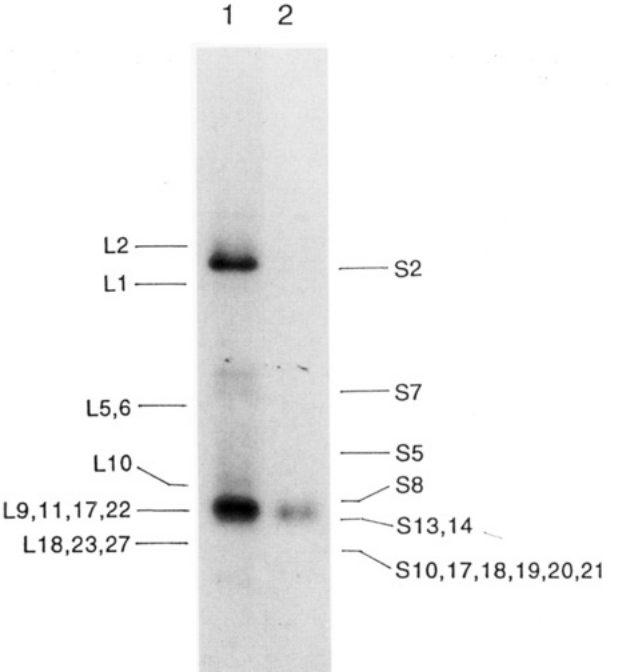

FIGURE 6: Electrophoretic analysis of proteins cross-linked to AcMet-[s^{4}U]tRNA$_2^{fMet}$. Covalent complexes between AcMet-[^{32}P][s^{4}U]-tRNA$_2^{fMet}$ and 50S or 30S subunits were isolated as in Figure 3a, digested with RNase T1, and subjected to SDS–PAGE according to Laemmli and Favre (1973). The gel was stained with Coomassie brilliant blue and subjected to autoradiography: lane 1, 50S-subunit proteins; lane 2, 30S-subunit proteins. The positions of a number of unmodified 50S- and 30S-subunit proteins are indicated.

Ribosomal Proteins Labeled by AcMet-[s^{4}U]tRNA$_2^{fMet}$. When AcMet-[s^{4}U]tRNA$_2^{fMet}$–50S subunit complexes, isolated as in Figure 3a, were digested exhaustively with RNase T1 and analyzed by one-dimensional SDS–PAGE, two ^{32}P-labeled bands were detected on the autoradiogram (Figure 6, lane 1). Analysis of AcMet-[s^{4}U]tRNA$_2^{fMet}$–30S subunit complexes under the same conditions revealed the presence of a single ^{32}P-labeled band (Figure 6, lane 2). When identical digests were resolved by two-dimensional PAGE, it was confirmed that AcMet-[s^{4}U]tRNA$_2^{fMet}$ labeled two proteins in the 50S subunit and a single protein in the 30S subunit (not shown). The pattern of protein labeling revealed by one- and two-dimensional PAGE is therefore consistent with that of the undigested tRNA–protein complexes illustrated in Figure 5.

