Mechanism of 4-Nitrophenol Oxidation in *Rhodococcus* sp. Strain PN1: Characterization of the Two-Component 4-Nitrophenol Hydroxylase and Regulation of Its Expression

Masahiro Takeo, a Masumi Murakami, Sanae Niihara, Kenta Yamamoto, Munehiro Nishimura, Dai-ichiro Kato, and Seiji Negoro

Department of Materials Science and Chemistry, Graduate School of Engineering, University of Hyogo, 2167 Shosha, Himeji, Hyogo 671-2201, Japan.

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4-Nitrophenol (4-NP) is a toxic product of the hydrolysis of organophosphorus pesticides such as parathion in soil. *Rhodococcus* sp. strain PN1 degrades 4-NP via 4-nitrocatechol (4-NC) for use as the sole carbon, nitrogen, and energy source. A 5-kb EcoRI DNA fragment previously cloned from PN1 contained a gene cluster (*nphRA1A2*) involved in 4-NP oxidation. From sequence analysis, this gene cluster is expected to encode an AraC/XylS family regulatory protein (*nphR*) and a two-component 4-NP hydroxylase (*NphA1* and *NphA2*). A transcriptional assay in a *Rhodococcus* strain revealed that the transcription of *nphA1* is induced by only 4-NP (of several phenolic compounds tested) in the presence of *nphR*, which is constitutively expressed. Disruption of *nphR* abolished transcriptional activity, suggesting that *nphR* encodes a positive regulatory protein. The two proteins of the 4-NP hydroxylase, *NphA1* and *NphA2*, were independently expressed in *Escherichia coli* and purified by ion-exchange chromatography or affinity chromatography. The purified *NphA2* reduced flavin adenine dinucleotide (FAD) with the concomitant oxidation of NADH, while the purified *NphA1* oxidized 4-NP into 4-NC almost quantitatively in the presence of FAD, NADH, and *NphA2*. This functional analysis, in addition to the sequence analysis, revealed that this enzyme system belongs to the two-component flavin-diffusible monooxygenase family. The 4-NP hydroxylase showed comparable oxidation activities for phenol and 4-chlorophenol to that for 4-NP and weaker activities for 3-NP and 4-NC.

Nitroaromatic compounds are important building blocks for the synthesis of chemical compounds such as dyes, explosives, and herbicides and therefore are used in large quantities in chemical industries. However, they are generally toxic to living organisms and easily bioconverted into more mutagenic and carcinogenic aromatic amines under anaerobic environments (41). One such compound, 4-nitrophenol (4-NP), is known to be very toxic and has often been detected in soil as a hydrolysis product of organophosphorus pesticides, parathion and methylparathion, especially in developing countries. For instance, in India, more than 20 mg kg⁻¹ of 4-NP was detected in soils of tea fields (R. K. Jain, Institute of Microbial Technology, India, personal communication). The U.S. Environmental Protection Agency has listed 4-NP as a priority pollutant (17). Therefore, much attention has been paid to its biodegradability in the environment and its biodegradation has been extensively studied for the last two decades.

4-NP can be degraded aerobically through two different pathways via 4-nitrocatechol (4-NC) or hydroquinone (HQ) (48). In the former pathway, 4-NP is first oxidized into 4-NC, which is further converted into 1,2,4-benzenetriol, followed by the cleavage of the aromatic ring. In the latter pathway, it is first oxidized into HQ, which is subjected to the cleavage of the aromatic ring. Then both resulting ring-cleaved compounds, \(\gamma\)-hydroxymuconic semialdehyde and maleylacetate, are metabolized into tricarboxylic acid cycle intermediates via \(\beta\)-ketoadipate. *Arthrobacter* sp. strain JS443, *Arthrobacter aurescens* TW17, *Pseudomonas* sp., *Rhodococcus* sp., and *Serratia* sp. were reported to employ the 4-NC pathway (11, 15, 28, 37, 38), while *Moraxella* sp., *Nocardia* sp., *Burkholderia cepacia* RKJ200, *Arthrobacter protophormiae* RKJ100, and *Pseudomonas* spp. were reported to use the HQ pathway (5, 11, 30, 40, 48). In these pathways, the initial degradation starts with hydroxylation (monooxygenation) of the 4-NP aromatic ring.

