plexed enzyme and is also in quite good agreement with our rate constant for the faster phase of association to the binary complex (7.4×10^6 M^{-1} s^{-1} at 20 °C and pH 6.0). Dunn et al. (1978) also reported evidence from stopped-flow fluorescence measurements of NADPH binding to LCDHFR that the enzyme exists in two conformers only one of which binds NADPH efficiently. This is consistent with the proposed binding of MTX to two enzyme forms in Scheme III.

Registry No. DHFR, 9002-03-3; MTX, 59-05-2; 3-deazaMTX, 34561-27-8.

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Photochemical Cross-Linking of $tRNA_1^{Arg}$ to the 30S Ribosomal Subunit Using Aryl Azide Reagents Attached to the Anticodon Loop†

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ABSTRACT: The 2-thiocytidine residue at position 32 of $tRNA_1^{Arg}$ from *Escherichia coli* was modified specifically with three photoaffinity reagents of different lengths, and the corresponding *N*-acetylarginyl-$tRNA_1^{Arg}$ derivatives were cross-linked to the P site of *E. coli* 70S ribosomes by irradiation. Covalent attachment was dependent upon the presence of a polynucleotide template and exposure to light of the appropriate wavelength. From 4% to 6% of the noncovalently bound tRNA became cross-linked to the ribosome as a result of photolysis, and attachment to the P site was confirmed by the reactivity of arginine in the covalent complexes toward puromycin. Analysis of the irradiated ribosomes by sucrose-gradient sedimentation at low Mg^{2+} concentration revealed that the tRNA was associated exclusively with the 30S subunit in all cases. Two of the *N*-acetylarginyl-$tRNA_1^{Arg}$ derivatives were attached primarily to ribosomal proteins whereas the third was cross-linked mainly to 16S RNA. Partial RNase digestion of the latter complex demonstrated that the tRNA had become attached to the 3′ third of the rRNA molecule. In addition, the tRNA–rRNA bond was shown to be susceptible to cleavage by hydroxylamine and mercaptoethanol.

Affinity labeling provides an effective methodology for investigating the molecular anatomy of ligand binding sites in complex cellular structures such as ribosomes and other components of the translational apparatus (Jakoby & Wilchek, 1977). In this approach, the ligand is first derivatized with an appropriate reactive group. The modified ligand is then allowed to associate with its binding site and a covalent cross-link between the two is established either chemically or photochemically. The use of photolabile substituents is particularly advantageous as the ligand can be positioned and tested for functional activity prior to covalent attachment. Further, by utilization of probes of different lengths and reactivities, binding site constituents located in the immediate vicinity, as well as in the general neighborhood, of the derivatized residue(s) can be identified. Transfer RNAs are well suited for such studies as they contain a number of minor bases that can be modified specifically with the required reagents (Kuechler & Ofengand, 1979). Moreover, because the three-dimensional structure of tRNA is known (Kim, 1979), cross-linking from a variety of positions within these molecules can in principle permit deduction not only of the composition but also of the spatial organization of the sites with which they interact in the course of protein biosynthesis.

Chemical and photochemical labeling techniques have already proved useful in defining the interaction of tRNAs with aminoacyl-tRNA synthetases (Budzik et al., 1975; Schoemaker et al., 1975), elongation factor Tu (Johnson et al., 1978; Duffy et al., 1981), and the ribosomal A and P sites (Kuechler & Ofengand, 1979). For example, the anticodon loop of

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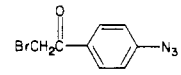
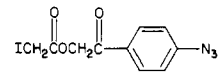
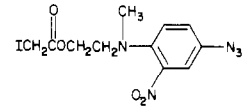
$tRNA_1^{Val}$ from *Escherichia coli* was recently shown to be in close proximity to 16S RNA in the 30S ribosomal subunit when the tRNA is located in the P site (Taylor et al., 1980). In this case, cmo^5U[1] in the 5'-position of the anticodon was photochemically cross-linked to C(1400) of the rRNA by formation of a cyclobutane linkage between the two bases (Prince et al., 1982; Ofengand & Liou, 1980). In order to further investigate the topography of the ribosome in the vicinity of the anticodon loop, we have introduced photolabile reagents at the s^2C residue that occurs at position 32 of *E. coli* $tRNA_1^{Arg}$, two bases before the 5' end of the anticodon (Chakraburtty et al., 1975). Previous modification of $s^2C(32)$ with monofunctional alkyl halides indicated that the presence of the substituents produced only minor alterations in the biological activity of the tRNA (Kruse et al., 1978). In the present study, we have attached three bifunctional photoaffinity probes of different lengths to $s^2C(32)$ in $tRNA_1^{Arg}$ after first inactivating the s^4U residue at position 8 by internally cross-linking it to C(13) (Ofengand et al., 1974). AcArg-$tRNA_1^{Arg}$ derivatives containing these modifications were then cross-linked to the P site of 70S ribosomes by irradiation, and the ribosomal components thus labeled were characterized.

Experimental Procedures

Materials. *E. coli* $tRNA_1^{Arg}$ and $tRNA_1^{Val}$ were purchased from Boehringer Mannheim and Subriden RNA, respectively; their stated amino acid acceptances ranged from 1000 to 1200 pmol/A_{260} unit. Unfractionated aminoacyl-tRNA synthetases were prepared from *E. coli* MRE600 by the method of Muench & Berg (1966). [^{3}H]Arginine (21 Ci/mmol), [^{14}C]arginine (333 mCi/mmol), [^{3}H]valine (37 Ci/mmol), iodo[^{14}C]acetamide (13 mCi/mmol), iodo[^{3}H]acetic acid (193 mCi/mmol), [γ-^{32}P]ATP (2600 Ci/mmol), Omnifluor, and Protosol were products of New England Nuclear. Poly(C,-G,A) was obtained from Miles Laboratories, ATP, Hepes, and puromycin were from Sigma, proteinase K was from EM Laboratories, Pipes, Bicine, Pronase, and RNase T_1 were from Calbiochem-Behring, and *E. coli* polynucleotide phosphorylase and T4 polynucleotide kinase were from P-L Biochemicals. Poly(C,G,U) was synthesized as described by Thang & Grunberg-Manago (1968) in reaction mixtures containing 4 mM CDP, 2 mM GDP, 4 mM UDP, and 1 unit/mL polynucleotide phosphorylase in 100 mM Tris-HCl, pH 8.5, and 5 mM $MnSO_4$; after incubation for 16 h at 37 °C, about 50% of the nucleoside diphosphates were incorporated into polymer. The 70S tight-couple ribosomes were isolated from *E. coli* MRE600 according to Debey et al. (1975). [^{14}C]Uracil-labeled 16S RNA was obtained as reported by Muto et al. (1974).

