Biochemical and Molecular Characterization of 1-Hydroxy-2-naphthoate Dioxygenase from Nocardoides sp. KP7*

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1-Hydroxy-2-naphthoate dioxygenase, which cleaves the singly hydroxylated aromatic ring, was purified from phenanthrene-degrading Nocardoides sp. strain KP7. The purified enzyme had a molecular mass of 45 kDa by SDS-polyacrylamide gel electrophoresis and 270 kDa by gel filtration chromatography. The apparent $K_m$ and $k_{cat}$ values of this enzyme for 1-hydroxy-2-naphthoate were 10 μM and 114 s⁻¹, respectively. One mole of molecular oxygen was consumed when 1 mol of 1-hydroxy-2-naphthoate was oxidized. This enzyme contained 1 mol of Fe(II)/mol of the subunit and was inactivated by o-phenanthroline. The enzyme that had been inactivated by o-phenanthroline was reactivated by incubating with FeSO₄ and ascorbic acid. Thus, Fe(II) was required for the enzyme to exhibit activity. The structural gene for this enzyme was screened from a cosmid library and then sequenced, the length of the 1-hydroxy-2-naphthoate gene being 1161 base pairs. The deduced amino acid sequence of this enzyme was different from those of other ring-cleaving dioxygenases that cleave the doubly hydroxylated aromatic ring.

Dioxygenases are a group of enzymes that catalyze the incorporation of two atoms of molecular oxygen into a substrate (1). These enzymes play an important role in the aerobic decomposition of aromatic compounds in bacteria. The aromatic ring of the substrate is generally hydroxylated by either a ring-hydroxylating dioxygenase or a ring-hydroxylating monooxygenase, and the intermediate possessing two hydroxyl groups is subsequently cleaved by a ring-cleaving dioxygenase (2, 3).

Dioxygenases cleaving an aromatic ring that possesses two adjacent hydroxyl groups are divided into two classes. One class of enzymes, the extradiol type, cleaves the ring at a bond proximal to one of the two hydroxylated carbon atoms. The other class of enzymes, the intradiol type, cleaves the aromatic ring between two hydroxylated carbon atoms (1). In the case of gentisate containing two hydroxyl groups at a position para to each other, the ring is cleaved between carbon 1 and carbon 2 by gentisate 1,2-dioxygenase, and the ring-fission mechanism is believed to resemble that of extradiol-type fission (4, 5).

Extradiol dioxygenases and gentisate 1,2-dioxygenase contain non-heme ferrous iron, whereas intradiol dioxygenases contain non-heme ferric iron (6). Non-heme iron is essential for the activity of these ring-cleaving dioxygenases.

In bacterial pathways for the degradation of phenanthrene via o-phenathlate, the initial substrate is transformed to 1-hydroxy-2-naphthoate, which is further oxidized by the ring cleavage enzyme, 1-hydroxy-2-naphthoate dioxygenase (Fig. 1) (7–10). The ring cleavage of singly hydroxylated 1-hydroxy-2-naphthoate is distinctive from other ring cleavage reactions in which the substrate always contains two hydroxyl groups. Despite its biochemically interesting properties, only a preliminary characterization of 1-hydroxynaphthoate dioxygenase has been presented (8, 10).

In this study, we purified and characterized 1-hydroxy-2-naphthoate dioxygenase from Nocardoides sp. strain KP7 and cloned and sequenced the structural gene for this enzyme.

EXPERIMENTAL PROCEDURES

Bacterial Strain—Nocardoides sp. strain KP7, which degrades phenanthrene via o-phenathlate (11), was used in this study.

Enzyme Assay—The activity of 1-hydroxy-2-naphthoate dioxygenase under standard conditions was spectrophotometrically measured at 300 nm in 50 mM Tris-HCl (pH 7.5) containing 0.1 mM 1-hydroxy-2-naphthoate at 25 °C. The absorbance of 1-hydroxy-2-naphthoate was a maximum at 340 nm, and the transformation of this substrate to trans-2-carboxybenzalpyruvate resulted in the appearance of another peak at 300 nm, as has been reported by Kiyohara and Nagao (8) and Barnsley (10). The difference between the extinction coefficient of trans-2-carboxybenzalpyruvate (the ring cleavage product of 1-hydroxy-2-naphthoate) and that of 1-hydroxy-2-naphthoate at 300 nm was determined to be 11.5 μM⁻¹ cm⁻¹. The specific activity of this enzyme is expressed as micromoles of trans-2-carboxybenzalpyruvate formed per min/mg of protein. The Michaelis-Menten kinetic parameters of this enzyme for 1-hydroxy-2-naphthoate were obtained by measuring the initial velocities that were determined under these conditions, except that various concentrations of the substrate were used. The inhibition of 1-hydroxy-2-naphthoate dioxygenase by substrate analogues was analyzed similarly in the presence of the analogues at concentrations of 1–1000 μM.

