2-Oxo-1,2-Dihydroquinoline 8-Monoxygenase: Phylogenetic Relationship to Other Multicomponent Nonheme Iron Oxygenases

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Received 21 January 1997/Accepted 13 March 1997

2-Oxo-1,2-dihydroquinoline 8-monoxygenase, an enzyme involved in quinoline degradation by Pseudomonas putida 86, had been identified as a class IB two-component nonheme iron oxygenase based on its biochemical and biophysical properties (B. Rosche, B. Tshisuaka, S. Fetzner, and F. Lingens, J. Biol. Chem. 270:17836-17842, 1995). The genes oxoR and oxoO, encoding the reductase and the oxygenase components of the enzyme, were sequenced and analyzed. oxoR was located approximately 15 kb downstream of oxoO. Expression of both genes was detected in a recombinant Pseudomonas strain. In the deduced amino acid sequence of the NADH (acceptor) reductase component (OxoR, 342 amino acids), putative binding sites for a chloroplast-type [2Fe-2S] center, for flavin adenine dinucleotide, and for NAD were identified. The arrangement of these cofactor binding sites is conserved in all known class IB reductases. A dendrogram of reductases confirmed the similarity of OxoR to other class IB reductases. The oxygenase component (OxoO, 446 amino acids) harbors the conserved amino acid motifs proposed to bind the Rieske-type [2Fe-2S] cluster and the mononuclear iron. In contrast to known class IB oxygenase components, which are composed of differing subunits, OxoO is a homomultimer, which is typical for class IA oxygenases. Sequence comparison of oxygenases indeed revealed that OxoO is more related to class IA than to class IB oxygenases. Thus, 2-oxo-1,2-dihydroquinoline 8-monoxygenase consists of a class IB-like reductase and a class IA-like oxygenase. These results support the hypothesis that multicomponent enzymes may be composed of modular elements having different phylogenetic origins.

Multicomponent oxygenases play an important role in the bacterial degradation of aromatic compounds. 2-Oxo-1,2-dihydroquinoline 8-monoxygenase catalyzes the second step of quinoline degradation by Pseudomonas putida 86: in a NADH-dependent oxygenation, 2-oxo-1,2-dihydroquinoline is converted to 8-hydroxy-2-oxo-1,2-dihydroquinoline (Fig. 1). As illustrated in Fig. 1, this enzyme system consists of two soluble protein components with four redox active centers, which constitute an electron transfer chain. Electrons are transferred from NADH via flavin adenine dinucleotide (FAD) and a chloroplast-type [2Fe-2S] cluster, which are located on the reductase component, to the substrate hydroxylating oxygenase component, which harbors Rieske-type [2Fe-2S] clusters and additional iron (26, 27).

Based on the number of its protein components and on its set of cofactors, the enzyme system belongs to the class IB multicomponent Rieske center nonheme iron oxygenases as defined by Batie et al. (1). However, it differs from known class IB enzymes, since the oxygenase component is a homomultimer and thus resembles class IA oxygenase components (27). This unusual property prompted us to investigate the phylogenetic relationship of multicomponent oxygenases.

Here we report the localization, expression, and comparative sequence analysis of the oxoO and oxoR genes, encoding the oxygenase and reductase components of 2-oxo-1,2-dihydroquinoline 8-monoxygenase from P. putida 86. Putative cofactor binding domains are located, and based on sequence alignments, the phylogenetic relationship of multicomponent oxygenases is discussed.

MATERIALS AND METHODS

Bacterial strains and plasmids. P. putida 86 had been isolated from soil by selective enrichment on quinoline as the carbon source (29). The recombinant strain P. putida mT-2 KT2440 (13/42) harbors the cosmids pCB119 with a 30-kb insertion of genomic DNA of P. putida 86 and has been described previously (2). Fragments of this insert were cloned in the vector plasmid pUC18 (23, 37) with Escherichia coli TGI1 (8) as the host strain. The plasmid pCB119 is a double cosmid and was a kind gift of Stephen T. Lam (Ciba-Geigy, Research Triangle Park, N.C.).

Media and growth conditions. For the preparation of plasmid DNA, overnight cultures in Luria-Bertani medium (28) with tetracycline, 50 μg/ml (for pCB119), or ampicillin, 100 μg/ml (for pUC18), were used. Cotranslational insertion of 2-oxo-1,2-dihydroquinoline was investigated by using mineral salt medium (7) with 2-gluteral succinate and 40-mg/liter 2-oxo-1,2-dihydroquinoline. Pseudomonas strains were grown at 30°C, and E. coli strains were grown at 37°C.

