Identification of the Site of Cross-Linking in 16S rRNA of an Aromatic Azide Photoaffinity Probe Attached to the 5′-Anticodon Base of A Site Bound tRNA

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ABSTRACT: The site of *Escherichia coli* 16S ribosomal RNA cross-linked to the 5′-anticodon base of A site bound *E. coli* valyl-tRNA was identified. Cross-linking was via the affinity probe 6-[(2-nitro-4-azidophenyl)amino]caproate (NAK) or 3-[[2-[(2-nitro-4-azidophenyl)amino]ethyl]dithio]propionate (SNAP) attached to the carboxyl group of the 5′-anticodon base 5-(carboxyethoxy)uridine via an ethylenediamine spacer [Gornicki, P., Ciesiolka, J., & Ofengand, J. (1985) *Biochemistry* (preceding paper in this issue)]. With both probes, RNase T_1 digestion of the isolated 16S RNA–tRNA covalent complex, 5′-^{32}P postlabeling, and gel electrophoresis yielded two oligonucleotides larger than any fragments from non-cross-linked tRNA or rRNA. Appearance of the oligomers was dependent on the presence of the probe on the tRNA. Unmodified tRNA in the A and/or P sites did not yield any product. The presence of elongation factor Tu in the incubation mixture was also required. Dithiothreitol (DDT) treatment of the SNAP-induced covalent complex prior to electrophoresis also abolished the oligomers. Only the larger of the two oligomers (present in a 3:1 ratio) was sequenced. The SNAP dimer was cleaved with DTT, and the rRNA and tRNA oligomers were separated and sequenced as monomers. The NAK dimer was sequenced without cleavage by taking advantage of the differences in electrophoretic mobility among sequence and/or composition isomers of the same length. In both cases, the rRNA oligomer was identified as $UACACACCG_{1401}$, and the nucleotide cross-linked was shown to be the C1400 residue. The expected tRNA modification site was also identified. Previously, the same C1400 residue was found directly cross-linked via cyclobutane dimer formation to the 5′-anticodon base of P site bound tRNA. The present results indicate that the single-stranded loop of rRNA from residues 1392 to 1407, conserved in all known small subunits rRNAs, is a prominent component of the ribosomal decoding site and suggest that the C1400 residue may be in a uniquely exposed position.

Although the sequence and secondary structure of the three *Escherichia coli* ribosomal RNAs are known in considerable detail (Noller, 1984; Brimacombe et al., 1983), the three-dimensional packing of these RNAs into a 220-Å diameter ribosomal particle is only poorly understood. Likewise, knowledge about functional roles for ribosomal RNA is still in its infancy. The frequently described interaction between complementary sequences of 5S RNA and the GTψC loop of tRNA [reviewed in Ofengand (1980)] has now been cast into serious doubt (Pace et al., 1982; Zagoroska et al., 1984; Pieler et al., 1984), at least with regard to 5S functional aspects. The close association of 23S RNA with the peptidyltransferase center [reviewed in Ofengand (1980)] has been recently verified by the finding that numerous rRNA mutations conferring resistance to antibiotics that affect peptide bond formation are located close to each other in the rRNA secondary structure [reviewed in Ofengand et al. (1984) and Noller (1984)] and near to the recently identified site of cross-linking of the 3′ end of tRNA (Barta et al., 1984). Resistance to thiostrepton which acts at the GTPase center (Cundliffe, 1980) has been localized to A1067 in 23S rRNA (Thompson et al., 1982), a contact site with EFG has been identified in the sequence 1055–1076 (Sköld, 1983), and a single cut by α-sarcin after G2661 blocks elongation factor dependent binding of tRNA (Chan et al., 1983).

The need for the base-pairing capability of the 3′ end of 16S RNA with the leader region of mRNA is well-known [reviewed by Gold et al. (1981)] and dimethylation of the m^6_2A residues in the loop near that end is responsible for resistance to kasugamycin (Hesler et al., 1972), an antibiotic that interferes with initiation of protein synthesis (Okuyama et al., 1971). Resistance to paromomycin, an antibiotic that induces miscoding, is produced by mutation of C1409 (Li et al., 1982), and the close contact of C1400 to the anticodon of P site bound tRNA [reviewed in Gornicki et al. (1985)] are other examples of an apparent functional role played by rRNA.

In order to gain further insight into the involvement of rRNA at the decoding site with respect to both function and tertiary structure, photoaffinity probes were placed at the anticodon of tRNA bound to both P and A sites, and the cross-linking prducts were examined for linkage to rRNA. Extensive cross-linking to 16S RNA from A site bound tRNA was found (Gornicki et al., 1985). In this work, the site of cross-linking was determined to the nucleotide level.

A preliminary account of part of this work has appeared (Ofengand et al., 1985).

Experimental Procedures

Materials. Materials not otherwise specified were obtained or prepared as described in Gornicki et al. (1984), Ciesiolka et al. (1985), or Gornicki et al. (1985). $tRNA^{Val}$ modified with NAK[1] or SNAP was prepared as described (Gornicki et al., 1985) except that the reagent excess was 50–70-fold for [^{14}C]NAK–SuNO and yielded 30–40% modification with a specific activity of 1500–1700 pmol of NAK incorporated per A_{260} unit of tRNA. For $tRNA^{SNAP}$, a 50-fold excess of SNAP–SuNO was added twice, and BD-cellulose chroma-

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[1] Abbreviations: DDT, dithiothreitol. All other abbreviations are as given in the preceding paper (Gornicki et al., 1985).

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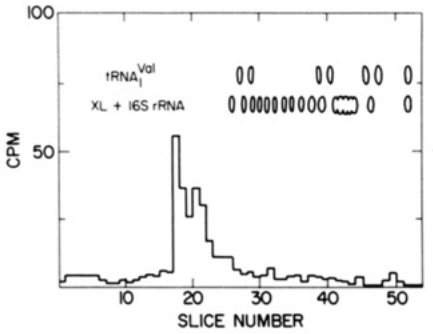

FIGURE 1: PAGE analysis of an RNase T_1 digest of the A-site 16S rRNA–tRNA covalent complex. 16S rRNA–tRNA$^{[14C]NAK}$ was recovered from an SDS–sucrose gradient like that of Figure 7 of Gornicki et al. (1985) by EtOH precipitation, digestion with RNase T_1 (25 Sanko units of RNase T_1 per A_{260} unit of rRNA per 40 μL of 100 mM Tris-HCl, pH 7.5, 4 h, 37 °C), and electrophoresed on 7 M urea-containing 20% PAGE as previously described (Gornicki et al., 1984). The upper two lanes show the position of oligonucleotides obtained by RNase T_1 digestion of *E. coli* tRNA$_1^{Val}$ and 16S RNA, respectively. They were visualized under UV light with a fluorescent screen. The lower graph shows the distribution of [^{14}C]NAK. Radioactivity was determined after solubilization of the gel slices in Soluene 350 (Gornicki et al., 1984). A background value of 12 cpm has been subtracted.

