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Evidence for Pyrimidine–Pyrimidine Cyclobutane Dimer Formation in the Covalent Cross-Linking between Transfer Ribonucleic Acid and 16S Ribonucleic Acid at the Ribosomal P Site†

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ABSTRACT: The covalent cross-linking between the anticodon of tRNA bound at the ribosomal P site and 16S ribosomal RNA which is induced by irradiation at wavelengths greater than 300 nm [Ofengand, J., Liou, R., Kohut, J., III, Schwartz, I., & Zimmermann, R. A. (1979) *Biochemistry 18*, 4322–4332] was shown not to involve any molecule of mRNA as a linker between tRNA and rRNA. After irradiation of a mixture containing oligo([^{3}H]G,U) (G:U = 1.1, average chain length 5), Ac[^{14}C]Val-tRNA, and ribosomes, isolation of the tRNA–ribosome complex by gel filtration in 0.1 mM Mg^{2+} followed by two cycles of dimethyl sulfoxide–sodium dodecyl sulfate denaturation and centrifugation on formamide–sucrose gradients gave a tRNA–rRNA complex with a mole ratio of Ac[^{14}C]valine to [^{3}H]guanosine of 5.6. In a similar experiment using oligo([^{3}H]$U_{1,2}$,G), the mole ratio of ^{3}H to ^{14}C was >50. Thus a minimum of 82% of the tRNA–rRNA complexes were free of any mRNA codons. The ribosome–tRNA covalent complex could be rapidly photolyzed by irradiation at 254 nm. With an incident light flux of 0.45 μEinstein min^{-1} mL^{-1}, the $T_{1/2}$ was 4 min. Both the ribosome and tRNA components which were photolytically split apart could again be cross-linked when reirradiated at 300 nm. Thus the cross-linking reaction was truly *photoreversible*. The kinetics of photolysis for *Escherichia coli* $tRNA_1^{Val}$ and $tRNA_1^{Ser}$ and *Bacillus subtilis* $tRNA^{Val}$ covalent complexes with ribosomes were virtually identical, suggesting a common structure for the cross-link in all three cases. The rate of photolysis at 254 nm was not affected by exposure to 0.1 M HCl at 23 °C for 15 min, ruling out oxetane structures for the cross-link. Photolysis of the *Escherichia coli* $tRNA^{Val}$–16S RNA complex could also be achieved by irradiation at 313 nm in the presence of the sensitizer 5-hydroxytryptamine or 5-hydroxytryptophan. No photolysis occurred when indole-3-carboxaldehyde was the sensitizer and almost none when no sensitizer was present. The sum of these properties is characteristic only of pyrimidine cyclobutane dimers, among the known photoinduced adducts of nucleic acids. Thus, we propose the structure of the cross-link to be such a dimer between the 5′-anticodon base of the tRNA and a pyrimidine in the 16S RNA.

Chemical and photochemical affinity labeling of macromolecules with large or small molecular weight ligands has become a commonly accepted tool in the biochemical armamentarium (Jakoby & Wilchek, 1977). Its utility in investigating the topography of the mRNA–tRNA–ribosome complex, the essential element in protein biosynthesis, is now well established (Kuechler & Ofengand, 1979; Ofengand, 1980). In our laboratory we have used photoactivatable derivatives of tRNA as photoaffinity probes to study the nature of the tRNA binding sites, the A and P sites, on the *E. coli* ribosome (Ofengand et al., 1980) in the belief that once the topography is known, the molecular processes involved in tRNA binding and translocation will become more evident.

As part of this program, we have recently described a unique photochemical cross-linking reaction which joins the anticodon of underivatized tRNA to the ribosome upon irradiation at wavelengths >300 nm (Schwartz & Ofengand, 1978; Ofengand et al., 1979). This cross-linking reaction is highly specific. It requires tRNA occupancy of the ribosomal P but not the

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A site, it links to the 3′ third (Zimmermann et al., 1979) of the 16S RNA but not to the 23S RNA or to the ribosomal protein, and only tRNAs with cmo^5U[1] or mo^5U at the 5′-anticodon position are competent for cross-linking. In view of the potential structural significance of such a direct and specific cross-link between tRNA and ribosomal RNA, the chemistry of the cross-link has been investigated further.

In this report, we prove that the cross-link is indeed directly between tRNA and rRNA without the participation of an mRNA linker, and we provide evidence to support the proposal that the structure of the cross-link is a pyrimidine–pyrimidine cyclobutane dimer.

Experimental Procedures

Chemicals. Ac[^{3}H]Val- and [^{14}C]Val-$tRNA_1^{Val}$ (*E. coli*), Ac[^{3}H]Val-$tRNA^{Val}$ (*B. subtilis*), Ac[^{3}H]Ser-$tRNA_1^{Ser}$ (*E. coli*), tight couple ribosomes, poly(U_2,G), and Sephacryl S-200 and S-300 were obtained or prepared as described previously (Ofengand et al., 1979). Dimethyl sulfoxide was redistilled and stored at −20 °C over molecular sieves. Formamide (Matheson, Coleman and Bell) was deionized just before use by shaking with 6 g of Bio-Rex RG501-X8 20–50-mesh mixed-bed resin (Bio-Rad) per 100 mL. 5-Hydroxytryptophan, 5-hydroxytryptamine (as the creatinine sulfate complex), and indole-3-carboxaldehyde were from Sigma.

Poly([^{3}H]$G_{1.1}$,U) and Poly([^{3}H]$U_{1.2}$,G). The polymers were prepared by reacting 7 mM UDP, 3.3 mM GDP, 0.15 M Tris, pH 8.7, 3 mM Mg^{2+}, 0.2 mM EDTA, and 4 units (Klee & Singer, 1967) per mL of *E. coli* polynucleotide phosphorylase (Type 25, P-L Biochemicals) at 37 °C. Polymerization was monitored by precipitation of aliquots with cold 5% Cl_3AcOH and filtration through cellulose nitrate membranes. Maximum insoluble radioactivity (83% of input) was reached in 60 min. The reaction was stopped at 120 min by dilution with 800 μL of 2% KOAc, pH 5, and extraction with 1 mL of phenol. The aqueous layer and wash were precipitated with 2 volumes of ethanol at −20 °C for 16 h, dissolved in 50 mM Hepes, pH 7.5, 50 mM NH_4Cl, and 0.5 mM Mg^{2+}, and chromatographed on a 1.5 × 26 cm column of Sephacryl S-200 in the same buffer. The sample eluted as a single symmetrical peak at 1.1 times the exclude volume of the column with 97% recovery; yield, 25–35 A_{260} units/mL of reaction mixture. The base composition was determined by enzymatic hydrolysis and high-pressure LC analysis (see below). The specific activities were for poly([^{3}H]$G_{1.1}$,U) 324 cpm/pmol of G residue and for poly([^{3}H]$U_{1.2}$,G) 152 cpm/pmol of U residue. The polynucleotides were almost as active as unlabeled poly(U_2,G) in the stimulation of noncovalent binding of AcVal-tRNA to ribosomes.

