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# Gentisate 1,2-Dioxygenase from *Xanthobacter polyaromaticivorans* 127W

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**A putative gentisate 1,2-dioxygenase was encoded in the dibenzothiophene degradation gene cluster (*dbd*) from *Xanthobacter polyaromaticivorans* 127W. The deduced amino acid sequence showed high sequence similarity with gentisate dioxygenases from *Pseudomonas alcaligenes* (AAD49427, 65% identical), *Bradyrhizobium japonicum* (NP_766750, 64%), and *P. aeruginosa* (ZP_00135722, 54%), and moderate similarity with 1-hydroxy-2-naphthoate dioxygenase from *Nocardioides* sp. KP7 (BAA31235, 33%) and salicylate dioxygenase from *Pseudaminobacter salicylatoxidans* (AAQ91293, 33%). The enzyme, GDOxp, was heterologously produced in *Escherichia coli* and purified to homogeneity. GDOxp formed a tetramer and exhibited high dioxygenase activity against 1,4-dihydroxy 2-naphthoate as well as gentisate, suggesting unusually broad substrate specificity. GDOxp easily released ferrous ion under unfavorable temperature and pH conditions to become an inactive monomer protein. An inactive monomer protein can reconstitute a tetramer structure and restore enzyme activity in a cooperative manner upon the addition of ferrous ion. Chymotryptic digestion and protein truncation experiments suggested that the N-terminal region is important for the tetramerization of GDOxp.**

**Key words:** gentisate 1,2-dioxygenase; *Xanthobacter*; gentisate; naphthoate; ferric ion

Dioxygenases are enzymes that catalyze dioxygen incorporation into various organic compounds. They play key roles in the degradation pathway of mono- and polycyclic aromatic hydrocarbons, including recalcitrant and detrimental compounds.[1–3] Aromatic ring dioxygenases contain nonheme ion and are classified into two classes. First-class enzymes are a two- or three-component system that consists of a reductase, (a ferredoxin), and large [$\alpha$] and small [$\beta$] subunits of a terminal oxygenase component ($\alpha_3\beta_3$ or $\alpha_3$).[3] These enzymes are mainly involved in the initial oxygenation step of aromatic substrates to dihydroxylated intermediates. The second class is made up of oligomeric dioxygenases, which are responsible for subsequent ring fission of dihydroxylated intermediates. Although the aromatic hydrocarbons are diverse in number of rings and modifications, they are degraded by way of a limited number of dihydroxylated intermediates, such as catechol (1,2-dihydroxylated benzene), protocatechuate (3,4-dihydroxybenzoate), and gentisate (2,5-dihydroxybenzoate). Therefore, the enzymes responsible for the ring fission of these compounds are the funnels in the biodegradation pathways of aromatic hydrocarbons. These ring-fission dioxygenases are further classified into three groups based on the cleavage site of substrates. The first group is intradiol dioxygenase, such as catechol 1,2-dioxygenase and protocatechuate 3,4-dioxygenase, which contain a ferric ion in the catalytic center of each subunit and cleave the aromatic ring between two vicinal hydroxyl groups to yield colorless *cis*, *cis*-muconic acid adducts.[4] The second group is extradiol dioxygenase, such as catechol 2,3-dioxygenase, protocatechuate 4,5-dioxygenase, and 2,3-dihydroxybiphenyl dioxygenase, which contain a catalytic ferrous ion and cleave the aromatic ring adjacent to two vicinal hydroxyl groups to yield yellow hydroxymuconic semialdehyde adducts.[5] The third group enzyme is apparently different from the two former groups because it degrades 1,4- or 2,5-dihydroxylated substrates such as gentisate (2,5-dihydroxybenzoate), homogentisate, and hydroquinone. Gentisate 1,2-dioxygenase (GDO, EC1.13.11.4) is included in this group, which cleaves the aromatic ring between the carboxyl and proximal hydroxyl groups in the aromatic ring to yield maleylpyruvate. It has been reported that the catalytic mechanism of GDO is basically similar