Earlier studies on 4-NP oxidation using cell extracts and partially purified cell fractions indicated that bacteria have monooxygenases capable of oxidizing NPs (25, 39). A membrane preparation of a *Moraxella* sp. oxidized 4-NP into HQ using molecular oxygen; the oxidation was dependent on the presence of NADPH (39), and the addition of flavin adenine dinucleotide (FAD) stimulated the reaction. In a different study, an extract of 4-NP-induced *Nocardia* sp. cells also oxidized 4-NP into 4-NC in the presence of molecular oxygen, NADH, and FAD (25). The enzymes catalyzing 4-NP oxidation, however, have not yet been purified. Later, a two-component 4-NP monooxygenase system, consisting of two proteins, an oxygenase component and a flavoprotein reductase component, was purified from *Bacillus sphaericus* JS905 (16). The reductase component reduced FAD with the concomitant oxidation of NADH, while the oxygenase component catalyzed two sequential oxidations from 4-NP into 1,2,4-benzenetriol through 4-NC using the reduced flavin. Hence, this monooxygenase is a key enzyme in the 4-NC pathway of JS905; however,
at present, the nucleotide and amino acid sequence data remain unpublished.

In our previous studies, we isolated a 4-NP-degrading bacterium, *Rhodococcus* sp. strain PN1, from activated sludge (43). This bacterium can degrade not only mono-NPs, including 4-NP, but also poly-NPs, such as 2,4-dinitrophenol and 2,4,6-trinitrophenol (picric acid) (1). Analyses of the metabolites in NP degradation revealed that PN1 degrades 4-NP through the 4-NC pathway, whereas it also degrades 2,4-dinitrophenol and picric acid via the corresponding hydride-Meisenheimer complexes (1, 13, 44). Thus, PN1 has at least two quite different pathways for NP degradation. The gene clusters involved in 4-NP degradation and picric acid degradation have been independently cloned from PN1 (13, 43). The gene cluster encoding 4-NP oxidation consists of three genes, *nphR*, *nphA1*, and *nphA2*, which were expected from the deduced amino acid sequences to encode an AraC/XylS family regulatory protein (9) and a 4-NP hydroxylase belonging to the two-component flavin-diffusible monooxygenase (TC-FDM) family (8, 43). Later, in a different study, another 4-NP degradation gene cluster (*npd*ABC), consisting of 4-NP monooxygenase genes (*npdA2*) and the subsequent hydroxyquinol 1,2-dioxygenase gene (*npdC*), was cloned from *Rhodococcus opacus* SAO101 and characterized (22). The conversion of 4-NP into maleylacetate via 4-NC was reported using the cell extracts of recombinant *Escherichia coli* including this gene cluster. In addition, a transcriptional assay by reverse transcription-PCR revealed that these three genes were transcribed in a unit and that the transcription was induced by 4-NP. Neither of these gene products has been purified, nor has the detailed regulation mechanism of the 4-NP oxidation been characterized. Very recently, Perry and Zybra cloned a 4-NP monooxygenase gene cluster (*npdA*ABC) from *Arthrobacter* sp. strain JS443 (29) that showed significant homology with the chlorophenol 4-monooxygenase gene cluster of *Arthrobacter chlorophenolicus* A6 (27). In this cluster, a flavin reductase gene (*npdA1*) and a monooxygenase gene (*npdA2*) are separated by a large putative regulatory gene (*npdR*) and transcribed divergently. RT-PCR analysis revealed that *npdA2* and *npdB* (encoding a hydroxyquinol 1,2-dioxygenase) are transcribed when JS443 is grown on 4-NP but not on a rich medium, suggesting that this gene cluster is involved in 4-NP degradation. The histidine-tagged product of *npdA1* showed NADH-dependent FAD reductase activity, while *E. coli* lysates including the *npdA2* gene product are capable of oxidizing 4-NP and a wide range of 4-substituted phenols, indicating that *NpdA1* and *NpdA2* also belong to the TC-FDM family in function as well as in sequence. This report suggested the presence of another novel 4-NP degradation pathway, in which 4-NP is converted into 1,2,4-benzenetriol via 1,4-benzoquinone and hydroxy-1,4-benzoquinone but not via HQ and 4-NC. Although the *npd* gene cluster has the putative regulatory gene, *npdR*, no analysis of the regulatory mechanism of gene expression has been performed.