Purification of 1-Hydroxy-2-naphthoate Dioxygenase—1-Hydroxy-2-naphthoate dioxygenase was purified from extracts of strain KP7. Cells were grown for 48 h at 30 °C in 10 liters of marine broth (Difco) containing 0.1% (w/v) phenanthrene. Cell extracts were prepared as described previously (12) and loaded into an anion-exchange column (TSKgel DEAE-5PW, 21.5 × 150 mm; Tosoh, Tokyo, Japan) fitted to a high-performance liquid chromatography system (Tosoh). The protein was eluted by a linear gradient of 0–0.5 M Na₂SO₄ in 300 ml of a 20 mM Tris-H₂SO₄ buffer (pH 7.5) at a flow rate of 5 ml/min. The eluate was collected in 5-ml fractions on ice. 1-Hydroxy-2-naphthoate dioxygenase was eluted at a salt concentration of 0.1 M. Pooled fractions containing the 1-hydroxy-2-naphthoate dioxygenase activity were adjusted to 14.6% saturation of ammonium sulfate at 4 °C, and the proteins that had been precipitated were removed by centrifugation at 27,700 × g for 30 min at 4 °C. The 1-hydroxy-2-naphthoate dioxygenase activity was recovered in the supernatant fluid. This supernatant was passed through a Millex-GV filter (0.45 μm pore size; Millipore) and loaded into a hydrophobic interaction column (TSKgel phenyl-5PW, 21.5 × 150 mm; Tosoh) that had been pre-equilibrated with a 20 mM Tris-H₂SO₄ buffer (pH 7.5) containing 0.6 M ammonium sulfate. Proteins were eluted from the column by a linear gradient from 0.6 to 0 M ammonium sulfate in 60 ml of the 20 mM Tris-H₂SO₄ buffer (pH 7.5) at a flow rate for 30 ml/min.

* This work was supported by the New Energy and Industrial Technology Development Organization. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB000735.

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of 1 ml min⁻¹. Pooled fractions containing active 1-hydroxy-2-naphthoate dioxygenase were concentrated to 80 µl in a 1.5-ml centrifuge tube fitted with an Ultrafree C3-LGC membrane (Millipore), loaded into a gel filtration column (TSKgel G3000SWXL, 7.8 x 300 mm; Tosoh), and eluted with a mobile phase of 20 mM Tris-H₂SO₄ (pH 6.8) containing 100 mM Na₂SO₄ at a flow rate of 0.5 ml min⁻¹. The absorbance of the protein effluent was monitored at 280 nm.

Measurement of Oxygen Uptake—The oxygen uptake catalyzed by 1-hydroxy-2-naphthoate dioxygenase was measured at 25 °C by an oxygen electrode (type 5/6 Oxygraph; Gilson, Villiers-le-Biel, France). Five µl of 10 mM 1-hydroxy-2-naphthoate was added to a cuvette containing 1.5 ml of 50 mM Tris-HCl (pH 7.5), and 36 µl of the enzyme solution (49 pmol) was subsequently added to the cuvette. The oxygen consumption was then measured, the initial oxygen concentration in the buffer being assumed to be 250 µM.

Determination of Iron Content—The iron content of 1-hydroxy-2-naphthoate dioxygenase was determined by using o-phenanthroline in the presence of 0.1 mM ascorbate (14).

Amino-terminal Sequencing—The amino-terminal sequence of the purified enzyme was determined by Edman degradation with an automated protein sequencer (Perkin-Elmer model 477).