tography was performed before aminoacylation. A 50% reaction yield was obtained as judged by the amount of A_{260} absorbance in the modified and unmodified tRNA peaks from the chromatogram. Aminoacylation was to a level of 1140 pmol/A_{260} unit. Isolation of Val-tRNA was by adsorption and elution from DEAE cellulose (Hsu et al., 1984). All procedures involving SNAP were done in the absence of mercaptans. The nonamer UACACACCG, which is common to all small subunit rRNAs, and the tRNA^{Val} oligomer CACCUCCCUcmo^{5}UACm^{6}AAG were isolated in the 5′-^{32}P-labeled form from *Artemia salina* 18S rRNA–tRNA covalent complexes as described by Ciesiolka et al. (1985). [^{14}C]NAK–SuNO (51.5 mCi/mmol) was prepared by the Isotope Synthesis Group of Hoffmann-La Roche, Inc., under the direction of Dr. Arnold Liebman.

Methods. A-site binding and cross-linking were done as described by Gornicki et al. (1985). Fifteen minutes of irradiation was used. Isolation of the cross-linked 16S RNA–tRNA by SDS–sucrose gradient centrifugation, RNase T_1 digestion, 5′-^{32}P labeling, and gel electrophoresis were performed as described (Ciesiolka et al., 1985; Gornicki et al., 1985) as were all other methods not otherwise specified. The minus enzyme lane of the sequencing gels used the conditions corresponding to U_2 incubation.

RESULTS

Initial Characterization of the Cross-Linked 16S RNA–tRNA Dimer. In order to assess the potential problems involved in isolation of a cross-linked oligonucleotide dimer by RNase T_1 digestion of the 16S RNA–tRNA covalent complex, the dimer was prepared with [^{14}C]NAK-modified tRNA so that *only* probe-modified oligomers would be detected. The 70S ribosome–tRNA covalent complex was disrupted by SDS and the rRNA–tRNA dimer isolated by SDS–sucrose gradient centrifugation (Gornicki et al., 1985). Following RNase T_1 digestion, the sample was analyzed by PAGE (Figure 1). The position of the ^{14}C radioactivity of the probe indicated that one or more rRNA oligonucleotides were cross-linked to the tRNA fragment. None of the radioactivity corresponded in position to that expected for the NAK-labeled tRNA oligonucleotide. An RNase T_1 digest of unmodified tRNA^{Val} was used in this experiment, but it is known from previous work

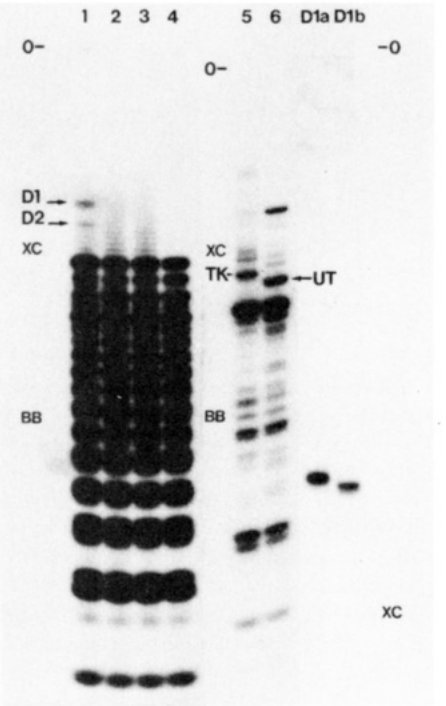

FIGURE 2: Identification and isolation of cross-linked rRNA–tRNA^{NAK} RNase T_1 oligonucleotide dimer by PAGE. Ribosomal A-site binding, cross-linking, SDS sucrose gradient isolation of rRNA–tRNA, RNase T_1 digestion, 5′-^{32}P labeling, and PAGE were done as described under Experimental Procedures using the preparative gel procedure. (Lane 1) Val-tRNA^{NAK} in A site and tRNA^{Val} in P site; (lane 2) as in lane 1 but with unmodified Val-tRNA in A site; (lane 3) as in lane 1 but with no A site tRNA; (lane 4) ribosomes only, without irradiation; (lane 5) tRNA^{NAK} digested with RNase T_1 and 5′-^{32}P labeled; (lane 6) unmodified tRNA treated as in lane 5; (lanes D1a and D1b) dimer D1 eluted, relabeled at the 5′ ends, and purified by electrophoresis. Only the final analytical gel is shown. D1 and D2, cross-linked oligonucleotide dimers; TK, NAK-modified RNase T_1 oligonucleotide from tRNA^{Val} (Gornicki et al., 1985); UT, the same oligonucleotide but unmodified. O, XC, and BB denote the origin, xylene cyanol FF, and bromphenol blue positions, respectively.

that DNP modification (similar to NAK) makes the slowest tRNA band run only slightly slower (Gornicki et al., 1984), and this was confirmed in a direct comparison of unmodified and NAK-modified T_1 oligonucleotides after 5′-^{32}P labeling (Figure 2, lanes 5 and 6). The size of the rRNA fragment or fragments was estimated to be about 10 residues in length, based on its relative position in the gel and the knowledge that a 15-mer of tRNA must be attached to it. The main virtue of this experiment, however, was not in precisely delineating the size of the oligomer but rather in demonstrating unequivocally that no dimers were produced that were smaller than the largest rRNA or tRNA oligomer.

Isolation and 5′-End Analysis of the rRNA Oligonucleotides Cross-Linked to tRNA^{NAK}. Figure 1 showed that the tRNA–rRNA cross-linked oligonucleotide dimer generated by T_1 RNase digestion was larger than any T_1 oligonucleotide from either 16S RNA or tRNA alone. On the basis of this information, the mixture of oligonucleotides produced by complete digestion of the cross-linked rRNA–tRNA with RNase T_1 was 5′-^{32}P labeled (10% yield) and displayed according to size by one-dimensional PAGE in 8 M urea (Figure 2, lane 1). Controls were the equivalent rRNA fractions from SDS–sucrose gradients of mixtures in which unmodified Val-tRNA was placed in the A site (lane 2) or no Val-tRNA was added (lane 3). Unincubated and unirradiated ribosomes were used as an additional control (lane 4). Lanes 5 and 6 show for comparison the distribution of the T_1 oligomers from