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to that of the extradiol dioxygenase group, and that the active center contains a 2-His-1-carboxylate facial triad that coordinates ferrous ion.[6] However, phylogenetic analyses indicate that these three groups of ring fission dioxygenases form structurally different clusters and originate from different ancestors.[1]

*Xanthobacter polyaromaticivorans* 127W is capable of degrading and growing on various 2-ring and 3-ring polycyclic aromatic hydrocarbons (PAHs) and heterocyclic aromatic compounds (HACs), such as dibenzothiophene.[7] It has been found that the strain degraded these aromatic compounds even under extremely low oxygen (ELO) conditions, where the dissolved oxygen concentration was lower than or equal to 0.2 ppm. We have reported information about the *dbd* gene cluster responsible for degradation of both PAHs and HACs from strain 127W, which is functional under ELO conditions.[8] The gene cluster contained a gene encoding putative gentisate 1,2-dioxygenase (*dbdB*), suggesting that gentisate is an intermediate in the PAHs and HACs degradation pathways of the strain. In this paper, we describe the production and characterization of recombinant GDO from strain 127W, GDOxp. GDOxp was found to be highly active against both gentisate and dihydroxynaphthoate. GDOxp had higher affinity to oxygen than other dioxygenase enzymes. The results also suggest that ferrous ion specifically functions in the formation of the active tetramer structure of GDOxp.

## Materials and Methods

*Cells and plasmids.* *X. polyaromaticivorans* 127W was isolated from bottom sludge of a crude oil reservoir tank in Fukui, Japan, for its ability to grow on dibenzothiophene (DBT) under ELO conditions.[7] *E. coli* JM109 and BL21 (DE3) (Stratagene, Heidelberg, Germany) were used in gene manipulation and protein production experiments respectively. Gene expression plasmids pET25b and pSTV28 were purchased from Novagen (Madison, WI) and Takara Bio (Ohtsu, Japan) respectively. All the recombinant cells were grown in L broth containing 50 mg/l ampicillin, unless otherwise stated. L broth contains 10 g/l Tryptone (Difco, Sparks, MD) and 5 g/l each of Yeast extract (Difco) and NaCl (pH 7.2).

*Prediction of secondary structure.* Fully automatic Jpred WWW server (Barton Group, Dundee, UK) was used in prediction of protein secondary structure.[9] A multiple amino acid sequence alignment of GDOxp and other related proteins was constructed using CLUSTAL W.[10]

*Construction of pET25b-GDOxp gene expression plasmid.* Genomic DNA of strain 127W was extracted and purified as previously described.[8] The *dbdB* gene was amplified by PCR with a combination of synthetic oligonucleotide primers, forward (5′-GAGGGCCACA*TATG*TCATTCGCG-3′) and reverse (5′-CCGAAGCGGGTCGACTTT*TCA*C-3′) primers, which were designed according to the gene sequences (accession no., AB121977). Another forward primer (5′-CTATACAGCGCTCCAT*ATG*GTGCTAAA-3′) was used in the production of dGDOxp with N-terminal peptide truncation from $S^2$ to $W^{29}$. Underlines represent *Nde*I and *Sal*I restriction sites respectively, and an initiation ATG codon and a sequence complementary to the termination TGA codon are shown in italics. PCR was performed in 30 cycles of 95 °C for 1 min, 58 °C for 1 min, and 74 °C for 1 min, using KOD plus DNA polymerase (Toyobo, Osaka, Japan) and a thermal cycler GeneAmp PCR system 2400 (PerkinElmer, Foster city, CA). Each DNA fragment was digested with *Nde*I and *Sal*I and ligated into the *Nde*I-*Sal*I gap of plasmid pET25b to create gene expression plasmid pET25b-GDOxp or pET25b-dGDOxp. Overexpression of the gene was induced by the addition of 0.5 mM IPTG to a mid-log phase culture ($OD_{660} = 0.5$) in L broth at 25 °C for 4 h.

