Two-Dimensional Gel Electrophoresis Technique for Determination of the Cross-Linked Nucleotides in Cleavable Covalent RNA-RNA Complexes. Application to *Escherichia coli* and *Bacillus subtilis* Acetylvalyl-tRNA Covalently Linked to *E. coli* 16S and Yeast 18S Ribosomal RNA†

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ABSTRACT: We have developed a new method which yields in a single step the site of cross-linking between two oligonucleotides covalently linked by a cleavable bond. The isolated duplex, labeled at both 5'-ends, is split randomly and then analyzed by diagonal gel electrophoresis with cleavage of the cross-link between the two gel dimensions. Digestion products which do not contain the cross-link migrate along the diagonal, while products resulting from cleavage of the cross-link migrate as off-diagonal products. The site of cross-linking is determined by analysis of both diagonal and off-diagonal products. This method was successfully applied to three different oligonucleotide duplexes isolated by T_1 RNase digestion from *Escherichia coli* tRNA covalently linked at the ribosomal P site to either *Escherichia coli* 16S RNA or yeast 18S RNA and from *Bacillus subtilis* tRNA cross-linked to *Escherichia coli* 16S RNA. The site of cross-linking was unambiguously localized to C_{1400} in *Escherichia coli* 16S RNA and to the equivalent position, C_{1626}, in yeast 18S RNA. Direct evidence was also provided for the participation of the wobble base (c)mo^5U_{34} of the tRNA in the cross-link. Furthermore, our results exclude the possibility of minor cross-linking sites at other positions. This new method is reliable, rapid, and easy to handle and should be applicable to any cleavable covalent RNA-RNA duplex. Furthermore, it is sensitive to certain aspects of the steric conformation of such covalent duplexes.

Formation of RNA-RNA cross-links is a promising tool for studying the relations between RNA molecules or for investigating their secondary or tertiary structure. Particular interest has focused on the study of the topography of ribosomal RNAs (Brimacombe et al., 1983) and on rRNA-tRNA contacts occurring on the ribosome (Ofengand et al., 1982). Both "zero-length" reagents such as mild UV irradiation (Zwieb & Brimacombe, 1980; Stiege et al., 1983; Ofengand et al., 1979) and non-zero-length chemical cross-linking agents have been used for this purpose. Among the latter class are nitrogen mustards (Stiege et al., 1982), psoralen derivatives (Rabin & Crothers, 1979; Thompson et al., 1981; Turner et al., 1982; Thompson & Hearst, 1983), and phenyldiglyoxal (Wagner & Garrett, 1978; Hancock & Wagner, 1982).

Wider application of these reagents has been hampered by the technical difficulties involved in determining the exact nucleotides involved in the cross-link. Thus, with all of the above reagents, except for the case described in the preceding paper (Ehresmann et al., 1984), only the oligonucleotide involved in the cross-link could be determined. In two cases, the exact nucleotides involved were inferred by consideration of the expected nucleotide specificity of the cross-linking reagents (Hancock & Wagner, 1982; Stiege et al., 1982). Even in the case of the reversible UV-induced cross-link between tRNA and rRNA, identification of the cross-linked residues was time-consuming and difficult (Prince et al., 1982; Ehresman et al., 1984). We therefore tried to develop a new method which would overcome these disadvantages and be applicable to any other cleavable covalent RNA-RNA adduct.

In this work, we have developed a new technology based on random hydrolysis of the covalent oligonucleotide duplex followed by two-dimensional gel electrophoresis, which yields the cross-linking site in both oligomers in one single step. The only requirement of this technique is that the cross-link should be cleavable between the two gel electrophoreses. We have applied this methodology to analysis of the covalent heterodimers formed between *Escherichia coli* tRNA and both *E. coli* 16S RNA and yeast 18S RNA and between *Bacillus subtilis* tRNA and *E. coli* 16S RNA (Ehresmann et al., 1984). We also used this method to investigate the possibility that low-yield cross-linking had occurred at other residues, since this could not be ruled out by any of the previous determinations (Prince et al., 1982; Ehresmann et al., 1984).

Experimental Procedures

Isolation of the Covalent tRNA-rRNA T_1 Oligonucleotide Duplex. The 5'-^{32}P-labeled covalent tRNA-rRNA T_1 oligonucleotide duplexes were isolated by one-dimensional gel electrophoresis as described in the preceding paper (Ehresmann et al., 1984).

Limited Alkaline Digestion. The covalent T_1 oligonucleotide duplexes plus 5 μg of unlabeled tRNA were dissolved in 10 μL of 50 mM carbonate buffer, pH 9.0, and 1 mM ethylenediaminetetraacetic acid (EDTA) as described by Donis-Keller et al. (1977). Incubation was for 15 min at 90 °C.

Two-Dimensional Gel Electrophoresis. The first dimension was run on 25% polyacrylamide slab gels (40 × 30 × 0.04 cm) containing 100 mM tris(hydroxymethyl)aminomethane–borate (Tris–borate) (pH 8.3), 0.25 mM Na_2EDTA, and 8 M urea. The weight ratio of acrylamide to bis(acrylamide) was 20:1. First-dimension gels were preelectrophoresed for 2 h at 1800 V and run at 1500 V until the bromophenol blue marker reached 13 cm (short migration) or 30 cm (long migration) from the origin. The gel, covered with saran wrap to prevent evaporation, was irradiated for 7 min under a 30-W Sylvania

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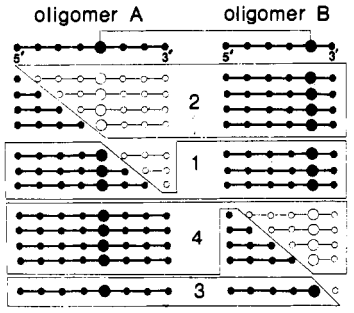

FIGURE 1: Diagram illustrating the four groups of products which are generated by random digestion of a covalent oligonucleotide duplex. Numbers 1–4 refer to the groups described in the text. Oligonucleotide length and the position of the cross-link are arbitrary. Nucleotides are indicated by small circles and cross-linked nucleotides by large circles. Products carrying the 5′-terminal label are indicated by solid circles and products lacking the 5′-terminal label by open circles. Only products with 5′-label are detected. Covalently linked products are enclosed in boxes.

germicidal lamp at a distance of 7 cm. The gel strip containing the digestion products was cut out and included at the top of a second 25% polyacrylamide slab gel for the second dimension. Electrophoresis at 800 V was terminated when the bromophenol blue marker had traveled 12 cm. After autoradiography, the resulting products were eluted according to the procedure described by Maxam & Gilbert (1977) and further analyzed.