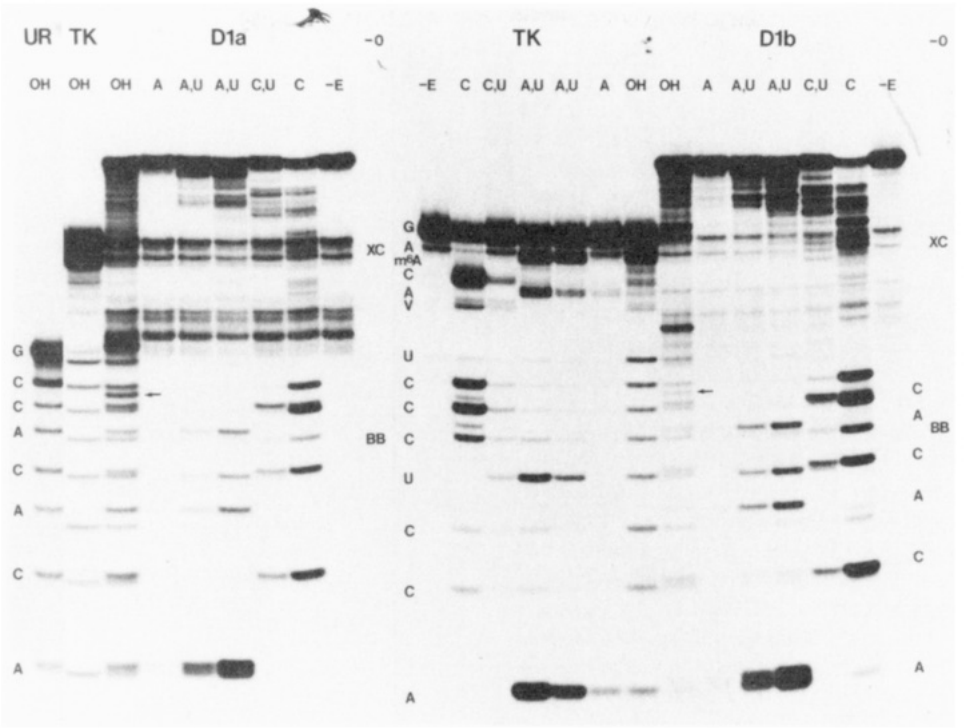

FIGURE 3: Sequencing gel analysis of dimer D1a and D1b. D1a and D1b, the isolated dimers from Figure 2; TK, the NAK-modified tRNA fragment from Figure 2; UR, unmodified rRNA nonamer UACACACCG. Oligomers were recovered from PAGE and sequenced enzymatically as described by Ciesiolka et al. (1985). (-E) No enzyme; (C) CL3; (C,U) *B. cereus*; (A,U) Phy M; (A) U_2; (OH) limited alkaline hydrolysis. XC and BB mark the tracking dye positions. The deduced sequence for each oligomer is shown. V is cmo^5U.

$tRNA^{NAK}$ and unmodified tRNA, respectively. Sequence analysis of TK and UT (see below) confirmed that these bands corresponded to the modified and unmodified forms, respectively, of the anticodon-containing T_1 oligonucleotide. These are the largest oligomers produced by RNase T_1 from this tRNA. Consequently, the slower moving bands should be incomplete digestion products. Indeed, when more RNase T_1 was used, these bands but not TK or UT, disappeared. Photolysis of $tRNA^{NAK}$ had no effect on the oligomer distribution (data not shown).

Only when Val-$tRNA^{NAK}$ was placed in the A site were there any oligonucleotides larger than those from the parent rRNA or tRNA. Two such bands were found, D1 and D2, in a ratio of 4:1 according to the ^{32}P labeling. When the experiment of lane 1 was repeated with an 80% labeling yield, the same pattern was obtained, and the ratio of D1 and D2 was 3:1. These ratios are not too dissimilar from the 60:40 ratio found with [^{14}C]NAK as the method of detection (Figure 1).

For sequence analysis, band D1 was excised, eluted, and relabeled at its 5′ end with ^{32}P to 60% of expected. The expected level was estimated from the amount of cross-linked complex applied to the first gel and the known efficiencies of recovery as deduced from model experiments. Purification of D1 by extended electrophoresis led to its separation into two bands, D1a and D1b. The relative electrophoretic mobility and radiochemical purity of D1a and D1b are shown in Figure 2 (the two right-hand lanes). The gel system was the same as for the other lanes, but the electrophoresis time was much extended as can be seen by comparing the XC dye markers. Rephosphorylation and electrophoresis of D2 plus the region between it and D1, where putative nonphosphorylated D2 would be expected, led to the appearance of two bands, one (D2′) intermediate in mobility between D1 and D2 and one like D2 (data not shown). The amount of ^{32}P in bands D1a plus D1b after relabeling and purification was 70% of the total from all of the relabeled D1 and D2 bands. This is similar to the percent found initially.

5′-end analysis (Ehresmann et al., 1984) of all four bands showed that both U and C were present in virtually equal amounts (data not shown). Since the expected tRNA oligomer ends in C, this result showed that the 5′ end of the rRNA oligomer was U and that all the bands apparently were cross-linked complexes. The cross-linked complex D1a was stable to 37 °C, 3 h, pH 3–8.5, according to PAGE analysis (not shown), thus allowing the use of these conditions for sequence analysis without cleavage of the cross-link.

Sequence Analysis of the Cross-Linked Oligonucleotides. The preceding results strongly indicated that the oligomers D1a, D1b, D2′, and D2 each contained one oligonucleotide from the tRNA and one from the rRNA. Since they were only found when NAK-modified tRNA was used, and the site of NAK modification was known, the tRNA oligonucleotide sequence should be CACCUCCCUcmo^{5}U*ACm^{6}AAG, where the asterisk refers to the NAK modification. Knowing the sequence of one oligomer of a dimer, we reasoned that it might be possible to deduce the other sequence by taking advantage of the fact that oligomers of equal length but different sequence have slightly different electrophoretic mobilities in 7 M urea high-resolution sequencing gels. This proved to be correct. In Figure 3 the dimers D1a and D1b were compared with the tRNA fragment alone, TK. TK was obtained by elution from the gel shown in Figure 2 and used without relabeling. Note that in order to reveal the slow-moving bands, the first residue was run off the gel. Other gels verified that the 5′ end of TK was C and that of the rRNA component of D1a and D1b was U, in agreement with the 5′-end analysis. The sequencing gel also showed that TK was the expected oligonucleotide. The fragment labeled UT in Figure 2 was similarly sequenced and shown to be the unmodified form of TK (data not shown). Modification of TK at the 10th nucleotide was confirmed by the gap in the ladder after the 9th residue. The minor band

just above the ninth residue was only seen in the alkaline ladder and is probably a chemical degradation product.