DNA techniques. Plasmid DNA was prepared by alkaline lysis (28) or by using the Midi kit (Qiagen, Inc., Chatsworth, Calif.). DNA fragments were isolated from agarose gels according to the instruction manual for the Qiaex II gel extraction kit (Qiagen). Restriction digestions, dephosphorylation of DNA fragments, and DNA ligation were performed as described by the enzyme suppliers (Eurugenetic, Seraing, Belgium; U.S. Biochemical Corp., Bad Homburg, Germany; and Pharmacia Biotech Inc., Freiburg, Germany). Transformation of E. coli TGI1 with recombinant pUC18 DNA was carried out by using CaCl2 (15). Cloned gene DNA was identified by DNA restriction of recombinant pCB119 or pUC18, agarose gel electrophoresis, Southern blotting, and hybridization with digoxigenin-labeled DNA probes by standard methods (28). Mixed digoxigenin-labeled oligonucleotides were synthesized on the basis of the N-terminal amino acid sequences of the two components of 2-oxo-1,2-dihydroquinoline 8-monoxygenase as determined by Edman degradation. The 23-mer ATG AA(A/G) GA(A/T) CA(A/G) ATG CA(A/T) CA(A/G) GT, designated “Red,” and the 17-mer ATG AA(A/T) CA(A/G) CCT AT(C/T) AT(C/T) CG, designated “Ox,” were used as probes for detection of the reductase and the oxygenase gene, respectively. The hybridization temperatures were 51°C for Red and 43°C for Ox. Stringent washes were performed at the same temperatures with 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate (SDS). For gene mapping of the recombinant cosmid, further DNA probes, fl.
Expression of recombinant 2-oxo-1,2-dihydroquinoline 8-monoxygenase. The recombinant clone *P. putida* mt-2 KT2440 (13/42), harboring the genes for 2-oxo-1,2-dihydroquinoline 8-monoxygenase on pCIB119, was tested for cometabolic conversion of 2-oxo-1,2-dihydroquinoline by clone 13/42 and the activities of both enzyme components of 2-oxo-1,2-dihydroquinoline 8-monoxygenase in crude extracts of clone 13/42 were comparable to those obtained with *P. putida* 86. Since neither substrate transformation nor reductase or oxygenase activity was detectable with the DNA recipient *P. putida* mt-2 KT2440, the recombinant cosmid DNA contained the genes 2oxoO and 2oxoR, encoding the functional oxygenase and reductase components of 2-oxo-1,2-dihydroquinoline 8-monoxygenase.

**Localization and cloning of the genes 2oxoO and 2oxoR.** Restriction fragments of the recombinant cosmid that hybridized with both of the oligonucleotide probes Ox and Red, which were deduced from the N termini of the oxygenase and the reductase of 2-oxo-1,2-dihydroquinoline 8-monoxygenase, were larger than approximately 20 kb. Thus, the 3.2-kb EcoRI fragment hybridizing with Ox and the 3.7-kb XmaI fragment hybridizing with Red were selected to subclone 2oxoO and 2oxoR separately in *E. coli* TG1 with pUC18 as a vector.

Restriction digestion of the recombinant cosmid with BglII and NsiI followed by hybridizations with the digoxigenin-labeled DNA probes f1, f2, f3, and Red resulted in the gene map shown in Fig. 2. 2oxoR is localized approximately 15 kb downstream of 2oxoO. The genes for quinoline 2-oxidoreductase are situated in between.

**Nucleotide sequences of 2oxoO, 2oxoR, and flanking regions.** The nucleotide sequences of 2oxoO and 2oxoR and the deduced amino acid sequences are shown in Fig. 3. Both translational ATG start codons were preceded by a putative ribosome binding sequence, 5'-GGAG-3'. The N-terminal amino acid sequences of purified oxygenase and reductase as determined by Edman degradation totally matched the corresponding amino acid sequences derived from the nucleotide sequences. 2oxoO encodes a protein of 446 amino acids. Its calculated molecular mass is 51.2 kDa, which corresponds with the molecular mass of 55 kDa determined by SDS-polyacrylamide gel electrophoresis (PAGE) (27). The 2oxoR product is 342 amino acids in length, and its deduced molecular mass of 37 kDa agrees with the molecular mass of 38 kDa estimated by SDS-PAGE. The G+C contents of 2oxoO and 2oxoR are 61.6 and 63.2%, respectively, and match the G+C content of 62.5% reported for the genome of *P. putida* biowar A (24). Codon usage in the 2oxoO and 2oxoR genes showed preferential usage of G and C in the third position.