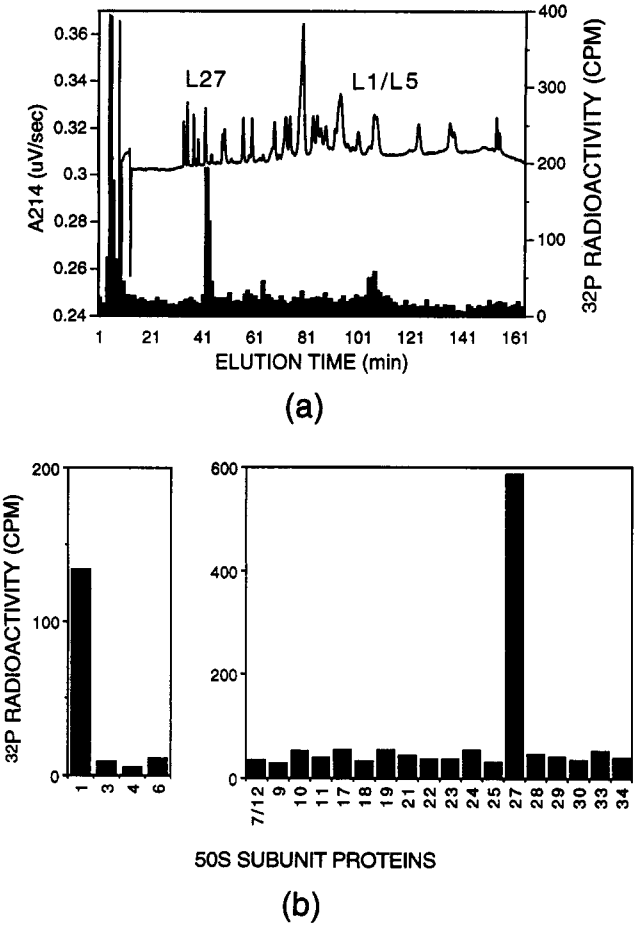

FIGURE 7: Analysis of 50S-subunit proteins cross-linked to AcMet-[s^4U]$tRNA_2^{fMet}$. (a) Covalent tRNA–50S-subunit protein complexes, isolated as in Figure 3a, were treated with RNase T1 and analyzed by reverse-phase HPLC. The continuous solid line depicts the absorbance profile of 50S-subunit proteins at 214 nm. The histogram shows the positions of ^{32}P-labeled proteins. (b) Covalent tRNA–50S-subunit protein complexes I and II, isolated as in Figure 5, were screened for reactivity to antibodies against 50S-subunit proteins (Gulle *et al.*, 1988). The histograms indicate the proteins that were labeled with ^{32}P.

Since one of the two 50S-subunit proteins labeled by AcMet-[s^4U]$tRNA_2^{fMet}$ migrated slightly more slowly than protein L1 on the one-dimensional polyacrylamide gel (Figure 6, lane 1) and was located "north-west" of protein L1 on the two-dimensional polacrylamide gel, we tentatively concluded that this protein was L1. Identification of the second protein, however, was impeded by the fact that it migrated to a position characteristic of many of the smaller 50S-subunit proteins in both gel systems. Analysis of RNase T1-digested tRNA–50S-subunit protein complexes by reverse-phase HPLC revealed that one of the two ^{32}P-labeled proteins coeluted with proteins L1 and L5, while the second one exhibited the same retention time as protein L27 (Figure 7a). Immunological assays confirmed that the 50S-subunit proteins labeled by P site-bound AcMet-[s^4U]$tRNA_2^{fMet}$ were L1 and L27 (Figure 7b).

When AcMet-[s^4U]$tRNA_2^{fMet}$–30S subunit complexes, isolated as in Figure 3a and treated with RNase T1, were analyzed by reverse-phase HPLC, only one ^{32}P-labeled 30S-subunit protein was observed (Figure 8). The retention time of the labeled protein corresponded to that of protein S19. Because the mobility of the ^{32}P-labeled band on SDS–PAGE agreed well with that expected for a covalent complex of S19 with a RNase T1-derived tetranucleotide from the tRNA, we

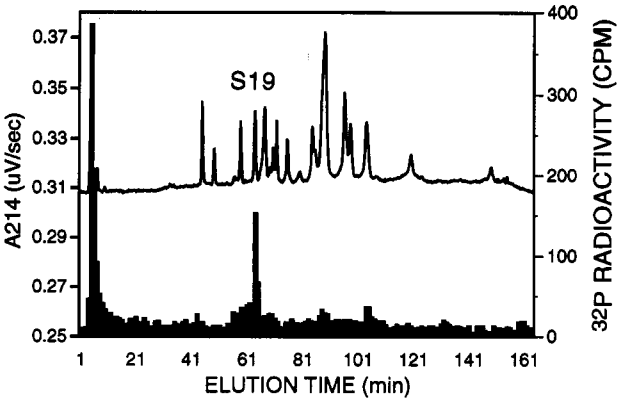

FIGURE 8: Identification of the 30S-subunit proteins cross-linked to AcMet-[s^4U]$tRNA_2^{fMet}$. Covalent [s^4U]$tRNA_2^{fMet}$–30S subunit complexes, isolated as in Figure 3a, were treated with RNase T1 and analyzed by reverse-phase HPLC. The continuous solid line depicts the absorbance profile of 30S-subunit proteins at 214 nm. The histogram shows the position of the ^{32}P-labeled 30S-subunit protein.