Internal Cross-Linking of tRNAs. Internal cross-links between residues $s^4U(8)$ and C(13) in $tRNA_1^{Arg}$ and $tRNA_1^{Val}$ were formed by irradiation at 350 nm and subsequent reduction with sodium borohydride (Ofengand et al., 1974). The fluorescence of the reduced, cross-linked bases was monitored with a Farrand spectrofluorometer using excitation and emission wavelengths of 395 and 440 nm, respectively.

Table I: Structures of Labeling Reagents

iodoacetamide (IA)	
p-azidophenacyl bromide (APA-Br)	
p-azidophenacyl iodoacetate (APAA-I)	
N-(4-azido-2-nitrophenyl)-*N*-methyl-2-aminoethyl iodoacetate (NAMA-I)	

Photoaffinity Reagents. [^{14}C]APA-Br (35 mCi/mmol) and [^{3}H]APAA-I (74 mCi/mmol) were prepared as previously described (Ofengand et al., 1977). NAMA-I was synthesized by a two-step procedure. First, a solution containing 2 g (16 mmol) of 4-fluoro-2-nitrophenyl azide (Fleet et al., 1972) and 3 g (40 mmol) of *N*-methylethanolamine in 50 mL of methanol was heated at 40 °C for 48 h with constant stirring and then chilled. Following the addition of 100 mL of chloroform, the resulting solution was washed with three 100-mL portions of distilled water, dried over $MgSO_4$, and concentrated under vacuum to give 3.8 g (97%) of *N*-(4-azido-2-nitrophenyl)-*N*-methyl-2-aminoethanol [IR (neat) 3400 (br), 2900, 2130, 1620, 1520, 1310, 1050, 880, 810 cm^{-1}; NMR ($CDCl_3$) δ 2.80 (s, 3 H, CH_3), 2.99 (br s, 1 H, OH), 3.23–3.40 (m, 2 H, CH_2), 3.66–3.79 (m, 2 H, CH_2), and 7.20–7.40 (m, 3 H, aromatic)]. Second, 1.18 g (5 mmol) of the latter compound was mixed with 0.93 g (5 mmol) of iodoacetic acid in 15 mL of dry ethyl acetate at 4 °C, and 1.03 g (5 mmol) of solid dicyclohexylcarbodiimide was added. This solution was stirred at 4 °C for 30 min, warmed to room temperature, stirred an additional 60 min, and filtered to remove the precipitated dicyclohexylurea. The filtrate was applied directly to 0.5 mm thick, 20 × 20 cm silica gel thin-layer chromatography plates, which were developed with 20% ether–chloroform to give 1.5 g (75%) of *N*-(4-azido-2-nitrophenyl)-*N*-methyl-2-aminoethyl iodoacetate (NAMA-I) as a red oil that could not be crystallized [IR (neat) 2970, 2130, 1740, 1530, 1270, 1140, 1100, 820 cm^{-1}; NMR ($CDCl_3$) δ 2.86 (s, 3 H, CH_3), 3.63 (s, 2 H, CH_2), 3.88 (t, J = 5.4 Hz, 2 H, CH_2), 4.30 (t, J = 5.4 Hz, 2 H, CH_2), 7.13–7.18 (m, 2 H, aromatic), and 7.35–7.39 (m, 1 H, aromatic); UV λ_{max} (EtOH) 430 nm (ϵ 1.50 × 10^3); mass spectrum, m/z 404.99517 (calcd for $C_{11}H_{12}N_5O_4I$: 404.99340)]. Use of ^{3}H-labeled iodoacetic acid in the second step permitted the synthesis of [^{3}H]NAMA-I with a specific radioactivity of 193 mCi/mmol. The molecular structures of these reagents are illustrated in Table I. The ester bonds in APAA-I and NAMA-I are in principle susceptible to cleavage with 1 M hydroxylamine (Hixson et al., 1980).

Derivatization of tRNAs. Alkylation of $tRNA_1^{Arg}$ and $tRNA_1^{Val}$ with IA, APA-Br, APAA-I, and NAMA-I was carried out by a modification of the procedure described by Schwartz & Ofengand (1974). Up to 1 A_{260} unit of tRNA in 100 μL of 10 mM KH_2PO_4, pH 7.4, and 80% Me_2SO (Eastman, redistilled) was incubated in the dark with a 200–300-fold molar excess of reagent for 1–4 h at 37 °C. This mixture was then extracted 3 times with ether to remove the bulk of the unreacted label. For quantitation of the reaction, the derivatized tRNA was precipitated with an equal volume of 10% trichloroacetic acid, and the precipitate was collected on a glass-fiber filter that was washed successively with 5 mL

[1] Abbreviations: cmo^5U, 5-(carboxymethoxy)uridine; s^2C, 2-thiocytidine; s^4U, 4-thiouridine; AcArg, *N*-acetylarginyl; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; APA-Br, *p*-azidophenacyl bromide; APAA-I, *p*-azidophenacyl iodoacetate; NAMA-I, *N*-(4-azido-2-nitrophenyl)-*N*-methyl-2-aminoethyl iodoacetate; IA, iodoacetamide; Me_2SO, dimethyl sulfoxide; (XL)tRNA, tRNA with an internal cross-link between $s^4U(8)$ and C(13); (XL)-$tRNA_{APA}^{Arg}$, (XL)$tRNA_1^{Arg}$ derivatized with APA-Br; (XL)$tRNA_{APAA}^{Arg}$, (XL)$tRNA_1^{Arg}$ derivatized with APAA-I; (XL)$tRNA_{NAMA}^{Arg}$, (XL)-$tRNA_1^{Arg}$ derivatized with NAMA-I; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride.

each of cold 5% trichloroacetic acid, 90% ethanol–10% trichloroacetic acid, 67% ethanol–33% chloroform, 50% ethanol–50% ether, and, finally, 100% ether to reduce the nonspecific binding of reagent to the filter disk. For preparative purposes, the modified tRNA was precipitated 5 times with ethanol after reaction and resuspended in 0.1% KOAc, pH 5.0. Substituents at the $s^2C(32)$ residue of $tRNA_1^{Arg}$ were removed by treatment with 25–50 mM 2-mercaptoethanol for 4 h at 37 °C in 50 mM Hepes–NaOH, pH 7.0, 10 mM $MgCl_2$, 50 mM KCl, and 50 mM NH_4Cl (Kruse et al., 1978).