Alkali-Cleaved Poly([^{3}H]$G_{1.1}$,U) and Poly([^{3}H]$U_{1.2}$,G). The polynucleotides prepared as described above (28 or 39 μg) were incubated in 1.2 mL containing 17 mM Hepes, pH 7.5, 17 mM NH_4Cl, 0.17 mM Mg^{2+}, and 100 mM KOH for 100 s at 60 °C (Bock, 1967) to cleave ca. 20% of the phosphodiester bonds in the polymer. The reaction was stopped by addition of 0.12 mmol of HCl and 0.07 mmol of Hepes, pH 7.5, to a final volume of 1.4 mL. This solution was used as the source of mRNA codons in the ribosomal incubations.

Nucleotide Analysis of Tritiated Polynucleotides and Specific Activity Determination. The polynucleotides were digested in 60 μL of a mixture containing 1–2 μg of polymer, 40 mM Tris, pH 7.4, 15 μg of pancreatic RNase, and 100 units of RNase T_1 (Sankyo) at 37 °C for 45 min. The mixtures were heated to 100 °C for 2 min and, after being cooled, were diluted with 20 μL of a solution containing 83 mM $MgCl_2$ and 200 mM potassium phosphate, pH 5.7. Twenty-microliter samples were analyzed by high-pressure LC on a 4.6 × 250 mm LiChrosorb (Merck) RP-18 10-μm column equilibrated and eluted with 50 mM potassium phosphate, pH 5.5 (Tyson & Wickstrom, 1980), at 1.5 mL/min and ca. 2000 psi. The elution positions were reproducibly 1.8 ± 0.1 mL for Up and 6.8 ± 0.2 mL for Gp, corrected for the 3.0 ± 0.1 mL dead volume of the column. The effluent was monitored at 260 nm by a Laboratory Data Control Spectromonitor III, and the detector output was displayed on a Soltec 210 recorder (full scale = 0.02*A*). The peaks were quantitated by integration and converted to picomoles by reference to standards (130 and 260 pmol) chromatographed under the same conditions. Base ratios and concentrations were obtained by calculation from the measured picomoles per 20 μL injected. Specific activities were obtained by counting aliquots of the same enzyme digest solution used for the high-pressure LC.

Irradiation at 254 nm. Samples were irradiated in the Rayonet RPR-100 photochemical reactor by using one or two of the 254-nm lamps (instead of the usual 16). The sample was contained in a 12-mm i.d. quartz tube mounted in the center of the reactor and irradiated at room temperature. No heating occurred at the low light fluences used. The sample was stirred with a magnetic flea during irradiation, and the tube was masked off at the liquid meniscus. In this configuration, the number of incident photons measured by potassium ferrioxalate actinometry (Jagger, 1967) was proportional to the volume as well as to the irradiation time in the range of sample volumes used in this work, 1–6 mL.

Irradiation at 313 nm. Samples (0.6 mL) were placed in a 1-cm light path × 0.4 cm stoppered quartz cuvette fitted with a gas bubbling tube and placed in the standard cell compartment of a Perkin-Elmer MPF-4 spectrofluorometer at room temperature. The sample was degassed for 5 min before irradiation and mixed during irradiation by gentle bubbling of 99.9995% N_2 (Matheson Purity, Matheson Co.). The sample was irradiated at 313 nm with a 20-nm slit width, the shorter wavelengths being removed by a solution filter of 1 cm of xylene (No. X-5, Fisher Scientific). There was 79% transmission at 313 nm, 10% transmission at 293 nm, and 1% transmission at 289 nm through this filter. Under these conditions, the amount of light incident on the sample was 0.102 μEinstein/min as determined by potassium ferrioxalate actinometry (Jagger, 1967).

tRNA–16S RNA Covalent Complexes. P site complexes between Ac[^{3}H]Val-tRNA and ribosomes were prepared and irradiated with 300-nm lamps plus the Mylar filter for 3 h as described previously (Ofengand et al., 1979). Forty-eight percent cross-linking was obtained. After EtOH precipitation, the pellet containing 37 A_{260} units of ribosomes and 440 pmol of Ac[^{3}H]Val-tRNA was dissolved in 122 μL of 16 mM Mes, pH 6.0, 82 mM NH_4Cl, 0.25 mM Mg^{2+}, 2.5 mM EDTA, and 1.6% $NaDodSO_4$ and incubated at 37 °C for 1 min with gentle shaking. The sample was then diluted with 300 μL of 20 mM Mes, pH 6.0, 100 mM NH_4Cl, 0.3 mM Mg^{2+}, and 3 mM EDTA (buffer A), applied to two 5–27% isokinetic sucrose gradients containing buffer A plus 0.1% $NaDodSO_4$, and centrifuged in the Spinco SW40 rotor at 31 000 rpm for 16 h at 20 °C. As expected (Schwartz & Ofengand, 1978), cross-linked Ac[^{3}H]Val-tRNA was found in the 16S RNA

[1] Abbreviations used: mo^5U, 5-methoxyuridine; cmo^5U, 5-(carboxymethoxy)uridine; AcVal- and AcSer-, *N*-acetylvalyl- and *N*-acetylseryl-, respectively; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate.

peak. The tRNA–16S RNA from both gradients was precipitated with 67% EtOH–2% KOAc (pH 5), dissolved in 0.5 mL of water, and desalted on an 0.9 × 29 cm column of Sephacryl S-200 equilibrated with 10 mM potassium phosphate buffers, pH 7.0. The peak fractions were pooled. The final specific activity was 1.2×10^5 cpm (19.7 pmol) per A_{260} unit of 16S RNA which is 29% pure assuming 67 pmol per A_{260} unit for 16S RNA.

$NaDodSO_4$–Dimethyl Sulfoxide Denaturation. Ac[^{14}C]-Val-tRNA–ribosome covalent complex, precipitated with 1–2 volumes of ethanol as described in the individual figure legends, was dissolved in 30 mM cacodylate buffer, pH 5.8, 3 mM EDTA, and 1.4% $NaDodSO_4$ and incubated at 37 °C for 2 min. Dimethyl sulfoxide was added to 68%, and incubation was continued for 30 min longer. This procedure did not liberate any acid-soluble ^{14}C radioactivity. The sample was then applied to a 5–27% isokinetic sucrose gradient containing 70% deionized formamide, 10 mM cacodylate, pH 5.8, 1 mM EDTA, and 0.1% $NaDodSO_4$ and centrifuged at 20 °C as specified in each case.

Other Methods. P site binding, irradiation at 300–350 nm, and assay for cross-link formation by nitrocellulose filtration at 0.1 mM Mg^{2+} were as previously described (Ofengand et al., 1979) except as noted in the individual experiments. Percent cross-linking is the ratio of covalently bound tRNA to noncovalently bound tRNA times 100. The concentrations of 5-hydroxytryptophan, 5-hydroxytryptamine, and indole-3-carboxaldehyde were determined spectrophotometrically; 5-hydroxytryptophan ϵ_{300} (EtOH) = 5755 Heinzelman et al., 1960), 5-hydroxytryptamine ϵ_{275} (H_2O) = 5480 (McMenamy & Oncley, 1958), and indole-3-carboxaldehyde ϵ_{302} = 13 500 (Morton & Fahmy, 1959).