*Construction of pSTV28-GroES/EL.* In order to promote proper folding of recombinant GDOxp in *E. coli* BL21(DE3), another gene expression plasmid, pSTV28-GroES/EL, was constructed and co-expressed in the cells. A gene locus containing sigma34-promoter, *groEL*, and *groES* (AE000487) was amplified by PCR with a set of primers and genomic DNA of *E. coli* BL21(DE3). The sequence of primers was: forward, 5′-GTGCTGATCAGAATTCTTTTTCTTTTTCCC-3′ and reverse, 5′-GTTTGTTTATGTCGACGAGGTGCAG-3′. Underlines show recognition sites *Eco*RI and *Sal*I respectively. The DNA fragment was digested with *Eco*RI and *Sal*I and ligated into the *Eco*RI-*Sal*I gap of pSTV28. Gene expression was induced as described above.

*Purification of the enzyme.*

*Production of GDOxp.* After induction of the gene for 2 h, cells were harvested by centrifugation, and suspended in 10 mM MOPS buffer (pH 7.2) containing 10% glycerol (buffer A). The cells were then disrupted by sonication with a model 450 sonifier (Branson Ultrasonic, Danbury, CT) on ice, and centrifuged at 20,000 *g* for 30 min to separate soluble (cell lysate) and insoluble (membrane and protein aggregates) fractions. The soluble fraction was used in the purification of GDOxp.

*Fractionation by ammonium sulfate.* The soluble fraction was further fractionated by stepwise increase in the concentration of ammonium sulfate. Proteins that were soluble at 30% and insoluble at 50% saturation of ammonium sulfate were collected by centrifugation at 20,000 *g* for 30 min (4 °C), and dissolved in an appropriate volume of buffer A. The protein solution was dialyzed against 10 mM MOPS buffer (pH 7.2) for 16 h. After dialysis, sediments were removed by centrifugation and the supernatant was pooled.

*Anion-exchange chromatography.* Further purification steps were performed using a Fast Protein Liquid Chromatography system (Amersham Biosciences, Buck-

inghamshire, UK). A dialyzed sample was applied to a HitrapQ column (5 ml, Amersham) equilibrated with buffer A. GDOxp and dGDOxp were eluted in a linear gradient from 0 to 1 M NaCl in buffer A.

*Gel filtration chromatography.* Active fractions eluted from the HitrapQ column were pooled and loaded onto a Hiload 16/60 column containing Superdex200pg (Amersham) equilibrated with buffer A containing 100 mM NaCl.

*Analysis on ferrous ion content.* Ferrous ion contents of proteins were measured by the method of Zabinski *et al.*[4)]

*Enzyme assays.* Gentisate 1,2-dioxygenase activity was assayed spectrophotometrically by monitoring the formation of maleylpyruvate ($\lambda_{max} = 330$ nm) at 23 °C. The molecular extinction coefficient value of maleylpyruvate at 330 nm was 10.2 $mM^{-1}\,cm^{-1}$.[6)] The reaction mixture contained 0.33 mM of gentisate and an appropriate amount of enzyme in 20 mM PIPES (pH 7.5) buffer containing 20 μM $Fe(NH_4)_2(SO_4)_2$ and 2 mM cysteine otherwise denoted. The activity of GDOxp against other substrates (0.33 mM) was assayed similarly. The molecular extinction coefficient values of products from 5-aminosalicylate, 1-hydroxy 2-naphthoic acid, and 1,4-dihydroxy 2-naphthoic acid were 12.4 (1,5-dicarboxy-3-amino-2,4-pentadiene-1-one, $\lambda_{max} = 358$ nm), 11.5 (4-(2-carboxyphenyl)-2-oxobut-3-enoate, $\lambda_{max} = 300$ nm), and 9.2 $mM^{-1}\,cm^{-1}$ (4-hydroxy-(2-carboxyphenyl)-2-oxobut-3-enoate, $\lambda_{max} = 312$ nm) respectively.[6,11,12)] One unit of activity was defined as the amount of enzyme converting 1 μmol of substrate to a corresponding product per min. Kinetic parameters were determined from a Lineweaver–Burk plot of initial reaction velocity against different concentrations of the substrate. The $K_m$ value for oxygen was determined in the presence of 0.33 mM gentisate under various dissolved-oxygen conditions. Different dissolved-oxygen conditions were prepared by changing the incubation time in an anaerobic chamber, as described previously.[7)] As the incubation time increased, the oxygen concentration in the solution decreased. The Hill coefficient was determined from a sigmoidal reactivation curve of monomer GDOxp under different ferrous ion concentrations.[13,14)] The protein concentration was determined by the method of Bradford (BioRad Protein assay kit, BioRad Laboratories, Hercules, CA), with bovine serum albumin as a standard.

