Photoaffinity Labeling of the Ribosomal A Site with *S*-(*p*-Azidophenacyl)valyl-tRNA†

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ABSTRACT: *S*-(*p*-Azidophenacyl)valyl-tRNA, an analog of valyl-tRNA which has a photoaffinity label attached to its 4-thiouridine residue, was bound to the ribosomal A site at 10 m*M* Mg^{2+}. Binding was stimulated 25-fold by the presence of elongation factor EFTu. Photoactivation of the *p*-azidophenacyl group by irradiation resulted in covalent linking of 6% of the noncovalently bound tRNA to the ribosomes. Covalent linking was dependent on the simultaneous presence of ribosomes, poly(U_2,G), EFTu·GTP, required irradiation, and did not occur when *S*-(phenacyl)valyl-tRNA, a nonphotolyzable analog, replaced *S*-(*p*-azidophenacyl)valyl-tRNA. The attached tRNA was distributed approximately equally between both the 30S and 50S subunits. At the 30S subunit, 30% of the tRNA was bound to protein while 70% was linked to 16S RNA. At the 50S subunit, however, negligible binding to the 23S RNA was observed. More than 90% of the tRNA was attached to low molecular weight material according to sodium dodecyl sulfate–sucrose gradient analysis, and more than 87% of this fraction consisted of tRNA–protein complexes as assayed by phenol solubility and electrophoretic mobility before and after protease treatment. These results, in conjunction with our previous report (I. Schwartz and J. Ofengand (1974), *Proc. Natl. Acad. Sci. U.S.A. 71*, 3951) which showed that covalent linking of this same tRNA derivative at the ribosomal P site resulted in attachment solely to the 16S RNA, demonstrate that 16S, but not 23S or 5S rRNA, is an important component of the tRNA binding site in the region of the 4-thiouridine residue and furthermore show that ribosomal A and P sites are topologically distinct.

Recently we described the preparation of *Escherichia coli* valine tRNA which had been derivatized at the sulfur atom of its 4-thiouridine residue with APA-Br,[1] and its use as a photoaffinity probe of the ribosomal P site. Covalent linking of the APA-[^{3}H]valyl-tRNA occurred exclusively to the 16S RNA of the 30S subunit (Schwartz and Ofengand, 1974).

In view of the current interest in comparing structural and functional properties of the two tRNA binding sites, we have now used this same derivatized tRNA to study binding at the ribosomal A site. Except for recent reports by Cantor and coworkers (Hsiung et al., 1974; Hsiung and Cantor, 1974; Eilat et al., 1974a), previous chemical affinity and photoaffinity labeling studies (Bispink and Matthei, 1973; Oen et al., 1973, 1974; Pellegrini et al., 1974; Sopori et al., 1974; Czernilofsky et al., 1974; Hauptmann et al., 1974; Eilat et al., 1974b; Girshovich et al., 1974) have been limited to a study of the ribosomal P site because they employed aminoacyl-tRNAs derivatized at the amino group of the aminoacyl moiety, and did not use the special conditions needed for A site binding. Since APA-[^{3}H]valyl-tRNA is derivatized at the 4-thiouridine residue, and thus contains an unblocked amino group, it is a suitable probe of the ribosomal A site as well as the P site.

We report here the elongation factor EFTu-dependent binding of APA-[^{3}H]valyl-tRNA to the ribosomal A site. Irradiation of the noncovalent tRNA–ribosome complex resulted in covalent linking of the tRNA to both the 30S and 50S subunits, in contrast to the result found previously when binding at the P site was examined.

Materials and Methods

Chemicals. E. coli $tRNA_1^{Val}$, elongation factor EFTu, 3 × NH_4Cl-washed ribosomes, [^{3}H]valine, (14,800 cpm/pmol), 4-thiouridine, phenacyl bromide, and APA-[^{3}H]valyl-tRNA were obtained or prepared as described previously (Schwartz and Ofengand, 1974). $N_{1,3}$-Dimethyl-4-thiouracil was obtained from Cyclo Chemical Company and *S*-cyanoethyl-4-thiouridine was prepared as described previously (Ofengand, 1967). Pronase (grade B) was obtained from Calbiochem and treated as described by Hourcade et al. (1973). Acrylamide and *N,N'*-diallyltartardiamide were obtained from Bio-Rad Laboratories.

S-(Phenacyl)-4-thiouridine. The title compound was prepared by incubation of a 60% methanol solution containing 40 m*M* KPO_4 buffer (pH 7.4), 15.5 m*M* 4-thiouridine, and 20 m*M* phenacyl bromide at 30° for 20 min. The reaction mixture was streaked out on Merck 2 mm thick silica gel thin-layer chromatography (TLC) plates (1 ml/plate) and chromatographed in $CHCl_3$–MeOH (85:15), R_f 0.5. Elution was performed first by chromatography perpendicular to the original direction with methanol two times to concentrate the band in a small area, followed by removal of the silica gel to the tube and serial elution with 3 ml of dioxane until the 300-nm absorbance of the eluate was sufficiently low. The dioxane eluates were pooled and concentrated in a stream of air. All operations were performed in the dark or with yellow safelights. The uv spectrum of the

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[1] Abbreviations used are: PA, phenacyl; PA-Br, phenacyl bromide; APA, *p*-azidophenacyl; APA-Br, *p*-azidophenacyl bromide; APA-Srd, *S*-(*p*-azidophenacyl)-4-thiouridine; APA-ψrd, *p*-azidophenacylpseudouridine (the site of attachment of the APA group is unknown but is probably at the N_1 or N_3 atom); APA-tRNA or APA-Val-tRNA, tRNA or Val-tRNA, respectively, derivatized at the sulfur atom of its 4-thiouridine residue with the *p*-azidophenacyl group; MGA, 3,3-dimethylglutaric acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

product did not change with pH from 2 to 10 and showed two nearly equal peaks at 250 and 300 nm characteristic of the phenacyl and thiouridine chromophores, respectively. Further characterization is given in Results.