concluded that protein S19 was the sole 30S-subunit protein cross-linked to P site-bound AcMet-[s^4U]$tRNA_2^{fMet}$.

Cross-Linking Sites in [s^4U]$tRNA_2^{fMet}$. To determine which nucleotides of the tRNA formed cross-links with proteins L1, L27, and S19, covalent complexes of these proteins with 5′-^{32}P-labeled [s^4U]$tRNA_2^{fMet}$ were prepared as in Figure 5 and subjected to partial alkaline hydrolysis. When the alkaline digests were extracted with phenol, the free oligonucleotides partitioned to the aqueous phase while protein–oligonucleotide complexes were sequestered in the phenol phase. The free oligonucleotides were subsequently fractionated by PAGE alongside partial hydrolyzates of 5′-^{32}P-labeled *E. coli* $tRNA^{fMet}$ and [s^4U]$tRNA_2^{fMet}$ previously irradiated at 300 nm. The oligonucleotide ladder derived from the S19–tRNA complex contained no components over 46 residues in length (Figure 9). Larger tRNA fragments were evidently cross-linked to protein and had been removed from the aqueous phase during phenol extraction. We therefore concluded that s^4U at position 47 of [s^4U]$tRNA_2^{fMet}$ was cross-linked to protein S19.

A similar analysis of complexes containing proteins L1 and L27 was less definitive. In these instances, the ladder was diminished in intensity after nucleotide 16 or 17, but nonetheless was still detectable. Although the results suggested that nucleotide 17 or 17.1 was the site of cross-linking for both proteins L1 and L27, this inference was confirmed by an oligonucleotide protection assay. In this assay, the RNA moiety of the protein–tRNA complex is first annealed to a complementary oligodeoxyribonucleotide. The resulting heteroduplex is then digested with an appropriate RNase, and the RNA fragment protected by the oligonucleotide is characterized. The site at which the protein is attached can be identified by virtue of the fact that RNA fragments cross-linked to protein, or peptides derived from it, exhibit a lower mobility on polyacrylamide gels than the corresponding fragments isolated from free tRNA.

To identify the site of cross-linking of proteins L1 and L27 in [s^4U]$tRNA_2^{fMet}$, two oligodeoxyribonucleotides were used: a 19-mer complementary to nucleotides 1–18 and a 12-mer complementary to nucleotides 1–12, of the tRNA molecule. RNase T1 digestion of heteroduplexes between either the 19-mer or the 12-mer and free [5′-^{32}P]$tRNA_2^{fMet}$ produced 5′-^{32}P-labeled tRNA fragments 20 and 12 nucleotides long, respectively (Figure 10, lanes 2 and 3). Since the protein–tRNA complexes hybridized poorly with the complementary