Nucleotide Length Determination and 5′-Nucleotide Analysis. Each product was eluted and submitted to electrophoresis on 25% polyacrylamide slab gels. A limited alkaline digest of the uncleaved oligomers was used as reference. The 5′-terminal nucleotide was identified by total hydrolysis with RNase P_1, followed by one-dimensional thin-layer chromatography, as described in the preceding paper (Ehresmann et al., 1984).

Results

Method. The two-dimensional gel electrophoresis analysis takes advantage of the ability of a covalent oligonucleotide duplex containing two oligomers (A and B) encompassing m and n nucleotide residues, respectively, to be cleaved at the site of cross-linking located at position x from the 5′-end in oligonucleotide A and at position y from the 5′-end in oligonucleotide B. Both oligomers are ^{32}P labeled at their 5′-end. Labeling at the 3′-end would also be possible. The covalent duplex is then randomly digested with alkali, in such a way that no more than one cut is allowed in each covalent dimer. Four distinct sets of digestion products are then generated (Figure 1): (a) uncleaved oligonucleotide A covalently linked to labeled and unlabeled cleavage products from B; (b) uncleaved oligonucleotide B covalently linked to labeled and unlabeled cleavage products from A; (c) cleavage products from oligonucleotide A which no longer contain the cross-link; (d) cleavage products from oligonucleotide B which no longer contain the cross-link.

The digest mixture is submitted to two-dimensional gel electrophoresis under denaturing conditions. After the first dimension, the gel is irradiated for cleavage of the cross-link, and the strip containing the digestion products is included at the top of a second slab gel of identical composition. Fragments which no longer contain the cross-link have an unchanged electrophoretic mobility in both dimensions and migrate along a diagonal. Since the oligomers are labeled at their 5′-end, only the digestion products containing the 5′-terminal nucleotide are detected by autoradiography. The size of the A-derived products is expected to range between 1 and $x - 1$ and the size of the B-derived products between 1 and $y - 1$. The digestion products still containing the cross-link are released as pairs of oligomers after photoreversal and migrate as off-diagonal products in the second dimension. Again, only those products still containing the labeled 5′-terminal nucleotide are detected. The off-diagonal products can be classified into four different sets (Figure 1): Group 1 consists of pairs between uncleaved oligonucleotide B and cleaved products derived from A which carry the labeled 5′-terminal nucleotide. Both released products are visible. The length of the associated A-derived subproducts is expected to range between x and $m - 1$ nucleotides. The number of released uncleaved B oligomers expected is $m - x$. Group 2 consists of pairs between uncleaved oligonucleotide B and digestion products derived from oligomer A which do not contain the 5′-labeled nucleotide. Only the uncleaved oligonucleotide B is visible. The electrophoretic mobility in the first dimension of each off-diagonal B oligomer is determined by the nucleotide length of the associated A-derived product. Therefore, the resulting number of released B oligomers is determined by the number of nucleotide residues which can be split from the 5′-end to the cross-linking site in oligomer A. This number should be $x - 1$. Group 3 is composed of pairs between uncleaved oligonucleotide A and digestion products derived from B still carrying the labeled 5′-nucleotide. In this case, the length of the B-derived product is expected to range between y and $n - 1$ nucleotides, and the number of released uncleaved A oligomers is $n - y$. Group 4 consists of pairs between uncleaved oligonucleotide A and digestion products derived from B which do not contain the 5′-labeled nucleotide. The number of released A products is expected to be $y - 1$.

The total number of labeled fragments expected in the first dimension is determined by the parameters m, n, x, and y. The series of fragments will run from 1 to $x - 1$ or $y - 1$, whichever is greater, these fragments being the now un-cross-linked diagonal fragments. There may or may not then be a gap before the series resumes with the still cross-linked fragments that will be off-diagonal in the second dimension. The fragment size at which the series resumes will depend on the smallest of the following: $m + n + 1 - x$, $m + n + 1 - y$, $n + x$, $m + y$. Depending on the values for these parameters, the un-cross-linked fragments may overlap in size with the cross-linked ones in the first dimension. However, the second dimension will sort out the fragments.

The location of the cross-linking site in each oligonucleotide can then be deduced from analysis of both diagonal and off-diagonal products. This is simply achieved by a nucleotide length determination and an appropriate identification of each product (i.e., is the product derived from A or B?). Identification can easily be carried out by total P_1 digestion if the two oligomers have a different 5′-terminal nucleotide, while digestion with RNase A or U_2 can be used to discriminate A and B subproducts with an identical 5′-terminus. Nucleotide length is electrophoretically determined by reference to a statistical digest of the complete corresponding oligomer. Application of these rules is best illustrated by the examples of the next section.

Experimental Findings. The covalent T_1 oligonucleotide duplexes obtained from the heterodimers between *E. coli* AcVal-tRNA and *E. coli* 16S RNA or yeast 18S RNA and between *B. subtilis* AcVal-tRNA and *E. coli* 16S RNA were prepared by T_1 hydrolysis and one-dimensional gel electrophoresis (Ehresmann et al., 1984). They were randomly digested with alkali and analyzed by two-dimensional gel electrophoresis. Each duplex was analyzed in at least four different

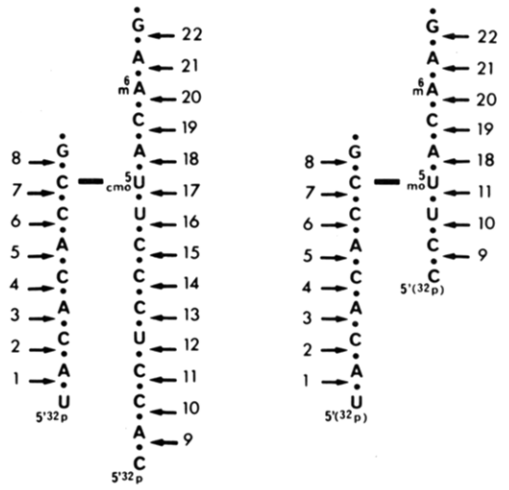

FIGURE 2: Nucleotide sequence of the T_1 oligonucleotide covalent duplex from (A) *E. coli* tRNA and *E. coli* 16S RNA and yeast 18S RNA and (B) *B. subtilis* tRNA–*E. coli* 16S RNA. Phosphodiester bonds are indicated by dots and the cross-link by a solid bar. Each cleavage position has been numbered. In each duplex, the tRNA oligonucleotide is on the right-hand side.

experiments with highly reproducible results. For reference in the subsequent discussion, the sequences of the subject T_1 oligonucleotides are shown in Figure 2.

