JOURNAL OF BACTERIOLOGY, Mar. 1991, p. 1690–1695
0021-9193/91/051690-06$02.00/0

Vol. 173, No. 5

Primary Structure of Xylene Monooxygenase: Similarities to and Differences from the Alkane Hydroxylation System

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Received 8 August 1990/Accepted 10 December 1990

Xylene monooxygenase, encoded by the TOL plasmid of *Pseudomonas putida*, catalyzes the oxidation of toluene and xylenes and consists of two different subunits encoded by *xylA* and *xylM*. In this study, the complete nucleotide sequences of these genes were determined and the amino acid sequences of the *xylA* and *xylM* products were deduced. The XylM sequence had a 25% homology with alkane hydroxylase, which catalyzes the ω-hydroxylation of fatty acids and the terminal hydroxylation of alkanes. The sequence of the first 90 amino acids of XylA exhibited a strong similarity to the sequence of chloroplast-type ferredoxins, whereas the rest of the XylA sequence resembled that of ferredoxin-NADP$^+$ reductases. Based on this information, the structure and function of xylene monooxygenase were deduced. XylM may be a catalytic component for the hydroxylation of the carbon side chain of toluene and xylenes and, as is the alkane hydroxylase protein, may be a membrane-bound protein containing ferrous ion as a prosthetic group. XylA may have two domains consisting of an N-terminal region similar to chloroplast-type ferredoxins and a C-terminal region similar to ferredoxin-NADP$^+$ reductases. The ferredoxin portion of XylA may contain a [2Fe-2S] cluster and reduce the oxidized form of the XylM hydroxylase. The activity determined by the C-terminal region of the XylA sequence may be the reduction of the oxidized form of ferredoxin by concomitant oxidation of NADH.

The TOL plasmid pWW0 of *Pseudomonas putida* encodes the enzymes for the oxidative catabolism of toluene and xylenes. These compounds are degraded by the progressive oxidation of a methyl side chain to carboxylic acid, followed by oxygenative cleavage of the aromatic ring. Xylene monooxygenase is the first enzyme in this pathway and oxidizes toluene and xylenes to (methyl)benzyl alcohols (26). The enzyme has a broad substrate specificity and also oxidizes (methyl)benzyl alcohols to (methyl)benzaldehydes and indole to indoxyl (5, 13). The enzyme consists of two different subunits encoded by *xylM* and *xylA*, respectively (8).

Although this enzyme activity could be detected in intact cells of *P. putida*(pWW0) or of *Escherichia coli* carrying *xylM* and *xylA*, we have not been successful in demonstrating its activity in vitro. In this study, we determined the complete nucleotide sequences of *xylM* and *xylA*. From the amino acid sequences of the *xylM* and *xylA* products deduced from the nucleotide sequences, possible functions and locations of these two-subunit proteins were suggested. On the basis of that information, we were able to demonstrate the membrane-bound activity of xylene monooxygenase in vitro.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *P. putida* PaW1 carrying the TOL plasmid pWW0 and *E. coli* K12ΔH1Δtrp carrying pGSH2836 have been described previously (5, 8). *P. putida* was grown in M9 minimal medium (5) containing 5 mM benzyl alcohol and a source of xylene vapors at 30°C, whereas *E. coli* was grown in L broth containing ampicillin (100 μg/ml) at 30°C.

DNA sequencing. The source of the DNA containing both *xylM* and *xylA* was pGSH2836 (5). The methods for DNA manipulations and for DNA sequencing with single-stranded M13 derivatives and with plasmid DNA have been described previously (7). Universal primers as well as 17-mer primers complementary to different regions of *xylA* and *xylM* DNA were synthesized on an Applied Biosystems model 381A DNA synthesizer and used for DNA sequencing.

Nucleotide sequence analysis. The microcomputer-assisted sequence analysis was done with the PC/GENE software package (Genofit/Intelligenetics, Geneva, Switzerland) developed by A. Bairoch. The search for proteins exhibiting sequence similarity to the *xylM* and *xylA* products was done by the method of Lipman and coworkers (12, 25) on the protein bank Swiss-Prot compiled by A. Bairoch. Statistic tests for the significance of a sequence similarity were performed as described previously (6).