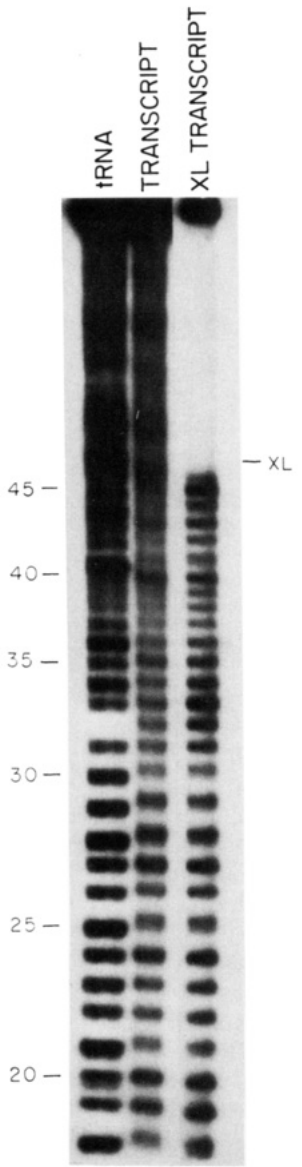

FIGURE 9: Identification of the site to which protein S19 is cross-linked in [s^4U]$tRNA_2^{fMet}$. 5′-^{32}P-labeled [s^4U]$tRNA_2^{fMet}$, both free and covalently bound to S19, was isolated as in Figure 5, subjected to limited alkaline hydrolysis, and extracted with phenol. Oligonucleotides that partitioned into the aqueous phase (XL TRANSCRIPT) were subjected to electrophoresis on a denaturing 20% polyacrylamide gel along with the alkaline ladders derived from 5′-^{32}P-labeled *E. coli* $tRNA^{fMet}$ (tRNA) and [s^4U]$tRNA_2^{fMet}$ transcripts (TRANSCRIPT). XL indicates the position of cross-linked nucleotide. The missing band in the alkaline ladder of *E. coli* $tRNA^{fMet}$ corresponds to the 2′-*O*-methylcytidine residue at position 32.