Preparation of Aminoacylated tRNA. From 0.8 to 1.6 A_{260} units of $tRNA_1^{Arg}$ was aminoacylated by incubation at 37 °C for 15 min in 1 mL of 100 mM Tris-HCl, pH 7.6, and 14 mM $MgCl_2$ containing 3 mM ATP, 14 μM ^3H- or ^{14}C-labeled arginine, and an appropriate amount of aminoacyl-tRNA synthetase. Arg-$tRNA_1^{Arg}$ was N-acetylated according to Haenni & Chapeville (1966), and the reaction was determined to be essentially complete by the $CuSO_4$–Tris assay of Schofield & Zamecnik (1968). When required, AcArg-$tRNA_1^{Arg}$ was labeled with ^{32}P at the 5′ terminus by a modification of the exchange reaction of Berkner & Folk (1979). Approximately 650 pmol of AcArg-$tRNA_1^{Arg}$ was incubated with [γ-^{32}P]ATP and polynucleotide kinase for 30 min at 37 °C in 50 mM imidazole, pH 6.6, 10 mM $MgCl_2$, 4.5 mM dithiothreitol, and 0.3 mM ADP.

Formation and Irradiation of AcArg-$tRNA_1^{Arg}$·Ribosome Complexes. AcArg-$tRNA_1^{Arg}$ was bound nonenzymatically to the ribosomal P site in 50 mM Hepes–NaOH, pH 7.6, 15 mM $MgCl_2$, and 100 mM NH_4Cl containing 750 pmol/mL of 70S tight couples, 140 pmol/mL of AcArg-$tRNA_1^{Arg}$, and 200–400 μg/mL of poly(C,G,A) or poly(C,G,U). After incubation at 37 °C for 15 min, noncovalent binding of AcArg-$tRNA_1^{Arg}$ to ribosomes was estimated from the radioactivity retained by nitrocellulose membranes when reaction mixtures were filtered in the presence of 20 mM Mg^{2+} (Schwartz & Ofengand, 1978). Association of the tRNA with the ribosomal P site was confirmed by the release of bound amino acid in the presence of 1 mM puromycin. Cross-linking of noncovalent 70S complexes containing $tRNA_1^{Arg}$ derivatized with APA or APAA was accomplished by irradiation for 4 h at 4 °C in a Rayonet RPR-100 photochemical reactor equipped with 350-nm lamps. Ribosomal complexes containing $tRNA_1^{Arg}$ derivatized with NAMA were irradiated with a Hanovia 450-W medium-pressure mercury lamp, housed in a quartz immersion well and surrounded by a Pyrex filter sleeve, that was placed 10 cm from the sample. Irradiated complexes were also treated with puromycin to verify that covalently bound AcArg-$tRNA_1^{Arg}$ remained functionally associated with the P site.

Isolation of Covalent AcArg-$tRNA_1^{Arg}$–Ribosome Complexes. After irradiation, ribosomal complexes were precipitated with 2 volumes of 95% ethanol, recovered by low-speed centrifugation, and resuspended in 10 mM Pipes–NaOH, pH 6.3, and 0.25 mM $MgCl_2$. This suspension was then layered onto a 12-mL 5–20% sucrose gradient in the same buffer with 100 mM NaCl and centrifuged at 28 000 rev/min for 12.5 h at 3 °C in a Beckman SW41 rotor. The gradient effluent was monitored for absorbance at 260 nm, and the acid-insoluble radioactivity in each fraction was collected on glass-fiber filters that were then counted in scintillation fluid containing 4 g of Omnifluor/L of toluene. The efficiency of cross-linking was taken as the amount of radioactivity sedimenting with the dissociated subunits divided by that bound to the ribosomes noncovalently prior to irradiation (Prince et al., 1979). Ribosomal subunits were precipitated from the appropriate gradient fractions with ethanol.

Cross-Linking to Ribosomal Proteins. To assess the covalent attachment of AcArg-$tRNA_1^{Arg}$ to ribosomal proteins, the subunits were resuspended in 10 mM Pipes–NaOH, pH 6.3, 0.25 mM $MgCl_2$, 2 M LiCl, and 4 M urea and left on ice for 24 h. Precipitated rRNA was then removed by centrifugation at 18 000 rev/min for 15 min in a Sorvall SS-34 rotor, and the supernatant, which contained the tRNA–protein complexes, was dialyzed against 5 mM KOAc, pH 5.8, and 4 M urea. Association of the tRNA with protein was verified by the susceptibility of the complexes to protease digestion (Schwartz et al., 1975). Portions of the dialyzed supernatant containing 1 mg of protein were incubated with proteinase K or Pronase at an enzyme:substrate ratio of 1:15 (w/w) for 25 min at 25 °C. These samples, together with aliquots of the undigested complex and of free AcArg-$tRNA_1^{Arg}$, were then made up to 5% glycerol and 0.002% bromophenol blue and subjected to electrophoresis in a discontinuous system consisting of a 1-cm stacking gel composed of 4% polyacrylamide and 0.16 M urea, and a 10-cm running gel composed of 15% polyacrylamide and 6 M urea, in 10 mM citric acid and 85 mM morpholinoethanesulfonic acid, pH 5.8, for 17 h at 2–3 mA/tube and at 20 °C (Stein & Varricchio, 1974). Gels were sliced into 2-mm sections and counted in a scintillation cocktail containing 4 g of Omnifluor and 40 mL of Protosol per liter of toluene after overnight incubation at 30 °C.

Cross-Linking to Ribosomal RNA. To assess the cross-linking of AcArg-$tRNA_1^{Arg}$ to rRNA, subunits were dissolved in 10 mM Pipes–NaOH, pH 6.3, and 10 mM $MgCl_2$ containing 0.1% sodium dodecyl sulfate and extracted with an equal volume of phenol. After a brief centrifugation, the aqueous phase was withdrawn and the phenol phase reextracted with the same buffer. The second aqueous phase was combined with the first, and the RNA was precipitated with ethanol. The RNA pellet was then suspended in the appropriate buffer and subjected to sucrose gradient analysis before and after treatment with RNase T_1, hydroxylamine, or mercaptoethanol as described in the text.

RESULTS

Derivatization of $s^2C(32)$ in $tRNA_1^{Arg}$. In order to alkylate specifically the s^2C residue at position 32 of $tRNA_1^{Arg}$, it was first necessary to sequester $s^4U(8)$, which also reacts with the photoaffinity reagents used in this work. To this end, $s^4U(8)$ was internally cross-linked to C(13) by irradiation of the tRNA at 350 nm (Ofengand et al., 1974). Reduction with $NaBH_4$ converts the photoproduct to a strongly fluorescent derivative, providing a convenient assay for the rate and extent of internal cross-link formation (see Figure 1). *E. coli* $tRNA_1^{Val}$, which contains $s^4U(8)$ and C(13), but no other thio bases, was used as a control.