Preparation of cell extracts and assay for xylene monooxygenase. *P. putida*(pWW0) cells were grown at 30°C overnight in 3 liters of M9 minimum medium containing 5 mM benzyl alcohol and a source of *m*-xylene vapors. They were centrifuged, washed once with 50 mM potassium phosphate buffer (pH 7.4), and resuspended in 80 ml of potassium phosphate buffer containing 5 mM dithiothreitol. The cells were disrupted by two passages through a precooled French press (SLM instruments, Urbana, Ill.) with a pressure difference of 76 MPa at the orifice. The cell extract thus obtained was centrifuged in a Sorvall SS34 rotor at 40,000 × *g* for 10 min, and the resulting supernatant was centrifuged at 200,000 × *g* for 2 h in a Beckman 60 Ti rotor. The pellet was suspended in 10 ml of 50 mM potassium phosphate buffer (pH 7.4) containing 20% (vol/vol) glycerol and 5 mM dithiothreitol. This membrane vesicle suspension was added to 10 ml of 100 mM potassium phosphate buffer (pH 7.4) containing 20 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate and 5 mM dithiothreitol; the final protein concentration was 3 mg/ml. The solution was gently stirred for 1 h at 4°C and then centrifuged at 200,000 × *g* for 1 h in a Beckman 60

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Ti rotor. The supernatant was called a CHAPS extract of the membrane fraction.

Xylene monooxygenase activity was monitored by either measuring spectrophotometrically the oxidation of NADH at 340 nm or measuring the consumption of oxygen in a Clark-type oxygen electrode. The reaction mixture for the spectrophotometric determinations consisted of 800 μl of 100 mM Tris-HCl (pH 8.0), 50 μl of a CHAPS extract, and 100 μl of 100 mM Tris-HCl buffer (pH 8.0) saturated in *m*-xylene or, for the reference reaction, 100 μl of Tris-HCl buffer alone. After 1 min of incubation, 50 μl of 10 mM NADH was added. For measuring the consumption of oxygen, the reaction occurred in 2 ml of 10 mM *N*,*N*-bis(2-hydroxyethyl)glycine/NAOH (pH 8.5), and 50 μl of 10 mM NADH was added after an incubation period of 2 min. The crude extract usually contained a high NADH oxidase activity independent of xylene monooxygenase. The activity of xylene monooxygenase was calculated as either the difference in the rate of oxidation of NADH in the presence or absence of *m*-xylene or the difference in the rate of the consumption of oxygen in the presence or absence of *m*-xylene. The activity of xylene monooxygenase was expressed as nanomoles of NADH oxidized per minute per milligram of protein or as nanomoles of oxygen consumed per minute per milligram of protein. Protein concentrations were determined by the bicinchoninic acid method (22) with the reagents supplied by Pierce (Rockford, Ill.).

Nucleotide sequence accession number. The sequence data presented in Fig. 1 will appear in the EMBL/GenBank nucleotide sequence data base under the accession number M37480.

RESULTS AND DISCUSSION

Nucleotide sequences of *xylM* and *xylA*. The complete DNA sequence of the *Sal*I-*Hin*dIII fragment (2,352 bp) of the TOL plasmid pWW0 containing both *xylM* and *xylA* (8) was determined (Fig. 1). The open reading frame analysis showed that *xylM* is most likely encoded in the frame which terminates at residue 1133. Several possible initiation codons exist in this frame, but only the one at nucleotides 27 to 29 is preceded by a putative Shine-Dalgarno sequence, AGGTG. If *xylM* starts at this residue, then the molecular weight of the *xylM* product is 41,650, which is larger than the size estimated from the product synthesized in maxicells (35 kDa [8]). As discussed below, XylM may be a membrane-bound protein, which could explain its abnormal behavior in sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

We have recently purified XylA (21a). The N-terminal sequencing data of the purified protein indicated that it contained equal amounts of two species whose N-terminal sequences were Lys-Lys-Ile-?-Gly-Leu-Phe-?-Pro-Pro and Lys-Ile-?-Gly-Leu-Phe-?-Pro-Pro-Pro. This result could best be interpreted such that the *xylA* gene starts at residue 1286 and that the primary product is cleaved before one of the two lysines at residues 6 and 7.