An autoradiogram of digestion products after the first dimension revealed the presence of a gap between the products which no longer contain any cross-link and the covalent cross-linked fragments. Short migration in the first dimension allowed recovery of all the un-cross-linked products, but a good resolution of the off-diagonal products in the second dimension required long migration in the first dimension. The two-dimensional gel electrophoretograms are shown in Figures 3A–6A. In all these samples, long migration was carried out in the first dimension, so that the shortest diagonal products were lost. Accompanying key diagrams are given for each electrophoretogram in Figures 3B–6B. The identity of each product was carefully verified by both 5′-terminal nucleotide analysis and nucleotide length determination. The products denoted by A were found to end with a 5′-terminal C residue and were therefore identified as being derived from the tRNA oligomer. The products denoted by B terminated with a 5′-terminal U residue and were thus assigned to subproducts from the rRNA oligomer. The associated numbers indicate the

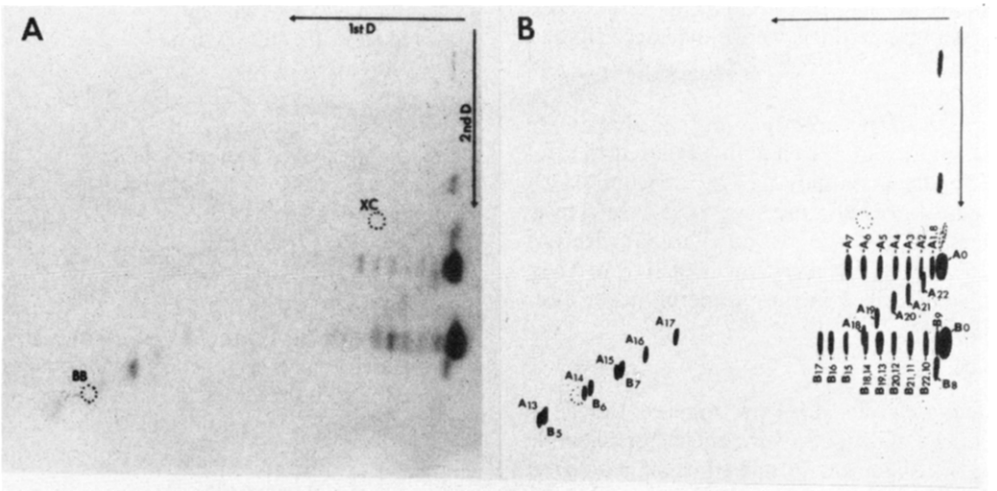

FIGURE 3: Two-dimensional gel electrophoresis of the covalent *E. coli* tRNA–*E. coli* 16S RNA oligonucleotide duplex. The covalent duplex was first randomly digested with alkali. The bromophenol blue dye migrated 28 cm in the first dimension and 12 cm in the second dimension. Photoreversal of the cross-link was carried out between the two dimensions. Each product was then excised, eluted, and submitted to nucleotide length determination and 5′-end nucleotide analysis. The autoradiogram is shown in (A) and the key diagram in (B). Products denoted by A have a 5′-[^{32}P]C residue and are derived from the tRNA pentadecamer. Products denoted by B have a 5′-[^{32}P]U residue and come from the rRNA nonamer. The associated number indicates the deduced position of the cleavage site in the oligonucleotide duplex according to the diagram in Figure 2.

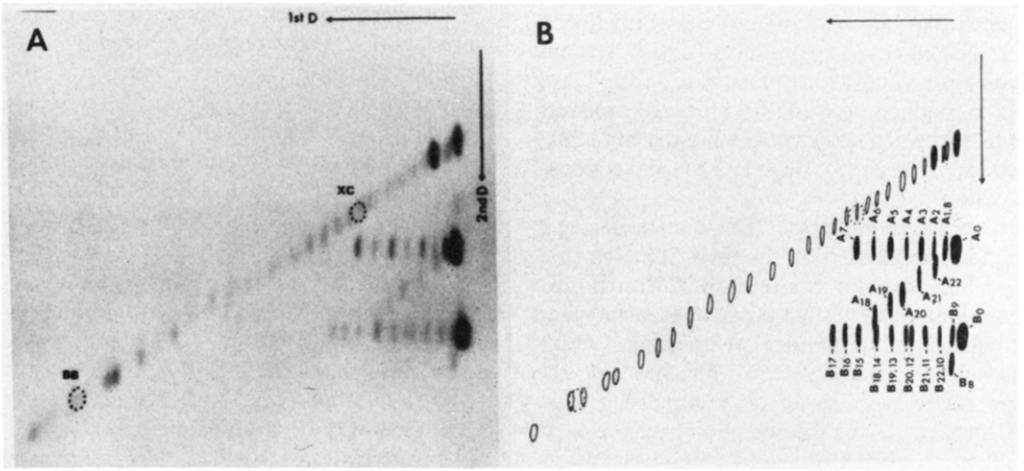

FIGURE 4: Two-dimensional gel electrophoresis of the covalent *E. coli* tRNA–yeast 18S RNA oligonucleotide duplex: (A) autoradiogram; (B) key diagram. Conditions as in Figure 3.

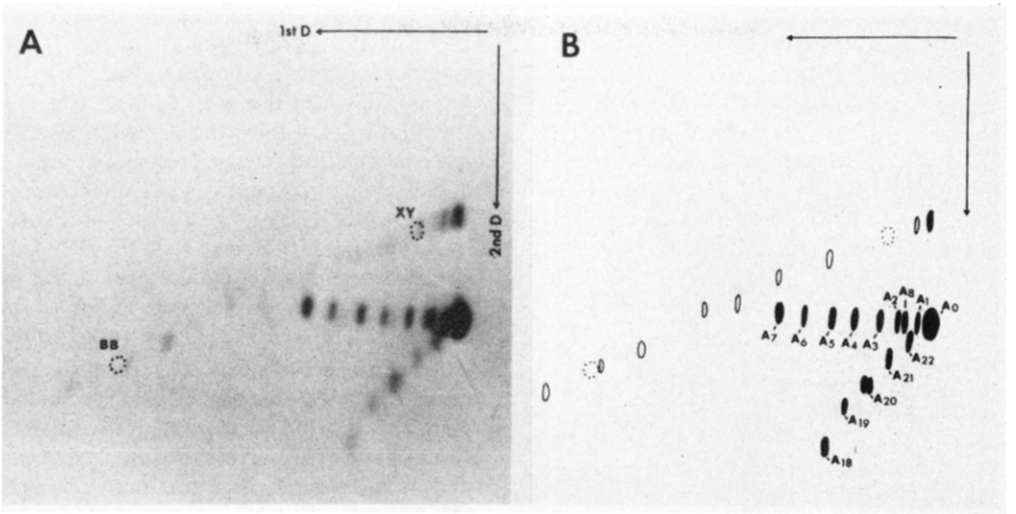

FIGURE 5: Two-dimensional gel electrophoresis of the covalent *B. subtilis* tRNA–*E. coli* 16S RNA duplex A: (A) autoradiography; (B) key diagram. Conditions as in Figure 3. Only the tRNA nonamer is 5′-labeled.