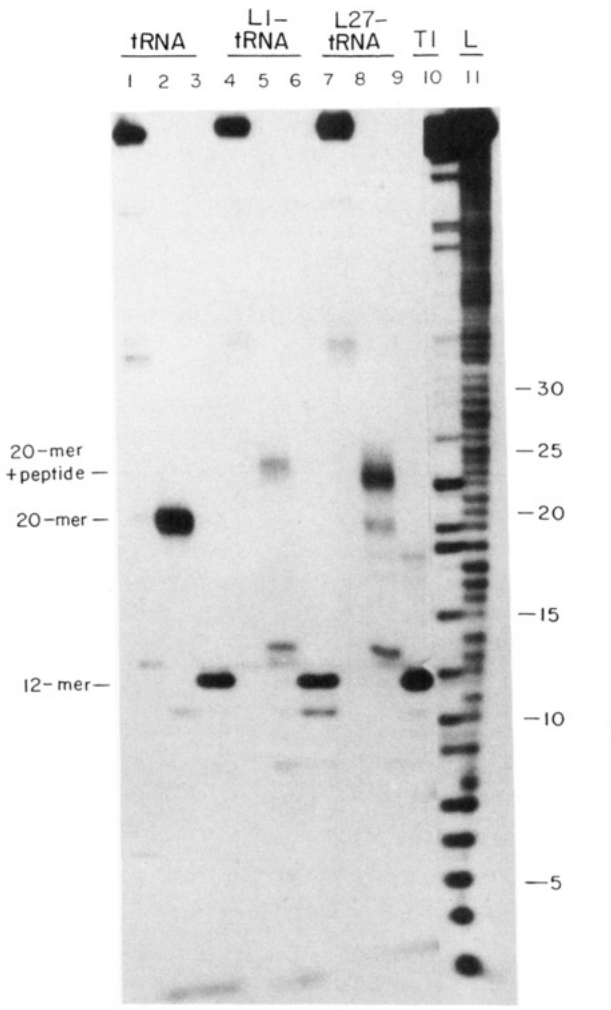

FIGURE 10: Identification of the sites of cross-linking of proteins L1 and L27 in [s^4U]$tRNA_2^{fMet}$. Covalent complexes of 5′-^{32}P-labeled [s^4U]$tRNA_2^{fMet}$ (tRNA) with proteins L1 and L27 were isolated as in Figure 5 and treated with proteinase K to yield L1–tRNA and L27–tRNA. Oligodeoxyribonucleotides complementary to either nucleotides 1–12 (12-mer) or nucleotides 1–18 (19-mer) of *E. coli* $tRNA_2^{fMet}$ were annealed to tRNA, L1–tRNA, and L27–tRNA, and the heteroduplexes were digested with RNase T1. The digestion products were then resolved on a denaturing 12% polyacrylamide gel: lanes 1, 4, and 7, untreated tRNA, L1–tRNA, and L27–tRNA, respectively; lanes 2, 5, and 8, products of RNase T1 digestion of heteroduplexes formed in the presence of the 19-mer; lanes 3, 6, and 9, products of RNase T1 digestion of heteroduplexes formed in the presence of the 12-mer; lane 10, products of partial digestion of 5′-^{32}P-labeled *E. coli* $tRNA^{fMet}$ with RNase T1; lane 11, alkaline ladder derived from 5′-^{32}P-labeled *E. coli* $tRNA^{fMet}$. Bands corresponding to the 12-nucleotide tRNA fragment (12-mer), the 20-nucleotide tRNA fragment (20-mer), and the 20-nucleotide tRNA fragment cross-linked to either protein L1 or L27 (20-mer + peptide) are indicated. The presence of free 20-mer in lane 8 may result from the instability of the L27–tRNA complex under the assay conditions.

oligonucleotides, they were treated with proteinase K prior to annealing. RNase T1 digestion of the heteroduplex between the 19-mer and the proteinase K-treated L1–[s^4U]-$tRNA_2^{fMet}$ complex yielded an RNA fragment that migrated more slowly than the expected 20-mer (Figure 10, lane 5). An RNA fragment of similar mobility was formed upon RNase T1 digestion of a heteroduplex consisting of the 19-mer and the proteinase K-treated L27–[s^4U]$tRNA_2^{fMet}$ complex (Figure 10, lane 8). In contrast, RNase T1 digestion of heteroduplexes containing either proteinase K-treated L1–[s^4U]-$tRNA_2^{fMet}$ or proteinase K-treated L27–[s^4U]$tRNA_2^{fMet}$ and the 12-mer resulted in RNA products (Figure 10, lanes 6 and 9) that migrated with exactly the same mobility as the dodecamer produced in the control reaction (Figure 10, lane 3). We interpret these observations to mean that the 12-mer protected an unmodified segment of the tRNA, whereas the 19-mer protected tRNA fragments covalently attached to peptides derived from L1 and L27. Thus, the sites to which the proteins cross-linked must be located between nucleotides 13 and 19 of the tRNA. As this segment of the $tRNA_2^{fMet}$ transcript can contain s^4U at only two positions (see Figure 2), we conclude that proteins L1 and L27 were cross-linked to nucleotide 17 or 17.1 or both.

DISCUSSION

Over the past few years, we have cross-linked a number of tRNAs containing the photolabile nucleosides $2N_3A$ and $8N_3A$ to the *E. coli* ribosome in an effort to delineate the topography

