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Cross-Linking of the Anticodon of P and A Site Bound tRNAs to the Ribosome via Aromatic Azides of Variable Length: Involvement of 16S rRNA at the A Site

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ABSTRACT: The topography of the ribosomal decoding site was explored by affinity labeling from the 5′-anticodon base, 5-(carboxymethoxy)uridine-34, of P or A site bound $tRNA_1^{Val}$. A nitrophenyl azide was attached to the carboxyl group of this nucleotide via side chains varying in length from 18 to 24 Å. Binding of acetylvalyl-tRNA to the P site was codon dependent and that of valyl-tRNA to the A site was both codon and elongation factor Tu (EFTu) dependent. Cross-linking to both A and P sites was irradiation, probe, codon, and, in the case of the A site, EFTu dependent. Putative P-site cross-linked aminoacyl-tRNA was reactive with puromycin. The yield of cross-linking was little affected by placement of the tRNA at the A or P site but varied considerably with the length and structure of the probe side chain. When the distance from the pyrimidine C-5 atom to the azide group was 23 Å, 42–45% cross-linking was obtained at each site, but when the distance was decreased to 18 Å, only 7–12% was found. Placing an S–S bond in the center of the 23-Å leash decreased the A-site yield to about half, while insertion of a CONH group decreased A-site cross-linking about 8-fold. P-site cross-linking was more sensitive to mercaptan quenching (50% at 0.5 mM) than was that at the A site (50% at >2.0 mM) but both were partially shielded from solvent. A-site cross-linking was mainly (≥60%) to the 16S rRNA with the remainder being to 30S protein(s). P-site cross-linking was only to 30S proteins. However, as the P-site cross-link was not completely stable to isolation, there may also be unstable cross-linking elsewhere. Sequence analysis of the cross-linked oligomer(s) is described by Ciesiolka et al. [Ciesiolka, J., Gornicki, P., & Ofengand, J. (1985) *Biochemistry* (following paper in this issue)].

Previous studies from this laboratory have shown that the anticodon loop of tRNA bound at the ribosomal P site can be cross-linked by cyclobutane dimer formation to a specific single-stranded loop of 16S rRNA (Ofengand et al., 1979; Ofengand & Liou, 1980). Cross-linking is between the 5′-anticodon base, 5-(carboxymethoxy)uridine-34 (cmo^5U-34)[1] in $tRNA_1^{Val}$, the tRNA used for most of these experiments, and C-1400 of 16S RNA (Prince et al., 1982; Ehresmann et al., 1984). Analogous cross-linking has been found in yeast (Ofengand et al., 1982; Ehresmann et al., 1984) and *Artemia salina* (Ciesiolka et al., 1985b) ribosomes to a cytidine residue which is the eukaryotic equivalent of C-1400. This cytidine is located in the center of a 16 nucleotide long sequence that has been conserved in all known small subunit rRNA primary structures [reviewed in Ofengand et al. (1984)]. The significance of this close association between P-site tRNA anticodon and 16S rRNA is so far not known, although the conservation of both sequence and proximity to the anticodon across all species studied is a strong indication of functional importance.

The involvement of rRNA at the decoding site indicated by these findings prompted us to ask what other segments of 16S RNA might also be involved. Although the secondary structure of this RNA is now well established (Noller, 1984; Brimacombe et al., 1983), its tertiary folding in the ribosome is little understood and the only functions so far known for 16S RNA are the well-established Shine–Dalgarno base-pairing interaction with mRNA [reviewed by Gold et al. (1981)] and the anticodon decoding site contact at C-1400 cited above. In order to locate additional regions of rRNA near the decoding site without the limitation of chemical or photochemical requirements for cross-linking, as in cyclobutane dimer formation, a nonspecific aryl azide photoaffinity probe was attached to the same tRNA nucleotide, cmo^5U-34. The

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[1] Abbreviations: EFTu, elongation factor Tu from *Escherichia coli*; cmo^5U-34, 5-(carboxymethoxy)uridine at position 34 of tRNA; NAK, 6-[(2-nitro-4-azidophenyl)amino]caproate; SuNOH, *N*-hydroxysuccinimide; NAK–SuNO, SuNOH ester of NAK; NAG, (2-nitro-4-azidophenyl)glycine; NAG–SuNO, SuNOH ester of NAG; NAL, *N*-(NAG)-β-alanine; NAL–SuNO, SuNOH ester of NAL; SNAP, 3-[[2-[(4-azido-2-nitrophenyl)amino]ethyl]dithio]propionate; SNAP–SuNO, the SuNOH ester of SNAP; EDC, 1-[3-(dimethylamino)-propyl]-3-ethylcarbodiimide; EDA, ethylenediamine; BD-cellulose, benzoylated DEAE-cellulose; Val-tRNA, valyl-tRNA; AcVal-tRNA; *N*-acetylvalyl-tRNA; $tRNA^{NAK}$ or $tRNA^{NAG}$, $tRNA^{Val}$ modified to contain a NAK or NAG group on the cmo^5U-34 residue as shown in Figure 1 of this paper; $tRNA^{SNAP}$ or $tRNA^{NAL}$, $tRNA^{Val}$ similarly modified to contain an NAL or SNAP group; Me_2SO, dimethyl sulfoxide; DNP, 2,4-dinitrophenyl; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RNase, ribonuclease; TCA, trichloroacetic acid.