Following irradiation and reduction, both $tRNA_1^{Val}$ and $tRNA_1^{Arg}$ exhibited a rapid increase in fluorescence and a concomitant decrease in reactivity to IA, an analogue of the labeling reagents APA-Br, APAA-I, and NAMA-I. While native $tRNA_1^{Val}$ can accept 1 mol of IA/mol of tRNA, over 85% of the irradiated material was unreactive at the fluorescence plateau, indicating that formation of the $s^4U(8)$–C(13) cross-link effectively blocked alkylation of $s^4U(8)$ (Figure 1a). Prior to irradiation, each mole of $tRNA_1^{Arg}$ accepted 2 mol of IA, presumably at $s^4U(8)$ and $s^2C(32)$. After induction of the $s^4U(8)$–C(13) cross-link, however, the incorporation of IA dropped by 50% (Figure 1b). We conclude that internal cross-linking prevents reaction at $s^4U(8)$ and specifically directs derivatization to $s^2C(32)$. Similar results

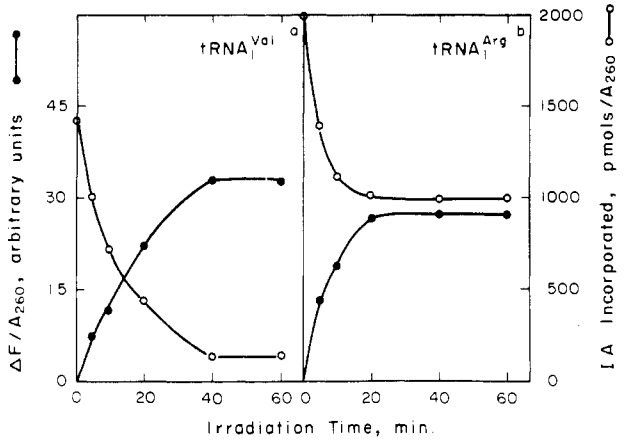


FIGURE 1: Formation of internal $s^4U(8)$–C(13) cross-links in $tRNA_1^{Val}$ and $tRNA_1^{Arg}$. Solutions of tRNA in 20 mM Bicine, pH 7.5, and 10 mM $Mg(OAc)_2$ were irradiated with 350-nm lamps using a $Pb(NO_3)_2$ filter to eliminate emissions below 330 nm (Ofengand et al., 1979). At each of the times indicated, two equal aliquots were withdrawn. One was reduced with sodium borohydride and assayed for fluorescence at 440 nm (Ofengand et al., 1974). The other was tested for the incorporation of [^{14}C]iodoacetamide as described under Experimental Procedures. (a) $tRNA_1^{Val}$. (b) $tRNA_1^{Arg}$. (●) Fluorescence increment per A_{260} unit of tRNA. (○) Iodoacetamide incorporated per A_{260} unit of tRNA.

Table II: Derivatization of Native and Internally Cross-Linked tRNAs

tRNA sample	incorporation (pmol/A_{260}) [^{3}H]APAA	[^{14}C]APA	[^{3}H]NAMA
$tRNA_1^{Val}$	1264		
$(XL)tRNA_1^{Val}$	215		
$tRNA_1^{Arg}$	2880		
$(XL)tRNA_1^{Arg}$	1590	1420	1441
$AcArg\text{-}(XL)tRNA_1^{Arg}$	1383	1502	1368

Table III: Release of Substituents by 2-Mercaptoethanol

derivatived tRNA	mercapto-ethanol	extent of derivatization (pmol/A_{260}) APAA	APA	NAMA
$tRNA_1^{Val}$	–	1529		
	+	1598		
$tRNA_1^{Arg}$	–	2929		
	+	1615		
$(XL)tRNA_1^{Arg}$	–	1821	1641	1618
	+	313	224	227

were obtained with APAA-I (Table II). In addition, internally cross-linked $tRNA_1^{Arg}$ [hereafter designated $(XL)tRNA_1^{Arg}$] also accepted 1 mol of APA and NAMA (Table II).

To verify that alkylation of $(XL)tRNA_1^{Arg}$ occurred at $s^2C(32)$, we took advantage of the observation that modifications at s^2C, but not at s^4U, can be removed by thiols such as 2-mercaptoethanol (Kruse et al., 1978). When $tRNA_1^{Val}$ and $tRNA_1^{Arg}$ were derivatized with APAA-I, the amount of APAA bound to $tRNA_1^{Val}$ was unchanged by treatment with mercaptoethanol whereas one of the 2 mol of APAA initially incorporated into $tRNA_1^{Arg}$ was released (Table III). Moreover, mercaptoethanol removed more than 85% of the substituent groups from $(XL)tRNA_1^{Arg}$ modified with APAA, APA, or NAMA [hereafter designated $(XL)tRNA_{APAA}^{Arg}$, $(XL)tRNA_{APA}^{Arg}$, and $(XL)tRNA_{NAMA}^{Arg}$, respectively].

Aminoacylation and Formation of Noncovalent Ribosomal Complexes. Although internally cross-linked $tRNA_1^{Val}$ and $tRNA_1^{Arg}$ could be aminoacylated to about the same extent as the native species, we found that the amino acid acceptance of derivatized $(XL)tRNA_1^{Arg}$ was only 25–30% that of the unsubstituted controls (see Table IV). Despite extensive effort, the poor charging was not improved by variations in the pH (6.7–8.5), by changes in the concentrations of Mg^{2+} (10–40 mM), amino acid (20–40 μM), or enzyme, or by the use of a high Mg^{2+}:ATP ratio (Kern et al., 1972). Because exposure of $(XL)tRNA_1^{Arg}$ to the derivatization conditions in the absence of reagent did not significantly alter its amino acid acceptance, we inferred that the introduction of bulky substituent groups at $s^2C(32)$ itself impaired aminoacylation. For this reason, $(XL)tRNA_1^{Arg}$ was routinely modified after it was charged and acetylated. As shown in Table IV for NAMA derivatives, aminoacylation does not interfere with modification although some deacylation occurred when $AcArg\text{-}(XL)tRNA_1^{Arg}$ was incubated with the photoaffinity reagent.

Table IV: Aminoacylation of $tRNA_1^{Arg}$ before and after Derivatization with NAMA

tRNA sample	extent of aminoacylation (pmol/A_{260})
$tRNA_1^{Arg}$	989[a]
$(XL)tRNA_1^{Arg}$	968[a]
$(XL)tRNA_1^{Arg}$, exposed to 80% Me_2SO	830[a]
$(XL)tRNA_{NAMA}^{Arg}$	245[a]
$AcArg\text{-}(XL)tRNA_{NAMA}^{Arg}$	751[b]

[a] Incorporation of [^{3}H]Arg into the indicated tRNA sample. [b] [^{3}H]Arg remaining after derivatization of $Ac[^3H]Arg\text{-}(XL)tRNA_1^{Arg}$ with NAMA.