Results

Absence of an mRNA Spacer from the 16S RNA–tRNA Covalent Complex. In our previous papers (Ofengand et al., 1979; Zimmermann et al., 1979), we showed that there was no protein spacer between the 16S RNA–tRNA covalent complex and that if there was a linking molecule of mRNA, the cross-links to 16S RNA and to tRNA could not be more than two nucleotides apart. This conclusion was based on the fact that cross-link formation with the triplet, GpUpU, was as efficient as with poly(U_2,G). However, for a further understanding of the nature of the cross-link, it was necessary to prove unequivocally that there was no mRNA linking the 16S RNA to the tRNA. Poly([^{3}H]$G_{1.1}$,U) was prepared by condensation of [^{3}H]GDP and UDP with polynucleotide phosphorylase as described under Experimental Procedures. Preliminary experiments using this polynucleotide as mRNA showed that the blank levels of radioactivity bound to ribosomes in the absence of irradiation were unacceptably high. Consequently, since we had already shown that the triplet, GpUpU, could effectively induce both binding and cross-linking, the poly($G_{1.1}$,U) was cleaved by alkali to an average chain length of five in order to reduce nonspecific binding effects. As will be shown below, this treatment was effective.

It was first necessary to show directly that the alkali-cleaved poly([^{3}H]$G_{1.1}$,U) was both necessary for and capable of supporting the cross-linking reaction (Figure 1). In this experiment, analysis was done by gel filtration at 0.1 mM Mg^{2+} where noncovalent complexes dissociate. Control experiments have shown that in the absence of irradiation, no tRNA is found at the elution position of the first peak even when poly(U_2,G) was present (Ofengand et al., 1980). In the absence of mRNA codons, only 3.4% of the Ac[^{14}C]Val-tRNA was found in the ribosome region of the column (peak 1) after

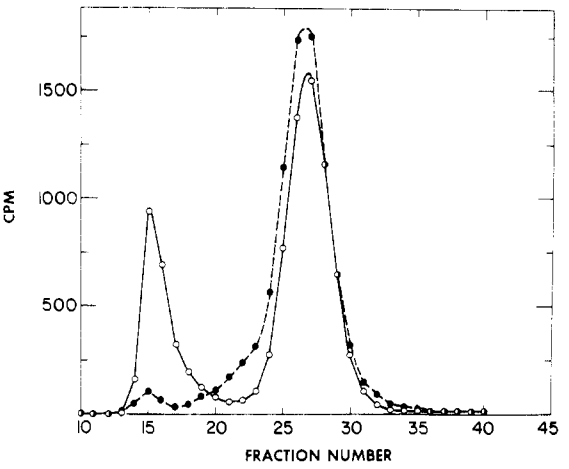

FIGURE 1: Gel filtration of irradiated tRNA–ribosome P site complexes with and without mRNA. The standard P site binding incubation was modified to contain 15 mM Mg^{2+}, 5.4 μg/mL alkali-cleaved poly([^{3}H]$G_{1.1}$,U), and 120 nM Ac[^{14}C]Val-tRNA. After 15 min at 37 °C, the mixture was irradiated (300-nm lamps without the Mylar filter) for 90 min, precipitated with 50% EtOH–2% KOAc, pH 5, dissolved in 50 mM Tris, pH 7.4, 50 mM KCl, and 0.1 mM Mg^{2+}, and chromatographed on a 1.5 × 28 cm column of Sephacryl S-300 in the same buffer at 4 °C; 1-mL fractions were collected and counted. (○) Plus or (●) minus alkali-cleaved poly($G_{1.1}$,U) in the incubation mixture; 27.7% or 3.4%, respectively, of the tRNA added was found in the first, or ribosome-associated, peak. The second peak corresponds in position to free AcVal-tRNA.

irradiation, while 27.7% of the ^{14}C became covalently attached when mRNA was present. For clarity, the [^{3}H]polynucleotide pattern, which showed considerable ^{3}H at the peak 1 position (approximately 36% of the total recovered) as well as elsewhere in the elution profile, has been omitted from the figure.

The peak 1 material containing ribosomes, Ac[^{14}C]Val-tRNA, and [^{3}H]polynucleotide from a larger scale reaction was treated with $NaDodSO_4$ to dissociate the 16S RNA from ribosomal protein. After denaturation at 37 °C in the presence of 64% dimethyl sulfoxide, the preparation was centrifuged through a 70% formamide-containing sucrose gradient (Pawson et al., 1977). The bottom part of this gradient is shown in the inset to Figure 2. Clearly the Ac[^{14}C]Val-tRNA–16S RNA had moved down the gradient, but the background of ^{3}H was still too high. Therefore, the ^{14}C peak was collected, freed of sucrose by precipitation, and again denatured and centrifuged (Figure 2). This second step reduced the ^{3}H background sufficiently to demonstrate that less than 18% of the Ac[^{14}C]Val-tRNA–16S RNA complexes contained even one guanosine residue of mRNA. The picomoles of ^{3}H was calculated for one G residue since at least one G must be present for every codon bound according to the coding properties for this tRNA. The same is true for cross-linking, for we have previously shown that poly(U) does not support cross-linking, whereas both poly(U_2,G) and GpUpU do (Ofengand et al., 1979).

An objection to this conclusion could be that while a G residue is necessary for codon-dependent binding, cross-linking might only involve the U residues of the codon. Consequently, if the [^{3}H]G residue were removed during the purification of the tRNA–rRNA complex by a contaminating nuclease, it would not be possible to detect the presence of cross-linked U residues derived from the codon. To meet this objection, the same polynucleotide was prepared, but with ^{3}H in the U moiety. Oligo([^{3}H]$U_{1.2}$,G) prepared as described above by mild alkaline hydrolysis, was used to direct Ac[^{14}C]Val-tRNA–ribosome complex formation. After irradiation, the covalent complex was isolated by gel filtration and purified