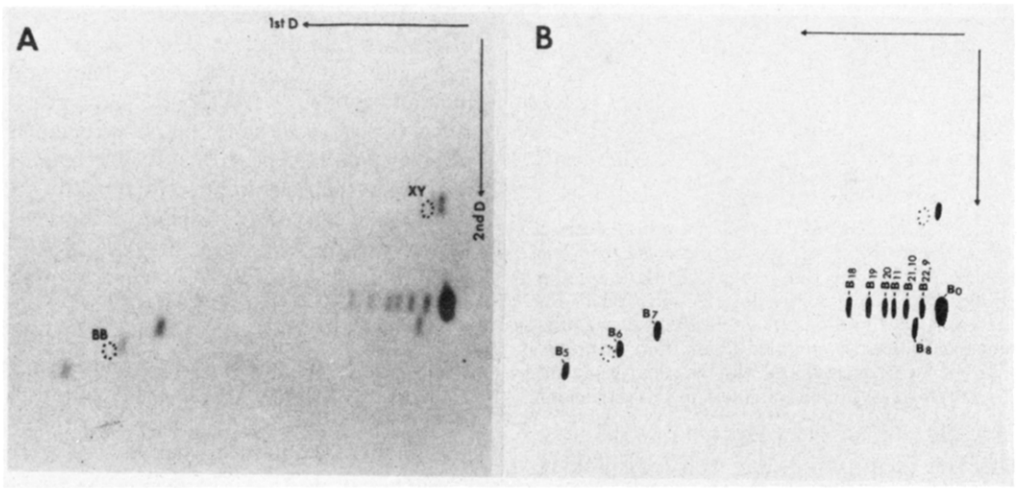

FIGURE 6: Two-dimensional gel electrophoresis of the covalent *B. subtilis* tRNA–*E. coli* 16S RNA duplex B: (A) autoradiography; (B) key diagram. Conditions as in Figure 3. Only the rRNA nonamer is 5′-labeled.

deduced cleavage position in the covalent duplex, according to the numbering system shown in Figure 2.

E. coli tRNA^{Val}*–E. coli 16S RNA.* The covalent T_1 oligonucleotide duplex was isolated in a high degree of purity due to the absence of other products migrating in that part of the gel [Figure 3A of Ehresmann et al. (1984)]. The two oligonucleotides were identified as pUpApCpApCpApCpCpG, a unique nonamer in the 16S RNA, and pCpApCpCpUpCpCpCpUpcmo^{5}UpApCpm^{6}ApApG, a pentadecamer of the tRNA containing the anticodon (Ehresmann et al., 1984). The resulting two-dimensional gel electrophoresis pattern is shown in Figure 3, and the nucleotide length analysis of the off-diagonal products is illustrated in Figure 7A.

The degradation products which did not contain a cross-link site and migrated along the diagonal were found to contain products derived from the tRNA oligomer ranging between one and seven residues. This observation indicates that the nucleotides in the tenth and eigth positions of the tRNA and rRNA oligomers, respectively, are both sites of cross-linking. No larger products were detectable.

The products which contained the cross-linking site and migrated as off-diagonal products could easily be identified. (i) The subfragments denoted A_{22}–A_{18} and their associated oligomers (B_{22}–B_{18}) belong to group 1 (uncleaved oligonucleotide B and 5′-subfragments from A). The length of the A-derived products ranged between 10 and 14 nucleotides (Figure 7A), thus confirming that cross-linking occurs at the tenth nucleotide residue in the tRNA pentadecamer. No smaller tRNA-derived product corresponding to a cross-link at the ninth residue could be detected. (ii) The only 5′-subfragment from B, B_8, belongs to group 3. This unique product derived from the rRNA nonamer contained eight nucleotides (Figure 7A, lane B), confirming the localization of the cross-link at position 8 of the rRNA oligomer. The fact that no shorter products derived from the rRNA oligomer could be detected even after long exposure of the autoradiogram indicates that the cross-linking site on the rRNA is unique. (iii) The set of uncleaved B oligomers arise from uncleaved B oligomer linked in the first dimension to either 5′- or 3′-subproducts from A. The nine mobility shifts observed indicate that nine nucleotide residues can be split from either the 5′- or the 3′-end of oligomer A until the cross-linking site corresponding to either the sixth or the tenth residue is reached. Since cross-linking at residue 6 would be inconsistent with the nine diagonal products found, this result provides further evidence for the localization of the cross-link at residue 10 in the tRNA oligomer. (iv) The products denoted A_1–A_7 are similarly due to uncleaved A oligomer associated with 5′- or 3′-fragments derived from the rRNA oligomer. The observed seven mobility shifts in conjunction with the number of diagonal products found confirm that the cross-linking site is localized at the eighth position in the rRNA oligomer.