To understand the mechanism of 4-NP oxidation in PN1 at both genetic and enzymatic levels, we first investigated the regulatory system for the expression of the 4-NP hydroxylase genes, *nphA1A2*. Then we purified each protein of the 4-NP hydroxylase using *Escherichia coli* expression systems and characterized their functions in 4-NP oxidation in vitro. The results showed that the hydroxylase oxidizes 4-NP and some phenolic compounds preferentially but its gene expression is specifically induced by 4-NP in the presence of a putative regulatory gene, *nphR*.

![FIG. 1. Physical map of pKP3 containing the 4-NP hydroxylase gene cluster of *Rhodococcus* sp. strain PN1 and its derivatives. A catechol 2,3-dioxygenase gene (*atdB*) from *Acinetobacter* sp. strain YAA (42, 45) was inserted into the StuI site and the EcoRV site of pKP3 as a reporter gene to construct pKP3N2 and pKP3N3, respectively. For the disruption of *nphR*, a tetracycline resistance gene (tet) from pBR322 (4) was introduced into the EcoRV site of pKP3N2 to construct pKP3N3. These derivatives were used to investigate the transcriptional activity of *nphA1* and *nphR* in *R. rhodochrous* ATCC 12674 in the presence or absence of various inducers.](http://jb.asm.org/content/126/10/7368.full.pdf)
introduced into the StuI site of pKP3 (44) in the same transcriptional direction as nphA1 to construct pKP3N3. Similarly, the amplified fragment was inserted into the EcoRV site of pKP3N in the same transcriptional direction as nphR to construct pKP3N5. Furthermore, to disrupt nphR in pKP3N2, a tetracycline resistance gene, which was amplified in the same way using another primer set, tetnrs50F (5'-TAAGGGTCGTAAGGCTTGAGTA-
AATCTAAC-3') and tetnrs50R (5'-ATGGCAGGTCTAAGGCTTGC-
ATTGAGGTCC-3'), was inserted into the EcoRV site of pKP3N2 to construct pKP3N3. These plasmids were introduced into Rhodococcus hosts by electroporation as described previously (43).

Assay for transcriptional activity. Recombinant R. rhodochrous ATCC 12674 harboring one of the pKP3N series plasmids was incubated in LB medium containing glycine (0.5 g liter⁻¹) and kanamycin for 24 h in the presence or absence of various phenolic compounds (phenol, 2-NP, 3-NP, 4-NP, 2-hydroxyphenylacetate [2-HPA], 3-HPA, 4-HPA, or 4-NC) at 0.3 mM for induction. Then the cells were harvested by centrifugation (7,000 × g for 10 min) and washed twice with 10 mM phosphate buffer (pH 7.0). The cells were resuspended with 5 ml of the same buffer and disrupted by ultrasonication using a TOMY UD-200 cell dismuter (TOMY, Kyoto, Japan) (output 6, 2 min, four times on ice). After centrifugation (24,900 × g for 30 min), the supernatant was used as a cell extract.

Preparation of E. coli cell extracts for protein purification. Recombinant E. coli JM109 harboring pUPNH-A1 or pUPNH-A2 (43) was cultured in the LB medium containing 1 mM isopropyl-β-thiogalactopyranoside (IPTG) and ampicillin at 37°C and 150 rpm on a rotary shaker. IPTG was added at 1 mM after an 8-h incubation, and further cultivation was continued for 16 h. E. coli cells were centrifuged (11,000 × g) at 4°C for 10 min, and washed twice with 10 mM phosphate buffer (pH 7.0). The cells were resuspended with 5 ml of the same buffer and disrupted by ultrasonication using a TOMY UD-200 cell dismuter (TOMY, Kyoto, Japan) (output 6, 2 min, four times on ice). After centrifugation (24,900 × g for 30 min), the supernatant was used as a cell extract.

Enzyme assay. (i) NAD(P)H-oxidizing activity. The assay cuvette for the standard reaction contained 0.1 mM FAD (or flavin mononucleotide [FMN]) and 0.2 mM NAD(P)H in 50 mM Tris-HCl buffer (pH 7.5) in a final volume of 1 ml. NAD(P)H-oxidizing activity was estimated by measuring the decrease in A340 for NADH (ε of 5,275 M⁻¹ cm⁻¹) or NADPH (ε of 4,898 M⁻¹ cm⁻¹) in the reaction mixture using a Hitachi spectrophotometer U-2900A (Hitachi, Tokyo, Japan).