Derivatization of $tRNA^{Val}$ with [^{14}C]-p-Azidophenacyl Bromide. Reaction and isolation were carried out as described previously (Schwartz and Ofengand, 1974) except that the concentration of [^{14}C]APA-Br (9.1 cpm/pmol, a gift of Dr. S. Hixson, University of Massachusetts) was reduced in half, the concentration of $tRNA^{Val}$ was 10 times higher, and the reaction time was doubled. Since the APA-Br was still present in a 100-fold molar excess the rate of reaction should be solely determined by the APA-Br concentration. The reaction time was doubled to compensate for the halved APA-Br concentration. These modified conditions were necessary in order to obtain sufficient tRNA-bound radioactivity with the limited amount of [^{14}C]APA-Br available. The final product, after reprecipitation to constant specific radioactivity, yielded 1265 pmol of [^{14}C]APA-tRNA/A_{260} unit, which compares favorably with the acceptance activity of 1230 pmol/A_{260} unit.

EFTu-Dependent Binding of APA-[^{3}H]valyl-tRNA to Ribosomes. EFTu-dependent binding mixtures contained in a total volume of 1.0 ml, 35–45 pmol of APA-[^{3}H]valyl-tRNA, 20 A_{260} units of 3 × NH_4Cl-washed ribosomes, 0.1 mg of poly (U_2,G), and 0.8 nmol of EFTu in 50 m*M* Tris (pH 7.4), 75 m*M* NH_4Cl, 75 m*M* KCl, 10 m*M* $Mg(OAc)_2$, 5 m*M* dithiothreitol, and 0.1 m*M* GTP. Ribosomes were activated by preincubation at 37° for 15 min before the addition of EFTu or tRNA. Two A_{260} units (40-fold excess) of uncharged $tRNA_1^{Val}$ was added before adding the Val-tRNA in order to block EFTu-independent binding (Ofengand and Henes, 1969; Ofengand et al., 1974). Mixtures were incubated for 30 min at 30°, at which time 47–50% of the Val-tRNA was bound. These conditions are slightly modified from Ofengand et al. (1974).

Irradiation of APA-[^{3}H]valyl-tRNA–Ribosome Complexes. Ribosomal A site binding mixtures were irradiated as described previously for P site binding complexes (Schwartz and Ofengand, 1974).

Calculation of Efficiency of Covalent Link Formation. The efficiency of covalent link formation was calculated by determining the fraction of added APA-[^{3}H]valyl-tRNA radioactivity which sedimented with the 30S and/or 50S subunit on a sucrose gradient. This value was divided by the fraction of total input tRNA added to the binding mixtures which became noncovalently bound to the ribosomal A site defined as EFTu-dependent binding, and determined by Millipore filtration (Schwartz and Ofengand, 1974). The value for efficiency was thus based on the amount of tRNA which was enzymatically bound to the ribosomal A site.

Acetic Acid Extraction and Pronase Digestion of Ribosome-tRNA Complexes. Acetic acid extraction was performed according to Hardy et al. (1969). Twenty A_{260} units of 50S subunit–tRNA complexes in 0.26 ml of 20 m*M* MGA buffer (pH 7.0)–10 m*M* $MgCl_2$ was chilled, adjusted to 0.2 *M* $MgCl_2$, and stirred vigorously. Two volumes of glacial acetic acid was added immediately. The mixture was stirred for 30 min and then centrifuged at 20,000*g* for 10 min. The precipitate was washed once with 0.2 *M* KOAc (pH 5) and 67% ethanol and recovered by centrifugation. The supernatant usually contained 10% of the total radioactivity and was discarded. The precipitate was suspended in 0.1 ml of 2 *M* urea–0.2 *M* Hepes buffer (pH 8.0) containing 0.1 mg of TPCK-trypsin (prepared by Dr. C.-Y. Lai of this Institute) by stirring for 10 min at 37°. An equal volume of a self-digested pronase solution (Hourcade et al., 1973) (50 mg/ml in 5 m*M* MGA (pH 7.0)) was then added and the samples were stirred at 37° for an additional 30 min. After digestion, sodium dodecyl sulfate was added to a final concentration of 0.5% and stirring continued at room temperature for 5 min. The solution was removed, and the empty tube was reextracted with 1 ml of 0.5% dodecyl sulfate and 0.1 *M* MGA (pH 7). An additional 10–12% of the cpm was recovered in this fraction. The overall recovery of input Cl_3CCOOH precipitable cpm ranged from 92 to 109%. The digest was then prepared for gel electrophoresis by addition of glycerol to 5%, Bromophenol Blue as a tracking dye, and running gel buffer to the same final concentration as in the running gel. All operations were carried out in a single centrifuge tube containing a magnetic flea to eliminate possible losses due to transfer and/or adhesion of precipitated material to the tube.

Gel Electrophoresis. Separations were performed using the pH 5.8–7 *M* urea system described by Stein and Varricchio (1974) modified by the use of *N,N'*-diallyltartardiamide as cross-linking agent (Anker, 1970). Slab gels were formed in a Hoefer electrophoresis cell, and electrophoresed using an Ortec Model 4100 power supply for approximately 18 hr with a room temperature cooling reservoir. The portion of the slab containing the radioactive samples was cut off, frozen, sectioned into 1-mm slices by hand, and then digested with 1 ml of fresh 2% periodic acid for 3 hr at 37° (Anker, 1970). Shaking was necessary to digest these 15% gels in a reasonable time. The slices were counted at 30% efficiency after addition of 10 ml of Hydromix (Yorktown Research, New Hyde Park, N.Y.). The remainder of the slab was fixed in 1 *M* acetic acid for 15 min, stained with 0.2% Methylene Blue in 0.4 *M* NaOAc (pH 5.5) for 60 min, and destained with running water. Staining markers, treated as above but without protease incubation, were purified valine tRNA (Krauskopf et al., 1972) and 5S RNA (Miles Laboratories), both from *E. coli*. A band moving faster than valine tRNA was found to be due to the dodecyl sulfate in the sample. The position of the markers has been corrected for swelling of the gel (approximately 30%) during the staining procedure.