The main feature of this experiment was the ability to distinguish two sets of fragments from oligomers D1a and D1b. Even the dinucleotides, corresponding to pCpA and pUpA, were distinct. The doublet nature of the bands was found at each size level up to seven residues, with the tRNA-derived fragment always slightly faster. There were no anomalous bands visible for the first seven residues, and D1a and D1b gave identical patterns over that region. Doublets ceased sharply at residue 8, where only the tRNA band was visible. The rRNA sequence could be easily read for the first seven residues as UACACAC. Moreover, since it was the product of a complete RNase T_1 digest, the nucleotide 5′ to the U residue must be G, and the 3′ terminal residue must also be G. The only such oligomer in *E. coli* 16S rRNA (Noller, 1984) is the sequence G_{1392}UACACACCG$_{1401}$. Comparison of the rRNA bands from the dimer with an alkaline ladder from authentic UACACACCG (UR) confirmed the position of all of the fragments and also showed that unmodified rRNA 8-mer moved slightly slower than the TK 8-mer. As there was no such band in the fragments from the dimer, there should be no cross-linking to residue 9, since that would have resulted in the appearance of an unmodified 8-mer band. Cross-linking to residues 1–7 in some of the molecules would not be detectable in the presence of molecules cross-linked at residue 8 unless it was extensive. This does not appear to be the case since there was no obvious diminution of band intensity in a steplike manner at some specific residue number less than 8. The only unexplained band is the one marked by the arrow. This band must be an rRNA fragment since its 5′ end was only U (data not shown). It was probably derived by chemical breakdown of the cross-link since there was no counterpart in the enzymatic digestion lanes.

Although D1a and D1b appear to contain the same two oligonucleotides cross-linked via the same residues, they differed in their behavior both to alkaline hydrolysis and to enzymatic digestion. The overall pattern of the slow-moving bands was quite different in the two cases. Possibly this behavior is due to linkage via the same nucleotides in the two dimers, but by a different chemistry, and therefore with a different chemical susceptibility.

The two bands obtained by relabeling of D2 did not yield interpretable sequence patterns. Enzymatic digestion under the same conditions used for Figure 3 gave very low yields of product oligomers. Although the expected tRNA sequence could be discerned by comparison with authentic TK, it proved impossible to obtain a convincing rRNA sequence from the dimers. Further attempts to work with this noncleavable dimer were abandoned when a pattern similar to the D1 and D2 dimer pattern was obtained with the cleavable cross-linking agent SNAP (see below).

Cross-Linking with tRNA^{SNAP}. Although it was possible to identify the oligonucleotide cross-linked to Val-tRNA^{NAK}, accurate determination of the actual nucleotide cross-linked required the use of a cleavable cross-linking agent. The reagent SNAP was chosen because it was almost identical in length to NAK with the same reactive aryl azide group (Gornicki et al., 1985), yet could be cleaved by SH reagents. Val-tRNA^{SNAP} was prepared and cross-linked at the A site as described in the preceding paper (Gornicki et al., 1985). As shown in that work, the SNAP-modified tRNA was almost as functional as the tRNA modified with NAK.

Digestion of the SDS-isolated rRNA–tRNA complex with RNase T_1, followed by 5′-^{32}P labeling yielded the oligonucleotides shown in Figure 4 (lanes 1–4). Lanes 1 and 2 show the pattern when cross-linking was done in the presence of EFTu and lanes 3 and 4 the results in its absence. Clearly oligomers D3 and D4 only were found when EFTu was present. Furthermore, prior treatment with DTT (lane 1) caused a marked diminution in the amount of D3 and D4, indicating that cross-linking was via the SNAP probe. These results are like those shown in Figure 7 of Gornicki et al. (1985), but whereas those results were obtained by using labeled cross-linked tRNA, these data came from ^{32}P-labeled oligonucleotides and thus show directly that the oligomers obtained were a consequence of SNAP cross-linking. In other experiments, the putative cross-linked 23S RNA from Figure 7 of Gornicki et al. (1985) was similarly examined with and without DTT treatment. No D3 and D4 bands were detected, and no difference in pattern due to DTT could be found.

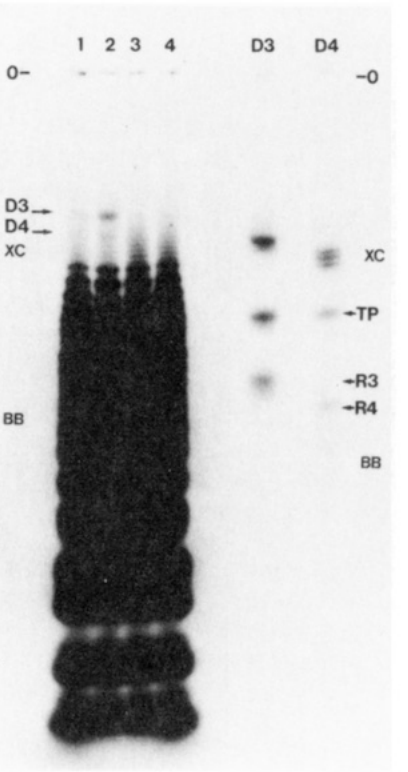

FIGURE 4: Characterization of the cross-linked rRNA–tRNA^{SNAP} oligonucleotides generated by T_1 RNase digestion. Ribosomal A-site binding, cross-linking, SDS–sucrose gradient isolation of rRNA–tRNA^{SNAP}, T_1 RNase digestion, 5′-^{32}P labeling (75%), and PAGE were done as described under Experimental Procedures. (Lanes 1 and 2) Plus EFTu; (lanes 3 and 4) minus EFTu; (lanes 1 and 4) incubated with 100 mM DTT, 23 °C, 2 h, after ^{32}P labeling but before PAGE; (lanes 2 and 3) no DTT treatment; (lanes D3 and D4) corresponding bands from lane 2 which were excised, soaked in 100 mM DTT, 23 °C, 30 min, and then polymerized at the top of a second slab gel. TP, oligonucleotide derived from tRNA; R3 and R4, rRNA-derived oligonucleotides from D3 and D4, respectively; O, XC, and BB, as in Figure 2.

In order to obtain the rRNA-derived oligomer, the D3 and D4 bands were excised, treated with DTT, and repolymerized onto a second gel. As shown in lanes D3 and D4, each band split into two bands, one of which (TP) had the same mobility in both cases. As shown below, this band came from the tRNA. R3 and R4 therefore must be derived from rRNA. The ratio of R3 to R4 was 3:1, like that when tRNA^{NAK} was used. All three oligomers, TP, R3, and R4, were sequenced enzymatically.

The slowest running bands near the XC dye marker consist of uncleaved dimer as well as contaminant oligonucleotides.