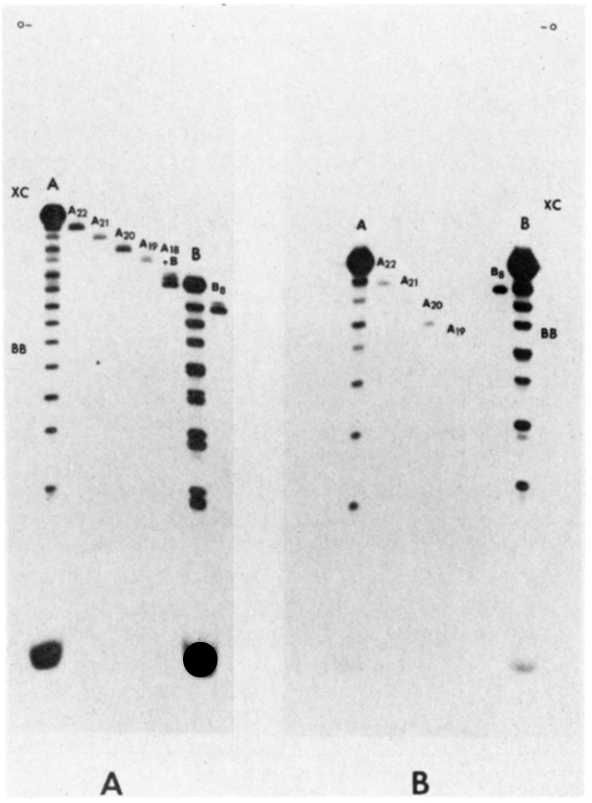

FIGURE 7: Nucleotide length determination of (A) products derived from the gel shown in Figure 4 and (B) products derived from the gels in Figures 5 and 6. The entire tRNA oligomer (lane A) and rRNA nonamer (lane B) were randomly digested with alkali for nucleotide length reference. Migration was on 25% polyacrylamide slab gels. The bromophenol blue dye migrated 11 cm from the origin. Product denoted A_{18} in (A) was contaminated with the rRNA nonamer ($B_{18,14}$ in Figure 3). Product A_{18} in (B) was lost in this particular sample.

All three procedures identify the same two cross-linking residues and allow us to unambiguously conclude that the cross-link is formed between C_{1400} in the 16S RNA and cmo^5U_{34} in the tRNA. In addition, our results show that the cross-link is restricted to a unique site. If the adjacent residue (C_{1399}) would have also participated in cross-link formation, an additional product containing seven nucleotides would have been found as an off-diagonal product. Note that inspection only of the diagonal products would not have distinguished between the presence or absence of a second cross-linking site, due to the presence of the seven-nucleotide partial cleavage product. This shows that one-dimensional analysis alone is inadequate. These results corroborate our previous findings (Ehresmann et al., 1984) and those of Prince et al. (1982) and, in addition, definitively exclude the possibility of a small amount of cross-link at C_{1399}. Identical results were found when the cross-link was formed in the presence of different codons [pGUU vs. poly(U_2,G)] or when different irradiation conditions were used (with or without a Mylar filter) (Ehresmann et al., 1984).

E. coli $tRNA^{Val}$–*Yeast 18S RNA*. The covalent T_1 oligonucleotide duplex was isolated by one-dimensional gel electrophoresis [Figure 3B of Ehresmann et al. (1984)] and shown to contain the tRNA pentadecanucleotide containing the anticodon and the universally conserved rRNA nonamer pUpApCpApCpApCpCpG (Ehresmann et al., 1984). This duplex was slightly contaminated by unrelated T_1 digestion products. Nevertheless, the off-diagonal products released by photolysis were pure. As shown in Figure 4, the resulting electrophoresis pattern was identical with that obtained with the homologous *E. coli* duplex. The single exception is the presence of a double band at position B_{20}–B_{12}, whose exact significance remains unclear. From the location of A_{20}, it seems likely that the band on the left is B_{20} and the one on the right B_{12}, but this has not been proven. The cross-linking site analyses yield the same conclusions as in the previous case and establish that cmo^5U_{34} in tRNA is cross-linked to C_{1626} in yeast 18S RNA. The absence of an off-diagonal seven-nucleotide product derived from the rRNA oligomer excludes the involvement of C_{1625} in the formation of the cross-link, and the absence of a nine-nucleotide tRNA subfragment similarly excludes U_{33}.

In some experiments, a faster moving covalent product was detected at a very low amount, which contained the incomplete cligomer pApCpApCpApCpCpG, lacking the 5′-terminal U (Ehresmann et al., 1984). Despite its low amount (about 2%) and its high degree of contamination by unrelated products, this minor duplex could be analyzed (data not shown). The results indicated that C_{1625}, not C_{1626}, was cross-linked in this particular product which lacked U_{1619}.

B. subtilis $tRNA^{Val}$–*E. coli 16S RNA*. Two distinct T_1 oligonucleotide duplexes could be fractionated by one-dimensional gel electrophoresis. They both contained the nonanucleotide pUpApCpApCpApCpCpG from the 16S RNA associated with the anticodon-containing nonanucleotide pCpCpUpmo^{5}UpApCpm^{6}ApApG arising from the tRNA but differed in their electrophoretic mobility and in their ability to be 5′-end labeled (Ehresmann et al., 1984). In the slower moving duplex, denoted A, only the tRNA nonamer was labeled, while only the rRNA nonamer was labeled in the faster moving duplex, denoted B. Both duplexes were obtained in a high degree of purity. The two-dimensional gel electrophoresis patterns of duplexes A and B are shown in Figures 5 and 6, respectively. Due to the fact that only one of the two oligomers was labeled, the resulting patterns were much simpler than those obtained when both oligomers were labeled.

(*i*) *Duplex A*. In this case, only the products derived from the tRNA nonamer are visible. Since long migration was done in the first dimension, the products which do not contain any cross-linking site were not recovered in the sample shown in Figure 5. Nevertheless, short migration in the first dimension did show three off-diagonal products ranging in size between one and three nucleotides (data not shown). This observation indicates that the fourth nucleotide (mo^5U) is part of the cross-link.

The off-diagonal products contain subfragments from the tRNA ranging between four and eight nucleotides (Figure 7B), thus confirming the localization of the cross-link to the fourth residue. If any cross-linking occurred to the third residue, a three-residue off-diagonal subproduct would have been found. The presence of a double band at position A_{20} was reproducibly observed. Its significance, if any, is not known.

There is no direct evidence in this case for the position of the cross-linked nucleotide in the rRNA oligomer, since the latter was unlabeled. Eight uncleaved tRNA bands were observed as off-diagonal products, which must have been associated with cleaved rRNA oligomers. Surprisingly, the mobility shifts appear to be irregular for some of them. It appears as if the mobility shifts differ when cleavage occurs 5′ or 3′ to the cross-linking site in the rRNA oligomer. In this situation, it is difficult to assign the exact cleavage position to the various products. Nevertheless, if we assume that the mobility shift resulting from cleavage at position 8 is the same as that observed in duplex B (Figure 6 below), then it becomes possible to discriminate among the different products. We

postulate that seven products arise from cleavage at positions 1–7 (bands A_1–A_7, Figure 5) and one from cleavage at position 8, even though A_1 and A_8 have the same number of nucleotides. This implies localization of the cross-link on either the second or the penultimate position of the rRNA nonamer. Since the second residue is A, and the cross-linking is known to be a pyrimidine–pyrimidine cyclobutane dimer (Ofengand & Liou, 1980), the penultimate C residue must be the site. While not rigorous, this analysis does rationalize the various observations and is in agreement with the demonstration by bisulfite modification that the C residue in the penultimate position is the site of cross-linking in duplex A (Ehresmann et al., 1984).