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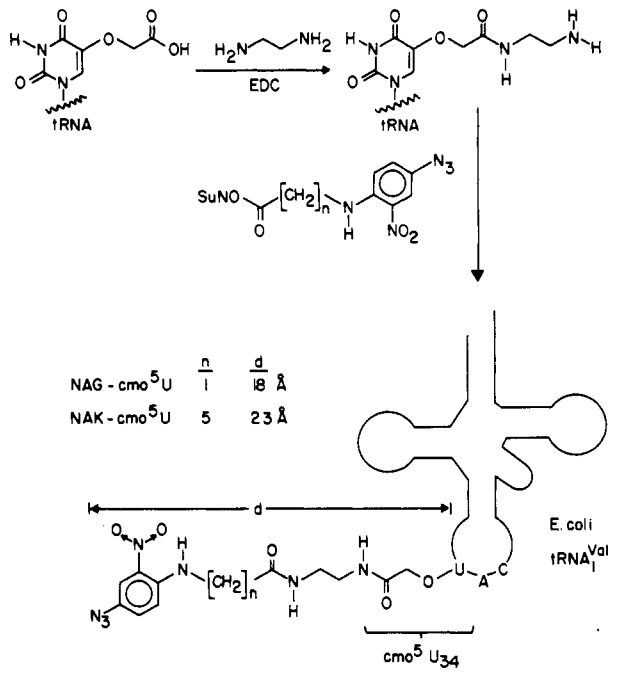

FIGURE 1: Scheme for addition of the nitrophenyl azide group to the cmo^{5}U-34 residue of *E. coli* tRNA$_1^{Val}$. Condensation with ethylenediamine was done at pH 4.0, and reaction with the SuNO ester of NAK or NAG was done at pH 8.1. Probe lengths are given for the most extended configuration.

length and structure of the alkyl chain leash was varied in order to allow us to explore in some detail the nature of other ribosomal components within range of the probes. When the appropriate probe was used, efficient cross-linking at both A and P sites was found. Cross-linking from A site bound tRNA was mainly to 16S RNA.

Preliminary accounts of part of this work have appeared (Gornicki et al., 1983; Ofengand et al., 1984, 1985).

Experimental Procedures

Materials. The following materials were obtained or prepared as described previously: *Escherichia coli* tRNA$_1^{Val}$, BD-cellulose, RNase T$_1$, EDA, EDC, acrylamide, bis(acrylamide), Me_2SO, urea, poly(C_2,A), anti-DNP antibody, [^{14}C]valine, and Soluene 350 (Gornicki et al., 1984); NAK–SuNO, NAG–SuNO, EFTu, tight couple *E. coli* ribosomes, puromycin, and cellulose nitrate membrane filters (Kao et al., 1983); poly(U_2,G), [^{3}H]valine, SDS, RNase-free sucrose, and Val-tRNA synthetase (Hsu et al., 1984). ε-DNP-lysine was from Sigma, and β-[^{3}H]alanine was from Amersham. SNAP–SuNO was a gift from New England Nuclear.

Buffers. Buffer A contained 10 mM potassium phosphate (pH 6.5) and 100 mM NaCl. Buffer B contained 50 mM Hepes (pH 7.5), 75 mM KCl, 75 mM NH_4Cl, and 7 mM $Mg(OAc)_2$. Buffer C contained 20 mM Mes (pH 6.0), 100 mM NH_4Cl, and 0.5 mM $Mg(OAc)_2$. Buffer D contained buffer C plus 100 mM LiCl and 3 mM EDTA.

(Ac)Val-tRNA^{NAK} and (Ac)Val-tRNA^{NAG}. *E. coli* $_1^{Val}$ was derivatized at cmo^{5}U-34 with ethylenediamine and then condensed with either NAK–SuNO or NAG–SuNO as illustrated in Figure 1. After aminoacylation and N-acetylation, the modified tRNA was purified by BD-cellulose chromatography. All procedures were as described by Gornicki et al. (1984) except that the ratio of reagent to EDA-modified tRNA was varied depending on the amounts available. For nonradioactive NAK–SuNO and NAG–SuNO, an 800–1400-fold excess of reagent gave an 80–90% yield of modified tRNA. Aminoacylation levels in picomoles per A_{260} unit were 1300 for tRNA^{NAG} and 1200–1500 for tRNA^{NAK}. Acetylation was always 98% or greater.

Table I: Characterization of Different Aromatic Azide Derivatives of tRNA^{Val}

derivative	length (Å)[a]	anti-DNP[b] B	XL	100(XL/B)
SNAP	24	91	54	59
NAK	23	87	47	54
NAL	23	77	42	55
NAG	18	69	48	68

[a] Measured from C_5 of U-34 to the N_3 in the maximally extended conformation. [b] Assayed as under Experimental Procedures with antibody excess. Values are percent of added Val-tRNA bound (B) or cross-linked (XL).

The ability of modified tRNA to bind and cross-link to anti-DNP antibody was used as a test for the intactness of the azidonitrophenyl moiety. Three different amounts of Ac-[^{3}H]Val-tRNA (0.5–4 pmol) were incubated with 25–35 pmol of antibody in 100 μL of buffer A for 10 min at 37 °C followed by 20 min at 0 °C. After an equal volume of buffer A or 0.6 mM DNP-Lys in buffer A was added, the mixture was incubated 10 min at 37 °C, chilled to 0 °C, and filtered through cellulose nitrate membranes with cold buffer A as a wash. The difference between the two samples was taken as the amount of tRNA specifically bound to the antibody. The amount of tRNA cross-linked was determined in a third tube which was irradiated as described below for ribosome cross-linking before addition of the DNP-Lys solution. The amount of tRNA cross-linked was taken as the difference between the irradiated and nonirradiated DNP-Lys-treated samples.