*CD spectrometry.* Far-UV (200–260 nm) circular dichroism (CD) spectra were obtained at 30 °C on a J-720W spectropolarimeter (Jasco, Tokyo). The sample contained proteins at 0.52 mg/ml (0.33 μM) in 10 mM MOPS buffer (pH 7.2). The optical path of the quartz cell was 2 mm. The mean molar ellipticity, $\theta$ [deg $cm^2\,dmol^{-1}$], was calculated using an average molecular weight of 110 for one amino acid residue.[15)]

*Limited proteolysis.* GDOxp was digested with chymotrypsin at 37 °C for 0 to 10 min in 10 mM MOPS buffer (pH 7.2) containing 2.5 mM DTT. The ratio of chymotrypsin to GDOxp in the reaction mixture was 1 to 100 (w/w). The protein bands visualized with Coomassie brilliant blue staining were transferred to a PVDF membrane and subjected to amino acid sequence analysis. The N-terminal amino acid sequence was determined with a pulsed liquid automated sequencing system Procise 491 (PerkinElmer).

## Results and Discussion

*Primary structure analyses of gentisate 1,2-dioxygenase (GDOxp) from* X. polyaromaticivorans *127W*

The amino acid sequence deduced from the nucleotide sequence of *dbdB* (AB121977)[8)] indicated that it encodes a gentisate 1,2-dioxygenase composed of 350 amino acid residues with a calculated molecular weight of 39.3 kDa and a pI value of 5.4. This protein was named GDOxp. GDOxp shared high sequence similarity with GDO from *Pseudomonas alcaligenes* (AAD49427, 65% identical), *P. aeruginosa* (ZP00135722, 54%), and *Bradyrhizobium japonicum* (NP766750, 64%), and moderate similarity with 1-hydroxy-2-naphthoate dioxygenase from *Nocardioides* sp. KP7 (BAA31235, 33%) and salicylate dioxygenase from *Pseudaminobacter salicylatoxidans* (AAQ91293, 33%). These GDO family proteins commonly have two sets of cupin domain, each comprising a six-stranded beta barrel structure predicted by the Jpred program (Fig. 1). A cupin domain typically contains a set of motifs, motif 1, $G(X)_5HXH(X)_{3,4}E(X)_6G$, and motif 2, $G(X)_5PXG(X)_2H(X)_3N$, and an intermotif region of various lengths.[16)] Two histidines, HXH in motif 1 and a histidine in motif 2, have been reported to be essential for dioxygenase activity, and all these histidines were completely conserved in GDOxp.[17)] However, the glutamic acid in motif 1 and the second glycine in motif 2 are commonly replaced in GDO with alanine (A114) and tryptophane or phenylalanine (W/F146) respectively. A highly conserved glutamic acid (E103) in motif 1 might constitute a 2-His-1-carboxylate facial triad that functions as a metal ligand. Although GDO has been classified in the bicupin family, which bears dual cupin domains, distinguished residues in motif 3 and motif 4 of the possible C-terminal domain were less conserved than those in motif 1 and motif 2 of the N-terminal domain.

*Overproduction and purification of GDOxp*

When GDOxp was overproduced in *E. coli* BL21 (DE3) cells harboring pET25b-GDOxp, it formed mostly an insoluble protein (Fig. 2A). We expected that a molecular chaperon would help GDOxp to fold properly to take soluble form. In fact, the co-expression systems of pET25b-GDOxp and pSTV28-GroES/EL in *E. coli* were successful in recovering, about half of the GDOxp in the soluble fraction. Soluble GDOxp was purified