Results

Reaction of p-Azidophenacyl Bromide with 4-Thiouridine. In our previous report (Schwartz and Ofengand, 1974), we presented evidence for adduct formation between APA-Br and 4-thiouridine based on the appearance of a new compound on TLC which possessed ir bands corresponding to azide, acetophenone ketone, and ribose hydroxyl groups. Assignment of the sulfur atom as the site of addition was based both on the literature for this class of reaction, and on the absence of the 330-nm uv absorption band which is characteristic of N-substituted 4-thiouridine (Kumar et al., 1973). Because the APA group absorbs strongly at 300 nm, it was not possible to observe the blue shift from 330 to 300 nm which is characteristic for addition to the sulfur atom of 4-thiouridine (Ofengand, 1967; Kumar et al., 1973).

However, by use of the analog, PA-Br, which has an absorption maximum at 250 nm, it was possible to obtain a satisfactory difference spectrum for the reaction between PA-Br and 4-thiouridine (Figure 1). The figure clearly shows the characteristic λ_{max} shift and decrease in intensity which we have previously observed both for cyanoethylation

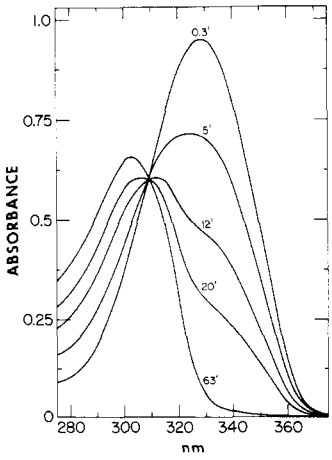

FIGURE 1: Reaction of 4-thiouridine with phenacyl bromide. Both sample and reference cell contained 3 ml of 40 mM KPO_4 buffer (pH 7.4) and 0.5 mM phenacyl bromide in water. After the base line was established, 3 μl of 4-thiouridine (57.3 mM) was added to the sample cell, and the difference spectrum immediately was recorded. Subsequent spectra were recorded at the times indicated while the sample remained in the spectrometer. The temperature was 23°. The pseudo-first-order rate constant, calculated from the change in absorbance at 340 nm, was 133 min^{-1} M^{-1}.

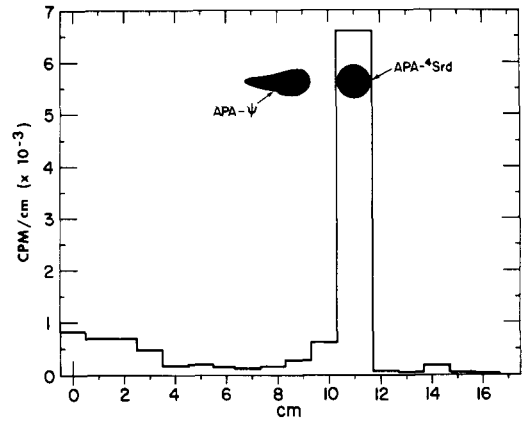

FIGURE 2: Nucleotide analysis of [^{14}C]APA-tRNA^{Val}. Digestion to nucleosides was accomplished in a mixture (0.33 ml) containing 0.1 mg of pancreatic RNase, 100 units of T_2 RNase (Sankyo), 280 units of alkaline phosphatase, 6.5 A_{260} units of tRNA (74,600 cpm), and 60 mM cacodylate buffer (pH 7.0). After 16 hr at 37°, 3.2 ml of acetone was added and the mixture chilled at 0° for 30 min. After centrifugation, the acetone supernatant contained 91% of the total radioactivity. The acetone solution was concentrated by evaporation, APA-Srd and APS-Ψrd were added as markers, and an aliquot (14,000 cpm) was chromatographed on 1 mm thick silica gel TLC plates in $CHCl_3$–MeOH (80:20). After drying, the location of the markers were visualized under uv light, and the radioactivity determined by scraping 1-cm wide sections of the strip into scintillation vials and counting under toluene scintillation fluid. The data are plotted as cpm divided by the width of the section in order to be analogous to a concentration term. Thus the *area* under a given section is proportional to its total count.

(Ofengand, 1967) and for reaction with iodoacetamide (Kumar et al., 1973). A good isosbestic point was obtained, indicating that only a single uv-absorbing species was formed. The rate constant calculated for the reaction under these conditions was 133 min^{-1} M^{-1}, which is extremely rapid for this class of reaction, the k for the analogous reaction with iodoacetamide being 10.5 min^{-1} M^{-1} at pH 10.

Derivatization at the sulfur atom was confirmed by the absence of a thioketone functional group in the product as shown by the iodine–azide spot test of Feigl, which is diagnostic for thiols and thioketones (Ofengand, 1967). Thus $N_{1,3}$-dimethyl-4-thiouracil was positive, but S-cyanoethyl-4-thiouridine was negative until the cyanoethyl group was removed by alkali. Similarly, PA-4-thiouridine was negative even at 20 times the concentration of $N_{1,3}$-dimethyl-4-thiouracil which gave a strong positive reaction.

Reaction of p-Azidophenacyl Bromide with tRNA^{Val}. In our prior report, we gave indirect evidence that only the 4-thiouridine residue of the tRNA had reacted with APA-Br. We now show this directly by the use of [^{14}C]APA-Br.[2] Derivatization of the tRNA was accomplished in the same way as before with the modifications described under Materials and Methods. The final product contained approximately 1 mol of the [^{14}C]APA group/mol of tRNA (specific activity 1.27 nmol/A_{260}). After digestion with nucleases and phosphatase to the nucleoside level, the digest was chromatographed together with unlabeled standards of APA-4-thiouridine and APA-pseudouridine (Figure 2).

These were the only two residues in tRNA^{Val} which showed any reactivity toward APA-Br in preliminary tests of nucleosides under more vigorous reaction conditions. Although pseudouridine did not react under the conditions used for tRNA derivatization, it was included since an example was recently described in which pseudouridine in tRNA was more reactive than when present as the free nucleoside (Yang and Söll, 1974), although the reverse is usually the case (Ofengand, 1971). In any event, it is clear from an inspection of the chromatogram that essentially all of the radioactivity was present as APA-4-thiouridine with less than 5% of that present in both nucleoside derivatives being in APA-pseudouridine. The radioactivity at the origin is probably due to incomplete digestion.