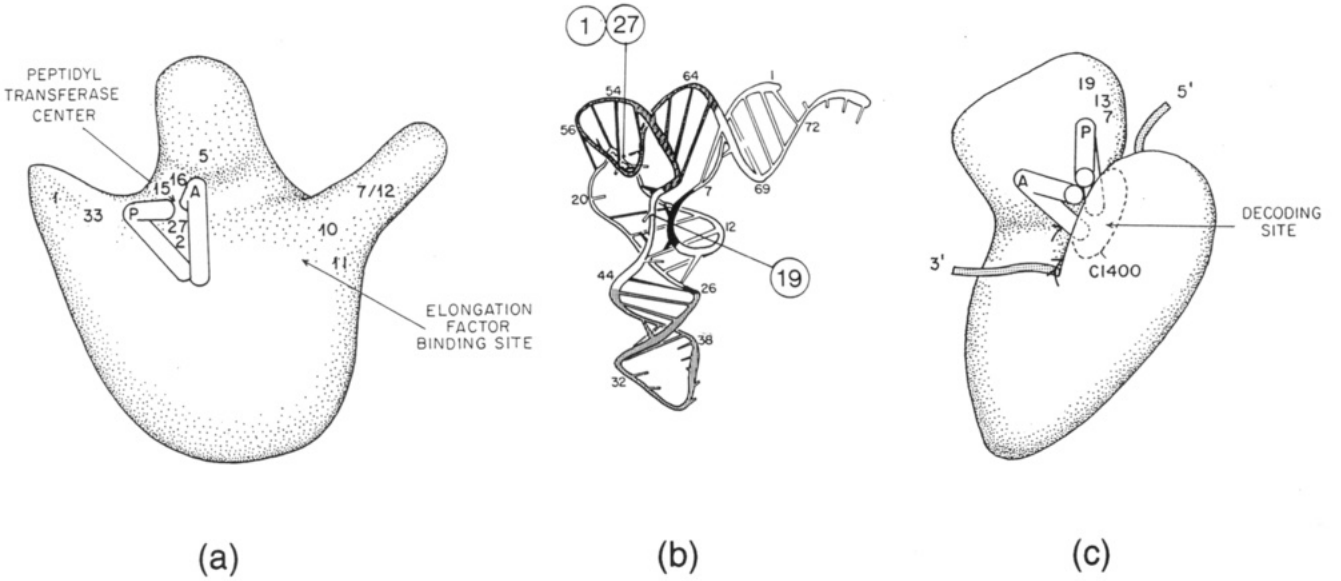

FIGURE 11: Topography of tRNA binding sites on the ribosome. The location of nucleotides cross-linked to proteins L1, L27, and S19 in the three-dimensional structure of yeast $tRNA^{Phe}$ is shown in b. Placement of the P site tRNA on the (a) 50S and (c) 30S subunits of the *E. coli* ribosome is predicated upon cross-links from the following positions in tRNA: $s^4U17/17.1$–L1, $s^4U17/17.1$–L27, s^4U47–S19 (this article); $2N_3A37$–S7 (Sylvers *et al.*, 1992); $2N_3A43$–S13 (Wower *et al.*, 1990); $2N_3A73$–L27 and $2N_3A76$–L27 (Wower *et al.*, 1988, 1989); and cmo^5U34–C1400 (16S rRNA) (Prince *et al.*, 1982). The A site tRNA is positioned as in the model of Wower *et al.* (1993). Numbers indicate the locations of proteins on the ribosomal subunits as determined by immune electron microscopy (Oakes *et al.*, 1986; Stöffler & Stöffler-Meilicke, 1986). The mRNA, represented as a shaded ribbon, is oriented on the 30S ribosomal subunit according to Olson *et al.* (1988).

of the A, P, and E sites (Wower *et al.*, 1988, 1989, 1990, 1993; Sylvers *et al.*, 1989, 1992). In these experiments, the azidoadenosines were incorporated at specific positions within the tRNA chain using controlled chemical or nucleolytic degradation, followed by enzymatic reconstruction. Here we report the use of tRNAs substituted with the uridine analog, s^4U, during their transcription to further define the structure of the ribosomal P site. Utilization of the photoreactive U analog enabled us to examine a different set of tRNA–ribosome contacts than in our previous work. As with $2N_3A$ and $8N_3A$, the selective activation of s^4U with near-UV light leads to the formation of short, covalent bonds with adjacent RNA or protein molecules (Favre *et al.*, 1969; Baltzinger *et al.*, 1979). Unlike the azidoadenosines, however, s^4U can be incorporated into transcripts either *in vitro* (Wollenzien *et al.*, 1991) or *in vivo* (Favre *et al.*, 1986). In this case, analysis of the resulting complexes entails identification of the cross-linked base(s) in the tRNA as well as the cross-linking target in the ribosome.