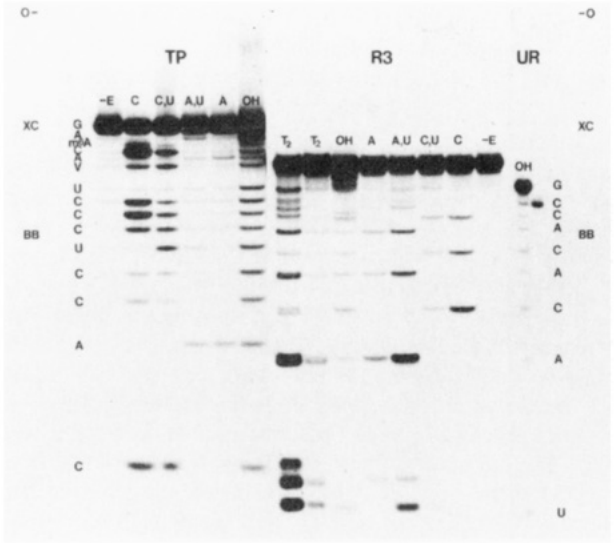

FIGURE 5: Sequencing gel analysis of oligonucleotide TP and R3. The fragments TP and R3 were released from dimer D3 by DTT treatment as in Figure 4. Recovery from the gel and enzymatic sequencing were as described in the legend to Figure 3. T_2 denotes a ladder generated by incubation of 0.08 A_{260} unit, primarily carrier tRNA, with 0.004 (right lane) and 0.04 (left lane) unit of RNase T_2 (Sigma) in 20 mM sodium citrate, 1 mM EDTA, and 7 M urea, pH 5.0, at 55 °C for 15 min. UR, the 16S nonamer UACACACCG. The deduced sequence for each oligomer is shown.

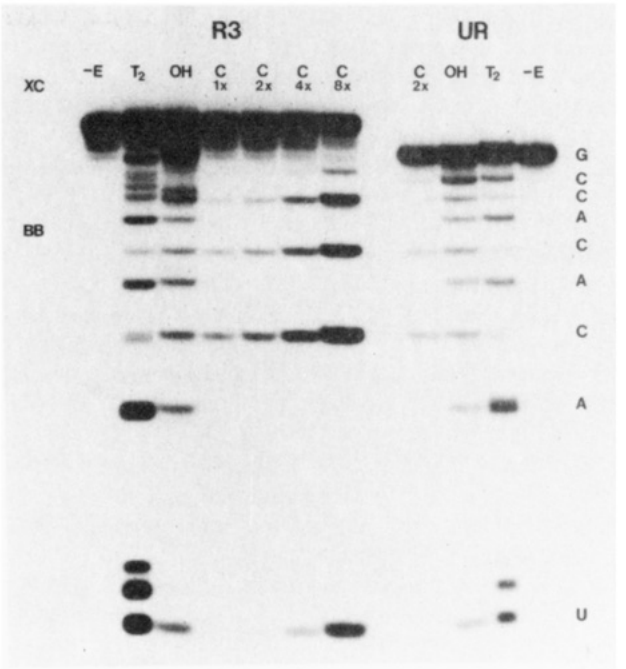

FIGURE 6: Sequencing gel comparison of R3 and UR. R3 and UR were obtained and digested with CL3, T_2, or alkali as above. T_2 (0.04 unit); CL3, 1x (0.5 unit/μg), 2x, 4x, and 8x. 2x corresponds to the amount used in Figures 3 and 5.

We have consistently observed that the –S–S– link in SNAP cannot be completely cleaved even with excess mercaptan and attribute this to oxidation to a noncleavable form during the lengthy purification and analysis process. Similar results were noted previously (Gornicki et al., 1985). In addition, contaminating oligomers which would not be cleavable should be found in this region. This is particularly evident in the case of D4 where extensive contamination can be expected by comparison of the D4 region in lane 2 to lanes 1, 3, and 4. This comparison also shows the virtue of being able to shift the position of the derived oligomer to an uncontaminated region of the gel by a specific treatment like mercaptan cleavage.

The analysis for R3 and TP is shown in Figure 5. The TP sequence was easily read as the expected one, CACCUCCCUcmo^{5}UACm^{6}AAG. The enzymatic sequencing lanes were almost blank at U-33 due to difficulty in cleavage 5′ to the modified base cmo^{5}U-34, and there was a gap between the 9th and 10th residues due to the presence of part of the SNAP moiety on the cmo^{5}U residue. The CL3 enzyme cut after the cmo^{5}U residue because U-derived nucleotides 5′ to A are recognized by this enzyme (D'Alessio, 1982). The m^{6}A residue was read anomalously. However, given the known sequence of tRNA^{Val} and the unambiguous portion of the gel, there can be no question about the tRNA sequence.

The sequence of R3 is equally clear for the first seven residues and corresponds to that determined above with Val-tRNA^{NAK}. Therefore, the major site of cross-linking with both NAK- and SNAP-modified tRNA is the same oligonucleotide. The anomalous bands above the seventh residue are most readily explained by modification at the eighth residue with various amounts of chemical degradation to faster moving components. There is no evidence for an 8-mer in the enzymatic sequencing lanes corresponding in position to the 8-mer from unmodified UACACACCG (UR). Each of the first seven bands in the sequencing gel matches exactly with corresponding bands from UR. There were no anomalous bands below the 7-mer as might have been expected if cross-linking had occurred to residues 5′ to the eighth nucleotide, suggesting that cross-linking was only to the eighth residue. This is similar to the results in Figure 3 but is more clearly evident since the tRNA bands are no longer present.

When R4 was similarly sequenced, the results were unclear. As was the case with tRNA^{NAK} above, the oligomer was only poorly digested under the same conditions used successfully in Figure 5. The T_2 ladder showed large gaps, and even alkali resulted in little cleavage. The reasons for this behavior are not known.

Confirmation of the Site of Cross-Linking. In order to confirm that cross-linking was only to the eighth residue, advantage was taken of the apparent failure of CL3 to cleave after a modified C residue (Figure 5, lane C). As shown in Figure 6, increasing the amount of CL3 8-fold did not yield any unmodified 8-mer. The band above the 7-mer which did appear did not comigrate with authentic unmodified 8-mer, run in a parallel set of lanes. Also, there was no band corresponding to unmodified 8-mer in the alkaline hydrolysis lane of R3. Thus, no evidence for any cross-linking to G1401 could be obtained.

There was complete correspondence between each of the three CL3-derived bands of R3 and those from the unmodified oligomer (UR), showing that no modifications had occurred to the first seven residues. By contrast, the difference between full-length modified and unmodified R3 was clearly evident.

This gel also illustrates the propensity of CL3 to cleave after U residues which are 5′ to A (D'Alessio, 1982). Cleavage after the 5′-U can be readily seen, especially at the higher CL3 concentrations.