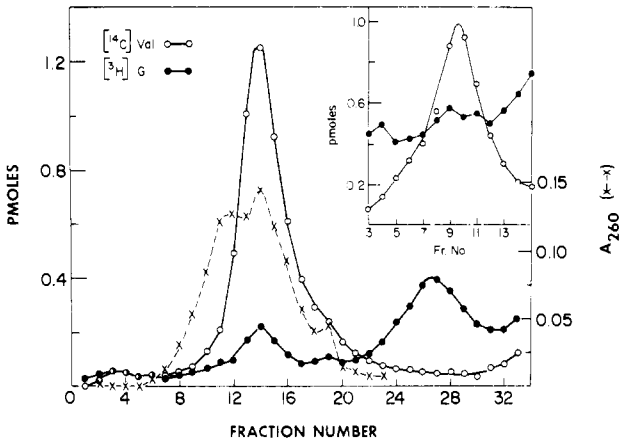

FIGURE 2: Formamide–sucrose gradient purification of Ac[^{14}C]-Val-tRNA–16S RNA covalent complexes from [^{3}H]G-containing codons. Ac[^{14}C]Val-tRNA–ribosome covalent complexes were prepared by incubation, irradiation, precipitation, and gel filtration exactly as in Figure 1. The first (ribosome-associated) peak was precipitated with ethanol as above, treated with $NaDodSO_4$, denatured with dimethyl sulfoxide, and layered on a formamide–sucrose gradient as described under Experimental Procedures. Centrifugation was in the Spinco SW56 rotor at 55 000 rpm for 17 h; 125-μL samples were collected and 30-μL samples were analyzed for ^{14}C and ^{3}H by double-channel counting. Part of this gradient is shown in the inset. Fractions 3–14, containing 83% of the total ^{14}C recovered from the gradient, were dialyzed vs. 20 mM cacodylate, pH 5.8, and then precipitated with 2.5 vol of ethanol at −20 °C for 16 h; 63% of the pooled ^{14}C was recovered. The precipitate was again denatured with $NaDodSO_4$–dimethyl sulfoxide and layered on a second formamide gradient. Centrifugation was as above but at 50 000 rpm; 78% of the applied ^{14}C was recovered. (○) ^{14}C; (●) ^{3}H; (×) A_{260}.

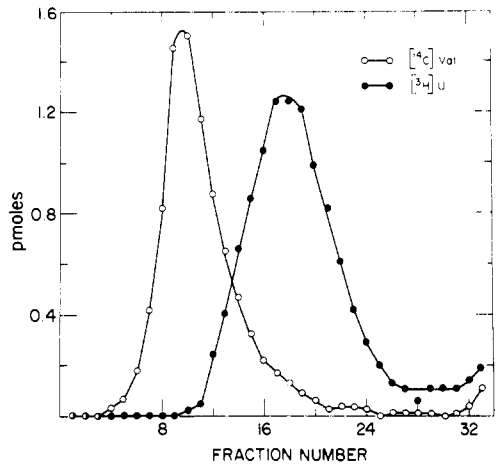

FIGURE 3: Formamide–sucrose gradient separation of tRNA–rRNA covalent complexes from [^{3}H]U-containing codons. Ac[^{14}C]Val-tRNA–rRNA covalent complexes were prepared by two sequential centrifugations through formamide–sucrose gradients as described in Figure 2 except that 4.2 μg/mL alkali-cleaved poly([^{3}H]$U_{1,2}$,G), 100 nM Ac[^{14}C]Val-tRNA, and irradiation *with* the Mylar filter for 180 min were used to prepare the complexes. The heaviest 76% of the ^{14}C peak from the second gradient was dialyzed and concentrated by EtOH precipitation. After a third $NaDodSO_4$–dimethyl sulfoxide treatment, the sample was again centrifuged through a formamide gradient; 80% of the applied ^{14}C was recovered. (○) ^{14}C; (●) ^{3}H.

by repeated gradient centrifugation exactly as described above. The results of the third successive gradient are shown in Figure 3. Clearly, the main part of the Ac[^{14}C]Val-tRNA–16S RNA peak (fractions 4–11) contains less than 1.5 mol of U residue per 100 mol of complex. Therefore, neither G nor U residues of the mRNA are to be found in the tRNA–rRNA covalent complex.

Cross-Linking Is Reversible by Irradiation at 254 nm. We have already shown that the bond linking the tRNA to 16S

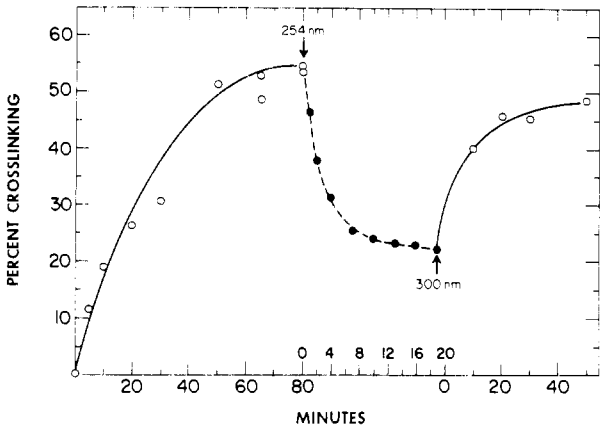

FIGURE 4: Reversibility of cross-link formation by UV light. Ribosomal P site complexes, formed with 20 μg/mL poly(U_2,G), 6.3 A_{260} units/mL tight couple ribosomes, and 230-nM AcVal-tRNA, were irradiated with 300-nm lamps without the Mylar filter. Samples (open circles) were assayed for cross-link formation. At 80 min, the sample was transferred to a quartz tube and irradiated at 254 nm as described under Experimental Procedures. The incident light intensity was 0.45 μEinstein min^{-1} mL^{-1}. Samples (filled circles) were assayed as before. At 20 min, the samples were transferred back to the original irradiation system and reirradiated. The level of noncovalent binding was 83, 77, and 67 nM for the first, second, and third irradiations, respectively.

RNA can be split with first-order kinetics by irradiation at 254 nm (Ofengand et al., 1979). We now show that not only can this photolysis be extended to ribosome–tRNA complexes but also that it is a fully *reversible* reaction. Figure 4 demonstrates that AcVal-tRNA–ribosome covalent complexes formed by irradiation with 300-nm lamps can be split simply by changing the irradiation wavelength to 254 nm. Complete cleavage was not obtained, however, probably because other nonreversible cross-links between the noncovalently bound tRNA and the ribosome were formed concomitantly with the photolysis of the preformed cross-link. The covalent link could be re-formed to the same extent simply by reirradiation at 300 nm. Thus, this experiment not only shows that the covalent link can be broken by 254-nm irradiation but also that the ribosomes were not damaged by either 300- or 254-nm irradiation, since they could not only rebind the tRNA but re-form the cross-link as well. Note that the ribosomes were limiting in this experiment. Therefore the same active ribosomes which were originally cross-linked and photolyzed could again be cross-linked when the irradiation wavelength was shifted back to 300 nm.