Table V: Noncovalent Binding of $tRNA_1^{Arg}$ Derivatives to 70S Ribosomes

tRNA sample	tRNA bound (%)[a] no template	poly-(C,G,A)	poly-(C,G,U)
$Ac[^3H]Arg\text{-}(XL)tRNA_1^{Arg}$	6	42	60
$Ac[^3H]Arg\text{-}(XL)tRNA_{APA}^{Arg}$	13	25	49
$Ac[^3H]Arg\text{-}(XL)tRNA_{APAA}^{Arg}$	13	27	42
$Ac[^3H]Arg\text{-}(XL)tRNA_{NAMA}^{Arg}$	11	25	46

[a] Percentage of total tRNA bound to 70S ribosomes at a molar tRNA:ribosome input ratio of 0.2:1. Data were averaged from several experiments.

$Ac[^3H]Arg\text{-}(XL)tRNA_1^{Arg}$ was bound nonenzymatically to the P site of 70S tight-couple ribosomes in the presence of poly(C,G,U) or poly(C,G,A). Although both polynucleotides contain codons complementary to the tRNA anticodon, I-C-G, poly(C,G,U) stimulated interaction to a greater extent than poly(C,G,A). In all cases, derivatized $AcArg\text{-}(XL)tRNA_1^{Arg}$ exhibited less message-directed, but more message-independent, binding than its unmodified counterpart (Table V). Optimal polynucleotide concentrations and tRNA:ribosome ratios were determined by titration in the presence of 15 mM Mg^{2+}. While a larger percentage of $AcArg\text{-}(XL)tRNA_1^{Arg}$ became bound to ribosomes at higher Mg^{2+} concentrations, nonspecific binding increased to an even greater extent.

Covalent Cross-Linking of Derivatized AcArg-(XL)-$tRNA_1^{Arg}$ to the Ribosomal P Site. Noncovalent $Ac[^3H]\text{-}Arg\text{-}(XL)tRNA^{Arg}$·70S ribosome complexes were irradiated, concentrated, and separated into 30S and 50S subunits by sucrose gradient centrifugation at 0.25 mM Mg^{2+}. The extent of covalent cross-linking was estimated from the fraction of the radioactivity that cosedimented with the subunits. As shown in Figure 2, covalent attachment of $AcArg\text{-}(XL)\text{-}tRNA_{NAMA}^{Arg}$ was nearly complete after 20 min of irradiation. For all three derivatives, a plateau was reached when 4–6% of the noncovalently bound tRNA became cross-linked (Table VI). The sucrose gradient profiles revealed that the tRNA was attached exclusively to the 30S ribosomal subunit in each

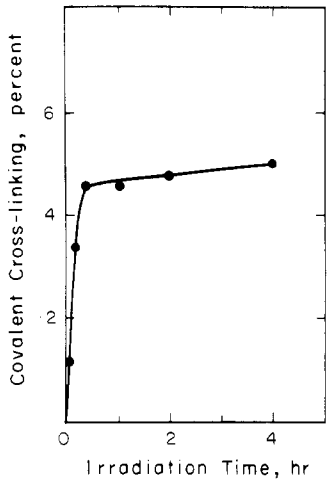


FIGURE 2: Kinetics of covalent $tRNA^{Arg}_{NAMA}$–ribosome cross-linking. Ac[^{3}H]Arg-(XL)$tRNA^{Arg}_{NAMA}$ was bound to the P site of 70S ribosomes in a 1-mL incubation mixture and irradiated at 350 nm. At each of the indicated times, samples of 100 μL were withdrawn and fractionated by sucrose gradient centrifugation. Covalent cross-linking was estimated from the fraction of Ac[^{3}H]Arg-(XL)$tRNA^{Arg}_{NAMA}$ associated with the ribosomal subunits. The data were corrected for a small amount of irradiation-independent radioactivity in the subunit region of the gradients. (●) Covalently cross-linked Ac[^{3}H]Arg-$tRNA^{Arg}_{NAMA}$ as a percentage of noncovalent tRNA·ribosome complexes.

case (Figures 3 and 4). Unmodified AcArg-(XL)$tRNA_1^{Arg}$ failed to undergo this reaction (Figure 3a). Figure 4 shows that cross-linking of AcArg-(XL)$tRNA^{Arg}_{NAMA}$ was dependent upon the presence of polynucleotide message and exposure to light of the appropriate wavelength. Attachment to the ribosomal P site was confirmed by the release of over 65% of the radioactive arginine from the covalently bound tRNA upon incubation with puromycin (Figure 4d). The cross-linking of AcArg-(XL)$tRNA^{Arg}_{APA}$ and AcArg-(XL)$tRNA^{Arg}_{APAA}$ also required the presence of polynucleotide and irradiation, and the resulting complexes exhibited a similar sensitivity to puromycin (data not shown).

Characterization of the Cross-Linked Complexes. To assess the cross-linking of the tRNA to ribosomal proteins, AcArg-(XL)$tRNA^{Arg}$–30S subunit complexes were dissociated

Table VI: Cross-Linking of Derivatized AcArg-(XL)$tRNA_1^{Arg}$ to Ribosomal Components

tRNA derivatized with	noncovalently bound AcArg-$tRNA^{Arg}$ cross-linked to ribosome (%)	covalently bound AcArg-$tRNA^{Arg}$ cross-linked to component (%)[a]	
		protein	16S RNA
APA-Br	4.8	83	17
APAA-I	4.1	78	22
NAMA-I	5.3	34	66

[a] tRNA–protein cross-linking was estimated from radioactivity in the nonaqueous phase after extraction of ribosomal subunits with phenol (Schwartz et al., 1975). tRNA–16S RNA cross-linking was estimated from radioactivity that remained in the aqueous phase and comigrated with 16S RNA upon subsequent sucrose gradient centrifugation.

and fractionated by either phenol extraction or exposure to 2 M LiCl and 4 M urea. In the former procedure, RNA remains in the aqueous phase, while proteins—including tRNA–protein complexes—partition to the phenol phase (Schwartz et al., 1975). In the LiCl–urea mixture, tRNA–protein adducts remain in solution, and high molecular weight RNA is precipitated (Traub et al., 1971). For purposes of quantitation, the phenol extraction procedure proved more reliable. Measurement of the acid-insoluble radioactivity in the phenol phase suggested that, on average, 83%, 78%, and 34% of the covalent cross-links were directed to 30S subunit proteins when photolysis was carried out with the APA, APAA, and NAMA derivatives of AcArg-(XL)$tRNA_1^{Arg}$, respectively (Table VI). The tRNA–protein adducts isolated by the LiCl–urea method were further analyzed by protease digestion and gel electrophoresis. As depicted in Figure 5a, the putative Ac[^{3}H]Arg-(XL)$tRNA^{Arg}_{APAA}$–protein complexes barely migrated into a 15% polyacrylamide gel at pH 5.8. After digestion with proteinase K or Pronase, however, only 25% of the radioactivity remained at the top of the gel whereas the main peak displayed very nearly the same mobility as free AcArg-$tRNA_1^{Arg}$ (Figure 5b,c). Although proteases can provoke extensive deacylation of acetylaminoacyl-tRNA (Schwartz et al., 1975), cleavage of the aminoacyl bond was limited to less than 40% in the present experiments by the use of a low enzyme:substrate ratio and relatively gentle incubation