Similarity of *xylA* product with chloroplast-type ferredoxin. A scan of the protein bank Swiss-Prot detected a similarity in the N-terminal sequence of the XylA protein with chloroplast-type ferredoxins. The alignment of the N-terminal sequence of XylA with the ferredoxin from Spirulina platensis (3) is presented in Fig. 2. Chloroplast-type ferredoxins contain [2Fe-2S] clusters associated with the invariant cysteines in the following structure: Cys-(four amino acids)-Cys-(two amino acids)-Cys (15). In XylA, these three cysteines are conserved. This conclusion prompted us to compare the rest of the XylA sequence with two ferredoxin-$NADP^+$ reductases, one from spinach and the other from a cyanobacterium (9, 27). As shown in Fig. 2, the XylA sequence could be aligned with these two ferredoxin-$NADP^+$ reductases and the similarity between XylA and these two ferredoxin-$NADP^+$ reductases was significant by the statistics test of Needleman and Wunsch (16). We concluded that the progenitor of XylA was formed by the fusion of a chloroplast-type ferredoxin and a ferredoxin-$NADP^+$ reductase. As discussed later, the function of the ferredoxin-$NADP^+$ reductase domain of XylA may be to reduce ferredoxin with the concomitant oxidation of NADH. If this is the case, the enzymatic activity of this domain should rather be termed NADH-ferredoxin reductase.

Similarity of *xylM* product to *alkB* product. Protein sequences similar to XylM were searched for in the protein bank Swiss-Prot, and 50 proteins exhibiting the highest scores for sequences similar to XylM were selected by the method of Lipman and coworkers (12, 25). However, their similarity to XylM was not significant when analyzed by the method of Needleman and Wunsch (16). From the observation that XylA is similar to the ferredoxins and ferredoxin-$NADP^+$ reductases, the function of XylM was postulated to be a hydroxylase, which catalyzes the incorporation of one atom of oxygen into the methyl group of toluene or xylenes. We therefore collected amino acid sequences of oxygenases and oxidases from Swiss-Prot, from recent publications, and from unpublished data and compared them with the sequence of XylM by the method of Needleman and Wunsch (16). A high degree of similarity was discovered between the amino acid sequences of XylM and alkane hydroxylase, the product of *alkB* encoded by the OCT plasmid of *Pseudomonas oleovorans* (11). The sequence of this protein had not been filed in Swiss-Prot when the search was done (April 1989). An alignment of XylM with AlkB is presented in Fig. 3.

The AlkB enzyme catalyzes the hydroxylation of the terminal carbon of alkanes and the ω-hydroxylation of fatty acids. This enzyme, however, does not hydroxylate the methyl side chain of toluene and xylenes (20) despite its similarity to XylM. Therefore, the amino acid residues important for the recognition of toluene and xylenes are apparently not present in the AlkB sequence. Alkane hydroxylase is an inner membrane-bound protein and is only active in vitro in the presence of phospholipids (20). In fact, this protein contains several hydrophobic regions (11) (Fig. 3). In the XylM protein, hydrophobic stretches are found in regions similar to those of AlkB (Fig. 3). This observation suggested that XylM is also membrane bound and that the mode of insertion of its polypeptide chain into the membrane is similar to that of AlkB. The substrates of both enzymes are hydrophobic and may be distributed into the membrane. The location of these enzymes in the membrane may therefore be advantageous for the efficient transformation of their hydrophobic substrates.

Functions of XylA and XylM deduced from their primary structures. Monooxygenases catalyze the reaction $S + O_2 + NAD(P)H + H^+ \rightarrow SO + H_2O + NAD(P)^+$, where S is a substrate. In this reaction, one atom of oxygen is incorporated into the substrate, while the other atom is reduced to H_2O (4, 17). This class of enzymes can be classified into two groups. The first group consists of single-subunit monooxygenases; they are usually flavoproteins. In these monooxygenases, all the reactions occur within one polypeptide. The mechanisms of the oxygenation of the substrate and the