Regeneration of the tRNA portion of the cross-linked complex could not be shown in this experiment since excess tRNA was present in the reaction mixture. Thus, new tRNA could have become cross-linked during the second 300-nm irradiation. This possibility was examined in a separate experiment. The covalent complex, formed by irradiation with 300-nm lamps, was freed from noncovalently bound tRNA by gel filtration in 0.1 mM Mg^{2+} (Figure 5A). According to the distribution of tRNA in the elution profile, 34% of the added tRNA had become cross-linked, which corresponded to the cross-linking of 37% of the ribosomes. Irradiation of this purified complex at 254 nm rapidly split off AcVal-tRNA with first-order kinetics (Figure 6). The released AcVal-tRNA was separated from nonphotolyzed tRNA by gel filtration (Figure 5B). The free tRNA peak was concentrated by lyophilization and used as the source of regenerated AcVal-tRNA, free of both unreacted AcVal-tRNA and of tRNA–ribosome complexes, for cross-linking to fresh ribosomes (Figure 7). Clearly the regenerated tRNA (solid circles) was as active as the untreated control tRNA (open

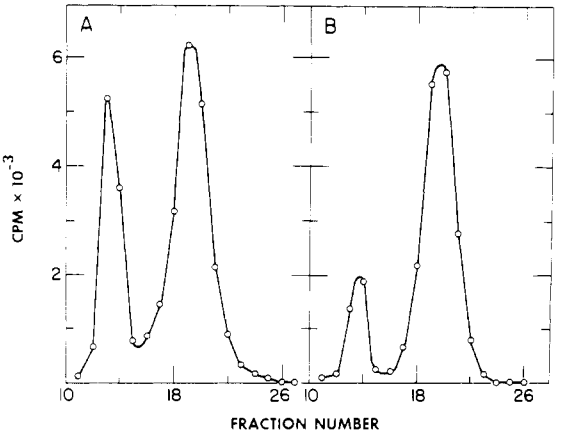

FIGURE 5: Separation of ribosome–tRNA complexes from tRNA by gel filtration. Cross-linked ribosome–tRNA complexes were prepared by incubation and irradiation (300-nm lamps without the Mylar filter) for 90 min as described under Experimental Procedures with 6.3 A_{260} unit/mL ribosomes and 172 nM AcVal-tRNA. (A) Separation of cross-linked from un-cross-linked tRNA. The reaction mixture was precipitated with 2% KOAc, pH 5, and 67% ethanol, redissolved in 50 nM Hepes, pH 7.5, 50 mM NH_4Cl, and 0.1 mM $MgCl_2$, and chromatographed on a 1.5 × 28 cm column of Sephacryl S-200 equilibrated with the same buffer. The first peak, which was the ribosome–tRNA complex, contained 34% of the total tRNA present. Overall recovery, 97%. The second peak corresponds in elution position to free AcVal-tRNA. (B) Separation of tRNA split off by UV irradiation from ribosome–tRNA complexes. After 10-min UV irradiation of the purified ribosome–tRNA complex of part A as described in Figure 6, the sample was rechromatographed on a 1.5 × 28 cm column of Sephacryl S-200 which was equilibrated with 5 mM Hepes, pH 7.5, 5 mM NH_4Cl, and 0.7 mM Mg^{2+}; 83% of the recovered tRNA was found at the free tRNA elution position. Overall recovery, 88%. Fractions 18–22 were combined and lyophilized to one-tenth the original volume.

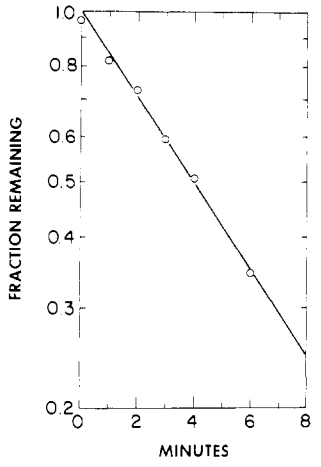

FIGURE 6: Kinetics of splitting of the ribosome–tRNA covalent link by 254-nm light. Fractions 12–14 of Figure 5A were cooled. The ribosome–tRNA complex (25 nM, A_{260} = 3.7) was placed in a quartz tube and irradiated with 254-nm lamps as described under Experimental Procedures. The incident light intensity was 0.45 μEinstein min^{-1} mL^{-1}. Samples were assayed for covalently bound tRNA by filtration at 0.1 mM Mg^{2+} as described under Experimental Procedures. The data have been corrected for the UV-resistant fraction (4%). $T_{1/2}$ was 4 min. There was no loss of Cl_3AcOH precipitable counts per minute as a result of the 10-min irradiation.

circles) for cross-linking. Therefore, both the ribosome and tRNA are *regenerated* by UV-induced splitting of the covalent complex.

Common Nature of the Cross-Link between 16S RNA and Three Different Reactive tRNAs. It has already been shown that tRNAs containing cmo^5U or mo^5U at their 5′-anticodon positions are capable of the cross-linking reaction described

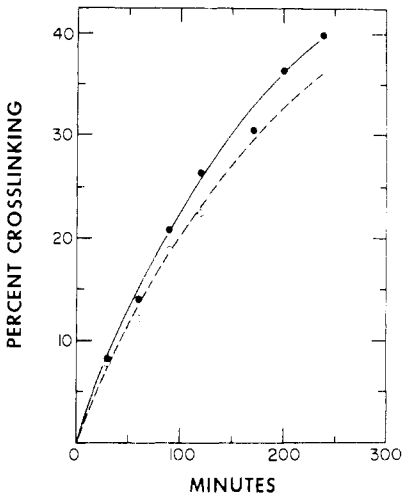

FIGURE 7: Cross-linking activity of AcVal-tRNA split off from the ribosome–tRNA complex by 254-nm light. Ribosomal P site complexes formed as in Figure 4 but at 8 mM Mg^{2+} were irradiated with 300-nm lamps plus the Mylar filter for the indicated times. Control untreated AcVal-tRNA (○) was added at 42 nM, and AcVal-tRNA split off from ribosomes by UV irradiation (●) was present at 40 nM. Regenerated AcVal-tRNA was prepared by irradiating the ribosome–tRNA complex fraction from the Sephacryl column of Figure 5A for 10 min under the conditions given in Figure 6, followed by purification from residual tRNA–ribosome complexes as shown in Figure 5B; 63% of control tRNA and 69% of the regenerated tRNA were noncovalently bound. No cross-linking was found after incubation in the absence of irradiation.

Table I: Rate of Photolysis of tRNA–Ribosome Covalent Complexes[a]

tRNA	anticodon[b]	rate constant[c] (min^{-1})
Val_1 (*E. coli*)	cmo^5U-A-C	1.27
Val (*B. subtilis*)	mo^5U-A-C	1.18
Ser_1 (*E. coli*)	cmo^5U-G-A	1.20

[a] Covalent ribosome–tRNA complexes were prepared by irradiation and freed of unreacted tRNA gel filtration as in Figure 5A. Photolysis with a light intensity of 0.22 μEinstein min^{-1} mL^{-1} and analysis was carried out as in Figure 6 and the rate constant evaluated from a semilogarithmic plot (Wang, 1962) of the data after correction for the blank value. [b] Sequence data for *E. coli* $tRNA_1^{Val}$ and $tRNA_1^{Ser}$ from Barrell & Clark (1974) and for *B. subtilis* $tRNA^{Val}$ from H. Ishikura (personal communication). [c] The maximum rate constant was obtained by photolysis of various dilutions of the sample until the rate constant did not increase upon further dilution.

here (Ofengand et al., 1979). These tRNAs include *E. coli* $tRNA_1^{Val}$ and $tRNA_1^{Ser}$ and *B. subtilis* $tRNA^{Val}$ and $tRNA^{Thr}$. The first three of these were selected for the following study as representative of cmo^5U-containing vs. mo^5U-containing tRNAs and of cmo^5U-containing tRNAs with different adjacent anticodon bases. The relevant anticodons are listed in Table I, together with the rate constant for photolysis of their respective tRNA–30S complexes. Clearly, the rate of photolysis of the three tRNA–ribosome complexes was essentially identical. In this connection, note that the reactive tRNA base is cmo^5U in *E. coli* $tRNA_1^{Val}$, but mo^5U in *B. subtilis* $tRNA^{Val}$. Also, while the codon for $tRNA_1^{Val}$ is GU(G,A,U) [poly(U_2,G) was used], that for Ser_1 is UC(G,A,U) [poly(U,C,A) was used]. The virtually constant rate of photolysis suggests that the 5′-anticodon base of tRNA is directly connected to the same base of 16S RNA in all three cases.