(ii) Oxidizing activity toward phenolic compounds. Oxidizing activity toward phenolic compounds (4-NP, 3-NP, 2-NP, phenol, 4-chlorophenol, 4-C, 4-HPA, 3-HPA, and 2-HPA) was determined by measuring the decrease in substrate concentration by high-performance liquid chromatography (HPLC). The standard reaction mixture contained 1 mM NADH, 5 mM FAD, 1 mM DTT, 1 mM MgSO₄, 2.5% [vol/vol] glycerol, pH 7.6 for NphA2. The bacterial pellicle was resuspended in 5 ml of the same buffer, sonicated with the cell disruptor (output 6, 1 min, six times, on ice), and then centrifuged (11,000 × g for 4°C for 30 min). The supernatant was used as a cell extract for protein purification.

For the preparation of a cell extract including histidine-tagged NphA2 (His-NphA2), recombinant E. coli JM109 harboring pPHNA2 (pQE80L [Qiagen, Tokyo, Japan] plus nphA2) were cultured in LB medium containing ampicillin at 37°C and 150 rpm on a rotary shaker. IPTG was added at 1 mM after an 8-h incubation, and further cultivation was continued for 16 h. The cells were harvested by centrifugation (7,000 × g for 4°C for 10 min), washed and resuspended with TD buffer, and disrupted as described above.

Purification of NphA1 by ion-exchange chromatography. The cell extract containing NphA1 was applied to a HiTrap Q-Sepharose column (column volume, 5 ml [GE Healthcare BioScience, Tokyo, Japan]) pre-equilibrated with 10 mM Tris-HCl buffer (pH 7.5) and 150 mM NaCl. The column was extensively washed with 10 mM Tris-HCl buffer. Proteins on the column were eluted with a linear NaCl gradient from 100 mM to 400 mM NaCl in TD buffer. Fractions containing NphA1 were pooled, desalted, and concentrated as described above.

Purification of NphA2 by ion-exchange chromatography and His-NphA2 by affinity chromatography. The cell extract containing NphA2 was applied to a Q-Sepharose fast-flow column (resin from GE Healthcare BioScience, column volume, 1.5 cm inside diameter by 30 cm) preequilibrated with 25 ml of lysis buffer (20 mM sodium phosphate buffer containing 500 mM NaCl, pH 7.8) and then washed with TDE buffer. Proteins on the column were eluted with a linear NaCl gradient from 0 mM to 500 mM NaCl in TDE buffer. Proteins containing NphA2 were pooled, desalted, and concentrated as described above.

For the purification of His-NphA2, binding buffer (20 mM sodium phosphate buffer containing 500 mM NaCl, pH 7.8) was used for the cell extract preparation instead of lysis buffer. To a 5-ml disposable polyethylene column, 2.5 ml of Ni-nitrilotriacetic acid (NTA) resin (GE Healthcare BioScience) was added, and then the column was washed with 2.5 ml of TDE buffer. Proteins on the column were eluted with a linear NaCl gradient from 0 mM to 300 mM NaCl in buffer B. Proteins containing His-NphA2 were pooled, desalted, and concentrated as described above.

For the purification of His-NphA2, binding buffer (20 mM sodium phosphate buffer containing 500 mM NaCl, pH 7.8) was used for the cell extract preparation instead of lysis buffer. To a 5-ml disposable polyethylene column, 2.5 ml of Ni-nitrilotriacetic acid (NTA) resin (GE Healthcare BioScience) was added, and then the column was washed with 2.5 ml of TDE buffer. Proteins on the column were eluted with a linear NaCl gradient from 0 mM to 300 mM NaCl in buffer B. Proteins containing His-NphA2 were pooled, desalted, and concentrated as described above.