Gene Cloning and Sequencing—Two degenerate PCR primers (the N primer and C primer), which could collaboratively amplify a 75-bp-long DNA fragment encoding the amino-terminal 25 amino acid residues of 1-hydroxy-2-naphthoate dioxygenase, were designed. Their sequences were 5'-AC/TGAG/GC/TGCAG/AAGG/AGTC/GC/TCAG/GCG/CC'-3' for the N primer, and 5'-CAT/TGAG/AGG/AGTG/TGC/TGC/TGCAG/GCG/AG/ATCCA/3' for the C primer. Cloning of the amplified PCR product was carried out by using a PCR-Script™ SK(+)+ cloning kit (Stratagene Cloning Systems, La Jolla, CA), and the nucleotide sequence of the cloned PCR product was determined by using a Taq DyeDeoxy terminator cycle sequencing kit and a 373A DNA sequencer (Perkin-Elmer).

Purification and Characterization of 1-Hydroxy-2-naphthoate Dioxygenase—1-Hydroxy-2-naphthoate dioxygenase was induced by phenanthrene in strain KP7; the specific activity of the enzyme in an extract of strain KP7 was 8.4 µmol min⁻¹ mg⁻¹ of protein after growth on marine broth containing 0.1% (w/v) phenanthrene, whereas it was 1.0 µmol min⁻¹ mg⁻¹ of protein after growth on marine broth without phenanthrene. This enzyme was purified from extracts of strain KP7 grown on phenanthrene as summarized in Table I. The enzyme activity was eluted from a DEAE column at a Na₂SO₄ concentration of 0.14 M, and separation of the pooled DEAE fractions by a phenyl column eluted the enzyme activity at an ammonium sulfate concentration of 0.27 M. 1-Hydroxy-2-naphthoate dioxygenase was further separated by gel filtration chromatography, the purified sample giving a single protein band by SDS-polyacrylamide gel electrophoresis (Fig. 2). The molecular mass of 1-hydroxy-2-naphthoate dioxygenase evaluated by gel filtration chromatography, SDS-polyacrylamide gel electrophoresis, and purified sample giving a single protein band by SDS-polyacrylamide gel electrophoresis was 45 kDa (Fig. 2). The homohexamer of the 45-kDa subunit constituted an active enzyme.

The oxygenation activity of this enzyme was polarographically measured at 25 °C; when 50 nmol of 1-hydroxy-2-naphthoate was added, 54 nmol of molecular oxygen was estimated to be consumed. It was thus concluded that 1 mol of the oxygen molecule was incorporated when 1 mol of 1-hydroxy-2-naphthoate was transformed.

The apparent Km and kcat values of purified 1-hydroxy-2-naphthoate dioxygenase for 1-hydroxy-2-naphthoate measured at 25 °C were 10 µM and 114 s⁻¹, respectively. The optimum pH of this enzyme was 7.5 when the activity was measured in a 0.1 M potassium phosphate buffer (pH 6–8), 0.1 M Tris-HCl buffer (pH 7–9), or 0.1 M glycine-NaOH buffer (pH 8.5–10.5) at 25 °C. The optimum temperature was 40 °C when the enzyme activity was measured in the 0.1 M Tris-HCl buffer (pH 7.5) containing 1 mM 1-hydroxy-2-naphthoate.

The oxidation of eight other compounds (gentisate, 3-hydroxyxanthanilate, 2-hydroxy-1-naphthoate, 3-hydroxy-2-naphthoate, salicylate, m-hydroxybenzoate, p-hydroxybenzoate, and protocatechuic acid) by this enzyme was spectrophotometrically examined at substrate concentrations of 0.1 and 1 mM in a 50 mM potassium phosphate buffer (pH 7.5) at 25 °C. None of them were transformed by this enzyme. When the inhibition of 1-hy-
1-Hydroxy-2-naphthoate Dioxygenase activity by each of these compounds was examined, only 3-hydroxy-2-naphthoate was found to be a competitive inhibitor with a $K_i$ value of 22 $\mu$M. None of the other compounds showed any inhibitory effect when the concentration of the substrate (1-hydroxy-2-naphthoate) was 10 $\mu$M and that of the tested compounds was 0.1 or 1 mM.

Effects of Chelators and Metals on the Activity of 1-Hydroxy-2-naphthoate Dioxygenase—The effect of various metals and metal chelators on the activity of the purified enzyme was examined (Table II). 1-Hydroxy-2-naphthoate dioxygenase was inactivated by 0.1 mM o-phenanthroline, which is the chelator specific to ferrous iron, but none of the other chelators and metals had any strong influence on the activity of this enzyme. Reactivation of the enzyme that had been inactivated by o-phenanthroline was examined with several metals in the presence of 0.1 mM ascorbate (Table III). Only Fe(II) was effective in reactivating the inactivated 1-hydroxy-2-naphthoate dioxygenase.