(*ii*) *Duplex B.* In this case, only the products from the rRNA nonamer were visible. Diagonal products were analyzed as ranging between one and seven nucleotides, indicating that the eight residue was cross-linked to the tRNA nonamer. The penta- to heptanucleotides are shown on the diagonal of the electrophoretogram of Figure 6.

The off-diagonal products contain only one cleaved rRNA oligomer (B_8), which was identified as an octamer (Figure 7), providing further evidence for the localization of the cross-link at the penultimate residue (C_{1400}). The absence of a seven-residue off-diagonal product excludes the possibility of a second cross-linking site at the adjacent C_{1399}. There is no direct evidence for localization of the cross-linked residue on the tRNA oligomer. Six uncleaved rRNA bands were observed, again with irregular mobility shifts. Making the assumption that mobility shifts resulting from cleavage at positions 22–18 were the same in both duplexes A and B, it was possible to correlate bands and cleavage positions. In this case, products resulting from cleavage at positions 9 and 22 as well as at positions 10 and 21 comigrate. It is not clear whether products B_{11} and B_{20} have a different mobility or whether cleavage at position 20 yields a double band, as observed in duplex A (Figure 5). With these assumptions, we conclude that the cross-link in the tRNA nonamer is located on either the fourth residue (mo^5U) or the sixth residue (C).

These analyses unambiguously show that mo^5U_{34} in the tRNA oligomer is part of the cross-link in duplex A and that C_{1400} in the rRNA oligomer is in the cross-link in duplex B. They also suggest that these residues are covalently linked to C_{1400} and mo^5U_{34} in duplexes A and B, respectively.

Electrophoretic Mobility of the Duplexes. A surprising observation was made about the electrophoretic mobility of the covalent duplexes in the first dimension. In particular, the mobility shift observed for cleavage at positions 22–18 of the *E. coli* tRNA oligomer differs from the other ones. In order to get a more precise idea of this behavior, we plotted the first-dimension electrophoretic mobility of the various cleaved duplexes as a function of their total nucleotide length (Figure 8A). The electrophoretic mobility not only was a function of nucleotide length but also depended on which RNA strand of the covalent duplex was cleaved. In particular, removal of nucleotide residues from the 3′-strand of the tRNA oligomer did not have the same effect on the mobility of the resulting compounds as did cleavage of the three other strands. An identical behavior was found when the yeast rRNA containing duplex was examined.

The *B. subtilis* tRNA–*E. coli* rRNA duplexes A and B also showed anomalous behavior when their electrophoretic mobility in the first dimension was plotted as a function of their nucleotide length (Figure 8B,C). It seems clear that the electrophoretic behavior is not simply a function of nucleotide length but that there is also a strong dependence on which of the four strands of the duplex is being cleaved. Furthermore, the slope of the lines in Figure 8 for duplexes A and B differs considerably from that observed for both duplexes containing *E. coli* tRNA.

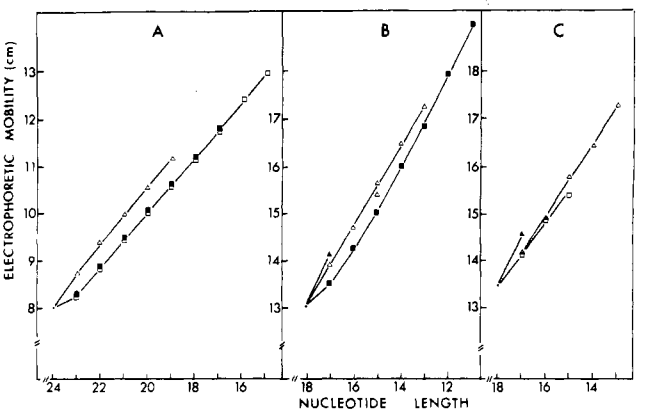

FIGURE 8: Electrophoretic mobility of covalent digestion products arising from random hydrolysis of (A) *E. coli* tRNA–*E. coli* 16S RNA and *E. coli* tRNA–yeast 18S RNA duplexes, (B) *B. subtilis* tRNA–*E. coli* 16S RNA duplex A, and (C) *B. subtilis* tRNA–*E. coli* 16S RNA duplex B. Electrophoretic mobility was directly measured on the autoradiogram of the first dimension. Identification of the various products in the first dimension was deduced from the known position of their photolyzed products in the two-dimensional electrophoresis pattern. Mobility (in centimeters) was plotted as a function of nucleotide length, according to the location of cleavage sites: (□) 5′-strand of the tRNA oligomer (cuts 9–17, panel A; cuts 9–11, panels B and C); (△) 3′-strand of the tRNA oligomer (cuts 18–22); (■) 5′-strand of the rRNA nonamer (cuts 1–7); (▲) 3′-strand of the rRNA nonamer (cut 8). The solid dot is the position of the uncleaved duplex.

Discussion

In this work, we have described a new method which allows determination of the site of cross-linking between two oligonucleotides linked by a cleavable covalent bond. The method is based on random hydrolysis of the covalent oligonucleotide duplex followed by two-dimensional gel electrophoresis. Such covalent oligonucleotide duplexes can easily be obtained by ribonuclease digestion (i.e., total T_1 RNase hydrolysis) of the covalent RNA dimer of interest, followed by fractionation by gel electrophoresis using comparison of the fractionation patterns of both uncleaved and cleaved RNA dimers (Ehresmann et al., 1984), or by the two-dimensional procedure developed by Zwieb & Brimacombe (1980). After the sequences of the individual cross-linked oligomers are obtained (Ehresmann et al., 1984), the nucleotide residues involved in the cross-link can be determined by analysis of both the diagonal and off-diagonal products.

A limitation inherent in all ladder methods of sequencing oligonucleotides is the inability to detect the presence of a second oligomer of the same sequence as the first one but one or more residues shorter. This deficiency is particularly important when cross-link sites are being determined, since in such a case it is quite important to know whether a unique site is involved or whether up- or downstream residues also cross-link, even at low yield. The method described here circumvents this problem by analysis from both ends of the oligomer. The 5′-labeled *diagonal* products set a maximum distance from the 5′-end for the cross-link site but do not allow detection of sites closer to the 5′-end. The 5′-labeled *off-diagonal subfragments* set the maximum distance from the 3′-end to the cross-link site but do not detect sites closer to the 3′-end. The combination of the two analyses thus allows exact determination of the cross-link site or sites. The number of off-diagonal original-length oligomers provides a confir-

mation of the analysis since that number indicates the distance of the cross-link site from *either* the 3′- or the 5′-end.