In the present work, s^4U was randomly incorporated into a precursor of *E. coli* $tRNA_2^{fMet}$, which contained U instead of C at position 17 of the D loop, by *in vitro* transcription with T7 RNA polymerase. The s^4U-substituted pre-$tRNA_2^{fMet}$ was then trimmed at the 5′-end by the RNA component of RNase P to yield mature [s^4U]$tRNA_2^{fMet}$. Although [s^4U]-$tRNA_2^{fMet}$ synthesized *in vitro* lacks the normal base modifications at positions 20, 32, 54, and 55, its specific amino acid acceptance was similar to that of $tRNA_2^{fMet}$ transcribed in the absence of s^4U and that of native *E. coli* $tRNA^{fMet}$. This observation is in accord with the findings of Sampson and Uhlenbeck (1988) and Samuelson *et al.* (1988), who found that unmodified transcripts of yeast $tRNA^{Phe}$ and mycoplasma $tRNA^{Gly}$ can be aminoacylated to approximately the same extent as their native counterparts. NMR studies showed that the yeast $tRNA^{Phe}$ transcripts acquire a three-dimensional structure identical to that of native yeast $tRNA^{Phe}$ (Hall *et al.*, 1989).

One cannot assume that all of the [s^4U]$tRNA_2^{fMet}$ transcripts form the correct three-dimensional structure, however, as the hydrogen-bonding properties of s^4U differ from those of U (Saenger & Suck, 1971). Nonetheless, the similar amino acid acceptance of unsubstituted and s^4U-substituted $tRNA_2^{fMet}$ suggests that most of the latter transcripts do indeed fold into the proper conformation. This may be due to the fact that 10 of the 13 U residues in $tRNA_2^{fMet}$ are located in non-base-paired regions; only U50, U27, and U24 are base-paired. In the case of U50, which pairs with G64 in the T stem, the substitution of U by s^4U should not lead to a distortion of the helix because the geometry of the s^4U–G pair is similar to that of the U–G pair (Psoda *et al.*, 1974; Riehl *et al.*, 1984). In contrast, the configuration of the D or anticodon stems could be perturbed by the incorporation of s^4U at position 24 or 27, respectively, as the geometry of the s^4U24–A11 or s^4U27–A43 pair may differ somewhat from the U24–A11 or U27–A43 pair (Saenger & Suck, 1971). However, these transcripts should only constitute a small fraction of the population of [s^4U]$tRNA_2^{fMet}$ transcripts and would not be expected to affect the aminoacylation assays significantly. It is also possible that RNase P does not cleave improperly folded pre-[s^4U]$tRNA_2^{fMet}$ transcripts, since it appears to recognize primarily features of the secondary and/or tertiary structure of tRNA rather than its primary structure (Abelson, 1979; Altman *et al.*, 1982). Pre-tRNA molecules with the wrong conformation may therefore be screened out in the processing step. The efficiency with which AcMet-[s^4U]$tRNA_2^{fMet}$ bound to 70S ribosomes, as well as the near-quantitative participation of the bound material in the puromycin reaction, provides additional evidence of the structural and functional integrity of the tRNA transcripts.

We have determined that P site-bound [s^4U]$tRNA_2^{fMet}$ formed cross-links to proteins L1 and L27 from position 17 or 17.1 and to protein S19 from position 47. As nucleosides 17 or 17.1 and 47 are located on opposite sides of the tRNA molecule, the cross-linking pattern indicates that the P site tRNA is sandwiched between the two ribosomal subunits, with the D loop side facing the 50S particle and the T loop side facing the 30S particle (Figure 11). This interpretation

is in agreement with our earlier models of the tRNA–ribosome complex (Wower *et al.*, 1989, 1991, 1993) and is, of course, based on the assumption that the tRNA retains a conformation close to or identical with that determined by X-ray diffraction (Kim *et al.*, 1974). All but five of the 77 bases composing *E. coli* $tRNA^{fMet}$ are engaged in stacking interactions (Woo *et al.*, 1980). The bases within s^4U-substituted $tRNA_2^{fMet}$ that became cross-linked to ribosomal proteins are members of this category. Other s^4U residues within the tRNA may fail to react with ribosomal proteins because they are sequestered in hydrogen-bonded regions, form intramolecular bonds similar to that between s^4U8 and C13 in *E. coli* $tRNA^{Phe}$ (Favre *et al.*, 1969), or cross-link to rRNA. Experiments aimed at identifying the s^4U residues attached to the 16S and 23S rRNAs are underway.