Binding of APA-[^{3}H]valyl-tRNA to the Ribosomal A Site. Binding to the A site was defined as binding to the ribosome in 10 mM Mg^{2+} which was dependent on addition of elongation factor Tu. Such a dependence for the binding of APA-[^{3}H]valyl-tRNA is shown in Figure 3. By adding excess unacylated tRNA^{Val} in order to depress nonenzymatic binding (Ofengand and Henes, 1969; Ofengand et al., 1974), a 25-fold stimulation of binding was observed upon addition of EFTu. The addition of 10^{-4} M sparsomycin, a peptidyl transferase inhibitor (Pestka, 1971), did not alter the extent of EFTu-dependent binding (data not shown), demonstrating that no appreciable translocation and oligovaline synthesis had occurred.

Irradiation of such a tRNA–ribosome complex resulted in covalent link formation between the tRNA and ribosome. The kinetics were essentially the same as for the P site reaction described previously (Schwartz and Ofengand, 1974), i.e., the reaction was complete after 4 hr of irradiation. The efficiency of covalent link formation was lower, however, being only 5–6% of that noncovalently bound, while at the P site 15–20% of the noncovalently bound tRNA became covalently attached to the ribosomes (Schwartz and Ofengand, 1974). These relatively low yields are characteristic for photoaffinity labeling reactions (Hsiung et al., 1974; Hsiung and Cantor, 1974; Bispink and Matthei, 1973; Girshovich et al., 1974) and presumably reflect competition for the reactive nitrene between ribosomal components and solvent. In the case of aromatic azides, intramolecular ring expansion reactions (Knowles, 1972) may also decrease the yield.

Identification of the Sites of Covalent Linking of APA-[^{3}H]Val-tRNA. The APA-[^{3}H]Val-tRNA-70S ribosome

[2] The [^{14}C]APA-Br was a geneous gift of Dr. S. Hixson, Department of Chemistry, University of Massachusetts.

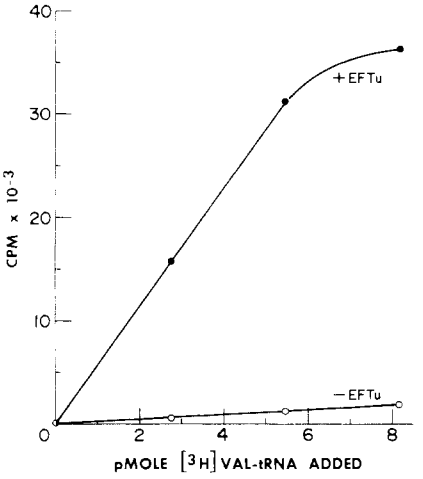

FIGURE 3: Elongation factor dependent binding of APA-[^{3}H]Val-tRNA to ribosomes. Ribosomal A site binding was carried out in a volume of 0.2 ml containing 10 mM Mg^{2+}, 50 mM Tris (pH 7.4), 75 mM NH_4Cl, 75 mM KCl, 5 mM dithiothreitol, 2 A_{260} units of 3 × NH_4Cl-washed ribosomes, and 0.1 mg/ml of poly(U_2,G) using the indicated amounts of APA-[^{3}H]Val-tRNA in the presence or absence of 0.08 nmol of EFTu and 0.05 mM GTP; 0.4 A_{260} unit of uncharged $tRNA^{Val}$ was added to block nonenzymatic binding. Mixtures containing ribosomes minus tRNA and EFTu were preincubated at 37° for 15 min to activate the ribosomes. Following addition of tRNA and EFTu, mixtures were incubated for 30 min at 30°, filtered through Millipore filters, and counted. Minus ribosome blanks were measured at each tRNA concentration and have been subtracted. (○) Binding mixture in the absence of added EFTu; (●) binding mixture plus added EFTu.

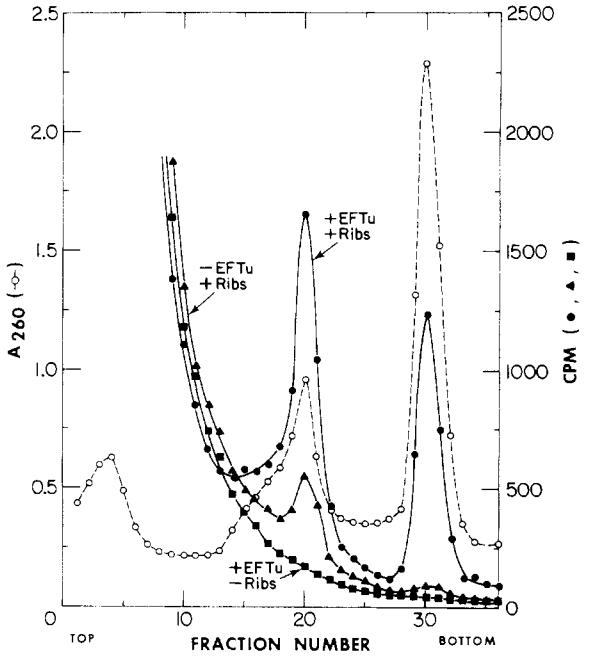

FIGURE 4: Sucrose gradient separation of 30S and 50S subunits following covalent attachment of APA-[^{3}H]Val-tRNA. A site binding mixtures in a total volume of 1.0 ml as described under Materials and Methods were incubated for 30 min at 30° and then irradiated at 0° for 4 hr. The mixtures were dialyzed overnight against 2 l. of 10 mM Tris (pH 7.0) 50 mM KCl–6 mM β-mercaptoethanol–0.3 mM $MgCl_2$, applied to 36 ml of a 10–30% sucrose gradient in the same buffer, and centrifuged for 16 hr at 24,000 rpm in an SW 27 rotor; 1.0-ml fractions were collected and the A_{260} and radioactivity of each fraction were determined. (○) A_{260}; (●) A site binding mixture incubated in the presence of EFTu; (▲) A site binding mixture minus EFTu and GTP; (■) A site binding mixture minus ribosomes.