Iron Content of Purified 1-Hydroxy-2-naphthoate Dioxygenase—1-Hydroxy-2-naphthoate dioxygenase was found to contain 1.15 mol of iron/mol of the subunit.

Amino-terminal Sequence of 1-Hydroxy-2-naphthoate Dioxygenase and Cloning and Nucleotide Sequence of Its Structural Gene—The 53-residue amino-terminal sequence of purified 1-hydroxy-2-naphthoate dioxygenase was determined by automated Edman degradation to be Asn-Ser-Ser-Asn-Thr-Gly-Ala-Val-Leu-Glu-Ala-Glu-Val-Leu-Glu-Ala-Phe-Asp-Arg-Arg-Ala-Glu-Gln-Tyr-Leu-Ary-Gly-Gln-Trp-Ile-Ala-Glu-Glu-His-Leu-Met-Arg-Ala-Ile-Gly-Gly-Pro-Arg-Pro-Pro-Glu-Ile-Pro-Tyr-Trp-Glu. Based on this amino acid sequence, two degenerate PCR primers (the N primer and cC primer) that would allow amplification of the 75-bp-long product were designed as described under “Experimental Procedures.” The primer set was used to amplify a PCR product corresponding to the size when total DNA from strain KP7 was used as a template. The 75-bp-long PCR product was cloned in the pCR-ScriptTM SK(+) plasmid, and the nucleotide sequence of this PCR product was determined by cycle sequencing. The amino acid sequence deduced from the DNA sequence of this PCR product was in agreement with the amino-terminal sequence of 1-hydroxy-2-naphthoate dioxygenase. This result indicates that the amplified product corresponded to the partial sequence of the 1-hydroxy-2-naphthoate dioxygenase gene.

The pLAFR3-based cosmids library of strain KP7 was constructed, and colonies of the library were screened by the ECL-labeled PCR product that had been amplified from the 75-bp-long fragment. Restriction maps of the seven positive clones were constructed (Fig. 3). All positive clones were found to contain three BamHI fragments of 1.0, 1.8, and 6.2 kb in size. The N and cC primers amplified the 75-bp-long fragment only when the 6.2-kb BamHI fragment was used as a template. This result indicates that the 6.2-kb BamHI fragment encoded the amino-terminal region of 1-hydroxy-2-naphthoate dioxygenase. The 6.2-kb BamHI fragment was then subcloned into the BamHI site of pACYC184 to construct pMK191.

Four PCR primers were used to localize the 75-bp region on the 6.2-kb BamHI fragment. Two of these were the primers used for amplifying the 75-bp fragment (the N primer and cC primer), whereas the other two were complementary to the...
FIG. 3. Restriction maps of the seven positive clones and mapping strategy of the 1-hydroxy-2-naphthoate dioxygenase gene. The solid line indicates the restriction sites (Bgl, BglII; BmA, BamHI; M, MluI; H, HindIII; N, NotI; Sp, SpI; St, StuI; and V, EcoRV). Open boxes indicate DNA inserts of positive clones, and arrows indicate the PCR primers used. Combination of the V1 and cC primers amplified a 3.6-kb-long fragment, whereas combination of the V2 and N primers amplified a 2.6-kb-long fragment. Thus the 5'-end of the 1-hydroxy-2-naphthoate gene was located 3.6 kb left from the left BamHI site of pMKT191.

FIG. 4. Nucleotide sequence of the 1-hydroxy-2-naphthoate dioxygenase gene. The deduced amino acid sequence is also presented, and the putative Shine-Dalgarno sequence is underlined.
pACYC184 sequences flanking the BamHI cloning site (the V1 and V2 primers; Fig. 3). Either the N primer or cC primer was used first, and either the V1 or V2 primer was used second. Long fragment PCR amplification, using a combination of the first and second primers, was then carried out. One of these combinations (the V1 and cC primers) amplified a 3.6-kb-long fragment, whereas an other combination (the V2 and N primers) amplified a 2.6-kb-long fragment. Thus, the location and orientation of the 5′-region of the 1-hydroxy-2-naphthoate dioxygenase gene was determined on a map of the 6.2-kb BamHI fragment (Fig. 3).