We have successfully applied this methodology to analysis of the covalent heterodimers formed between *E. coli* AcVal-tRNA$_I^{Val}$ and both *E. coli* 16S RNA and yeast 18S RNA and between *B. subtilis* AcVal-tRNA^{Val} and *E. coli* 16S RNA. These tRNAs can be specifically cross-linked at the ribosomal P site of 70S or 80S ribosomes (Ofengand et al., 1979, 1982) by pyrimidine–pyrimidine cyclobutane dimer formation (Ofengand & Liou, 1980; Ofengand et al., 1982) between a residue in 16–18S RNA and (probably) the wobble base of the tRNA (cmo^{5}U$_{34}$ or mo^{5}U$_{34}$). Recent work of Prince et al. (1982) identified the residues as C$_{1400}$ of *E. coli* 16S RNA and the 5′-base of the anticodon (cmo^{5}U$_{34}$) of *E. coli* tRNA^{Val}. Using bisulfite modification, we were able to demonstrate that C$_{1400}$ in *E. coli* 16S RNA or the equivalent C$_{1626}$ in yeast 18S RNA was cross-linked to *E. coli* tRNA and that *B. subtilis* tRNA was also cross-linked to C$_{1400}$ in *E. coli* 16S RNA (Ehresmann et al., 1984). Nevertheless, neither our previous study nor the work of Prince et al. (1982) excluded cross-linking at a low extent from another position of the rRNA nonamer or the tRNA oligomer.

The various covalent oligonucleotide duplexes were labeled and isolated as described in the preceding paper (Ehresmann et al., 1984). They were submitted to random digestion with alkali and analyzed by two-dimensional gel electrophoresis, taking advantage of the fact that the pyrimidine–pyrimidine cyclobutane dimer can be photolyzed at 254 nm (Ofengand et al., 1979; Ofengand & Liou, 1980). Our results directly confirmed the indirect analysis of Ofengand et al. (1979) that the wobble bases of both *E. coli* and *B. subtilis* tRNAs were part of the cross-link. They further unambiguously demonstrated that cross-linking is restricted to one unique site, namely, between cmo^{5}U$_{34}$ of *E. coli* tRNA and C$_{1400}$ of *E. coli* 16S RNA or C$_{1626}$ of yeast 18S RNA, or between mo^{5}U$_{34}$ of *B. subtilis* tRNA and C$_{1400}$ of *E. coli* 16S RNA. In no case was it possible to detect any trace (>1%) of product resulting from cross-linking at another position, with one minor exception. In the aberrant situation in which the yeast 18S rRNA chain had been cleaved at position U$_{1619}$ prior to cross-linking, the adjacent C$_{1625}$ was found cross-linked.

The case of the two distinct duplexes isolated from the heterodimer between *B. subtilis* tRNA and *E. coli* 16S RNA is puzzling. They differ by both their electrophoretic mobility and their 5′-labeling behavior (Ehresmann et al., 1984), but surprisingly, their nucleotide lengths and cross-link sites were identical. The two-dimensional gel analysis also showed surprising differences between these complexes and the two duplexes containing the *E. coli* tRNA oligomer. In the latter case, an increased electrophoretic mobility was observed only for those products cleaved in the 3′-strand of the tRNA oligomer, while in both *B. subtilis* tRNA duplexes electrophoretic mobility was mostly increased by cleavage of the 3′-strand of the rRNA oligomer. Therefore, the two duplexes containing *B. subtilis* tRNA appear to differ not only from one another but also from those containing *E. coli* tRNA. The reasons for these differences are unclear, although we speculate that they may be related to the absence of the COOH group from the 5′-anticodon base in *B. subtilis* tRNA and/or to the difference in length between the oligomers derived from the two tRNAs. In any event, the observation that the electrophoretic mobility of the random products not only is a function of their nucleotide length but also is affected by the location of the cleavage site is most probably related to the steric conformation of the various duplex fragments.

This two-dimensional procedure has proven to be a powerful and sensitive technique, which is both rapid and easy to handle. It is a general and convenient method to determine cross-linking sites in cleavable covalent RNA–RNA complexes. Its greatest advantage is to yield the site of cross-linking in both oligomers in one step and to detect the presence of minor cross-linking sites. Due to its absence of nucleotide specificity, it can be easily applied to any other kinds of RNA–RNA covalent duplexes with the only limitation that the cross-link be cleavable. The most straightforward application is to other photoreversible covalent complexes, such as those obtained with psoralens (Wollenzien et al., 1978). It is, however, not limited to photoreversible complexes. As an example, bis(glyoxal) derivatives can induce covalent cross-links between nonpaired guanosine residues (Wagner & Garrett, 1978) which are reversible under mild alkaline conditions. In this case, random hydrolysis with ribonuclease P$_1$ would be preferable to alkaline digestion, and soaking the gel strip in mild alkali should reverse the cross-link after the first-dimension electrophoresis. Other modifications of the procedure could be imagined for different cross-links. In addition, because high specific activity can be obtained by 5′-labeling, low amounts of material can be analyzed. A high degree of purity is not even required, since only products arising from covalently linked oligomers are released as off-diagonal products. In this case, however, analysis of the diagonal products is not possible, and one self-checking aspect is sacrificed.

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Registry No. pUpApCpApCpApCpCpG·pCpApCpCpUpCpCpCpUpcmo^{5}UpApCpm^{6}ApApG, 88156-53-0; pUpApCpApCpApCpCpG·pCpCpUpmo^{5}UpApCpm^{6}ApApG, 87953-06-8.

References

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

Donis-Keller, H., Maxam, A. M., & Gilbert, W. (1977) *Nucleic Acids Res. 4*, 2527–2538.

Ehresmann, C., Ehresmann, B., Millon, R., Ebel, J.-P., Nurse, K., & Ofengand, J. (1984) *Biochemistry* (preceding paper in this issue).

Hancock, J., & Wagner, R. (1982) *Nucleic Acids Res. 10*, 1257–1269.

Maxam, A. M., & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 560–564.

Ofengand, J., & Liou, R. (1980) *Biochemistry 19*, 4814–4822.