complex was dissociated in low Mg^{2+} and the 30S and 50S subunits were separated on a sucrose gradient. The results are given in Figure 4 and Table I. When tRNA was bound to ribosomes in the presence of EFTu, the covalently bound tRNA was partitioned about equally between the 30S and 50S subunit. In the absence of EFTu, the amount of tRNA covalently linked was much lower since there was little noncovalent binding in the absence of EFTu (Figure 3). The *efficiency* of covalent linking in the absence of EFTu, however, was 15–20% and all the covalently bound tRNA sedimented with the 30S subunit. This result was expected since we had previously shown that irradiation of nonenzymatically bound tRNA (i.e., tRNA presumably bound to the P site) resulted in covalent linking of the tRNA exclusively to the 16S RNA of the 30S subunit (Schwartz and Ofengand, 1974). In seven different experiments, 47–56% of the tRNA was found covalently bound to the 30S subunit and 44–53% to the 50S subunit after correction for the contribution of EFTu-independent binding to the covalent linking.

Covalent linking was dependent on the simultaneous presence of EFTu-GTP, poly(U_2G), and ribosomes. Overall, there was a ninefold decrease in linking if irradiation was omitted, and a fivefold decrease if PA-Val-tRNA, an analog lacking the azido group, replaced APA-Val-tRNA (Table I). The irradiation-independent binding of APA-Val-tRNA appeared specific for the 50S subunit, while irradiated PA-Val-tRNA was attached equally to both subunits. Probably two different mechanisms are involved. The unirradiated APA-Val-tRNA may be acting as a chemical affinity probe since the reaction requires the presence of EFTu·GTP and poly(U_2,G) (cf. lines 2 and 3 vs. 4) and it is well known that aromatic azido groups can be readily displaced by attacking nucleophiles. On the other hand, since the linking of PA-Val-tRNA required irradiation, a photochemical reaction not involving nitrenes may be taking

Table I: Requirements for Covalent Linking to the Ribosomal A Site.[a]

tRNA	Reaction Mixture	% Bound		
		Total	30S	50S
APA-Val-tRNA	Complete	2.7	1.6	1.1
	−EFTu·GTP	0.4	0.4	0.0
	−Poly(U_2,G)	0.0	−0.1	0.1
	−Irradiation	0.3	0.0	0.3
PA-Val-tRNA	Complete	0.6	0.3	0.3

[a] Procedures were as described in the legend to Figure 4 with omissions or substitutions as noted. The amount of tRNA which was covalently bound was calculated from the radioactivity under the 30S and 50S peaks after correction for minus ribosome blanks like those in Figure 4; 30 pmol of APA-Val-tRNA or of PA-Val-tRNA were initially present in each reaction mixture, of which 47 and 38%, respectively, were noncovalently bound. The results are presented as percent of *input* tRNA, not as the percent of noncovalently bound tRNA, in order to properly display the component omission experiments. The data have been corrected for a blank of 0.1% found only in the 50S subunit when unirradiated PA-Val-tRNA was used.

place in this instance (Escher and Schwyzer, 1974). Note that the wavelengths of irradiation used here are outside of the absorption band of the phenacyl group, as was the case in the experiments of Escher and Schwyzer. Such a reaction might account for the linking of PA-Val-tRNA to both subunits as was the case for APA-Val-tRNA, since the distribution of cross-links might be more dependent on gross orientation of the probe and less dependent on the exact kind of photochemical activation taking place.

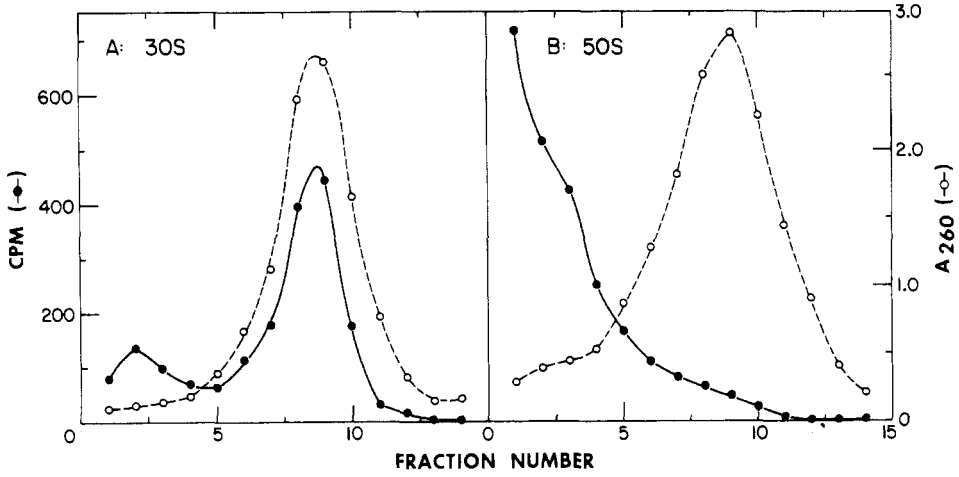

FIGURE 5: Sodium dodecyl sulfate gradient separation of ribosomal protein and RNA from the 30S and 50S subunit-tRNA covalent complexes. 30S and 50S subunit-tRNA covalent complexes were isolated from the sucrose gradient of Figure 3 by adjusting the $MgCl_2$ concentration to 10 m*M* and centrifugation at 55,000 rpm in a type 65 rotor for 5.5 hr. The pelleted subunit-tRNA complexes were resuspended in 10 m*M* Tris (pH 7)-10 m*M* $MgCl_2$ to an A_{260} of 20-25 for the 30S subunit and 60-70 for the 50S subunit; 0.1-0.2 ml was layered on 4 ml of a 5-20% gradient containing 20 m*M* cacodylate (pH 5.8), 100 m*M* NaCl, 1 m*M* EDTA, and 0.5% dodecyl sulfate. After centrifugation at 50,000 rpm for 3 hr in an SW 56 rotor, 0.25-ml fractions were collected and the A_{260} and Cl_3CCOOH-precipitable radioactivity determined. The top of the gradient is at the left. (A) 30S subunit, (B) 50S subunit.