The nucleotide sequences of flanking regions of 2oxoO and 2oxoR were analyzed for further gene regions. The N-terminal-region-encoding part (coding for 272 amino acid residues) of a putative open reading frame was identified 53 nucleotides from the first enzyme of the quinoline degradation pathway in *P. putida* 86 (2). In this study, the expression of 2-oxo-1,2-dihydroquinoline 8-monoxygenase, the second enzyme in this degradation pathway, was investigated, using the same recombinant strain. The rate of cometabolic transformation of 2-oxo-1,2-dihydroquinoline by clone 13/42 and the activities of both enzyme components of 2-oxo-1,2-dihydroquinoline 8-monoxygenase in crude extracts of clone 13/42 were comparable to those obtained with *P. putida* 86. Since neither substrate transformation nor reductase or oxygenase activity was detectable with the DNA recipient *P. putida* mt-2 KT2440, the recombinant cosmid DNA contained the genes 2oxoO and 2oxoR, encoding the functional oxygenase and reductase components of 2-oxo-1,2-dihydroquinoline 8-monoxygenase.

**RESULTS**

Expression of recombinant 2-oxo-1,2-dihydroquinoline 8-monoxygenase. The recombinant clone *P. putida* mt-2 KT2440 (13/42), harboring the cosmid vector pCIB119 with a 30-kb insertion of genomic DNA of *P. putida* 86, was reported to carry and express the genes for quinoline 2-oxidoreductase, the first enzyme of the quinoline degradation pathway in *P. putida* 86 (2). In this study, the expression of 2-oxo-1,2-dihydroquinoline 8-monoxygenase, the second enzyme in this degradation pathway, was investigated, using the same recombinant strain. The rate of cometabolic transformation of 2-oxo-1,2-dihydroquinoline by clone 13/42 and the activities of both enzyme components of 2-oxo-1,2-dihydroquinoline 8-monoxygenase in crude extracts of clone 13/42 were comparable to those obtained with *P. putida* 86. Since neither substrate transformation nor reductase or oxygenase activity was detectable with the DNA recipient *P. putida* mt-2 KT2440, the recombinant cosmid DNA contained the genes 2oxoO and 2oxoR, encoding the functional oxygenase and reductase components of 2-oxo-1,2-dihydroquinoline 8-monoxygenase.

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**FIG. 2.** Partial restriction map for the cloned 2oxoO and 2oxoR regions. 2oxoMSL encodes the medium, small, and large subunits of quinoline 2-oxidoreductase (2). f1, f2, f3, ox, and red are the DNA probes used for the hybridization experiments (bars show locations but are not to scale). Restriction sites: B, BglII; E, EcoRI; H, HindIII; N, NsiI; S, SphI; X, XmaI. The hatched areas represent the regions that have been sequenced. The arrows indicate the positions and the direction of transcription of the genes 2oxoO, 2oxoR, 2oxoMSL, 2oxoMSL, and 2oxoR.

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downstream of the stop codon of oxoO. Comparison with DNA and protein databases revealed homologies between the encoded protein (amino acid identity of about 30%) and a number of hydrolases, which are involved in the microbial degradation of aromatic compounds, catalyzing the hydrolysis of meta cleavage products. 2,6-Dioxo-6-phenylhexa-3-enoate hydrolase (encoded by bphD [12]) and 2-hydroxymuconic semialdehyde hydrolase (encoded by dmpD [22]), both from P. putida, were among the top matches.

Upstream of oxoO, part of another potential open reading frame, which ended 242 nucleotides in front of the oxoO start codon, was identified. The deduced amino acid sequence (244 residues) showed similarity to transcription-regulatory (DNA-binding) proteins encoded by the araC-xylS family (25). For instance, an amino acid identity of 32% was calculated for the protein ThcR, which is assumed to regulate the degradation of thiocarbamates in a Rhodococcus sp. (17).

The flanking DNA sequences 0.5 kb upstream and 2 kb downstream of oxoR showed no relevant similarity to any sequences available in DNA databases.

**DISCUSSION**

The structural genes for 2-oxo-1,2-dihydroxyquinoline 8-monoxygenase, a two-component nonheme iron oxygenase system involved in quinoline degradation by P. putida 86, were sequenced and analyzed. From the following observations we concluded that oxoO and oxoR code for its oxygenase and reductase components: (i) The N-terminal amino acid sequences deduced from the nucleotide sequences of oxoO and oxoR were identical to the N-terminal sequences of the enzyme components as determined by Edman degradation; (ii) The predicted molecular masses of oxoO and oxoR corresponded to the molecular masses of the enzyme components estimated by SDS-PAGE (27); and (iii) Functional expression of both enzyme components was detected as in vivo and in vitro activity in a recombinant clone of P. putida mt-2 KT2440 that harbors both oxoO and oxoR from P. putida 86.