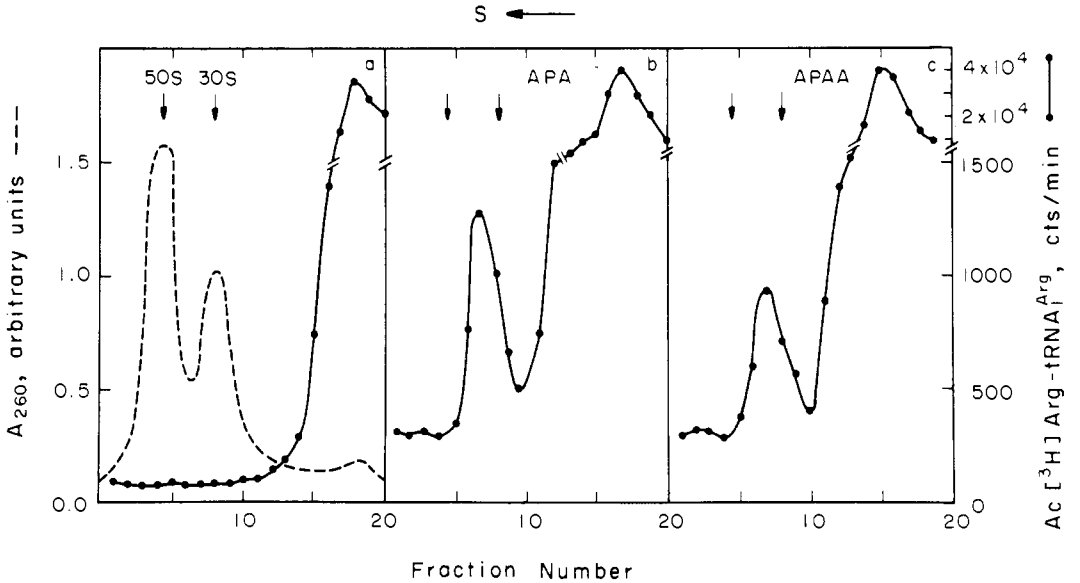


FIGURE 3: Cross-linking of derivatized AcArg-(XL)$tRNA_1^{Arg}$ to the 30S ribosomal subunit. Noncovalent Ac[^{3}H]Arg-(XL)$tRNA_1^{Arg}$·70S ribosome complexes were formed in the presence of poly(C,G,A), irradiated for 4 h with 350-nm lamps, and fractionated on 5–20% sucrose gradients in 0.25 mM Mg^{2+}. Reaction mixtures contained (a) underivatized Ac[^{3}H]Arg-(XL)$tRNA_1^{Arg}$, (b) Ac[^{3}H]Arg-(XL)$tRNA^{Arg}_{APA}$, and (c) Ac[^{3}H]Arg-(XL)$tRNA^{Arg}_{APAA}$.

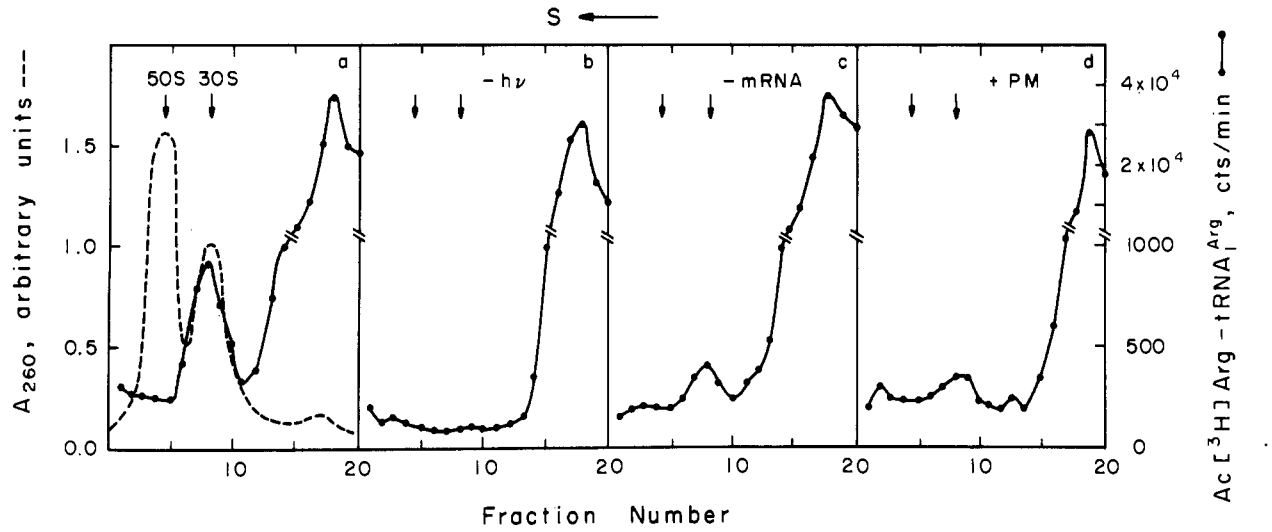


FIGURE 4: Effects of irradiation, template, and puromycin on complexes containing AcArg-(XL)$tRNA^{Arg}_{NAMA}$. Noncovalent Ac[^{3}H]Arg-(XL)$tRNA^{Arg}_{NAMA}$·poly(C,G,A)·70S ribosome complexes were irradiated with a medium-pressure mercury lamp and analyzed as in Figure 3. (a) Complete system; (b) unirradiated; (c) poly(C,G,A) omitted; (d) complete system treated with puromycin after irradiation.