In view of the fact that protein L27 has already been attached to positions 56, 73, and 76 of P site-bound tRNA *via* short-range cross-links (Wower *et al.*, 1989; Abdurashidova *et al.*, 1990), the labeling of L27 from positions 17 or 17.1 as reported here provides additional evidence that both the 3′-end and the central fold of tRNA are in contact with this protein. Interestingly, previous cross-linking studies indicated that the same regions of tRNA are in close proximity to protein L2, which has been cross-linked to positions 17 and 73 of P-site tRNA (Wower *et al.*, 1989; Abdurashidova *et al.*, 1990). These observations are mutually supportive, as proteins L2 and L27 are close neighbors in the 50S subunit (Traut *et al.*, 1986; Walleczek *et al.*, 1988). As illustrated in Figure 11a, we believe that the coaxial acceptor/T stem helix is located in the valley between the central protuberance and the L1 ridge, where proteins L2 and L27 have been mapped by immune electron microscopy (Stöffler & Stöffler-Meilicke, 1986; Oakes *et al.*, 1986).

The labeling of protein L1 from P site-bound tRNA has been observed in two cases, one of which involved yeast $tRNA_m^{Met}$ derivatized at t^6A37 with a 17-Å probe and the other involved lupine $tRNA_m^{Met}$ derivatized at $acp^3U20.1$ with either a 10- or 18-Å probe (Podkowinski & Gornicki, 1989, 1991). Owing to the lengths of these probes, the site of reaction was necessarily at some distance from the derivatized base in the tRNA. As a consequence, it was not possible to reach an unequivocal conclusion about the proximity of L1 to the P site. Because the bonds that result from the photolysis of s^4U are short, cross-linking of L1 from position 17 or 17.1 of $[s^4U]$-$tRNA_2^{fMet}$ indicates that the D loop of at least some of the tRNA molecules is in the proximity of this protein. Although the L1–$[s^4U]tRNA_2^{fMet}$ complex is a minor component of the total cross-linked material, it is possible that L1 extends farther toward the central protuberance than is generally indicated by its position in the immune electron microscopy map of the 50S subunit (see Figure 11a).

Protein S19 was previously cross-linked to s^4U of A site-bound *E. coli* $tRNA^{Phe}$ *via* a 9-Å probe and to acp^3U47 of P site-bound *E. coli* $tRNA^{Phe}$ *via* a 20-Å probe (Ofengand *et al.*, 1981; Lin *et al.*, 1984). The cross-linking pattern indicated that the D loop side of the A site tRNA faces the T loop side of the P site tRNA, as proposed by Sundaralingam (1975). However, because the probes used in these experiments were quite long, it was not possible to discriminate conclusively between the two possible arrangements of the tRNAs relative to the ribosomal subunits [see Ofengand *et al.* (1984)]. This ambiguity can now be resolved by the cross-linking of protein S19 from s^4U47 of P site $[s^4U]tRNA_2^{fMet}$ described here, as well as by the labeling of proteins S13 and S19 from $2N_3A43$ in the anticodon arm of yeast $tRNA^{Phe}$ (Wower *et al.*, 1990), since the cross-links generated by photolysis of s^4U and $2N_3A$ are only 2–4-Å in length. As shown in Figure 11c, the P site tRNA must be situated to the "right" of the A-site tRNA on the interface side of the 30S subunit in such a way that the central fold is directed toward the head of the subunit where proteins S13 and S19 have been mapped. On the 50S subunit, the P site tRNA is positioned in the valley between the L1 ridge and the central protuberance to the "left" of the A site tRNA (Figure 11a). This arrangement places the A site closer to the L7/L12 stalk and is in accord with the fact that the binding site for EF-Tu, which transports the tRNA to the A site, is located at the base of the L7/L12 stalk.

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