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      10|       20|       30|       40|       50|       60|       70|       80|       90|      100|      110|      120|
TCGACAATAATAAAGTAGGTGGATATATGGACACGCTTCGTTATTACCTGATTCCTGTTGTTACTGCTTGCGGGCTGATCGGATTTTACTATGGTGGCTATTGGGTTTGGCTTGGGGCGG
                          MetAspThrLeuArgTyrTyrLeuIleProValValThrAlaCysGlyLeuIleGlyPheTyrTyrGlyGlyTyrTrpValTrpLeuGlyAlaAla
      130|      140|      150|      160|      170|      180|      190|      200|      210|      220|      230|      240|
CAACATTCCCTGCACTGATGGTGCTTGATGTCATTTTACCGAAGGATTTTTCGGCCAGAAAGGTAAGTCCCTTTTTCGCAGACCTTACCCAGTATTTGCAGTTACCATTAATGATCGGTC
  ThrPheProAlaLeuMetValLeuAspValIleLeuProLysAspPheSerAlaArgLysValSerProPheAlaAspLeuThrGlnTyrLeuGlnLeuProLeuMetIleGlyLeu
      250|      260|      270|      280|      290|      300|      310|      320|      330|      340|      350|      360|
TATATGGGCTCCTTGTCTTCGGAGTTGAAAACGGGCGTATCGAACTTAGTGAGCCGTTACAAGTGGCAGGGTGCATTCTTTCTTTGGCTTGGCTTAGTGGTGTGCCAACTCTTCCGGTTT
  TyrGlyLeuLeuValPheGlyValGluAsnGlyArgIleGluLeuSerGluProLeuGlnValAlaGlyCysIleLeuSerLeuAlaTrpLeuSerGlyValProThrLeuProValSer
      370|      380|      390|      400|      410|      420|      430|      440|      450|      460|      470|      480|
CGCATGAGTTGATGCATCGTCGCCACTGGTTGCCTCGGAAAATGGCGCAGCTATTGGCTATGTTTTATGGTGATCCGAACCGAGACATTGCCCATGTCAACACGCATCACCTTTACTTAG
  HisGluLeuMetHisArgArgHisTrpLeuProArgLysMetAlaGlnLeuLeuAlaMetPheTyrGlyAspProAsnArgAspIleAlaHisValAsnThrHisHisLeuTyrLeuAsp
      490|      500|      510|      520|      530|      540|      550|      560|      570|      580|      590|      600|
ATACGCCTCTCGATAGCGATACTCCGTACCGTGGTCAGACAATTTACAGTTTCGTGATCAGTGCGACAGTTGGTTCCGTCAAAGATGCGATAAAGATTGAGGCTGAAACTTTACGTAGAA
  ThrProLeuAspSerAspThrProTyrArgGlyGlnThrIleTyrSerPheValIleSerAlaThrValGlySerValLysAspAlaIleLysIleGluAlaGluThrLeuArgArgLys
      610|      620|      630|      640|      650|      660|      670|      680|      690|      700|      710|      720|
AAGGACAGTCACCGTGGAATTTGTCCAACAAAACATATCAATATGTCGCACTTCTGCTCGCTCTGCCTGGCTTGGTTTCTTATCTGGGCGGGCCAGCATTAGGGTTGGTTACGATTGCTT
  GlyGlnSerProTrpAsnLeuSerAsnLysThrTyrGlnTyrValAlaLeuLeuLeuAlaLeuProGlyLeuValSerTyrLeuGlyGlyProAlaLeuGlyLeuValThrIleAlaSer
      730|      740|      750|      760|      770|      780|      790|      800|      810|      820|      830|      840|
CGATGATTATTGCGAAAGGGATAGTCGAGGGTTTTAATTACTTTCAGCACTATGGTTTAGTACGCGATTTAGATCAGCCTATCCTCCTGCACCACGCGTGGAATCATATGGGAACAATTG