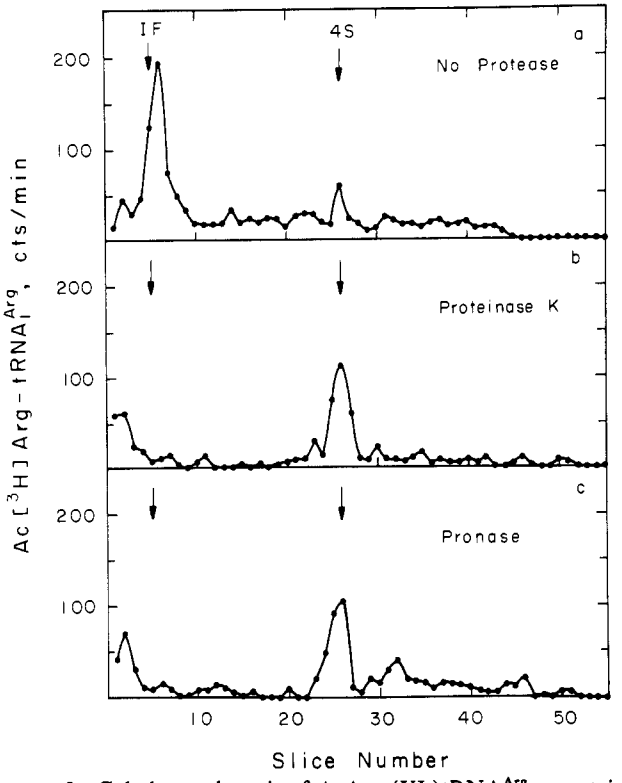


FIGURE 5: Gel electrophoresis of AcArg-(XL)$tRNA^{Arg}_{APAA}$–protein complexes after digestion with proteases. Ac[^{3}H]Arg-(XL)-$tRNA^{Arg}_{APAA}$–protein complexes were isolated by the LiCl–urea method. Samples containing 1 mg of protein in 5 mM KOAc, pH 5.8, and 4 M urea were incubated with (a) no addition, (b) 67 μg of proteinase K, and (c) 67 μg of Pronase for 25 min at 25 °C. The digests were subjected to electrophoresis in discontinuous polyacrylamide gels as described under Experimental Procedures. After sectioning at 2-mm intervals, the gel slices were immersed in an Omnifluor–Protosol–toluene cocktail and assayed for radioactivity. IF marks the interface between the stacking and running gels, and 4S the position to which free AcArg-(XL)$tRNA^{Arg}_1$ migrated.

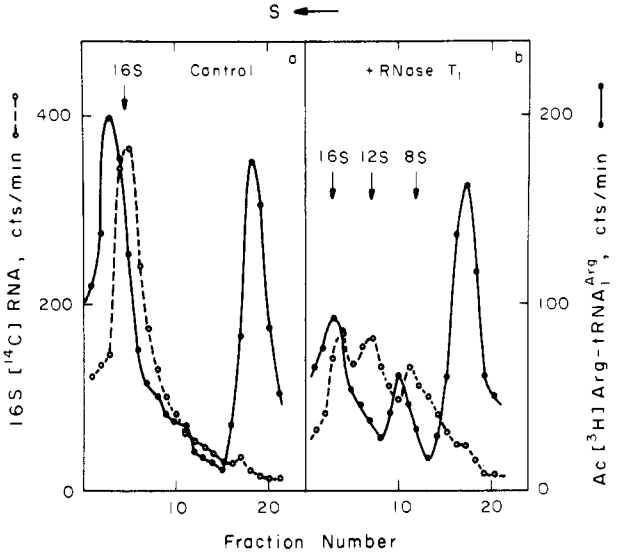


FIGURE 6: Partial hydrolysis of AcArg-(XL)$tRNA^{Arg}_{NAMA}$–16S RNA complex with RNase T_1. Ac[^{3}H]Arg-(XL)$tRNA^{Arg}_{NAMA}$–16S RNA (55 μg) was mixed with ^{14}C-labeled 16S RNA (20 μg) in 300 μL of PMK buffer (10 mM potassium phosphate, pH 6.2, 20 mM $MgCl_2$, 350 mM KCl), incubated for 10 min at 40 °C, and chilled on ice. After adjustment of the pH to 7.2 with K_2HPO_4, the mixture was divided into two equal portions. Sample a was untreated while sample b was incubated with RNase T_1 at an enzyme:substrate ratio of 1:450 (w/w) for 15 min at 0 °C. Both mixtures were then centrifuged through 3–15% sucrose gradients in PMK buffer for 15 h at 32000 rev/min in a Beckman SW41 rotor, and the acid-insoluble radioactivity in each fraction was measured.

conditions. The results thus confirm that the AcArg-(XL)-$tRNA^{Arg}$ in the LiCl–urea extract was associated with protein.

Covalent attachment of tRNA to 16S RNA was estimated from the radioactivity present in the aqueous phase of phenol-extracted AcArg-(XL)$tRNA^{Arg}$–30S subunit complexes. As the aqueous phase also contained some free AcArg-(XL)$tRNA^{Arg}$, tRNA–16S RNA cross-linking was generally quantitated only after the complexes were separated from the tRNA by sucrose gradient centrifugation (see Figure 6a). Whereas tRNA–16S RNA adducts amounted to 20% or less of the total cross-linking when the tRNA was modified with APA or APAA, 65% of AcArg-(XL)$tRNA^{Arg}_{NAMA}$ became covalently bound to 16S RNA under the same conditions (Table VI). The region of the 16S RNA to which the tRNA became cross-linked was determined by RNase digestion of the covalent tRNA–16S RNA complex. In the presence of 20 mM Mg^{2+}, partial hydrolysis of 16S RNA with RNase T_1 produces fragments of 12 S and 8 S that correspond to the 5′ 60% and 3′ 40% of the intact rRNA molecule, respectively (Muto et al., 1974). Figure 6a shows that untreated Ac[^{3}H]Arg-(XL)$tRNA^{Arg}_{NAMA}$–16S RNA sedimented slightly ahead of the