Sensitized Photolysis of the tRNA–16S RNA Cross-Link at 313 nm. Thymine cyclobutane dimers in DNA (Charlier & Hélène 1975; Hélène & Charlier, 1977) and uridine dimers

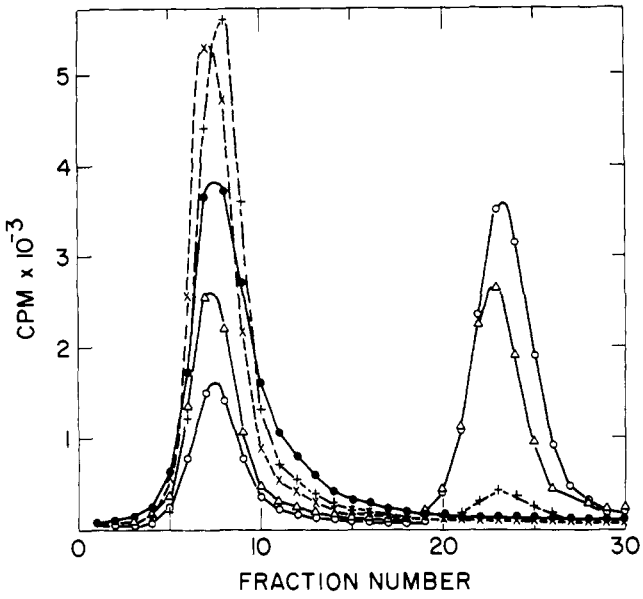

FIGURE 8: Sensitized photolysis of tRNA–RNA complexes by irradiation at 313 nm. Ac[^{3}H]Val-tRNA–16S RNA covalent complexes, prepared as described under Experimental Procedures, were irradiated for 30 min at 313 nm at a concentration of 9.6 nM (total A_{260} = 0.49) in 32 mM potassium phosphate buffer, pH 7, containing 1 mM sensitizers as indicated below. The entire sample (0.6 mL) was applied to a 5–27% isokinetic sucrose gradient in buffer A plus 0.1% Na-DodSO$_4$ and centrifuged in the Spinco SW40 rotor (20 °C, 31 000 rpm, 17 h). Collection was from the bottom of the tube. (○) 5-Hydroxytryptophan; (●) indole-3-carboxaldehyde; (△) 5-hydroxytryptamine; (×) 5-hydroxytryptophan or 5-hydroxytryptamine in the absence of light; (+) no sensitizer added.

Table II: Indole-Sensitized Photolysis of the tRNA–16S RNA Adduct at 313 nm[a]

sensitizer	concn (mM)	irradiation time (min)	percent photolysis	$T_{1/2}$ (min)	average $T_{1/2}$ (min)
none		30	4.5	452	442
		60	9.2	431	
indole-3-carboxaldehyde	1	30	0.2	10400	26 000
	1	60	0.1	41 600	
5-hydroxytryptamine	1	20	61.1	14.7	21.0
	1	30	53.3	27.3	
	1	30[b]	0[b]	∞[b]	
	2	30	64.3	20.2	20.2
5-hydroxytryptophan	1	20	54.3	17.7	16.7
	1	30	73.7	15.6	
	2	20	45.7	22.7	20.1
	2	30	69.8	17.4	
	4	60[b]	0[b]	∞[b]	
	4	60[c]	0[c]	∞[c]	
	6	120	100		

[a] Covalent tRNA–16S RNA adducts were prepared and irradiated as described in the legend to Figure 8 except that the indicated irradiation times were used; 1-min irradiation equals 0.10 μEinstein incident on the sample. $T_{1/2}$ was calculated as $[0.693t]/[-\ln(1 - \text{fraction split at time } t)]$. Fraction split was determined by sucrose gradient centrifugation (see Figure 8) to separate free tRNA from the tRNA–16S RNA adduct. [b] Incubation in the irradiation apparatus with the shutter closed. [c] Irradiation with O_2 in place of N_2.

in RNA (Chen et al., 1976) can be photolyzed in the presence of certain indole-containing compounds by irradiation at 313 nm, a wavelength at which the indole sensitizer absorbs but the cyclobutane dimer does not. Only certain indole derivatives possess this property, among them 5-hydroxytryptamine (Charlier & Hélène, 1975) and 5-hydroxytryptophan (Chen et al., 1976; Hélène & Charlier, 1971a,b). Other indoles such as indole-3-carboxaldehyde cannot sensitize the photolysis of uridine cyclobutane dimers (Chen et al., 1976). The reason for this specificity is thought to lie in the electron-donating properties (active species) vs. electron-accepting properties (inactive species) of the particular indole derivative (Chen et al., 1976).

We have used this property of pyrimidine cyclobutane dimers to be selectively photolyzed only in the presence of electron-donating indoles as a way to further characterize the tRNA–16S RNA cross-link. As shown in Figure 8, irradiation of the tRNA–RNA adduct in the presence of 5-hydroxytryptophan or 5-hydroxytryptamine readily split off the tRNA from the adduct while no photolysis occurred in the presence of indole-3-carboxaldehyde. In all three cases, the concentration of indole was sufficiently high to absorb >97% of the incident radiation. There was a small amount of photolysis in the absence of sensitizer, but in the absence of irradiation, there was no photolysis, even after incubation with 5-hydroxytryptophan or 5-hydroxytryptamine. There results are summarized in Table II along with additional data which confirm the specificity of the photolytic reaction. In particular, lines 7 and 13 show that irradiation is required, line 14 demonstrates that O_2 can completely quench the reaction, and line 15 shows that all of the adduct can be split by sufficient irradiation.

Resistance of 254-nm Photolysis to Acid Treatment. Because oxetanes as well as cyclobutane dimers can be photolytically reversed by 254-nm light (Fisher & Johns, 1976; Varghese, 1975), treatment with acid was used to distinguish between these two possibilities. Oxetanes are very unstable and readily undergo acid-catalyzed ring opening to the hydroxy derivatives (Wang, 1971, 1976). These latter derivatives, in which the two pyrimidines are joined by a single C–C bond from C_4 to C_5 or C_6, are not susceptible to photolysis at 254 nm (Wang, 1976; Rhoades & Wang, 1970). Consequently, to test for the presence of oxetanes in the tRNA–rRNA adduct, the adduct was treated at 23 °C for 15 min with 0.1 N HCl, a condition expected to open any oxetane rings (A. J. Varghese, personal communication), and then irradiated at 254 nm (Table III). It is clear that the acid treatment had no effect on the subsequent ability of the cross-link to be photolyzed at 254 nm and thus that oxetanes were not present.