Could the EFTu-dependent linking to the 30S subunit be entirely due to translocation to the P site of part of the tRNA bound initially at the A site? Although the sparsomycin results cited above show that no appreciable translocation occurred, only 15% of the A site bound material need migrate to account for the results obtained since the efficiency of cross-linking at the P site is six times better than at the 50S A site. In order to eliminate this possibility, the amount of tRNA present in the P site after incubation with EFTu was determined by its reactivity with puromycin. The amount of EFTu-dependent puromycin-reactive material thus becomes a measure of the extent of translocation which had occurred during the binding reaction. (Recall that the amount of EFTu-independent covalent linking was low). The conditions used were those described in Figure 4, except that Phe-tRNA and poly(U) replaced valyl-tRNA and poly(U_2,G) because puromycin does not react well with valyl-tRNA. The efficiency of the puromycin reaction was determined to be 0.5 in a separate incubation in which a large excess of EFG was added and deacylated tRNA was omitted since it inhibits translocation. The maximum amount of EFTu-dependent Phe-puromycin formed was 0.09 pmol when 16 pmol of Phe-tRNA was bound in an EFTu-dependent reaction. This corresponds to 1.1% translocation during the A site binding reaction, which is only 8% of the amount needed to account for covalent linking to the 30S subunit. Even if the putative rate of translocation of Val-tRNA were twice that of Phe-tRNA, only 16% of the covalent linking observed could be due to translocation. Clearly 30S subunit linking does indeed occur from the A site.

Distribution of the Covalently Attached [^{3}H]Val-tRNA between Protein and RNA. The 30S and 50S subunit-tRNA covalent complexes were isolated and rerun on a dodecyl sulfate containing sucrose gradient in order to separate the ribosomal proteins from the RNA. The results are shown in Figure 5. In the case of the 30S subunit (Figure 5A), some radioactivity was detected at the top of the gradient which corresponds to covalent attachment of tRNA to the ribosomal proteins. This material does not come from contamination with free tRNA or tRNA-50S subunit covalent complexes, which yields all low molecular weight material on treatment with dodecyl sulfate (Figure 5B), since 30S subunits, repurified through a second sucrose gradient, gave the same results after dodecyl sulfate gradient analysis as presented in Figure 5A. This result is in contrast to the previous P site binding study (Schwartz and Ofengand, 1974), in which all the radioactivity associated with the 30S subunit was attached to the 16S RNA. Since these 30S subunits contained tRNA covalently linked in both EFTu-dependent and independent reactions, it was necessary to first subtract the EFTu-independent linking. If all of this linking is assumed to be due to P site linking to the 16S RNA, then it can be calculated that 30% of EFTu-dependent 30S bound tRNA was attached to protein, and 70% was attached to 16S RNA.

With the 50S subunit, however, virtually all of the radioactivity was found at the top of the gradient (Figure 5B). Thus, almost all of the tRNA was covalently linked to ribosomal protein and/or 5S RNA. There was negligible binding to the 23S RNA. Since 5S RNA did not migrate appreciably into the gradient under the conditions employed, it was not possible to differentiate between covalent linkage to protein or 5S RNA by this method. The complex was insoluble (>90% of the radioactivity was in the precipitate) after extraction with 67% acetic acid as described by Hardy et al. (1969) for removal of ribosomal proteins, but tRNA-protein complexes might also have precipitated under these conditions. Although all of the bulk rRNA could be readily solubilized by extraction of the acetic acid precipitate with 6 *M* urea-0.1 M MGA buffer (pH 7.0) at 0° for a few minutes, typically 25% of the radioactivity remained in the pellet and could only be solubilized by the use of 0.5% dodecyl sulfate and 0.1 *M* MGA (pH 7); 0.1% dodecyl sulfate at pH 7 was slightly less effective and 0.5% sarkosyl (pH 7) left 15% of the radioactivity behind. These results indicated that the tRNA might at least in part be attached to protein.

In order to be able to use susceptibility to protease digestion as a criterion for attachment to ribosomal proteins, a procedure was devised in which the 50S ribosomal subunit was first broken up by extraction with 67% acetic acid to remove most of the ribosomal proteins (Hardy et al., 1969),

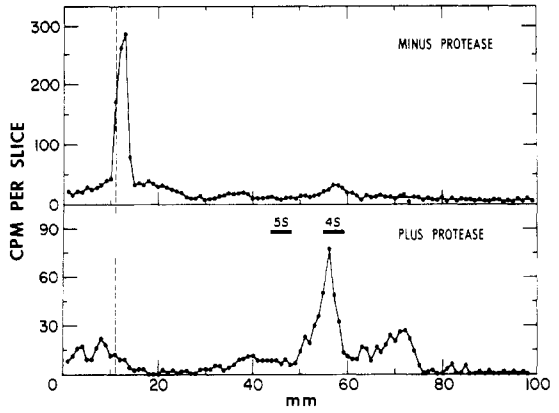

FIGURE 6: Gel electrophoresis analysis of the Val-tRNA–50S complex. Sample preparation, gel electrophoresis, and analysis were performed as described under Materials and Methods; 200-μl samples were loaded onto 1-cm stacking gels and 10-cm running gels. The beginning of the running gel is indicated by the dashed line. The positions of 5S RNA and valine tRNA (4 S) markers are indicated. The recovery of radioactivity from the gel was 98 and 80% for the minus and plus protease, respectively.

and the pellet then was digested with protease. Finally, any remaining material was solubilized by addition of dodecyl sulfate to 0.5% and the mixture was then analyzed by electrophoresis on polyacrylamide gels. This procedure was chosen in order to detect tRNA–5S covalent complexes as well as tRNA–protein complexes. The completeness of the solubilization both in the presence and absence of protease was verified by further washing of the tube with 0.5% dodecyl sulfate (pH 7). Typically, 10% of the radioactivity was found in the acetic acid extract, 10% in the dodecyl sulfate final wash, and 80% in the digest. Overall recoveries were from 92 to 109%. Although the vigorous digestion procedure found necessary caused extensive (65–70%) hydrolysis of the aminoacyl bond, the results are still meaningful since the extent of alkaline hydrolysis should be equal for all types of Val-tRNA covalent complexes.