Other Modified tRNAs. tRNA$^{[3H]NAL}$ was prepared by first reacting NAG–SuNO with β-[^{3}H]alanine at pH 8 followed by condensation of the carboxyl group with SuNOH in the presence of dicyclohexylcarbodiimide and finally reacting with EDA-derivatized tRNA$_1^{Val}$ as described above for reaction with NAK–SuNO and NAG–SuNO. tRNA^{SNAP} was prepared similarly from SNAP–SuNO. Both tRNAs were aminoacylated ([^{14}C]valine was used with tRNA$^{[3H]NAL}$) and purified by BD-cellulose chromatography. All SH-containing reagents were omitted when working with SNAP. Modification levels were tested by binding and cross-linking to anti-DNP antibody just as for tRNA^{NAK} and tRNA^{NAG} (see Table I).

Ribosomal P- and A-Site Binding and Cross-Linking. P-site binding reactions contained, per milliliter, 120 pmol of Ac-[^{3}H]Val-tRNA, 7 A_{260} units of *E. coli* 70S ribosomes, 50 μg of poly(U_2,G), 50 mM Hepes (pH 7.5), 50 mM NH_4Cl, and 7 mM $Mg(OAc)_2$. Incubation was at 37 °C for 20 min. A-site binding mixtures contained, per 0.95 mL of buffer B, 90 pmol of Val-tRNA, 50 μg of poly(U_2,G), 0.24 μmol of GTP, and 0.42 nmol of EFTu. After 10 min at 37 °C, 3 A_{260} units of *E. coli* tRNA$_1^{Val}$ was added and then 7 A_{260} units of 70S ribosomes. The mixture was incubated 10 min more at 30 °C. Binding was assayed by nitrocellulose membrane filtration at 20 mM Mg^{2+} as described previously (Ofengand et al., 1979). Correction was made for modified tRNA binding to filters in the absence of ribosomes. Codon-independent ribosome binding was measured with poly(C_2,A) in place of poly(U_2,G). A and P site bound tRNA were cross-linked by irradiation with visible light at 0 °C (Kao et al., 1983). Assay was by nitrocellulose membrane filtration at 0.1 mM Mg^{2+} (Ofengand et al., 1979) or by sucrose gradient separation of subunits in buffer C.

Separation of Ribosomal Subunits and Ribosomal RNAs and Proteins by Sucrose Gradient Centrifugation. Ribosomal subunits were separated by centrifugation (SW40 rotor, 27 000

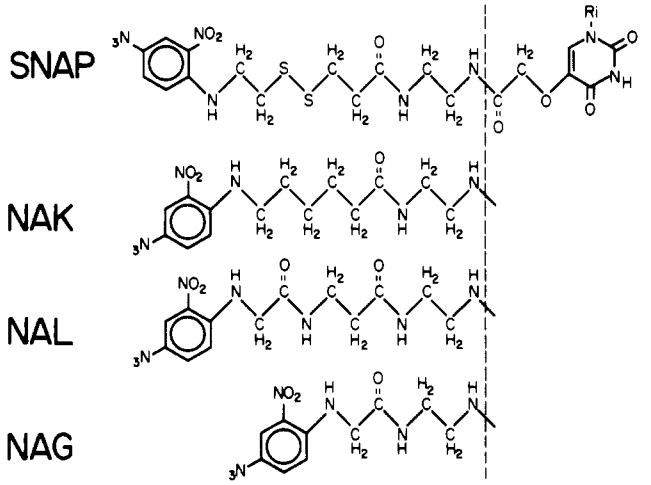

FIGURE 2: Structure of various aromatic azide derivatives used in this work. In each case, the most extended conformation is shown. The distance from the azide group to the C_5 of U-34 is given in Table I.

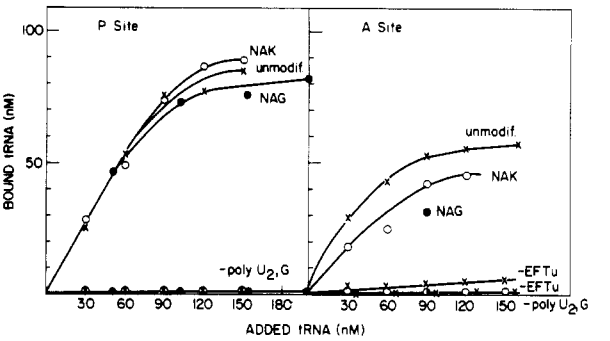

FIGURE 3: Noncovalent binding of NAK- and NAG-modified Val-tRNA and AcVal-tRNA to ribosomal A and P sites, respectively. Binding of the various tRNAs were measured as described under Experimental Procedures. (Left panel) P-site binding of AcVal-tRNA. (○) NAK; (●) NAG; (×) unmodified. (Upper curves) Plus poly-(U_2,G); (lower curve) minus poly(U_2,G). (Right panel) A-site binding of Val-tRNA. (○) NAK; (●) NAG; (×) unmodified. (Upper curves) Plus EFTu; lower curves, minus EFTu or minus poly(U_2,G). The upper minus EFTu curve applies only to unmodified tRNA, while the lower minus curve applies to all other situations.

rpm, 4 °C, 17.5 h) through a 12–37% (w/v) isokinetic sucrose gradient in buffer C. For further analyses, subunits were precipitated with 0.1 volume of 20% KOAc (pH 5), 0.01 volume of 1 M $Mg(OAc)_2$, and 2 volumes of EtOH at −20 °C for 0.5 h or more. The precipitate was dissolved in 8 M urea in buffer C, incubated at 37 °C for 5–7 min, diluted with buffer C to 6 M urea, and centrifuged through the same 12–37% isokinetic sucrose gradient in buffer C plus 6 M urea (SW40 rotor, 39 000 rpm, 15 °C, 20 h). For separation of RNAs and proteins without prior subunit separation, solid urea was added to the cross-linking reaction mixture to 8 M and then processed as described above. For SDS disruption of ribosomes, the incubation/cross-linking mixture was precipitated with 67% EtOH and then treated with 2% SDS, 100 mM LiCl, and 10 mM EDTA at 37 °C for 5 min. After 3-fold dilution with buffer D, the mixture was centrifuged as above in buffer D plus 0.1% SDS (SW40 rotor, 30 000 rpm, 15 °C, 16 h). One A_{260} unit was taken equal to 25 pmol of 70S ribosomes and 67 pmol of 30S ribosomes or 16S rRNA.