DISCUSSION

Site and Probe Specificity of Cross-Linking. Although cross-linking of Val-tRNA^{NAK} to 16S RNA was shown to be A site specific in the preceding paper (Gornicki et al., 1985), it was necessary to show directly that the oligonucleotides isolated and sequenced in this work were a result of that cross-linking. The experiment of Figure 2 shows that although the P site was occupied at all times with unacylated tRNA^{Val}, which cross-links at the P site as effectively as AcVal-tRNA

(C. Oste and J. Ofengand, unpublished results), the oligomers D1 and D2 only appeared when Val-tRNA^{NAK} occupied the A site. The same was true when tRNA^{SNAP} was used. The larger oligomers appeared when EFTu was used to bind Val-tRNA^{SNAP} to ribosomes, but not in its absence (Figure 4). In addition, that experiment verified the SNAP dependence of the oligomers D3 and D4 by showing that they disappeared after treatment with S–S bond cleaving reagents.

The cross-linking pattern was similar with both modified tRNAs. A major band was found, which upon sequencing turned out to be the same oligomer in both cases, and there was also a minor band. The electrophoretic mobility of the bands were the same in the two cases and were also similar or the same as the two bands found initially when [^{14}C]NAK was used to track their location.

Identification of the Oligonucleotide Cross-Linked to tRNA^{NAK} and tRNA^{SNAP}. The sequencing gels of Figures 3 and 5 unambiguously identified the same seven-nucleotide sequence, UACACAC, as being the 5′-terminal portion of the rRNA sequence involved in cross-linking to both modified tRNAs. Examination of the *E. coli* 16S rRNA sequence reveals that this oligomer occurs at only two loci, namely, in the conserved sequence G_{1392}*UACACAC*CG_{1401} (A) and in another sequence G_{1222}*CUACACAC*G_{1231} (B) (Noller, 1984), the oligomer in question being italicized in both cases. The evidence in support of oligomer A is as follows. First, as the oligomers were all RNase T_1 digestion products, the 5′ ends of A and B would be different, U and C, respectively. When both tRNA^{NAK} and tRNA^{SNAP} were used, U was found by direct 5′-end analysis in approximately equal amount to the tRNA 5′-C. In order to get 5′-U from oligomer B, it would be necessary to postulate an adventitious nuclease cut between the C and U residues. However, the data of Figure 8 in Gornicki et al. (1985) show that all of the rRNA cross-linked to either tRNA^{NAK} or tRNA^{SNAP} behaved like a full-length complex. Cleavage at C_{1223} would have resulted in the release of a 319-residue fragment coupled to the tRNA, and this was clearly not observed. The small amount of cleavage product found with tRNA^{SNAP} is more consistent with cleavage of the –S–S bond in the cross-link and release of tRNA than with release by denaturation of a tRNA linked to a sizable piece of rRNA. Moreover, partial cleavage should have produced a pCpU dinucleotide in addition to pCpA and pUpA, as well as another set of higher oligomers, yet none were observed in the sequencing gels of Figures 3 and 5. Also, there was no evidence for any 5′-C in the rRNA moiety sequenced in Figure 5. Therefore, it appears that oligonucleotide A is the only one cross-linked to both tRNA species.

A surprising feature was the inability to sequence either one of the minor bands by the methods that were successful for the major components. The reasons for this remain unclear.

Identification of the Cross-Linked rRNA Nucleotide. The sequencing gel of Figure 3 shows doublet bands for oligomers 1–7 but none for the 8-mer. Moreover, there is no band corresponding in position to the reference rRNA 8-mer. This strongly suggests that all of the oligomer is cross-linked to the eighth nucleotide and that there is no cross-linking to the 3′-G residue. The fact that the rRNA oligomers 1–7 show no obvious step change in relative amount suggests that there is little or no cross-linking to residues 1–7. This is not a strong argument, however, as it is difficult to quantitate bands from a sequencing gel. The sequencing gel of Figure 5 provides better evidence. There was no indication of an unmodified rRNA 8-mer in the C lanes, as expected if cross-linking to G1401 had occurred, and there was no sign of any fragments shorter than eight with SNAP moieties attached. By contrast, there were multiple additional bands migrating above the 7-mer position. All of these results suggest unique cross-linking to residue 8, C1400.

Confirmation of this result was obtained by more extensive digestion with CL3 in direct comparison with the unmodified oligomer. No evidence for an unmodified 8-mer could be found by digestion of R3 with CL3, while UR readily yielded this fragment. It could be argued that modification at G1401 might also block cleavage after C1400. However, that would also have to be true for the *B. cereus* enzyme since no 8-mer was found in the C,U lane of Figure 5, and G1401 modification would not explain the absence of an 8-mer band in the alkaline hydrolysis lane of R3. Cross-linking to any of the first seven residues was ruled out by the perfect correspondence of the CL3-generated 3-mer, 5-mer, and 7-mer with that from the unmodified oligomer. Modification would have resulted in a clear electrophoretic difference, as shown by the distinctive separation between the unmodified and modified full-length oligomer, which is even longer than the CL3 fragments.

Significance of This Cross-Linking Site. It was a major surprise to find that these 23–24 Å long nonspecific photoaffinity probes attached to the 5′-anticodon base of A site bound tRNA cross-linked to a major extent only to the same residue of rRNA, C1400, which was previously shown to be within 4 Å of the 5′-anticodon base of P site bound tRNA (Ciesiolka et al., 1985 and references therein). In our previous work, unique cross-linking to C1400 was explained by the stereochemical constraints of cyclobutane dimer formation. The selectivity exhibited by the relatively nonspecific probes used in this study implies that the other nucleotides of the ribosomal RNA are largely inaccessible to such probes at the decoding site. This point is reinforced by the fact that although tRNA^{SNAP} and tRNA^{NAK} differed in cross-linking yield almost 2-fold (Gornicki et al., 1985), they both cross-linked to the same nucleotide.

A comparison of the accessibility of rRNA in the C1400 region as revealed by this technique with results obtained by other methods is difficult because most other accessibility studies have been done on free, albeit "native" rRNA. Despite strong evidence that the secondary structure of free 16S RNA is close to that in the ribosome (Van Stolk & Noller, 1984), tertiary structural interactions may be different and in functionally important regions may dominate. Nevertheless, the rRNA studies which have been done agree well with our results. For example, the region CCGm^{4}CmCC-1404 has never been found to be reactive toward single-strand-specific chemical reagents (Woese et al., 1980; Douthwaite et al., 1983a; Hogan et al., 1984) even when Gram-positive prokaryotes and eukaryotes are included, with the exception of a mild reaction of G1401 in *B. stearothermophilus*. Indeed, the cobra venom nuclease cuts after the C1400 and G1401 equivalents in yeast rRNA (Douthwaite et al., 1983a) are suggestive of a structured region there. Nevertheless, the adjacent G1405 appears highly exposed in rRNA (Douthwaite et al., 1983a) as well as in active and inactive 30S subunits (Herr et al., 1979). Likewise, C1397 (Woese et al., 1980; Kop et al., 1984) and A1398 (Douthwaite et al., 1983a; Van Stolk & Noller, 1984) are both reactive residues in native 16S RNA. Despite this exposure to chemical reagents, there was no evidence for cross-linking to any of these three residues. Possibly, formation of the 70S couple which shields G1405 (Herr et al., 1979) has the same effect on C1397 and A1398, as well as on A1394 and C1395 which are similarly exposed in 16S rRNA (Woese et al., 1980; Douthwaite et al., 1983a; Kop et al., 1984; Van

Stolk & Noller, 1984). If virtually all of the single-stranded residues of this conserved sequence were to be shielded in the 70S couple, A1396 being unreactive even in 16S RNA (Douthwaite et al., 1983a; Van Stolk & Noller, 1984), one is left to speculate about the mechanism by which C-1400 become uniquely exposed to react with an aryl azide probe as well as to form a cyclobutane dimer.