Sequence analysis of nphRA1A2 gene products. In our previous study, we isolated from PN1 a 5-kb EcoRI DNA fragment (the insert of pKP3N in Fig. 1) that conferred the activity to oxidize 4-NP onto a host strain, R. rhodochrous ATCC 12674 (43, 44). Nucleotide sequence analysis revealed that it contained at least three genes named nphR, nphA1, and nphA2 (Fig. 1). BLAST searches (2) using the deduced amino acid sequences showed that the nphR gene product (NphR) shares quite high identities with the putative AraC/XylS family (hereafter, AraC type) transcriptional regulatory proteins (9) of Rhodococcus sp. strain RHA1 (99% identity, accession no. YP_703831 (24) and Nocardia farcinica IFM 10152 (74%, YP_119268 (14), respectively. Putative functions for these proteins were assigned during the annotation of the whole genome, but these functions have not been experimentally confirmed. NphR also shows moderate identities to some members of the AraC-type regulatory proteins, CadR (31%), NikR (28%), and HpaA (24%), which have been shown to be positive regulators for the expression of the 2,4-dichlorophenoxacyclic acid degradation genes (cadABK) in Bradyrhizobium sp. strain HW13 (21), the nitrophenolase gene (nita) in R. rhodochrous J1 (23), and the 4-hydroxyphenylacetate 3-hydroxylation genes (hpaBC) in E. coli W (ATCC 11105) (33, 35). Recombinant NphR has a DNA-binding helix-turn-helix motif (pfam00165) conserved among the AraC-type regulators at its C-terminal region (at amino acids [aa] 284 to 328 in NphR).

The homology search also revealed that NphA1 and NphA2 were pooled, buffer exchanged with 50 mM potassium phosphate buffer (pH 7.5), and concentrated as described above.

Enzyme assay. (i) NAD(P)H-oxidizing activity. The assay cuvette for the standard reaction contained 0.1 mM FAD (or flavin mononucleotide [FMN]) and 0.2 mM NAD(P)H in 50 mM Tris-HCl buffer (pH 7.5) in a final volume of 1 ml. NAD(P)H-oxidizing activity was estimated by measuring the decrease in A340 for NADH (ε of 5,275 M⁻¹ cm⁻¹) or NADPH (ε of 4,898 M⁻¹ cm⁻¹) in the reaction mixture using a Hitachi spectrophotometer U-2900A (Hitachi, Tokyo, Japan).
share significant identities with the large subunits and the small subunits of the putative two-component aromatic ring hydroxylases of *Rhodococcus* sp. strain RHA1 (99%, YP_703830; 98%, YP_703829) (24) and *N. furcinea* IFM 10152 (89%, YP_119267; 78%, YP_119266) (14), respectively. Their putative functions were also assigned during the annotation of the whole genome. The genes encoding these putative hydroxylases are clustered with the corresponding AraC-type regulator genes described above. This sequence analysis revealed that *nphRA1A2*-like gene clusters are well conserved in the genomes of these high-G+C-content, gram-positive bacteria. *NphA1* also shows remarkable identities to the 4-coumarate 3-hydroxylase (Sam5 [78%, ABC88666]) of *Saccharothrix espanaensis* (3), the phenol hydroxylase large subunits (PheA [61%, AAC38324] and PheA1 [52%, AAF66546]) of *Geobacillus thermodenitrificans* A2 (6) and *Geobacillus thermoglucosidasius* A7 (7), and the 4-HPA 3-hydroxylase large subunits (HpaB [53%, Q48440] and HpaB [52%, Q57160]) of *Klebsiella oxytoca* (10) and *E. coli* W ATCC 11105 (33, 46). These hydroxylases can oxidize phenol and/or 4-substituted phenols.

In contrast, *NphA2* shows considerable identities to the hydroxylase component B (MobC [55%, BAD08312]) of *Bacillus* sp. strain JF8 (26), the naphthalene-inducible monoxygenase small subunit (NimA [46%, AAL61657]) of *Rhodococcus aethereovorus* I24 (31), the phenol hydroxylase component B (46%, YP_145483) of *Thermus thermophilus* H88, and the phenol hydroxylase component B (PheA2 [45%, AAF66547]) of *G. thermoglucosidasius* A7 (7, 20). *NphA2* was reported to be an NADH/flavin oxidoreductase in the phenol hydroxylase system of *A7* (20). Therefore, *NphA2* was expected to enhance the hydroxylase activity of *NphA1* by reducing flavins with the concomitant oxidation of pyridine nucleotides such as NAD(P)H and supplying the reduced flavins to *NphA1*.