Ofengand, J., Liou, R., Kohut, J., III, Schwartz, I., & Zimmermann, R. E. (1979) *Biochemistry 18*, 4322–4332.

Ofengand, J., Gornicki, P., Chakraburtty, K., & Nurse, K. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2817–2821.

Prince, J. B., Taylor, B. H., Thurlow, D. L., Ofengand, J., & Zimmermann, R. A. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5450–5454.

Rabin, D., & Crothers, D. M. (1979) *Nucleic Acids Res. 7*, 689–703.

Stiege, W., Zwieb, C., & Brimacombe, R. (1982) *Nucleic Acids Res. 10*, 7211–7229.

Stiege, W., Glotz, C., & Brimacombe, R. (1983) *Nucleic Acids Res. 11*, 1687–1706.

Thompson, J. F., & Hearst, J. E. (1983) *Cell (Cambridge, Mass.) 32*, 1355–1365.

Thompson, J. F., Wegnez, M. R., & Hearst, J. E. (1981) *J. Mol. Biol. 147*, 417–436.

Turner, S., Thompson, J. F., Hearst, J. E., & Noller, H. F. (1982) *Nucleic Acids Res. 10*, 2839–2849.
Wagner, R., & Garrett, R. A. (1978) *Nucleic Acids Res. 5*, 4065–4075.
Wollenzien, P. L., Youvan, D. C., & Hearst, J. E. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1642–1646.
Zwieb, C., & Brimacombe, R. (1980) *Nucleic Acids Res. 8*, 2397–2411.

Reconstitution of the Membrane-Bound, Ubiquinone-Dependent Pyruvate Oxidase Respiratory Chain of *Escherichia coli* with the Cytochrome *d* Terminal Oxidase†

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ABSTRACT: Pyruvate oxidase is a flavoprotein dehydrogenase located on the inner surface of the *Escherichia coli* cytoplasmic membrane and coupled to the *E. coli* aerobic respiratory chain. In this paper, the role of quinones in the pyruvate oxidase system is investigated, and a minimal respiratory chain is described consisting of only two pure proteins plus ubiquinone 8 incorporated in phospholipid vesicles. The enzymes used in this reconstitution are the flavoprotein and the recently purified *E. coli* cytochrome *d* terminal oxidase. The catalytic velocity of the reconstituted liposome system is about 30% of that observed when the flavoprotein is reconstituted with *E. coli* membranes. It is also shown that electron transport from pyruvate to oxygen in the liposome system generates a transmembrane potential of at least 180 mV (negative inside), which is sensitive to the uncouplers carbonyl cyanide *p*-(trichloromethoxy)phenylhydrazone and valinomycin. A transmembrane potential is also generated by the oxidation of ubiquinol 1 by the terminal oxidase in the absence of the flavoprotein. It is concluded that (1) the flavoprotein can directly reduce ubiquinone 8 within the phospholipid bilayer, (2) menaquinone 8 will not effectively substitute for ubiquinone 8 in this electron-transfer chain, and (3) the cytochrome *d* terminal oxidase functions as a ubiquinol 8 oxidase and serves as a "coupling site" in the *E. coli* aerobic respiratory chain. These investigations suggest a relatively simple organization for the *E. coli* respiratory chain.

Many of the components of the *Escherichia coli* aerobic electron-transport chain have now been identified. The respiratory chain is branched at both the substrate side and the oxygen side. There are several flavoprotein dehydrogenases, including the pyruvate oxidase flavoprotein, that feed electrons into the chain [see Bragg (1979)]. Ubiquinone 8 is clearly an important component, as demonstrated by the use of ubiquinone-deficient strains in studies of the NADH, succinate, and D-lactate oxidase systems (Cox et al., 1970; Wallace & Young, 1977; Downie & Cox, 1978). The respiratory chain contains two terminal oxidases. Both the cytochrome *d* terminal oxidase complex and the cytochrome *o* terminal oxidase complex have now been purified to homogeneity. The cytochrome *d* complex contains cytochromes b_{558}, a_1, and *d* and has two subunits as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)[1] (Miller & Gennis, 1983). The cytochrome *o* complex is a *b* type cytochrome with absorption peaks at 555 and 562 nm, which has four subunits by SDS–PAGE analysis (Kranz & Gennis, 1983; Matsushita et al., 1983) and has been shown to function as a "coupling site" in vitro with ubiquinol 1 (UQ-1) as a reductant (Matsushita et al., 1983; Kita et al., 1982). Together, these two terminal oxidases account for most of the cytochromes that have been identified by spectroscopic and electrochemical methods of analysis of the membranes (Shipp, 1972; Scott & Poole, 1982; Reid & Ingledew, 1979; Van Wielink et al., 1982; Pudek & Bragg, 1976; Hendler et al., 1975). One additional component, cytochrome b_{556}, has also been purified (Kita et al., 1978), and this cytochrome has been suggested to be part of the "*o* branch" (Kita & Anraku, 1981), though this has yet to be rigorously demonstrated.

Pyruvate oxidase is a flavoprotein dehydrogenase that has been isolated from *E. coli* (Williams & Hager, 1966; O'Brien et al., 1976). The enzyme catalyzes the oxidative decarboxylation of pyruvate to yield acetate plus CO_2 and is located on the inner surface of the cytoplasmic membrane (Shaw-Goldstein et al., 1978). Previous work has shown that when *E. coli* membrane vesicles are supplemented with the purified flavoprotein, pyruvate-driven oxygen consumption is observed (Shaw-Goldstein et al., 1978; Deeb & Hager, 1964; Cunningham & Hager, 1975). The pure enzyme does not react rapidly with oxygen, but significant oxidase activity is manifested in the presence of *E. coli* membranes. Pyruvate oxidase flavoprotein, like other *E. coli* flavoprotein dehydrogenases (Jaworowski et al., 1981; Haldar et al., 1982; Schryvers et al., 1978), appears to couple to the aerobic respiratory chain of the cytoplasmic membrane.

The pyruvate oxidase flavoprotein is unique among these dehydrogenases in that it is a peripheral membrane protein and easily dissociates from the membrane in a water-soluble form. This has been an advantage in studying the enzymology and lipid-binding properties of the enzyme (e.g., Schrock &

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[1] Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; UQ, ubiquinone; MK, menaquinone; HQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; CCCP, carbonyl cyanide *p*-(trichloromethoxy)phenylhydrazone; TPP, thiamin pyrophosphate. In UQ and MK, the number of isoprene units in the side chain is indicated by a number following the abbreviation.

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