Electrophoresis. Formamide gel electrophoresis was performed on 29 × 14 × 0.15 cm 7% polyacrylamide gels [acrylamide–bis(acrylamide), 19:1 w/w] containing 98% formamide (BRL; redistilled nucleic acid grade) and 10 mM NaH_2PO_4, pH 6.0. Precipitated tRNA–rRNA samples were dissolved in 30 μL of 99% formamide, 10 mM NaH_2PO_4, pH 6.0, and 10% sucrose, heated at 50 °C for 2 min and electrophoresed as 1.5-cm bands at 400 V for 10 h at 23 °C with circulation of the electrolyte buffer. After electrophoresis, the 16S RNA and tRNA lanes were stained with Stains-all (Eastman), the [^{32}P]DNA marker lanes were autoradiographed, and the sample lanes were cut into 0.5-cm slices, treated with 1 mL of Soluene 350 Gel Solubilizer (Packard) for 48 h at 23 °C, and then counted in 10 mL of Bray's solution. The DNA fragment markers (generously donated by M. Siddiqui of this Institute) were approximately sized by comparison with standards on nondenaturing polyacrylamide gels.

RESULTS

tRNA Modification. Nitrophenyl azide probes of various lengths were coupled to the cmo^{5}U-34 residue of *E. coli* $_1^{Val}$ in order to investigate the topography of the ribosome in the vicinity of the decoding site. The procedure was the same as that used previously (Gornicki et al., 1984) except that the SuNOH ester was varied. The probes used are indicated in the schematic flow diagram of Figure 1 and in Figure 2. The NAK and NAG probes differed only in their alkyl chain length, the azido group of the NAK-modified tRNA being 23 Å from the pyrimidine ring, while the NAG probe was 5 Å shorter. As will be seen below, this small difference in length nevertheless has a major effect on cross-linking yield. The SNAP probe was used because it could be cleaved by SH reagents and was only slightly longer than NAK. However, the S–S link in the center does confer a small amount of additional rigidity to the molecule. NAL was the same length as NAK but possessed extra constraints due to the CONH group in the center of the alkyl leash.

When DNP-modified tRNA made in exactly the same way was analyzed by T_1 RNAse digestion and polyacrylamide gel electrophoresis, a single DNP-containing oligonucleotide was found and in the position expected for derivatization at cmo^{5}U-34 (Gornicki et al., 1984). An exact determination of the site of NAK and SNAP modification was made by sequence analysis of the isolated T_1 oligonucleotide (Ciesiolka et al., 1985a).

The intactness of the azidonitrophenyl group of the modified tRNAs was tested by binding and cross-linking to anti-DNP antibody. Good cross-reactivity of the azidonitrophenyl group with the DNP combining site is known to occur, and cross-linking with an efficiency of approximately 43% can be obtained (Fisher & Press, 1974). As indicated in Table I, from 70 to 90% of the modified tRNAs could be bound to antibody, and a cross-linking efficiency of 54–68% was obtained. Clearly there is no evidence for any probe decomposition during the tRNA labeling, aminoacylation, and purification process.

Binding and Cross-Linking Activity of the Modified tRNAs. As shown in Figure 3, both NAK- and NAG-modified tRNAs were as competent as unmodified tRNA for specific P-site binding and almost as good as untreated tRNA for A-site binding. Both P-site binding and A-site binding were totally dependent on added mRNA, and the A-site reaction was additionally completely dependent on added EFTu. Under the conditions of these assays, the ribosomes were about 50% active for P-site binding and 35% active at the A site. Nevertheless, when ribosomes were in excess, that is, at 30–50 nM tRNA, essentially all the tRNA could be bound at either ribosomal site.

Cross-linking activity of the modified tRNAs was initially tested at the P site. Since low cross-linking efficiency was expected for these nitroaryl azides, analysis was done by su-

Table II: Specificity of P-Site Cross-Linking of NAK- and NAG-Modified $tRNA^{Val}$ [a]

	pmol cross-linked/pmol of ribosome	
	NAK	NAG
complete	0.170 (35)	0.025 (5.7)
plus puromycin[b]	0.019	0.006
poly(C_2,A) in place of poly(U_2,G)	0.026	0.006

[a] Ribosomal P-site binding, cross-linking, and sucrose gradient analysis were performed as described under Experimental Procedures. Noncovalent binding was 0.49 and 0.44 pmol of tRNA/pmol of ribosomes, for AcVal-$tRNA^{NAK}$ and AcVal-$tRNA^{NAG}$, respectively. All cross-linking was to the 30S subunit. Values in parentheses are percent cross-linking. [b] Puromycin (3 mM) at 37 °C for 6 min *after* irradiation but before centrifugation.