In conclusion, these sequence analyses suggest that *NphA1* and *NphA2* are an oxygenase component and a flavin reductase component, respectively, in the 4-NP hydroxylase system of PN1, while *NphR* is likely an AraC-type regulatory protein for the expression of *nphA1A2*.

**Mechanism of regulating 4-NP oxidation in PN1.** In our previous study, *R. rhodochrous* ATCC 12674 cells harboring pKP35 (*nphRA1A2*) (Fig. 1) grown in the presence of 4-NP degraded 0.3 mM of 4-NP completely in only 1 h, whereas those grown in the absence of 4-NP took 8 h to achieve complete degradation of the same concentration with a 2-h lag period (43). This fact indicated that the DNA fragment on the plasmid encoded a regulatory system for 4-NP degradation. In order to understand the regulatory mechanism, the transcriptional activity of *nphA1* was first measured using the cell extract of the recombinant *Rhodococcus* strain harboring pKP35 with a reporter C23O gene in *nphA1* (Fig. 1), which was grown in the presence of various phenolic compounds for induction (Table 1). When 4-NP was used as an inducer, the cell extract showed more than 120-fold-higher C23O activity than that without induction. In contrast, the transcriptional activity upon induction by other phenolic compounds was quite low and similar to that without induction. Therefore, of the compounds tested, 4-NP was found to be the only inducer for *nphA1* expression in the presence of *nphR*.

To examine the effect of *nphR* on the transcription of *nphA1*, we disrupted the gene by inserting a tetracycline resistance gene from pBR322 (4) into the EcoRV site of pKP35 to construct pKP33 (Fig. 1). The cell extract of a recombinant *Rhodococcus* strain harboring pKP33 showed no detectable transcriptional activity irrespective of the presence of any inducers, the activity levels detected on November 11, 2017 by guest http://jb.asm.org/ Downloaded from
introduced into the BamHI site of pQE80L to construct pQPHNA2, resulting in the addition of 12 aa residues (MRG SHHHHHHGS) to the N terminus of NphA2. This trial was very successful. That is, His-NphA2 was successfully purified in large amounts by passing the cell extract of E. coli JM109 harboring pQPHNA2 through an affinity column of Ni-NTA resin. The size estimated by SDS-PAGE was 20 kDa, which is consistent with the size calculated from the amino acid sequence (20.6 kDa). The native molecular mass estimated by gel filtration analysis was approximately 40 kDa (data not shown), indicating that it forms a dimeric structure. For convenience, His-NphA2 was used for further studies.

Substrate specificity and kinetic parameters of His-NphA2.

From sequence analysis, NphA2 was expected to be an NAD(P)H/flavin oxidoreductase in the 4-NP hydroxylase system. Thus, NAD(P)H-oxidizing activity was evaluated in the presence of FAD or FMN using the purified His-NphA2. Maximal activity for the oxidation of NADH was achieved in the presence of FAD (6.6 ± 0.2 μmol min⁻¹ mg-protein⁻¹). No activity was detected when NADPH was used instead of NADH. FMN was also unable to replace FAD. This result shows that NphA2 reduces FAD with the concomitant oxidation of NADH and that this enzyme recognizes these substrates strictly. The corresponding proteins of the 4-NP monoxygenases of B. sphaericus JS905 (16) and Arthrobacter sp. strain JS443 (29) also prefer the combination of NADH and FMN; this enzyme also reduces FAD and 4-NP-oxidizing activity was completely lost at 50 μM. The typical spectrum for flavins was not detected from the protein uses FAD as a substrate but not as a prosthetic group.

Substrate specificity and kinetic parameters of His-NphA2.

As shown in Fig. 2, 0.3 mM of 4-NP was almost quantitatively oxidized by NphA1 in the presence of NADH, FAD, and His-NphA2. Enzymatic conversion of 4-NP was carried out using the purified NphA2 and His-NphA2 (data not shown). In contrast, the phenol hydroxylase component B, PheA2, of G. thermoglucosidius A7 displays a spectrum typical for a flavoprotein, with maxima at 376 nm and 455 nm and characteristic shoulders around 355 nm and 485 nm (20). This protein showed 38% amino acid sequence identity to NphA2, uses FAD as a substrate in addition to a prosthetic group, and has been suggested to employ the “ping pong bi bi” kinetic mechanism for FAD reduction (20).