  MetIleIleAlaLysGlyIleValGluGlyPheAsnTyrPheGlnHisTyrGlyLeuValArgAspLeuAspGlnProIleLeuLeuHisHisAlaTrpAsnHisMetGlyThrIleVal
      850|      860|      870|      880|      890|      900|      910|      920|      930|      940|      950|      960|
TGCGCCCGCTGGGTTGCGAAATTACTAACCATATCAATCATCATATTGACGGCTATACACGGTTCTATGAGTTGCGTCCGGAAAAAGAAGCCCCGCAGATGCCTTCGCTCTTTGTGTGTT
  ArgProLeuGlyCysGluIleThrAsnHisIleAsnHisHisIleAspGlyTyrThrArgPheTyrGluLeuArgProGluLysGluAlaProGlnMetProSerLeuPheValCysPhe
      970|      980|      990|     1000|     1010|     1020|     1030|     1040|     1050|     1060|     1070|     1080|
TCCTTCTAGGGCTTATTCCGCCTCTTTGGTTCGCTCTCATTGCAAAACCAAAGTTGAGAGACTGGGACCAGCGGTACGCAACTCCAGGTGAGCGCGAACTGGCTATGGCTGCAAATAAAA
  LeuLeuGlyLeuIleProProLeuTrpPheAlaLeuIleAlaLysProLysLeuArgAspTrpAspGlnArgTyrAlaThrProGlyGluArgGluLeuAlaMetAlaAlaAsnLysLys
     1090|     1100|     1110|     1120|     1130|     1140|     1150|     1160|     1170|     1180|     1190|     1200|
AAGCGGGATGGCCACTGTGGTGTGAAAGTGAACTGGGTCGGGTGGCTAGCATTTGATAATACAGGCAGTCTAAACCGTTTCGACTGAGACCGTAGATAAGCGAAGAGCGAACGAACTCTA
  AlaGlyTrpProLeuTrpCysGluSerGluLeuGlyArgValAlaSerIle
     1210|     1220|     1230|     1240|     1250|     1260|     1270|     1280|     1290|     1300|     1310|     1320|
CGGTCTCTTTTTGAAAAAAACGTTCCAAATGGGACAAAGTTTCCGTTTTTTCTAATTAACGGGCGTCTAACTGAGCGATGGGTTTATGAATGAGTTTTTTAAGAAAATCTCTGGTTTATT
                                                                  MetAsnGluPhePheLysLysIleSerGlyLeuPhe
     1330|     1340|     1350|     1360|     1370|     1380|     1390|     1400|     1410|     1420|     1430|     1440|
TGTGCCGCCTCCGGAATCTACCGTTTCAGTCAGAGGGCAGGGGTTTCAGTTTAAGGTGCCACGCGGGCAAACCATTCTGGAAAGCGCTCTGCATCAAGGAATTGCCTTTCCGCATGATTG
  ValProProProGluSerThrValSerValArgGlyGlnGlyPheGlnPheLysValProArgGlyGlnThrIleLeuGluSerAlaLeuHisGlnGlyIleAlaPheProHisAspCys
     1450|     1460|     1470|     1480|     1490|     1500|     1510|     1520|     1530|     1540|     1550|     1560|
CAAAGTCGGATCTTGTGGGACATGTAAATATAAACTGATATCTGGCAGGGTCAATGAGTTGACCTCTTCTGCTATGGGTCTGAGTGGCGATCTGTATCAGTCCGGCTATCGTTTGGGTTG
  LysValGlySerCysGlyThrCysLysTyrLysLeuIleSerGlyArgValAsnGluLeuThrSerSerAlaMetGlyLeuSerGlyAspLeuTyrGlnSerGlyTyrArgLeuGlyCys
     1570|     1580|     1590|     1600|     1610|     1620|     1630|     1640|     1650|     1660|     1670|     1680|
TCAATGCATACCAAAAGAAGATCTCGAGATAGAGCTAGACACAGTGCTCGGGCAGGCGTTAGTTCCAATAGAAACGAGTGCCTTGATTAGTAAGCAGAAACGGCTGGCGCACGATATAGT
  GlnCysIleProLysGluAspLeuGluIleGluLeuAspThrValLeuGlyGlnAlaLeuValProIleGluThrSerAlaLeuIleSerLysGlnLysArgLeuAlaHisAspIleVal