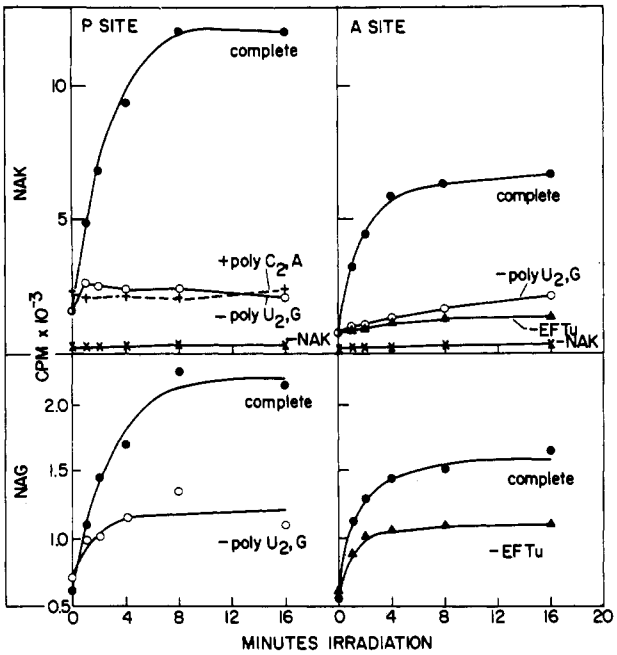

FIGURE 4: Kinetics and specificity of ribosomal A- and P-site cross-linking for NAK- and NAG-modified tRNA. P- and A-site binding and cross-linking were performed as described under Experimental Procedures. Values given are the cpm bound to filters with no blank subtractions. (●) Complete system; (○) minus poly(U_2,G); (+) poly(C_2,A) replaced poly(U_2,G); (×) unmodified tRNA; (▲) minus EFTu.

crose gradient centrifugation (Hsu et al., 1984). This also allowed determination of the subunit that was cross-linked. The results (Table II) showed that both $tRNA^{NAK}$ and $tRNA^{NAG}$ could be cross-linked and that only the 30S subunit was involved (see also Figure 6). Cross-linking was at the P site since puromycin treatment *after* cross-linking released most of the AcVal from the cross-linked tRNA. If the residual radioactivity found in the presence of the nonspecific mRNA, poly(C_2,A), is taken as a blank, then virtually all of the codon-dependent cross-linking was releasable by puromycin. Although the same results were obtained for both tRNAs, the cross-linking efficiencies were markedly different, 35% for NAK and 5.7% for NAG, respectively. Thus, the 5-Å difference in length is quite important in determining the accessibility to ribosomal components.

Kinetics and Specificity of the Cross-Linking Reaction. A more detailed examination of cross-linking at both P and A sites is shown in Figure 4. Assays were performed by filter binding, and the actual cpm bound to the filter is indicated so that the magnitude of the background subtraction can be assessed. In all cases, the reaction was over after a 5–10-min irradiation, which is consistent with previously determined

Table III: Cross-Linking of Modified $tRNA^{Val}$ to Ribosomal P and A Sites[a]

modified tRNA	dependency	site	pmol of tRNA/pmol of ribosomes		% cross-linking
			bound	cross-linked	
NAK	irrad	P	0.56	0.22	39
	codon	P	0.56	0.21	38
NAK*	irrad	P	0.46	0.015	3.3
NAG	irrad	P	0.47	0.035	7.4
	codon	P	0.47	0.021	4.5
none	irrad	P	0.49	<0.001	<0.2
NAK	irrad	A	0.27	0.13	48
	codon	A	0.26	0.10	38
	EFTu	A	0.27	0.11	41
NAK*	irrad	A	0.20	0.004	2.0
SNAP	irrad	A	0.44	0.13	30
NAL	irrad	A	0.15 (0.13)	0.01 (0.01)	7 (8)
NAG	irrad	A	0.18	0.021	12
	EFTu	A	0.18	0.011	6.1
none	irrad	A	0.30	<0.001	<0.2

[a] Binding and cross-linking were performed as under Experimental Procedures. The values in the absence of the stated dependency have already been subtracted. The actual values for NAK and NAG can be obtained from Figures 3 and 4, respectively. The blank values for SNAP and NAL were similar. NAK*, (Ac)Val-$tRNA^{NAK}$ prephotolyzed under standard conditions in the absence of ribosomes. Values for NAL in parentheses were obtained by measuring [^{3}H]NAL. Other values were obtained from Ac[^{3}H]Val-tRNA.

kinetics for nitroaryl azide probes in this irradiation apparatus (Ofengand et al., 1977; Kao et al., 1983).

A summary of cross-linking yields from this and similar experiments is given in Table III. Since the choice of dependency, i.e., polynucleotide, EFTu, probe, or irradiation, affects the percent cross-linking calculation when the yield is low, as in the case of NAG, Table III lists all of the values. Note that there is little effect when good cross-linking was obtained, as with NAK. As already indicated in Figure 3, both NAK and NAG cross-linked to the P site, NAK being 5–8 times more efficient. The reaction was codon, probe, and irradiation dependent, and prephotolysis reduced cross-linking 12-fold. Equal or better cross-linking occurred at the A site despite the lower noncovalent binding. The reactions showed the same dependencies and in addition required EFTu. The difference between NAK and NAG at the A site varied from 4- to 7-fold, the principal cause for variation being uncertainty about the apparent EFTu-dependent reaction. Prephotolysis reduced A-site cross-linking 24-fold. Note that although SNAP, NAK, and NAL were almost the same length (Figure 2), they not only bound with different efficiency to the A site but most strikingly cross-linked with different yields. The greatest difference was between NAK and NAL where a 7-fold change was found.