Multicomponent Rieske center nonheme iron mono- and dioxygenases are known as enzymes that participate in bacterial degradation of aromatic compounds. They consist of two or three soluble proteins that constitute an electron transport chain, transferring electrons from NAD(P)H via flavin and [2Fe-2S] centers to a non-heme-bound mononuclear iron as the site of dioxygen activation (1, 16). The substrate-hydroxylating terminal oxygenases always harbor Rieske-type [2Fe-2S] centers and mononuclear iron. Conserved amino acid motifs that are supposed to coordinate these cofactors (1, 13, 16, 14) were identified in the oxygenase component of 2-oxo-1,2-dihydroxyquinoline 8-monoxygenase and are indicated in Fig. 3.
Class I reductase components contain flavin (flavin mononucleotide in class IA and FAD in class IB) and a chloroplast-type [2Fe-2S] center. In class II enzymes, the flavin (always FAD) and the [2Fe-2S] center (IIA, chloroplast type; IIB, Rieske type) are located on separate components. Class III systems harbor FAD and chloroplast-type [2Fe-2S] in the reductase as well as Rieske-type center in an additional component. This biochemical classification is supposed to have a strong evolutionary basis (10, 18, 19).

In order to investigate the evolutionary relationship between reductase and oxygenase components from different classes, dendrograms of amino acid sequences had been established (18, 38). As shown in Fig. 4A, the oxygenase components of each distinct class form a separate branch, thus confirming an evolutionary basis for their biochemical grouping. However, although 2-oxo-1,2-dihydroquinoline 8-monoxygenase was classified as a class IB system (27), OxoO shows only distant relatedness to class IB oxygenase sequences. Surprisingly, it turned out to be most related to class IA oxygenases, which form a separate branch. The assignment to class IA is supported by the previous observation that the native oxygenase component of 2-oxo-1,2-dihydroquinoline 8-monoxygenase, like all known class IA oxygenases (26, 27), is a homomultimer, whereas class IB oxygenases are composed of differing subunits.

On the other hand the dendrogram of reductases (Fig. 4B) revealed that the amino acid sequence of OxoR is most similar to the sequences of a branch of class IB reductases and there is a far distance to IA reductases, which again belong to a distinct group. The dendrogram supports the evolutionary divergence between group IA and IB reductases. In order to develop a more comprehensive understanding of the phylogenetic relationship of multicomponent oxygenases, the dendrograms of the sequences of DmpP, MmoC, and XylA are also shown in Fig. 4. Obviously, these reductases are related to class IB and class III reductases. In spite of this sequence similarity, these multicomponent oxygenase systems do not belong to any of the classes as defined by Batie et al. (1), because their oxygenase components do not contain a Rieske center.

The evolutionary divergence between group IA and IB reductases is also illustrated by the different arrangements of their cofactor binding sites (Fig. 5): the amino acid sequence for OxoO was aligned with the class IB reductase sequences of BenC (19), XylZ (11), and CbaC (9). The overall amino acid identity of the sequences was 19.5%. OxoO and XylZ appeared most similar, with 32.6% amino acid identity. In the N-terminal regions of the reductases, the conserved amino acid motif CXXCXXCC_24–34C indicates the presence of a chloroplast-type [2Fe-2S] cluster (16, 19). Downstream, sequence motifs homologous to known FAD- and NAD-binding domains (5, 18, 19) were found. The arrangement of the chloroplast-type [2Fe-2S], flavin-, and NAD-binding sites (Fig. 5B) is conserved in class IB and class III (5) and is even found in the reductase component MnoC of methane monoxygenase from Methylococcus capsulatus (31), which shows 32.5% amino acid identity with OxoR. As indicated in Fig. 5B, the arrangement of the cofactor binding sites is changed in class IA reductases. This fact has been attributed to an evolutionary divergence by alternative fusions of the distinct modular domains (5, 18).

Thus, 2-oxo-1,2-dihydroquinoline 8-monoxygenase consists of a class IA-like oxygenase and a class IB-like reductase. Consequently, the biochemical classification of the enzyme system as a whole (class IB) does not correspond to the genetic relationships of the individual components. The unusually far
distance (15 kb) between the genes ooxO and ooxR also may hint at independent origins of the genes. An example of independently organized component genes was reported by Chang and Zylstra (4), who found that the reductase and oxygenase
genes of phthalate dioxygenase from *Pseudomonas cepacia* DBO1 are located on separate operons. All these findings indicate that modular protein elements have been combined during evolution. Whereas many mutations may be necessary for the development of a novel catalytic specificity, the genetic rearrangement and functional combination of catalytically active protein modules may open up novel metabolic pathways in a few steps.

ACKNOWLEDGMENTS

We thank Birte Kruckwett for assistance. This work was supported by the Fonds der Chemischen Industrie.

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