Discussion

As the direct covalent linking of tRNA to 16S RNA by long-wavelength UV irradiation was a very unexpected finding as well as one of considerable potential importance for our understanding of the interactions between tRNA and ribosomal RNA at the ribosomal decoding site, it was necessary to first definitively establish the number of macromolecules involved in this cross-link. The evidence for lack of involvement of a ribosomal protein(s) was previously supplied (Ofengand et al., 1979; Zimmermann et al., 1979).

The evidence against an mRNA spacer has been documented in this paper. More than 82% of the complexes were free of mRNA when [^{3}H]G-labeled mRNA was used, assuming only one G residue per codon. If, however, it is recalled that the G:U base ratio was 1.1 and the average chain length was calculated to be 5, then the average oligonucleotide containing either a GUU or GUG codon would consist of, on the average, 2.6 G and 2.4 U residues. Consequently, it is likely that there should be 2.6 G residues per complex, and the

Table III: Effect of Acid Treatment on Photolysis at 254 nm[a]

irradiation time (min)	percent photolysis	
	control	acid-treated
0		0
2	61	71
4	80	83

[a] tRNA-16S RNA adduct (9.3 pmol), prepared as described under Experimental Procedures, was incubated for 15 min at 23 °C in 0.75 mL containing 75 μmol of HCl and 1.5 μmol of potassium phosphate, pH 7. The sample was then neutralized with 75 μmol of NaOH and adjusted to 2% KOAc, pH 5, 0.92 mL. After precipitation with 2 volumes of EtOH at −20 °C for 30 min, the pellet was dissolved in 0.24 mL containing 64% Me_2SO, 30 mM potassium phosphate, pH 6.5, and 10 mM EDTA and incubated for 30 min at 37 °C. The sample was then diluted with H_2O to 0.75 mL and irradiated at 254 nm (0.22 μEinstein min^{-1} mL^{-1}) as described under Experimental Procedures for the times indicated. The fraction photolyzed was determined as in Table II except that formamide–sucrose gradient centrifugation was used as in the legend to Figure 2. For the control, tRNA–rRNA adduct was only added after the neutralized 2% KOAc mixture was prepared. It was also incubated at 23 °C for 15 min and processed in exactly the same way as the acid-treated sample.

fraction of complexes free of mRNA codons can then be reasonably estimated to be as high as 93%. This calculation does not consider the possible partial hydrolysis of acetylvaline from the tRNA. To the extent that this occurs, the true percentage figure would be even higher. Moreover, when [^{3}H]U-labeled poly($U_{1,2}$,G) was used, an even better separation of the [^{3}H]U residue from the ^{14}C-labeled complex was obtained. If only the fast-sedimenting 63% of the ^{14}C-containing complex is considered (fractions 4–11), more than 98% of the complexes were free of even a single residue of U. There can be no doubt, therefore, that the cross-link described in this paper is directly between the tRNA and the 16S RNA.

These results also show that no cross-linking of mRNA to rRNA occurs under these irradiation conditions, even in the absence of tRNA cross-linking to either RNA. The purification steps used in Figures 1–3 would not separate mRNA–rRNA from the tRNA–rRNA adducts, and these mRNA–rRNA complexes would then be counted as contributing mRNA codons to the rRNA–tRNA adduct. However, very little of this was found, in contrast to the results of others who have used shorter wavelength irradiation (Schenkman et al., 1974; Fiser et al., 1975; Margaritella & Kuechler, 1978).

The ability to be rapidly split by short-wavelength UV light with *regeneration* of the original reactants is a characteristic feature of pyrimidine–pyrimidine cyclobutane dimers. Although a variety of nucleotide–nucleotide adducts are known (see below), some of which can be photolyzed at 254 nm, the only ones known to regenerate the original reactants upon photolysis are the cyclobutane dimers of pyridimines (Fisher & Johns, 1976) and the analogous oxetane adducts of pyrimidines (Varghese, 1975). Thus, it was quite important to be able to demonstrate the reversibility of the photolysis of the tRNA–rRNA adduct, as shown in Figures 4–7. Cyclobutane dimers are readily distinguished from oxetanes since the latter are unstable in aqueous solution (Wang, 1971, 1976), normally being immediately converted to bipyridimine adducts which are resistant to photolysis at 254 nm (Rhoades & Wang, 1970; Wang, 1976). However, since one oxetane structure was isolated and crystallized (Varghese, 1975), a more rigorous test of the presence of oxetanes in the rRNA–tRNA adduct was carried out by treating the adduct with acid under conditions known to open oxetane rings, followed by irradiation at 254 nm. As was shown in Table III, the acid treatment had no effect, demonstrating the absence of any oxetane-like structures. Thus, it is most likely that the structure of the cross-link is a pyrimidine–pyrimidine cyclobutane dimer. Such a structure can explain the tRNA specificity for cross-linking which we reported previously (Ofengand et al., 1979). As we discussed in that report, the failure of tRNAs possessing C, f^5U, or G in the 5′-anticodon position to be cross-linked can be understood in terms of their known inability to form cyclobutane dimers.

The behavior of the tRNA–rRNA adduct to sensitized photolysis at 313 nm is a further indication that the structure is a cyclobutane dimer. Irradiation at this wavelength in the absence of sensitizer had almost no effect, but electron-donating indoles such as serotonin (5-hydroxytryptamine) and 5-hydroxytryptophan sensitized the cross-link to rapid photolysis. Electron-accepting indoles such as indole-3-carboxaldehyde were inert. These properties are just those expected of cyclobutane dimers (Chen et al., 1976). The sensitized photolysis experiments were done under magnesium-free low salt conditions in order to minimize any noncovalent aggregation of rRNA and tRNA since the indoles used as sensitizers are known to sensitize cyclobutane dimer formation in addition to photolysis under the proper steric conditions (Charlier & Hélène, 1975). In our case, however, sufficient irradiation gave 100% photolysis (see Table II).

Other known types of nucleotide adducts have properties different from those of the tRNA–rRNA complex described in this paper. The classes of bipyrimidine photoproducts derived from pyrimidine radicals (Wang, 1976; Cabrera-Juarez & Setlow, 1977) are unlikely candidates because not only are they resistant to photolysis at 254 nm (Wang, 1976; Cabrera-Juarez & Setlow, 1977) while the tRNA–rRNA adduct was sensitive but also their formation should be blocked by free-radical quenching agents while tRNA–rRNA cross-linking was not (Ofengand et al., 1979). The bipyrimidines derived from oxetanes or azetidines (Wang, 1976) are also not likely candidate structures since, as mentioned above, they are also resistant to short-wavelength (225–290 nm) irradiation (Wang, 1976; Patrick, 1970). Moreover, while they can be photolyzed at 313 nm (Patrick, 1970), the tRNA–rRNA complex was quite resistant (Table II). Other more complex adducts like those described by Sarpotdar & Burr (1978) are ruled out by their failure to be monomerized by short-wavelength UV light.