     1690|     1700|     1710|     1720|     1730|     1740|     1750|     1760|     1770|     1780|     1790|     1800|
CGAGATGGAAGTAGTGCCCGATAAGCAGATAGCCTTCTACCCCGGCCAGTATGCAGATGTAGAATGTGCAGAATGCTCTGCTGTAAGGAGTTATTCTTTTTCCGCTCCGCCCCAACCTGA
  GluMetGluValValProAspLysGlnIleAlaPheTyrProGlyGlnTyrAlaAspValGluCysAlaGluCysSerAlaValArgSerTyrSerPheSerAlaProProGlnProAsp
     1810|     1820|     1830|     1840|     1850|     1860|     1870|     1880|     1890|     1900|     1910|     1920|
CGGCTCCCTGAGCTTCCATGTTCGCCTTGTCCCAGGTGGAGTTTTCAGTGGTTGGCTATTTGGTGGCGATCGTACAGGAGCGACACTAACCCTGCGAGCGCCTTATGGACAGTTCGGGCT
  GlySerLeuSerPheHisValArgLeuValProGlyGlyValPheSerGlyTrpLeuPheGlyGlyAspArgThrGlyAlaThrLeuThrLeuArgAlaProTyrGlyGlnPheGlyLeu
     1930|     1940|     1950|     1960|     1970|     1980|     1990|     2000|     2010|     2020|     2030|     2040|
CCATGAGAGCAATGCCACGATGGTCTGCGTAGCCGGCGGAACGGGGCTTGCTCCAATTAAATGTGTTTTGCAGAGCATGACCCAGGCCCAGCGAGAGCGTGATGTGTTGTTGTTCTTTGG
  HisGluSerAsnAlaThrMetValCysValAlaGlyGlyThrGlyLeuAlaProIleLysCysValLeuGlnSerMetThrGlnAlaGlnArgGluArgAspValLeuLeuPhePheGly
     2050|     2060|     2070|     2080|     2090|     2100|     2110|     2120|     2130|     2140|     2150|     2160|
AGCTCGTCAACAACGTGACCTATATTGCCTCGACGAAATAGAAGCGCTGCAACTCGATTGGGGTGGGCGCTTCGAGCTTATTCCAGTTTTGTCCGAAGAGTCTTCTACGTCGTCATGGAA
  AlaArgGlnGlnArgAspLeuTyrCysLeuAspGluIleGluAlaLeuGlnLeuAspTrpGlyGlyArgPheGluLeuIleProValLeuSerGluGluSerSerThrSerSerTrpLys
     2170|     2180|     2190|     2200|     2210|     2220|     2230|     2240|     2250|     2260|     2270|     2280|
AGGGAAACGTGGCATGGTAACCGAGTATTTTAAGGAGTACCTCACTGGGCAGCCTTATGAAGGATACCTTTGCGGGCCGCCCCCTATGGTGGACGCTGCCGAGACCGAGCTCGTTCGACT
  GlyLysArgGlyMetValThrGluTyrPheLysGluTyrLeuThrGlyGlnProTyrGluGlyTyrLeuCysGlyProProProMetValAspAlaAlaGluThrGluLeuValArgLeu
     2290|     2300|     2310|     2320|     2330|     2340|     2350|
TGGTGTTGCGCGGGAATTAGTGTTTGCGGACCGTTTTTATAATAGACCTCCTTGCTAGCAGTAGCAGAAGCT
  GlyValAlaArgGluLeuValPheAlaAspArgPheTyrAsnArgProProCys
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FIG. 1. Nucleotide sequences of *xylM* and *xylA*. The nucleotide sequence of the 2,352-bp *Sal*I-*Hin*dIII segment of the TOL plasmid pWW0 containing *xylM* (nucleotides 27 to 1133) and *xylA* (nucleotides 1286 to 2335) is presented. The amino acid sequences of the *xylM* and *xylA* products deduced from the DNA sequence are also shown. Putative Shine-Dalgarno sequences of *xylM* and *xylA* are underlined.