The results of gel electrophoresis of this material both before and after proteolysis gave the results shown in Figure 6. It is clear from the figure that while the radioactivity barely penetrated the running gel before the treatment with protease <20% could be found at the top of the gel after protease digestion. Most of the radioactivity was found at the tRNA position but an appreciable fraction moved somewhat faster. This is probably due to a contaminating amount of nuclease in the trypsin used, since preliminary gels run with self-digested Pronase alone never showed any radioactivity moving faster than 4S RNA, while untreated Pronase gave a similar pattern to that shown in the figure. The 15% fraction found in the 5S RNA region presumably corresponds to Val-tRNA carrying incompletely digested tails of positively charged peptides.

No radioactivity was found in the region of the gel expected for a 4S–5S covalent complex either in the absence or presence of proteolytic digestion, suggesting that all of the 50S low molecular weight material which was attached to tRNA corresponded to ribosomal protein(s).

In order to confirm this finding by a method involving less manipulation, partitioning of tRNA–50S covalent complexes between a phenol–water layer was carried out (Table II). While it was possible that tRNA–protein complexes might be extracted into the aqueous phase, it was highly unlikely that any 5S-tRNA complexes would be found in the phenol layer. Suitable controls showed the distribution of

Table II: Phenol Solubility of [^{3}H]Val-tRNA–50S Complexes.[a]

Extraction Time (min)	Phase	[^{3}H]Val-tRNA Plus Ribosomes cpm	%	[^{3}H]Val-tRNA–50S Complexes cpm	%
1	Aqueous	8870	95.9	901	15.9
	Phenol	380		4748	
5	Aqueous	8167	95.7	827	18.1
	Phenol	370		3748	

[a] [^{3}H]Val-tRNA–50S complexes isolated by ethanol precipitation from a sucrose gradient as in Figure 4 or a mixture of [^{3}H]Val-tRNA plus 70S ribosomes were freshly precipitated with 2 volumes of ethanol from 50 m*M* cacodylate–20 m*M* $MgCl_2$ (pH 5.8) in order to remove any free valine. The samples were resuspended in 50 m*M* cacodylate buffer (pH 5.8)–10 m*M* $MgCl_2$; 0.3-ml aliquots (13–16 A_{260} units) were added to 2 ml of 2% KOAc (pH 5.3); 2.5 ml of redistilled phenol, previously equilibrated with the aqueous phase, was added, and the mixture was shaken vigorously in a Vortex mixer at room temperature for 1 or 5 min as indicated. After separation of the aqueous layer with care to leave the interface material behind, a second 2 ml of KOAc was added, and the process repeated. Both aqueous layers were combined; 1- and 2-ml aliquots from the aqueous phase and duplicate 1-ml aliquots from the phenol layer were mixed with 10 ml of Bray's solution and counted. Quench corrections were determined with a [^{3}H]valine internal standard.

free Val-tRNA between the two layers. The table shows clearly that at least 83% of the Val-tRNA was attached to protein, even when the extraction was extended to two 5-min periods at room temperature. This estimate is a minimum figure for two reasons. First, the dodecyl sulfate gradients of Figure 5 showed that 5–10% of the tRNA might be attached to 23S rRNA which would be found in the aqueous layer, and, second, the attachment of tRNA to ribosomal protein might slightly alter its distribution coefficient between the phenol and water layers. The conclusion from these experiments is that at least 85–90% of the covalent linkage to the 50S subunit was to ribosomal protein(s).

Discussion

The marked difference in the labeling pattern which was obtained when tRNA was bound enzymatically with EFTu vs. that found previously for nonenzymatic binding (Schwartz and Ofengand, 1974) underscores the topological differences between these two modes of binding. EFTu-dependent binding to the ribosome is generally accepted as a measure of A site binding and in confirmation of this, 86% of the EFTu-dependent binding was blocked by 0.4 m*M* tetracycline (unpublished results). Since nonenzymatic binding cannot be assigned to the P site without additional evidence, in our previous work (Schwartz and Ofengand, 1974) we relied on the use of a large excess of ribosomes, the lack of inhibition by tetracycline and the marked difference in covalent labeling patterns between enzymatic and nonenzymatic binding reactions to show that nonenzymatic covalent linking was only occurring from the P site. We have now confirmed this conclusion (unpublished results) by showing that 40% of the APA-Val-tRNA bound nonenzymatically and isolated as ribosome–tRNA complexes can react with puromycin. Since this amount of reaction was unaffected by the presence or absence of tetracycline, or by the addition or omission of G factor plus GTP, the incompleteness of the reaction appears to be due to an intrinsic deficiency in the formation of Val-puromycin and not to partial binding at the A site. In agreement with this view, 85% of *N*-acetyl-APA-Val-tRNA bound nonenzymatically

reacted with puromycin and gave covalent linking patterns identical in type and extent with nonenzymatically bound APA-Val-tRNA. Furthermore, if EFTu-independent binding to the A site had occurred in our P site experiments, some covalent linking to 50S subunits should have been detected, but none was observed.

Although the existence of two functionally distinct sites has long been known, this study is the first direct demonstration of a *structural* distinction between the two sites related explicity to a fixed, as opposed to a mobile, region of the tRNA molecule. This finding suggests that alternative models which have been proposed for the two tRNA binding sites in which the two sites are not distinctly A or P sites but can alternatively function as either one (Woese, 1970; Pestka, 1972) are likely to be incorrect. Hsiung et al. (1974) and Hsiung and Cantor (1974) have recently used tRNA with a photoaffinity label attached to the aminoacyl end to probe the peptidyl transferase center when tRNA was bound to either the ribosomal P or A site. In that case no difference in the labeling pattern was found when the tRNA was bound at either site, the major labeled products in both cases being proteins L11 and L18. There is apparently sufficient flexibility in the combination of the terminal CCA end of the tRNA molecule plus the lengthy probe used by these workers to allow the aminoacyl moiety to react at the same place when the tRNA is bound either at the P or A site. In addition, the choice of probe seems to be important since chemical affinity labeling with bromoacetyl Phe-tRNA bound to the A and P sites did show differences in the proteins reacted (Eilat et al., 1974a). Moreover, Bispink and Matthei (1973) earlier reported exclusive labeling of the 23S RNA from the P site using a photoaffinity probe attached to the aminoacyl end of tRNA which was shorter than the one used by Hsiung et al. (1974) or Hsiung and Cantor (1974), and recently Girshovich et al. (1974) reported that an intermediate length photoaffinity probe reacted 65% with 23S RNA and 35% with 50S proteins.