Mercaptoethanol Inhibition of NAK and NAG Cross-Linking. In view of rather similar levels of cross-linking at the A and P site for NAK, and also for NAG although at a much lower level than for NAK, we hypothesized that NAK cross-linking at both P and A sites might be to the same locus, while NAG cross-linking would be to a different site. One way to test this was to determine an inhibition curve for cross-linking as a measure of the accessibility of the probe to solvent. Different sites were expected to show different accessibilities. For example, binding of the azidonitrophenyl group to the DNP combining cavity of antibody resulted in a close fit with exclusion of solvent, as evidenced by the failure of even 2 mM mercaptoethanol to inhibit (Figure 5). On the other hand, the cross-linking of a phenyl azide probe to ribosomal protein S19 (Hsu et al., 1984) was readily blocked (50% at 0.1 mM) as shown by the dashed line in the Figure.

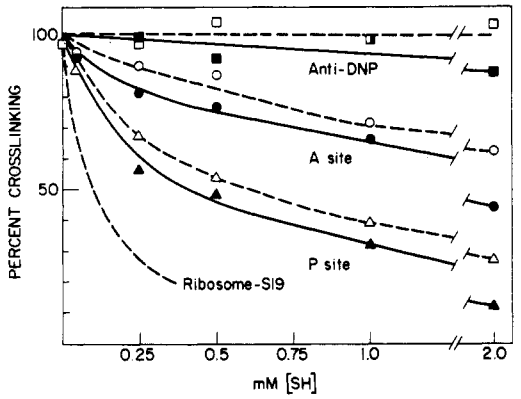

FIGURE 5: Inhibition of cross-linking by mercaptoethanol. Binding and cross-linking of (Ac)Val-tRNA^{NAK} and (Ac)Val-tRNA^{NAG} to ribosomal A (circles) and P (triangles) sites and to anti-DNP antibody (squares) were performed and assayed as described under Experimental Procedures in the presence of the indicated amounts of mercaptoethanol. Binding and cross-linking in the absence of mercaptoethanol, taken as 100%, was an indicated in Table III. Mercaptoethanol had no effect on binding. The dashed line represents inhibition of the cross-linking of azidophenacyl-4-thiouridine in tRNA^{Phe} to protein S19 at the ribosomal A site (Hsu et al., 1984). (Solid symbols) NAK; (open symbols) NAG.

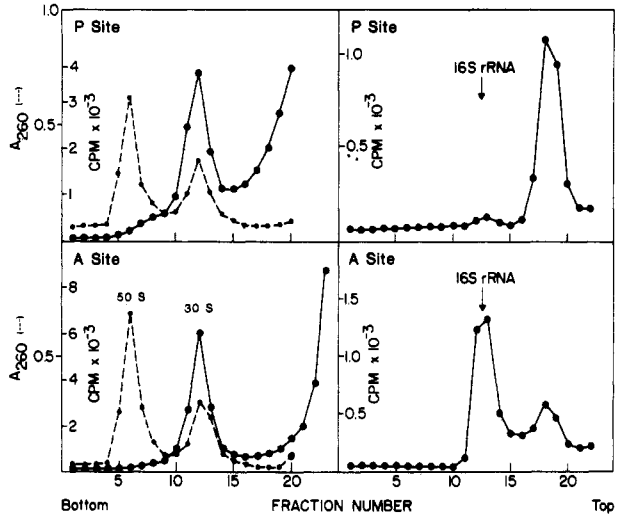

FIGURE 6: Localization of the sites of cross-linking of P and A site bound tRNA^{NAK}. Binding, cross-linking, and separation of ribosomal components were performed and assayed as described under Experimental Procedures. (Upper panels) P site; (lower panels) A site. (Left panels) Separation of ribosomal subunits at 0.5 mM Mg^{2+}. (Right panels) Dissociation of 30S subunits obtained as in the left panel with 8 M urea and centrifugation in 6 M urea. (Solid line) cpm of [^{3}H]valine; (dashed line) A_{260}.

The results with NAK and NAG were surprising. Despite the 4–8-fold difference in cross-linking efficiency, NAK and NAG at the same site had an almost identical quenching curve. The two sites were different, however, the A-site reaction being 4–8-fold more resistant than that at the P site. However, even the P-site reaction was about 5-fold more resistant than cross-linking to S19. These results suggest that at a given site, NAK and NAG, despite their differing length and cross-linking efficiency, may be reacting with the same components. The results also indicate that the A-site contact area is different from that at the P site and further that while both areas are partially shielded from solvent, the A site is more shielded than the P site.

Location of Cross-Linking Sites on the Ribosome. As shown in Figure 6, the site of cross-linking of tRNA^{NAK} at either P site or A site was exclusively to the 30S subunit. However, the components of the 30S subunit which were

Table IV: Evidence for Stability of the tRNA^{NAK}–Ribosomal A Site Cross-Link and Instability of the P-Site Cross-Link[a]

site bound	100(pmol of tRNA/pmol of ribosomal component) 70S[b]	30S[c]	16S[d]	low M_r[e]
P	31	14 (15)	<0.1	15
A	25	20 (23)	15	8
A	18	ND	11[f]	ND

[a] Binding, cross-linking, and separation of ribosomal components were performed and assayed as under Experimental Procedures and as shown in Figure 6. ND, not determined. [b] All 70S cross-linking was irradiation dependent and was measured by the filter assay. [c] Calculated from the cpm/A_{260} ratio after separation of ribosomal subunits by sucrose gradient centrifugation at 0.5 mM Mg^{2+} as in Figure 6. The value in parentheses is the sum of the next two columns. [d] Calculated from the cpm/A_{260} ratio after urea gradient separation of the 30S fraction into 16S RNA and proteins. [e] Calculated from footnote *d* and the distribution of cpm between 16S RNA and low molecular weight components. This fraction could contain 16S RNA–tRNA degradation products, as well as protein–tRNA covalent adducts. [f] Calculated from the cpm/A_{260} ratio after direct analysis of cross-linked 70S ribosomes on a urea gradient.

cross-linked differed in the two cases. Sixty percent of the A-site cross-link was to 16S rRNA, while all of the P-site cross-link was to low molecular weight material, presumably 30S protein (Figure 6 and Table IV). As shown in Table IV, most of the A-site cross-linking to the 70S as assayed by filter binding could be recovered after sucrose gradient centrifugation, and the same proportion of 16S rRNA to 70S cross-linking was obtained when the cross-linked reaction mixture was treated immediately with urea and analyzed on a urea-containing gradient. Thus, the A-site cross-link appeared stable, like all previous aryl azide induced ribosome and protein cross-links studied in this laboratory.