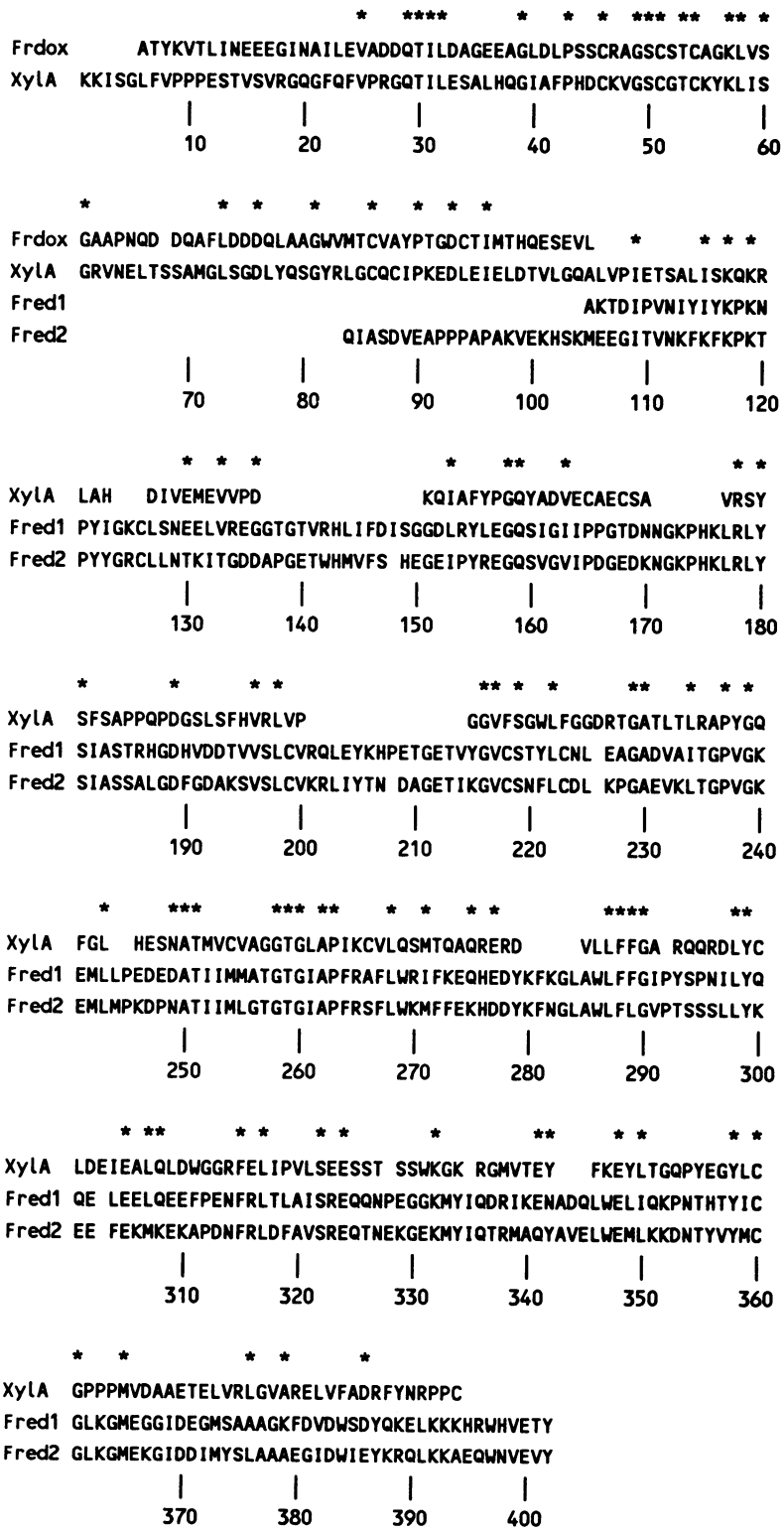

FIG. 2. Comparison of the amino acid sequence of XylA with that of ferredoxin from *S. platensis* (Frdox [4]) and those of ferredoxin-NADP reductases from spinach (Fred1 [9]) and from *Spirulina* sp. (Fred2 [27]). * indicates the positions at which XylA contains residues identical to those of one of the compared sequences.

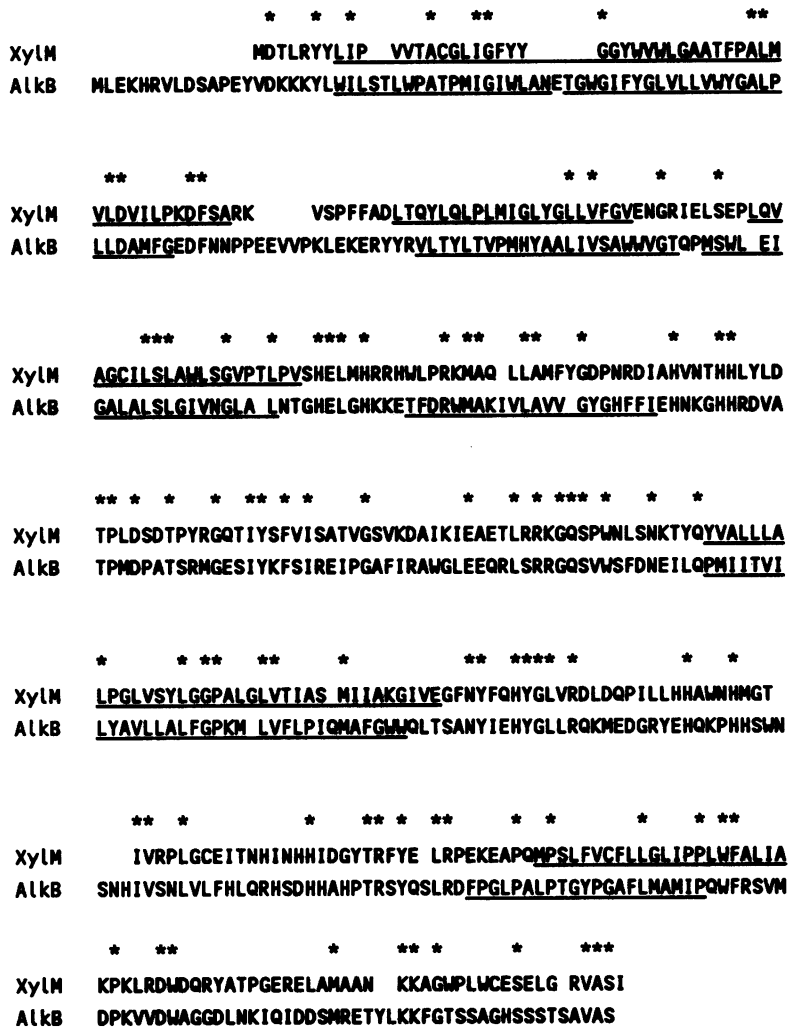

FIG. 3. Comparison of the amino acid sequence of XylM with that of AlkB. The data for the AlkB sequence were taken from reference 11. * indicates residues that are identical between XylM and AlkB. Possible membrane-associated polypeptides are underlined. They were detected by published methods (2, 19) by using the HELIXMEM and RAOARGO programs in the software package PC/GENE.

reduction of oxygen to H_2O by this group of enzymes have well been characterized (21, 24).