Nucleotide sequencing of the 1-hydroxy-2-naphthoate dioxygenase gene (phdI; phd stands for phenanthrene degradation) was initiated by using a primer complementary to the 75-bp fragment and further extended by using primers designed from the determined sequence. The length of the 1-hydroxy-2-naphthoate dioxygenase gene was 1161 bp, and the deduced amino acid sequence of the enzyme was 387 amino acids long (Fig. 4). The deduced amino acid sequence in the amino-terminal region showed a perfect match with the sequence determined by the Edman degradation. The deduced molecular mass (43 kDa) from the amino acid sequence of this enzyme was in agreement with the size evaluated by SDS-polyacrylamide gel electrophoresis (45 kDa). The amino acid sequence of this enzyme has no significant similarity to any other dioxygenases or to other reported proteins in protein data bases.

Expression of 1-Hydroxy-2-naphthoate Dioxygenase in Escherichia coli—The 2.8-kb StuI-BamHI-digested fragment from pMKT191 (Fig. 3) was subcloned into pUC18 to construct pMKT280. The SpI-BamHI fragment of pMKT280 downstream of the phdI gene was removed by digesting the pMKT280 plasmid by SpI and BamHI and ligating the digested DNA after the Klenow treatment. The pMKT290 plasmid thus constructed was used to transform E. coli JM109, and a cell extract prepared from this transformant contained the 1-hydroxy-2-naphthoate dioxygenase activity of 20 μmol min⁻¹ mg of protein⁻¹. Thus it was confirmed that the 1161-bp-long sequence encoded 1-hydroxy-2-naphthoate dioxygenase.

DISCUSSION

Substrates of ring-cleaving dioxygenases generally carry two hydroxyl groups at a position either ortho or para to each other (3). The biochemical reason for the requirement of two hydroxyl groups for ring cleavage is not yet understood. A model for the mechanism of extradiol dioxygenases has recently been proposed, and it was suggested that two hydroxyl groups of the substrate would interact with the catalytic iron, deprotonation of one of these hydroxyl groups being the first step of ring cleavage (16). 1-Hydroxy-2-naphthoate dioxygenase is unique among ring-cleaving dioxygenases because it can cleave a singly hydroxylated aromatic ring.

3-Hydroxy-2-naphthoate was a competitive inhibitor of 1-hydroxy-2-naphthoate dioxygenase, whereas 2-hydroxy-1-naphthoate and salicylate exhibited no effect on the enzyme activity. Comparing the structures of these compounds, a fused ring structure and the position of the carboxyl group seem to be important for binding to the substrate-binding pocket of this enzyme.

The previously characterized ring-cleaving dioxygenases required Fe(II) or Fe(III) for their activities. Fe(II) is required for extradiol-type ring-cleaving dioxygenases and gentisate 1,2-dioxygenase, whereas Fe(III) is required for intradiol-type ring-cleaving dioxygenases (1). In this study, we have demonstrated that 1-hydroxy-2-naphthoate dioxygenase also required Fe(II) for its activity, as has been suggested by Barnsley (10). However, the amino acid sequence of this enzyme was not related to that of any previously characterized ring-cleaving enzyme. 1-Hydroxy-2-naphthoate dioxygenase cleaved the aromatic ring between the carboxylated and hydroxylated carbons of the substrate. Such cleavage has also been observed in the reaction catalyzed by gentisate 1,2-dioxygenase. The subunit size of gentisate 1,2-dioxygenase from Comamonas acidovorans and C. testosteroni was approximately 40 kDa, which is close to that of 1-hydroxy-2-naphthoate dioxygenase (4, 5). Although 1-hydroxy-2-naphthoate dioxygenase was not able to cleave the ring of gentisate, it is still possible that the structure and catalytic mechanism of 1-hydroxy-2-naphthoate dioxygenase are similar to those of gentisate 1,2-dioxygenase. In this respect, it will be of interest to determine the primary structure of gentisate 1,2-dioxygenase.

Acknowledgments—We are grateful to Shigetoh Miyachi for support. We also thank Atsushi Saitoh, Jun Inoue, and Satoshi Odo for valuable discussions and Yukiko Itazawa for technical assistance.

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