Surprisingly, the P-site cross-link was unstable, about half of the cross-linked material detected by filter assay being lost upon sucrose gradient centrifugation (Table IV). The material remaining with the 30S fraction was all low molecular weight after urea gradient centrifugation (Figure 6). Incubation of the isolated 30S fraction, with or without added 6 M urea, over the course of 20 h at 23 °C led to the gradual conversion of half of the remaining radioactive material, initially totally bound to cellulose nitrate filters, into a filterable form. This incubation treatment did not, however, affect the total TCA-precipitable cpm (data not shown).

Characterization of the Cross-Linked rRNA–tRNA^{NAK} and rRNA–tRNA^{SNAP}. The 16S RNA–tRNA covalent complex formed by irradiation of A site bound Val-tRNA^{NAK} or Val-tRNA^{SNAP} could also be isolated by direct SDS disruption of cross-linked tRNA–ribosome complexes. Centrifugation through an SDS–sucrose gradient separated the rRNAs from each other and from non-cross-linked tRNA. Figure 7 shows an SDS–sucrose gradient analysis of the cross-linked ribosome–tRNA^{SNAP} complex. Results with the tRNA^{NAK} complex were similar. All of the 16S RNA cross-linking was EFTu dependent and thus must have come from A site bound tRNA. The apparent cross-linking to 23S RNA was an artifact since (a) there was no cross-linking to the 50S subunit (data not shown; but see Figure 6 for the analogous case with NAK) and (b) sedimentation of the "23S" fraction through a 6M urea sucrose gradient resulted in all of the radioactivity being converted to small molecular weight material, while >75% of a similarly treated "16S" fraction resedimented with a 16S RNA UV marker (data not shown). The yield of cross-linked 16S RNA complex, 0.10 pmol of tRNA/pmol of rRNA, was the same as the EFTu-dependent cross-linking per picomole ribosomes calculated by the filter assay. In order

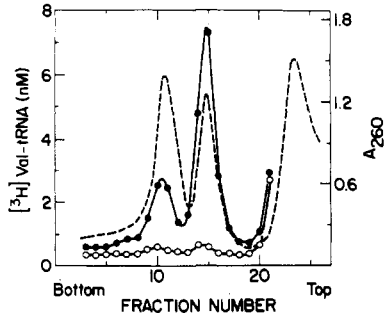

FIGURE 7: EFTu dependence of Val-tRNA^{SNAP} cross-linking to 16S rRNA. Complexes at the A site with and without EFTu were formed, irradiated, treated with SDS, and run on SDS–sucrose gradients as described previously (Ciesiolka et al., 1985a,b; Gornicki et al., 1985). (Filled circles) Plus EFTu; (open circles) minus EFTu; (dashed line) A_{260}. There was 0.10 EFTu-dependent pmol of tRNA cross-linked per picomole of 16S RNA.

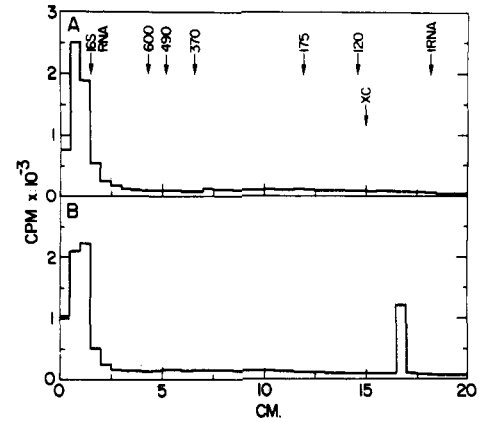

FIGURE 8: Formamide–PAGE analysis of the integrity of the 16S RNA–tRNA^{NAK} and 16S rRNA–tRNA^{SNAP} complexes. The precipitated cross-linked complexes of 16S RNA–[^{3}H]Val-tRNA^{NAK} (panel A), 9730 cpm, and 16S RNA–[^{3}H]Val-tRNA^{SNAP} (panel B), 9190 cpm, were electrophoresed as described under Experimental Procedures. The 16S RNA and tRNA markers were located by staining, the 5′-^{32}P-labeled DNA markers (120–600 nucleotides as indicated) were detected by autoradiography, and XC is the position of xylene cyanol FF. The sample lanes were sliced and counted for [^{3}H]valine. Recoveries were 71% and 89% for panels A and B, respectively. The tRNA-like peak in panel B is 17% of the recovered radioactivity.

to verify that cross-linking was via the SNAP probe, the complex was treated with 25 mM DTT at 37 °C for 30 min prior to running the SDS gradient. This reduced both the 16S peak and 23S peaks by 70–73% (data not shown). Incomplete cleavage most probably is due to oxidation of the –S–S– bridge to a form not cleavable by thiols. Similar incomplete cleavage has also been noted in other cross-links formed with this reagent (Ciesiolka et al., 1985a; R. Denman and J. Ofengand, unpublished results).