Monooxygenases of the second class are composed of two or three different subunits. One subunit is a hydroxylase which catalyzes the oxygenation of substrates. The alkane hydroxylation system which hydroxylates the terminal carbon of alkanes and of long-chain fatty acids, for example, is made of three components: a terminal hydroxylase, a rubredoxin which contains a nonheme iron, and an NADH-rubredoxin reductase which is a flavoprotein (1, 20, 23). The hydroxylase component contains ferrous ion and catalyzes the hydroxylation of alkanes and long-chain fatty acids (20). The electrons required for the hydroxylation are transferred from rubredoxin-NADPH reductase to rubredoxin and then from rubredoxin to the hydroxylase component. According to the sequence similarities of XylM to AlkB and of XylA to ferredoxins and ferredoxin-$NADP^+$ reductases, we propose that the XylM component of this enzyme is membrane bound, contains ferrous ion in the catalytic center, and exhibits a hydroxylase activity, while the XylA component contains two functional domains, a ferredoxin- and an NADH-ferredoxin-reductase domain, and transfers electrons to the XylM subunit.

Demonstration of xylene monooxygenase activity in vitro. The sequencing data of *xylM* suggested that the XylM component of xylene monooxygenase is membrane bound. To examine this hypothesis, we prepared a cell extract of *P. putida*(pWW0) as described in Materials and Methods. The extract was centrifuged at 200,000 × *g* to separate a membrane fraction (pellet) and a soluble fraction (supernatant). In the soluble fraction, we did not detect any xylene monooxygenase activity (<5 nmol of NADH oxidized per min per mg of protein). The activity in the membrane fraction could not be measured because of the high activity of NADH oxidase independent of xylene monooxygenase. However, we could demonstrate xylene monooxygenase activity in a CHAPS extract (80 nmol of NADH oxidized per min per mg of protein or 75 nmol of oxygen consumed per min per mg of protein). The addition of purified XylA increased the xylene monooxygenase activity by 80%. This result suggested that a fraction of XylA was membrane bound in our preparation.

Precarious partnerships between hydroxylase and electron

transfer components of oxygenases. Although the hydroxylase component of xylene monooxygenase and that of the alkane hydroxylase system probably derived from the same progenitor, the electron transfer components of these enzymes are evolutionarily unrelated—the amino acid sequences of the chloroplast-type ferredoxin family and the rubredoxin family are completely different (3, 10). This observation indicates that, during their evolutionary courses, either XylM or AlkB has changed, at least once, its partnership with electron donors.

Therefore, the multicomponent monooxygenase systems could best be classified if hydroxylase components and electron transfer components were considered separately. In cytochrome P-450 monooxygenase systems, all the hydroxylase components, namely, the cytochromes P-450, were shown to be members of a one-gene superfamily (15). This group of the hydroxylases is, however, not related to the hydroxylase component of xylene monooxygenase, XylM. In contrast, the electron transfer component of xylene monooxygenase, XylA, is evolutionary related to the electron transfer component of the cytochrome P-450 monooxygenase systems, namely, NADPH–cytochrome P-450 reductase: the C-terminal portion of XylA was similar to ferredoxin-$NADP^+$ reductase (this study) and ferredoxin-$NADP^+$ reductase was similar to NADPH–cytochrome P-450 reductase (18).

We have recently found that a subunit of toluate 1,2-dioxygenase, the product of *xylZ* encoded in the TOL plasmid pWW0, has an amino acid sequence very similar to that of XylA. Furthermore, we found that the product of *benC*, a component of benzoate 1,2-dioxygenase from *Acinetobacter calcoaceticus* isofunctional to XylZ, resembles XylA (16a). The N-terminal and C-terminal portions of XylZ and BenC also exhibited sequence similarity with chloroplast-type ferredoxins and ferredoxin-$NADP^+$ reductase, respectively. These observations further support the hypothesis that XylA has a structure equivalent to the fusion of a chloroplast-type ferredoxin and a ferredoxin-$NADP^+$ reductase.

ACKNOWLEDGMENTS

We thank A. Bairoch, C. Hartnett, E. Neidle, and L. N. Ornston for useful discussions. We also thank F. Rey for expert secretarial assistance.

This work was supported by a grant from the Swiss National Science Foundation to S.H.

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