Oxidation activities of NphA1 toward 4-NP and other phenolic compounds.

In order to confirm the function of NphA1, enzymatic conversion of 4-NP was carried out using the purified NphA1 in the presence of NADH, FAD, and His-NphA2. As shown in Fig. 2, 0.3 mM of 4-NP was almost quantitatively converted into 4-NC within 20 min. In contrast, 4-NP never decreased in the same reaction mixture without NphA1 or one of the other components (NADH, FAD, or His-NphA2) (data not shown). Hence, NphA1 is the oxygenase component in the 4-NP hydroxylase system. During the 4-NP oxidation experiment using NphA1, inhibition of 4-NP oxidation was observed at >10 μM FAD and 4-NP-oxidizing activity was completely lost at >50 μM. In contrast, concentrations below 1 μM were less effective, and thus the optimal concentration was determined to be 5 μM. More than 80% of the 4-NP-oxidizing activity of NphA1 could be kept for at least a week in the presence of 20% (vol/vol) glycerol at −20°C. The optimal pH for 4-NP oxidation was 8.0.

To determine the substrate specificity of NphA1, enzymatic conversion of several phenolic compounds (phenol, 2-NP, 3-NP, 2-HPA, 3-HPA, 4-HPA, and 4-NC) was carried out in the reaction mixture with the same composition. As shown in Fig. 2, NphA1 degraded phenol and 4-chlorophenol as rapidly as it did 4-NP, while it reduced 4-NC and 3-NP more slowly. No decreases in concentrations for 2-NP, 2-HPA, 3-HPA, and 4-HPA were observed (data not shown). In the phenol, 4-chlorophenol, and 3-NC oxidation reactions, major metabolites were detected by HPLC, whose retention times on the HPLC chromatograms were consistent with those of catechol, 4-chlorocatechol, and 4-NC, respectively. Some metabolites, probably being 1,2,4-benzenetriol or its oxidized forms, were also detected in the 4-NC oxidation reaction, but we were unable to exactly identify them due to the overlapping of some other
peaks originated from the reaction mixture components on the chromatogram.

**DISCUSSION**

In this study, we clarified the mechanism by which PN1 oxidizes 4-NP. In this process, a two-component aromatic ring hydroxylase encoded by *nphA1* and *nphA2* is produced when its expression is induced by 4-NP in the presence of *nphR*; this hydroxylase carries out the oxidation of 4-NP to 4-NC in the presence of NADH and FAD. The kinetic and spectral studies suggest that FAD is used as a substrate but not a prosthetic group. This is one of the characteristics of the TC-FDM family enzymes.

Figure 3a summarizes the gene clusters (*nph*, *npc*, and *npd*) for 4-NP oxidation that have been cloned from three bacteria, *Rhodococcus* sp. strain PN1, *R. opacus* SAO101, and *Arthrobacter* sp. strain JS443 (22, 29, 43). The genetic organizations of these clusters are quite different in each microorganism. The *nph* and *npc* gene clusters include an AraC-type regulatory gene (*nphR* and *npcR*) and a LysR-type regulatory gene (ORF1), respectively, while the *npd* gene cluster includes a large MalT-DnrI-fused-type regulatory gene (*npdR*). Although the *npc* and *npd* gene clusters were transcribed in the cells grown on 4-NP (22, 29), little is known about the regulatory systems for their expression, including the functions of ORF1 and *npdR*. We demonstrated in the present study that *nphR* encodes a positive regulatory protein for the expression of *nphA1* and that 4-NP is the only molecule capable of functioning as an inducer (of the several compounds tested).