Cross-linking to C1400 from the A site is stereochemically reasonable. According to the model of Fuller & Hodgson (1967), the best stereochemical arrangement for both tRNA anticodons interacting with sequential mRNA codons is as a 3′ stack. A 5′-stack arrangement such as has been suggested by Woese (1970) and Lake (1977) would not only require reorientation of the tilt of the anticodon stem base pairs (Mizuno & Sundaralingam, 1978) but also has been shown to be unlikely for ribosome-bound tRNA by photo-cross-linking between the tRNA anticodon loop and mRNA (Matzke et al., 1980; Steiner et al., 1984) and by chemical modification (Douthwaite et al., 1983b) and fluorescence (Odom et al., 1978; Paulsen et al., 1982) experiments. The 3′-stack arrangement places the 5′-anticodon bases of the two tRNAs ca. 22 Å apart (Fuller & Hodgson, 1967). If C1400 is positioned within 4 Å of the P-site 5′-anticodon base (in order to account for cyclobutane dimer formation), then a 23-Å probe at the A site should be just sufficient to reach it.

Accessibility of only C1400 would also provide an explanation for the observed strong dependence of cross-linking on the length of the probe (Gornicki et al., 1985). The drastic 4–5-fold decrease in cross-linking yield upon shortening the probe by 5 Å can be understood in terms of a dramatic reduction in the probability of an approach to within covalent bonding distance. This implies the existence of some conformational flexibility of the anticodon loop of tRNA at the decoding site. Evidence for such flexing was obtained previously (Ofengand & Liou, 1981). In that work, P-site cross-linking via cyclobutane dimer formation was markedly inhibited by base pairing between the tRNA anticodon base involved in the cross-link and its matching codon base. Since changing one base of a codon triplet should not affect the orientation of rRNA, it was concluded that at least the 5′-anticodon base of the tRNA anticodon loop was mobile, and only when *not* constrained by codon base pairing could it properly stack on the C1400 residue of 16S rRNA so as to form a cyclobutane dimer upon irradiation.

These structural considerations may also explain why P-site cross-linking did not involve any 16S RNA component. If little else but C1400 is accessible, even the short probe placed on the 5′-anticodon base would be too long for a high probability of reaction with the nearby C1400 residue.

In support of the results described in this paper, recent experiments have shown that it is possible to cross-link both unmodified $tRNA^{Val}$ in the P site and Val-$tRNA^{SNAP}$ in the A site to the same rRNA oligonucleotide, yielding an oligonucleotide trimer after RNase T_1 digestion (J. Ciesiolka and J. Ofengand, unpublished results). Moreover, the order of cross-linking to rRNA does not affect the results. It is not yet known, however, whether C1400 is involved in both of the cross-links.

References

Barta, A., Steiner, G., Brosius, J., Noller, H. F., & Kuechler, E. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3607–3611.

Brimacombe, R., Maly, P., & Zwieb, C. (1983) *Prog. Nucleic Acid Res. Mol. Biol. 28*, 1–48.

Chan, Y.-L., Endo, Y., & Wool, I. G. (1983) *J. Biol. Chem. 258*, 12768–12770.

Ciesiolka, J., Nurse, K., Klein, J., & Ofengand, J. (1985) *Biochemistry 24*, 3233–3239.

Cundliffe, E. (1980) in *Ribosomes: Structure, Function, and Genetics* (Chambliss, G., Craven, G., Davies, J., Davis, K., Kahan, L., & Nomura, M., Eds.) pp 555–581, University Park Press, Baltimore, MD.

D'Allesio, J. M. (1982) in *Gel Electrophoresis of Nucleic Acids: A Practical Approach* (Rickwood, D., & Hames, B. D., Eds.) pp 173–197 (see 185), IRL Press Ltd., Oxford and Washington, DC.

Douthwaite, S., Christensen, A., & Garrett, R. A. (1983a) *J. Mol. Biol. 169*, 249–279.

Douthwaite, S., Garret, R. A., & Wagner, R. (1983b) *Eur. J. Biochem. 131*, 261–269.

Ehresmann, C., Ehresmann, B., Millon, R., Ebel, J.-P., Nurse, K., & Ofengand, J. (1984) *Biochemistry 23*, 429–437.

Fuller, W., & Hodgson, A. (1967) *Nature (London) 215*, 817–821.

Gold, L., Pribnow, D., Schneider, T., Shinedling, S., Singer, B. S., & Stormo, G. (1981) *Annu. Rev. Microbiol. 35*, 365–403.

Gornicki, P., Nurse, K., Hellmann, W., Boublik, M., & Ofengand, J. (1984) *J. Biol. Chem. 259*, 10493–10498.

Gornicki, P., Ciesiolka, J., & Ofengand J. (1985) *Biochemistry* (preceding paper in this issue).

Helser, T., Davies, J. E., & Dahlberg, J. E. (1972) *Nature (London), New Biol. 235*, 6–9.

Herr, W., Chapman, N. M., & Noller, H. F. (1979) *J. Mol. Biol. 130*, 433–449.

Hogan, J. J., Gutell, R. R., & Noller, H. F. (1984) *Biochemistry 23*, 3322–3330.

Hsu, L., Lin, F.-L., Nurse, K., & Ofengand, J. (1984) *J. Mol. Biol. 172*, 57–76.

Kop, J., Kopylov, A. M., Magrum, L., Siegel, R., Gupta, R., Woese, C. R., & Noller, H. F. (1984) *J. Biol. Chem. 259*, 15287–15293.

Lake, J. A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1903–1907.

Li, M., Tzagoloff, A., Underbrink-Lyon, K., & Martin, N. C. (1982) *J. Biol. Chem. 257*, 5921–5928.

Matzke, A. J. M., Barta, A., & Kuechler, E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5110–5114.

Mizuno, H., & Sundaralingam, M. (1978) *Nucleic Acids Res. 5*, 4451–4461.

Noller, H. F. (1984) *Annu. Rev. Biochem. 53*, 119–162.