Likewise, the participation of adenine or guanine residues in the 16S RNA is unlikely since all addition reactions known for purines involve radical mechanisms (Elad, 1976a,b), while radical quenching agents did not block tRNA–rRNA cross-linking, as mentioned above. The reaction of purines in nucleic acids appears to require local denaturation (Ben-Ishai et al., 1973), so that it is unlikely that the cross-linkable partners could be protected from the quenching agents by structural effects. Moreover, while purine adducts can be photoreversed by >290-nm light plus sensitizer, they are stable to >260-nm light in the absence of sensitizer (Salomon & Elad, 1974).

Other adducts of pyrimidines involving hydroxy, amino, or SH groups, such as the side chain of cysteine (Varghese, 1973; 1976), are also unlikely either for reasons of stability to 254-nm photolysis, failure to regenerate reactants, or failure to react in the presence of oxygen. Adduct formation with amino acid side chains of ribosomal proteins has been ruled out in the present case by the protease digestion experiments described previously (Zimmermann et al., 1979).

In our previous report (Ofengand et al., 1979), we pointed out that the relationship of the 5′-anticodon base of the tRNA

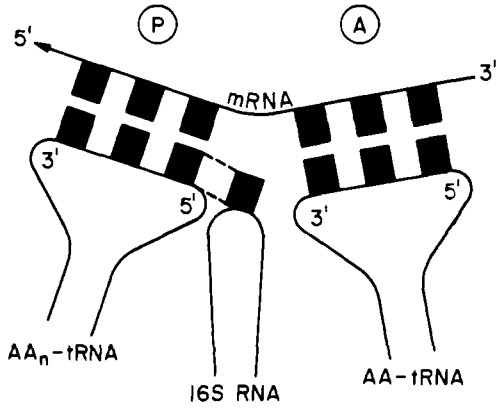

FIGURE 9: Schematic diagram of tRNA in the P site interacting with 16S RNA while engaged in codon translation (Ofengand et al., 1980). AA_n-tRNA, peptidyl tRNA in the P site; AA-tRNA, aminoacyl-tRNA in the A site. The arrangement of tRNA and mRNA shown on the figure is dictated by the fact that mRNA is translated from the 5′ to the 3′ end (Schweet & Heintz, 1966) and codon–anticodon base pairing is antiparallel (Ofengand, 1977). The arrow shows the direction of mRNA movement past a stationary ribosome. A loop of 16S rRNA is inserted such that one of its pyrimidine bases is brought sufficiently close to the 5′-anticodon base of the P site bound tRNA to allow cyclobutane dimer formation (dashed lines).

to the cross-linking base of the 16S RNA should be strictly defined by the stereochemistry of the P site, and thus be the same for any tRNA occupying the P site. It was possible to show in this work that the stereochemistry of the cross-link is very likely the same in all three of the cases examined. This conclusion is based on the common rate of photolysis found for all three complexes (Table I) since it is known from previous studies (Herbert et al., 1969) that different geometrical isomers of cyclobutane dimers will photolyze at different rates. Unfortunately, in the absence of suitable standards, it is not possible to determine from these data which isomer is involved in the cross-link. This aspect of the problem is currently under study.

The close contact between the 5′ base at the tip of the tRNA anticodon and a residue of 16S RNA which is implicit in the finding of a direct cross-link upon UV irradiation suggests that some special structural arrangement exists between these two residues on the 30S subunit. The marked preference for cross-linking from the P site rather than the A site (Ofengand et al., 1979) indicates in addition that this should be a site-specific structure. A hypothesis for this interaction which would still allow codon–anticodon recognition to occur is shown in Figure 9. In this figure we assume that base pairing takes place at both P and A sites simultaneously, although there is no evidence for this [see Ofengand (1980) for further discussion]. A loop of 16S RNA must also be part of the decoding site since cross-linking, probably by cyclobutane dimer formation, can occur (dashed lines). We postulate that it may be necessary to stabilize, by base stacking, the anticodon of P site bound tRNA either in addition to normal base pairing or because of the lack of it; a loop of 16S RNA may be used for that purpose.

This model fits in well with the fact that it is necessary for mRNA to kink between the two adjacent codons in the P and A sites in order for two tRNA anticodons in the crystal structure conformation to form six antiparallel base pairs simultaneously with mRNA (Rich, 1974; Sundaralingam et al., 1975; Pongs, 1978). Furthermore, the consequence of this kinking is that the 5′-anticodon base in the P site has a different environment than the other two bases of the P site codon (Rich, 1974). This fact appears to be expressed as the ability to be cross-linked upon irradiation.

The recently described cross-linking of $tRNA^{Lys}$ and $tRNA^{Glu}$ to 16S RNA (Prince et al., 1979) has a number of similarities to the reaction described here and in our previous reports (Schwartz & Ofengand, 1978; Ofengand et al., 1979). Cross-linking was only to 16S RNA, and occurred from the P site. However, cross-linking yields were considerably lower, 5–6% instead of the 60–80% found in our studies (Ofengand et al., 1979; J. Ofengand, unpublished results), and no evidence for direct cross-linking between the tRNA and rRNA was provided. Moreover, reactivity at the A site was not tested while we have shown that the A site is essentially nonreactive (Ofengand et al., 1979). These authors also ascribed their cross-link to cyclobutane dimer formation, in their case between the 5′-anticodon base, mam^5s^2U, and a pyrimidine of the 16S RNA. This conclusion was based on our prior findings (Ofengand et al., 1979; J. Ofengand, unpublished results) and on their ability to photolyze the complex at 254 nm. However, their rate of photolysis was 150–200 times slower than the rates reported here, and no evidence was presented for the *reversibility* of the process. As noted above, reversibility is the most critical test of cyclobutane dimer formation. In view of the much slower rate of photolysis as well as the existence of a sulfur-containing residue in the 5′-anticodon position of these tRNAs, it seems more likely that their cross-link consists of a sulfur addition product to a 16S RNA residue. Similar sulfur addition products to pyrimidine residues are known to be photolyzable (Varghese, 1973; Paradiso et al., 1979), as are purine adducts (Elad, 1976a,b) under suitable conditions. Tests for photoreversibility and for oxygen quenching of cross-link formation (Varghese, 1973; Elad, 1976a,b) would be helpful in determining the likely structure involved but have not yet been reported. Such a cross-link would be quite interesting since the reactive atom, S_2, is located on the other side of the pyrimidine ring from the C_5–C_6 atoms involved in cyclobutane dimer formation. A different 16S RNA residue might be involved, and its position relative to the one involved in the cross-link described in this paper could be quite informative about the detailed orientation of the tRNA anticodon–16S RNA complex.

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