Figure 8 shows that both cross-links occurred to full-length rRNA. When the rRNA–tRNA^{NAK} complex isolated by SDS–sucrose gradient centrifugation was denatured with 99% formamide and 10 mM NaH_2PO_4, pH 6.0 (Grierson, 1982), and electrophoresed (panel A), all of the attached Val-tRNA was found in the high molecular weight region with little or no material migrating like cleavage products. When the rRNA–tRNA^{SNAP} complex was analyzed (panel B), less than 18% of the cross-linked tRNA–rRNA was in the form of a tRNA-like fragment. This may have arisen by cleavage of the –S–S– link in SNAP, releasing a modified tRNA. The slightly slower mobility of the radioactive band compared to the marker of unacylated and unmodified tRNA^{Val} would be consistent with the retarding effect of the retained half of the SNAP moiety (Figure 5) plus the loss of two negative charges due to both derivatization of the cmo^{5}U carboxyl group and the addition of the positively charged amino acid. The cross-linked complex also migrated slightly slower than control 16S RNA as noted previously by Zimmerman et al. (1979).

Discussion

In all ribosomal A-site cross-linking experiments, it is important to be sure that inadvertent translocation to the P site did not take place due either to contaminating amounts of EFG or to nonspecific translocation. Such problems have been reported in the past (Johnson & Cantor, 1980). In the current experiments this complication is ruled out by the demonstrated difference in cross-linking pattern between purported A-site reaction and direct, puromycin-sensitive, P-site cross-linking (Figure 6). Moreover, under the usual conditions for such reactions it is virtually impossible to translocate an aminoacyl-tRNA, only peptidyl-tRNAs being active.

The one-third of A site cross-linking to low molecular weight material either could be to 30S protein or could be due to degradation of the rRNA–tRNA covalent complex, either by nucleases or by decomposition of the covalent link itself. The fact that both 70S and 30S ribosomes gave the same fraction of total cross-linking to 16S rRNA suggests that nuclease cleavage is not a likely explanation. If cross-linking is indeed to 30S protein(s), then those regions should be near the rRNA cross-linking site and probably help make up the probe binding cavity.

The nature of the P-site cross-linked components is less clear. While it is most likely that the low molecular weight material obtained by urea treatment of the 30S complex is protein, it is nevertheless possible that it represents decomposition of a cross-link to 16S RNA. The half of the cross-linked material that was lost upon subunit separation by sucrose gradient centrifugation could have been to any ribosomal component of either subunit. Dissociation of 5S RNA–tRNA or protein–tRNA complexes could have occurred during that step. Alternatively, a labile covalent link to any ribosomal component could have decomposed during the time needed for centrifugation. These two processes are not necessarily mutually exclusive. Some support for the latter mode of decomposition was obtained by the finding that isolated 30S–tRNA complexes could lose still more tRNA in a time-dependent manner. The unstable P-site cross-linking is not likely to be due to noncovalent association of photolyzed tRNA^{NAK} since the magnitude of such an association, at least as assessed by filter binding, was less than 10% of the covalently bound tRNA^{NAK} at the P site, whereas 50% of the filter-bound tRNA was lost upon sucrose gradient centrifugation. This is at least 5 times the amount of the prephotolyzed blank shown in Table III.

The most surprising feature of these cross-linking results was the high yield of covalent product from a nitrophenyl azide probe. In our previous experiences with aryl azides, ≤15% yields were obtained (Ofengand et al., 1980; Schwartz & Ofengand, 1982; Kao et al., 1983; Hsu et al., 1984). Generally speaking, a high yield indicates that the azido function is not readily accessible to solvent since parallel reaction with buffer components or even with water are the primary reasons for consumption of the active nitrene intermediate. The 39–48% yields for NAK cross-linking at P and A sites, respectively, are indicative of a close fit into a hydrophobic cavity of some sort, since even when the azidonitrophenyl group was bound to the DNP-combining site of antibody, only 54–68% cross-linking was obtained (Table I). This conclusion is reinforced by the mercaptan inhibition experiment of Figure 5. In line with the increase in cross-linking yield from P to A site

(39–48%), mercaptan inhibition decreased in the same direction.

On the other hand, a low cross-linking yield does not necessarily indicate accessibility to solvent but could be due instead to a low probability of finding reactive atoms. This appears to be the situation with NAG cross-linking where, despite the 8-fold lower yield compared to NAK, mercaptan inhibition was about the same for both probes. In this case, we suggest that both probes react with the same locus but that the NAG probe, being 6 Å shorter, has only a low residence lifetime in the binding site. The same line of reasoning could be applied to the SNAP and NAL reagents (Table III) although no mercaptan inhibition was done in these two cases. Here we reason that the additional rigidity imposed by the S–S and CONH groups decrease the statistical probability of being in the right place enough of the time. Sequence analysis of the 16S RNA site cross-linked with NAG or SNAP would test this proposal. At least in the latter case, cross-linking at the A site was to the same nucleotide as NAK, despite the halved cross-linking yield shown in Table III (Ciesiolka et al., 1985a).

Location of the A cross-linking site in a hydrophobic pocket is consistent with the electron microscopic localization of C-1400 of 16S rRNA to a position deep in the cleft of the 30S subunit (Gornicki et al., 1984) since that residue is the one cross-linked to NAK at the A site (Ciesiolka et al., 1985a).

Registry No. NAG-SuNO, 60177-06-2; NAK-SuNO, 64309-05-3; SNAP-SuNO, 81705-07-9; $NH_2(CH_2)_2NH_2$, 107-15-3; [^{3}H]-N-$H_2(CH_2)_2CO_2H$, 73603-73-3; poly(C_2,A), 26182-06-9; poly(U_2,G), 26680-26-2.

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