Odom, O. W., Craig, B. B., & Hardesty, B. A. (1978) *Biopolymers 17*, 2909–2931.

Ofengand, J. (1980) in *Ribosomes: Structure, Function, and Genetics* (Chambliss, G., Craven, G., Davies, J., Davis, K., Kahan, L., & Nomura, M., Eds.) pp 497–529, University Park Press, Baltimore, MD.

Ofengand, J., & Liou, R. (1981) *Biochemistry 20*, 552–559.

Ofengand, J., Gornicki, P., Nurse, K., & Boublik, M. (1984) in *Alfred Benzon Symposium No. 19: Gene Expression, The Translational Step and Its Control* (Clark, B. F. C., & Petersen, H. U., Eds.) pp 293–315, Munksgaard, Copenhagen.

Ofengand, J., Ciesiolka, J., Gornicki, P., & Nurse, K. (1985) in *Cellular Regulation and Malignant Growth* (Ebashi, S., Ed.) pp 61–73, Japan Scientific Societies Press, Tokyo/Springer-Verlag, Berlin.

Okuyama, A., Machiyama, N., Kinoshita, T., & Tanaka, N. (1971) *Biochem. Biophys. Res. Commun. 43*, 196–199.

Pace, B., Matthews, E. A., Johnson, K. D., Cantor, C. R., & Pace, N. R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 36-40.
Paulsen, H., Robertson, J. M., & Wintermeyer, W. (1982) *Nucleic Acids Res. 10*, 2651-2663.
Pieler, T., Digweed, M., & Erdmann, V. A. (1984) in *Alfred Benzon Symposium 19: Gene Expression, The Translational Step and its Control* (Clark, B. F. C., & Petersen, H. U. Eds.) pp 353-376, Munskgaard, Copenhagen.
Sköld, S.-E. (1983) *Nucleic Acids Res. 11*, 4923-4932.
Steiner, G., Luhrmann, R., & Kuechler, E. (1984) *Nucleic Acids Res. 12*, 8181-8191.
Thompson, J., Schmidt, F., & Cundliffe, E. (1982) *J. Biol. Chem. 257*, 7915-7917.
Van Stolk, B. J., & Noller, H. F. (1984) *J. Mol. Biol. 180*, 151-177.
Woese, C. (1970) *Nature (London) 226*, 817-820.
Woese, C. R., Magrum, L. J., Gupta, R., Siegel, R. B., Stahl, D. A., Kop, J., Crawford, N., Brosius, J., Gutell, R., Hogan, J. J., & Noller, H. F. (1980) *Nucleic Acids Res. 8*, 2275-2293.
Zagorska, L., Duin, J. V., Noller, H. F., Pace, B., Johnson, K. D., & Pace, N. R. (1984) *J. Biol. Chem. 259*, 2798-2802.
Zimmermann, R. A., Gates, S. M., Schwartz, I., & Ofengand, J. (1979) *Biochemistry 18*, 4333-4339.

Resonance Raman Spectra of CN^--Bound Cytochrome Oxidase: Spectral Isolation of Cytochromes a^{2+}, a_3^{2+}, and $a_3^{2+}(CN^-)$

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ABSTRACT: Reduced cyanide-bound cytochrome oxidase in the absence of any oxygen gives a resonance Raman spectrum consistent with that expected for low-spin heme *a*. Thus, in contrast to prior reports, ligand binding of cytochrome a_3 to form a six-coordinate low-spin ferrous heme does not result in any unusual electronic structure, hydrogen bonding, environment, or conformation of the formyl group. It appears unlikely that there are any changes in this group in cytochrome a_3 that control the ligand affinity or redox potential in physiological forms of the ferrous enzyme. With the use of our difference spectrometer and by appropriately selecting the laser excitation frequency, we are able to isolate spectrally cytochromes a^{2+}, a_3^{2+}, and $a_3^{2+}(CN^-)$. The addition of a small amount of oxygen to a preparation of the cyanide-bound reduced enzyme results in a complex with the same Raman spectrum as that previously reported to originate from the cyanide-bound reduced complex. Any oxygen present in the sample leads to enzyme turnover resulting in a mixed valence state $[a^{2+}a_3^{3+}(CN^-)]$. The comparison between the data on the cyanide-bound reduced enzyme and the data on the CO-bound reduced enzyme illustrates that cyanide binding affects only the modes that respond to the spin state of the ferrous iron, while CO binding affects vibrational modes that respond to a π-electron density change as well.

Cytochrome oxidase is the terminal enzyme in the electron-transport chain in mitochondria. It reduces dioxygen to water and generates a proton gradient across the mitochondrial membrane. This enzyme has four redox centers, which consist of two heme *a* chromophores referred to as cytochromes *a* and a_3 and two Cu centers. The iron atom in cytochrome *a* is six-coordinate and low spin in both the 2+ and 3+ oxidation states and does not bind any external ligands. The iron atom in cytochrome a_3 is five-coordinate and high spin in the ferrous state and six-coordinate and high spin in the ferric (resting) state. O_2 binds to ferrous cytochrome a_3 where it is reduced to water. [For a detailed review, see Wikström et al. (1981)].

In order to understand the molecular mechanism of this enzyme's function, it is necessary to characterize the structure of both types of cytochromes in the various intermediates following the binding of molecular oxygen. However, study of the enzyme bound to the physiologically important ligand, O_2, is difficult by conventional resonance Raman methods because of its rapid reduction to water. In addition, for the interpretation of the resonance Raman spectra of O_2-bound complexes, it is necessary to separate the contributions of cytochromes *a* and a_3 and then find the sensitivity of the Raman lines to oxidation-state changes and spin-state changes as well as other ligand-induced and protein-induced perturbations. An approach to such an understanding is to study the spectra of the enzyme with alternate ligands such as carbon monoxide, cyanide, azide, or sulfide bound to the cytochrome a_3. In other papers we examined the geometry of the CO ligand bound to cytochrome a_3 (Argade et al., 1984a) and the behavior of the porphyrin modes upon CO binding (Argade et al., 1986). In this paper we focus on CN^- binding. CN^- inhibits the function of this enzyme; hence, the study of the CN^--bound complex has physiological importance as well.

Resonance Raman spectra of CN^--bound reduced cytochrome oxidase have been reported in the past, and the results were quite surprising. The mode associated with the C=O stretching vibration of the formyl group from cytochrome a_3, which is found at 1671 cm^{-1} for the oxidized enzyme and 1664 cm^{-1} for the reduced enzyme, was reported to disappear in the CN^--bound reduced enzyme (Salmeen et al., 1978) and in cyanide-bound mitochondria (Adar & Erecinska, 1979). In contrast, this mode is observed at 1660 cm^{-1} in reduced high-spin (2-methylimidazole) heme *a* model compounds and at 1644 cm^{-1} in the reduced low-spin [bis(imidazole)] heme

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