In the 4-NP oxidation reaction (Fig. 2a), the 4-NP hydroxylase converted 4-NP into 4-NC stoichiometrically. In contrast, in the 4-NC oxidation reaction (Fig. 2c), although it also showed weak oxidation activity for 4-NC, the decrease in 4-NC concentration almost stopped at 10 min, indicating that 4-NC inhibited the enzyme activity or deactivated the enzyme itself. Therefore, in the 4-NP oxidation, the 4-NC formed might inhibit further oxidation of 4-NC itself, resulting in the stoichiometric accumulation. As shown in Fig. 3b, the 4-NP monooxygenase of *R. opacus* SAO101 (NpcA and NpcB) (22) can carry out two sequential oxidations from 4-NP to 1,2,4-benzenetriol. In contrast, as shown here, the 4-NP hydroxylase of PN1 catalyzed the first oxidation mainly. Since PN1 doesn’t accumulate large amounts of 4-NC in 4-NP degradation (43), it should have another enzyme capable of degrading 4-NC. In our previous study, we isolated a mutant strain from PN1 by chemical mutagenesis (43), which accumulated 4-NC from 4-NP almost stoichiometrically. It is probable that this mutant lost a gene encoding such a 4-NC-degrading enzyme. Recently, we found a gene encoding a functional hydroxyquinol dioxygenase in PN1 (data not shown), which cleaves the aromatic ring of 1,2,4-benzenetriol to form maleylacetate in the 4-NC pathway (Fig. 3b). These facts strongly suggest that PN1 has
enzymes involved in the 4-NC pathway in addition to the 4-NP hydroxylase.

In the TC-FDM family, the 4-HPA 3-hydroxylase system (HpaB and HpaC) of *E. coli* W ATCC 11105 (8, 32–35, 46) has been purified and characterized in detail at both the genetic and enzymatic levels. The gene cluster (*hpaABC*) that encodes this enzyme system also contains the regulatory gene *hpaA*, which encodes an AraC-type positive regulatory protein (33, 35). Therefore, *hpaABC* is very similar in its gene organization to *nphRA1A2*; however, *hpaABC* is transcribed in the same direction, whereas *nphR* and *nphA1A2* are transcribed divergently. Generally, genes encoding the AraC-type regulatory proteins are located upstream of their target genes and transcribed in the same direction (9) like *hpaABC*. Thus, the genetic organization of *nphRA1A2* is one of the minor cases.

The amino acid sequence identities of HpaA, HpaB, and HpaC to NphR, NphA1, and NphA2 are 24%, 52%, and 25%, respectively. HpaC was found to be able to reduce FMN, FAD, and riboflavin with concomitant oxidation of NADH or NADPH, but the most efficient cofactors were found to be a combination of NADH and FMN (8), as described above. In contrast, NphA2 uses only a combination of NADH and FAD and shows no NADPH-oxidizing activity and no FMN-reducing activity. The *K₅₅* of NphA2 for NADH (58.1 μM) in the presence of FAD is comparable to that of HpaC (40 μM) in the presence of FMN, while that of NphA2 for FAD (271 μM) in the presence of NADH is much higher than those of HpaC for FMN (2.1 μM) or for FAD (3.1 μM). This value for FAD is also approximately 10 to 100 times higher than those of other NAD(P)H/flavin oxidoreductases listed by Kim et al. (19).

The oxygenase component, HpaB, was found to have a broad substrate range for phenolic compounds, since *E. coli* crude extracts containing both HpaB and HpaC were found to oxidize 4-HPA, 3-HPA, 3,4-dihydroxyphenylacetate, 2,5-dihydroxyphenylacetate, p- cresol, phenol, 4-chlorophenol, and others (32). Only 4-HPA, 3-HPA, and phenylacetate, however, can induce the expression of the *hpaBC* genes (35). These facts show that the 4-HPA 3-hydroxylase system has developed for HPA metabolism. Similar 4-HPA 3-hydroxylases and their genes can be found in many bacteria, because HPA metabolism is involved in aromatic amino acid metabolic pathways (http://www.genome.ad.jp/kegg/pathway/map/map01150.html).

In contrast, NphA1, coupled with NphA2, oxidizes 4-NP, phenol, 4-chlorophenol, 3-NP, and 4-NC, suggesting that it too has broad substrate specificity. HPAs were not oxidized by the 4-NP hydroxylase, however, nor were they able to function as an inducer for the expression of *nphA1A2* (Table 1). These differences in gene regulation and substrate specificity suggest that the 4-NP hydroxylase system might have been completely ramified for 4-NP oxidation from 4-HPA 3-hydroxylase systems, although both hydroxylase systems are expected to share the same origin, judging from their amino acid sequence identities. Very recently, the crystal structure of a different 4-HPA 3-monoxygenase (*HpaB*) of *Thermus thermophilus* HB8 was determined and its catalytic mechanism was discussed (18). To understand the catalytic mechanism of our enzyme, we are also trying to elucidate the structure through crystallographic study and X-ray analysis.

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