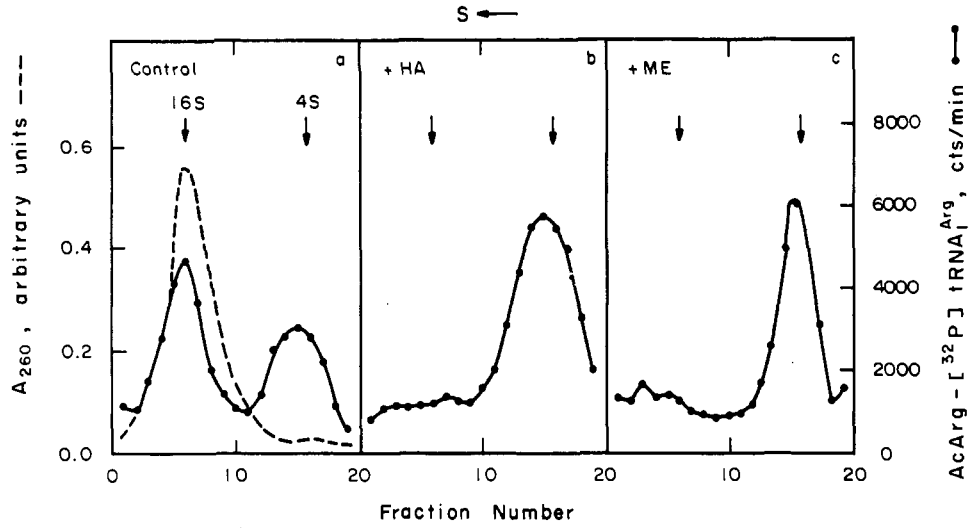


FIGURE 7: Cleavage of AcArg-(XL)tRNA$^{Arg}_{NAMA}$–16S RNA complex with hydroxylamine and mercaptoethanol. AcArg-(XL)tRNA$_1^{Arg}$ was labeled with ^{32}P at the 5′ terminus, derivatized with NAMA, and cross-linked to 70S ribosomes as described under Experimental Procedures. ^{32}P-labeled AcArg-(XL)tRNA$^{Arg}_{NAMA}$–16S RNA complexes were isolated and suspended in 10 mM Pipes–NaOH, pH 6.3, and 0.25 mM $MgCl_2$. Aliquots of 100 μL containing 10 μg of complex were incubated for 8 h at 25 °C in the presence of (a) no addition, (b) 1 M hydroxylamine, pH 7.2, and (c) 50 mM mercaptoethanol, pH 6.3, after which the RNA was precipitated with ethanol. Samples were fractionated on 5–20% sucrose gradients in 10 mM Pipes–NaOH, pH 6.3, 1 mM $MgCl_2$, and 100 mM NaCl for 16 h at 30000 rev/min in a Beckman SW41 rotor, and the acid-insoluble radioactivity in each tube was assayed.

16S RNA standard as observed for other tRNA–16S RNA adducts (Prince et al., 1979; Zimmermann et al., 1979). After digestion with RNase T_1, the Ac[^{3}H]Arg-(XL)tRNA^{Arg} moiety was found in association with the 8S, or 3′, fragment but not with the 12S, or 5′, fragment of the rRNA (Figure 6b). Comparable patterns were obtained when AcArg-(XL)tRNA^{Arg}–16S RNA complexes cross-linked via APA and APAA were analyzed in the same way (data not shown).

As illustrated by the sucrose-gradient profiles in Figure 7, the AcArg-(XL)tRNA$^{Arg}_{NAMA}$–16S RNA cross-link is susceptible to cleavage by hydroxylamine and mercaptoethanol. Incubation of the complex for 8 h at 25 °C resulted in the spontaneous release of 30–50% of the tRNA from the 16S peak (Figure 7a). After cleavage of the ester bond in the cross-linking reagent with 1 M hydroxylamine, removal of tRNA from the 16S complex was virtually complete (Figure 7b). Treatment of the tRNA–rRNA adduct with 50 mM mercaptoethanol also caused a quantitative shift of radioactivity from the 16S peak to the 4S region of the gradient (Figure 7c). The unique sensitivity of alkylated s^2C to thiols provides additional evidence that tRNA–16S RNA cross-linking is mediated by derivatization of s^2C at position 32 of (XL)tRNA$_1^{Arg}$ (Kruse et al., 1978).

Discussion

When positioned at the ribosomal P site, AcArg-tRNA$_1^{Arg}$ derivatized with any one of three different photoaffinity labels at $s^2C(32)$ in the anticodon loop can be cross-linked photochemically to the 30S subunit. In all cases, covalent attachment requires irradiation and the presence of an appropriate polynucleotide template. Although each reagent mediates cross-linking of the anticodon loop to both protein and RNA components of the 30S particle, the particular labeling pattern is dependent upon the nature of the probe. Thus, the APA and APAA derivatives react mainly with 30S subunit proteins whereas the NAMA derivative inserts mainly into 16S RNA. These differences may be attributable to variations either in the lengths of the reagents—the photochemical moieties of APA, APAA, and NAMA are situated approximately 9, 12, and 13 Å from the s^2C residue, respectively (Ofengand et al., 1977; S. S. Hixson, unpublished results)—or in the reactivities of the aryl nitrenes that are formed when they are irradiated. In any event, our results suggest that the portion of the 30S ribosomal subunit that surrounds the anticodon loop in the P site consists of an integrated ribonucleoprotein matrix and not exclusively of protein, on the one hand, or of RNA, on the other hand. Furthermore, they indicate that it may be possible to define the protein and RNA components of this neighborhood by the use of substituents at a single site in the tRNA.

The proximity of $s^2C(32)$ to 16S RNA is consistent with the finding that $cmo^5U(34)$, the 5′ anticodon base of tRNA$_1^{Val}$, can be cross-linked directly to C(1400) of the ribosomal RNA at the P site (Prince et al., 1982). As the $cmo^5U(34)$–C(1400) bond results from cyclobutane dimerization (Ofengand & Liou, 1980), the anticodon base must lie within 3–4 Å of the 16S RNA molecule. Moreover, it is significant that AcArg-tRNA$^{Arg}_{NAMA}$ cross-links to a segment of 16S RNA encompassing residues 918–1497 since $s^2C(32)$ precedes the 5′ residue of the anticodon by only two bases and should therefore be associated with the same ribosomal neighborhood as $cmo^5U(34)$.

There are two discrepancies between our work and that of Kruse et al. (1978), who earlier modified the s^2C residue in tRNA$_1^{Arg}$ with IA and a monofunctional spin-label derived from it. First, substitution was found to be highly specific for $s^2C(32)$ under their conditions even though $s^4U(8)$ was not blocked. In contrast, we concluded that both thio bases could be alkylated to about the same extent by IA and APAA-I (see Figure 1 and Table II). Derivatization could be directed specifically to $s^2C(32)$, however, by initially establishing an internal cross-link between $s^4U(8)$ and C(13) via irradiation at 350 nm (Ofengand et al., 1974). A second difference concerns the aminoacylation of tRNA$_1^{Arg}$ after modification of $s^2C(32)$. Whereas Kruse et al. (1978) found that alkylation at position 32 caused only a slight reduction in the rate of charging, we noted a sharp drop in specific amino acid acceptance following derivatization of (XL)tRNA$_1^{Arg}$ with the reagents used in the present study (see Table IV). This discrepancy may be attributable in part to the larger size of the photoaffinity labels compared to the groups introduced in the earlier work. On the basis of our results, we infer that substituents in the anticodon loop can indeed influence the rec-

ognition of $tRNA_1^{Arg}$ by arginyl-tRNA synthetase.

Removal of substituents from s^2C can be brought about by treatment with 2-mercaptoethanol (Kruse et al., 1978). In particular, we have shown that the release of APA, APAA, and NAMA from $s^2C(32)$ in $tRNA_1^{Arg}$ was over 85% complete after a short incubation with the thiol (Table III). 2-Mercaptoethanol also proved to be effective in removing tRNA from the cross-linked AcArg-(XL)$tRNA_{NAMA}^{Arg}$–16S RNA complex. In addition, the tRNA was released by cleavage of the ester linkage in NAMA or APAA with hydroxylamine (see Figure 7). The availability of these two methods for severing the bond between $tRNA_1^{Arg}$ and the ribosomal components with which it reacts should prove of value in future efforts to determine the precise site(s) in the 16S RNA, as well as the identity of the 30S subunit proteins, to which the tRNA is attached.

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