The differences we have found for tRNA bound either at the P or A site illustrate the sensitivity of the photoaffinity labeling method to tRNA orientation on the ribosome. Not only was the distribution of cross-links different, but the overall efficiency of covalent link formation was affected. When tRNA is bound in the P site, the sulfur atom of the 4-thiouridine residue possibly projects toward the 30S subunit thus resulting in a 15–20% efficiency and the absence of any linking to the 50S subunit. When bound in the A site, however, this residue is more likely directed toward the interface between the two subunits, accounting both for the approximately equal linking to each subunit and for the decreased overall efficiency of covalent bond formation due to increased exposure to solvent.

All of the covalent linking from the P site was to 16S RNA (Schwartz and Ofengand, 1974), and 35% of the linking from the A site was also to the 16S RNA. Clearly, rRNA is directly involved in the tRNA binding site. On the other hand, we have not found any evidence for involvement of 23S RNA in the area of the A and P sites that we have studied so far. All of the linking to the 50S subunit (50% of the total) was to ribosomal protein, as was 30% of the linking to the 30S subunit (15% of the total). The identity of the proteins involved is currently under investigation.

The fact that reaction with protein was observed at the A site underscores our failure to find reaction with any proteins at the P site, and makes it clear that the active nitrene generated by irradiation would react with ribosomal proteins at the active site if they were there. Reaction with ribosomal protein is to be expected on theoretical grounds (Knowles, 1972) and has also recently been shown by others using similar aromatic azides attached to the aminoacyl end of tRNA (Hsiung et al., 1974; Hsiung and Cantor, 1974; Girshovich et al., 1974).

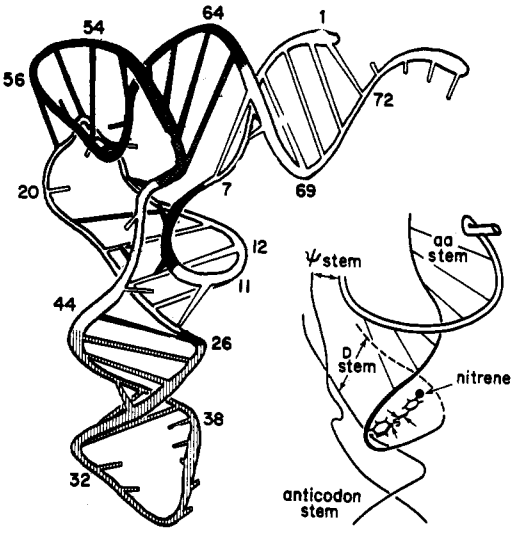

FIGURE 7: The proposed three-dimensional structure for yeast phenylalanine tRNA (Kim et al., 1974). Uridine-8 corresponds to the position of 4-thiouridine in *E. coli* valine tRNA. The inset shows a perspective drawing of part of the structure with the APA probe inserted in its most probable orientation. The pyrimidine ring of 4-thiouridine is at the left, the sulfur atom is indicated, and the solid ball (not to scale) is the nitrene atom. Bonds with free rotation which can affect the position of the nitrene are indicated by arrows.

Much earlier work has been done on the topography of ribosomal binding sites for tRNA by a variety of techniques, such as specific chemical (Noller and Chaires, 1972; Ginzburg et al., 1973) or immunological blocking (LeLong et al., 1974; Highland et al., 1974), deletion or supplementation of specific proteins (Randall-Hazelbauer and Kurland, 1972; van Duin et al., 1972) and protection by tRNA from some inactivating process (Noller and Chaires, 1972; Rummel and Noller, 1973). The current state of this work has been recently summarized (Pongs et al., 1974). However, all such indirect methods suffer from the disadvantage that on the one hand, a positive effect could be due to either direct action at the site or to an allosteric effect transmitted from a distant site, and, on the other hand, failure to find an effect may only mean that the interacting part of the ribosomal component is not involved in the active site. For example, anti-L18 and L25 do not inhibit tRNA binding (Highland et al., 1974), yet these proteins are known to be intimately associated with 5S RNA (Gray et al., 1973) which, in turn, is thought to interact directly with tRNA (Ofengand and Henes, 1969; Erdmann et al., 1973; Richter et al., 1973; Grummt et al., 1974). In addition, none of these approaches can give any information about what *region* of the tRNA is involved, while that is clearly a major advantage of the approach described in this paper.

The probable orientation of the photoaffinity probe with respect to the three-dimensional structure of tRNA is illustrated in Figure 7, using the model of yeast phenylalanine tRNA described by Kim et al. (1974) for illustrative purposes. The nitrene atom of the probe extends maximally 8.5 Å from the sulfur atom of the 4-thiouridine and at about a 120° angle from the C_4–S_4 vector. The APA group fits nicely into a pocket in the three-dimensional structure,

which no doubt accounts for the full retention of biological activity (Schwartz and Ofengand, 1974). A similar pocket exists in the model described recently by Robertus et al. (1974). As can be seen from the figure, the nitrene projects through the plane of the paper toward the rear, and thus those regions of the ribosome which become covalently attached must be within range when the noncovalent complex is formed. However, since it is not known if tRNA maintains its crystal structure on the ribosomal surface, the results obtained in this work cannot be used to construct a model of the tRNA–ribosome complex until these and similar probes are placed at different positions on the tRNA molecule, and the covalent linking patterns are correlated with each other. Such experiments are currently in progress.

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