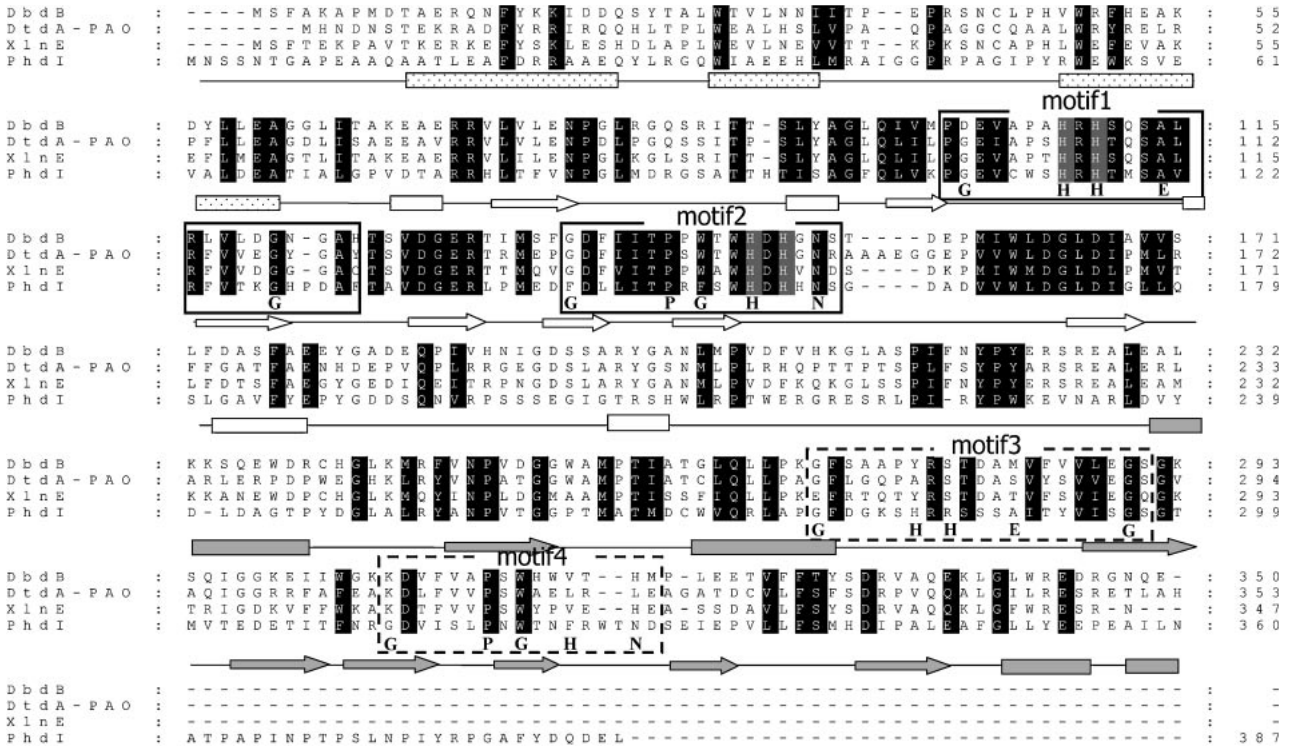


**Fig. 1.** Amino Acid Sequence Alignment of Selected GDO and HNDO.

GenBank accession numbers are as follows: DbdB (GDOxp), gentisate 1,2-dioxygenase from *X. polyaromaticivorans* 127W (AB121977); DtdA-PAO, gentisate 1,2-dioxygenase from *P. aeruginosa* (AE004674); XlnE, gentisate 1,2-dioxygenase from *P. alcaligenes* (AF173167); PhdI, 1-hydroxy 2-naphthoic acid dioxygenase from *Nocaridoides* sp. KP7 (D89987). Consensus amino acid residues among cupin family proteins are shown under the alignment. Cupin motifs are indicated by thick boxes in the N-terminal domain and by broken boxes in the putative C-terminal domain. Four histidine residues essential for enzyme activity[7] are shaded in gray. The secondary structure of GDOxp was predicted by the Jpred program,[9] and the results are shown by boxes (alpha-helix) and arrows (beta-sheet) under the sequence alignment. The external alpha-helix region and the N-terminal and C-terminal cupin domains are discriminated by dotted, open, and shaded symbols respectively.

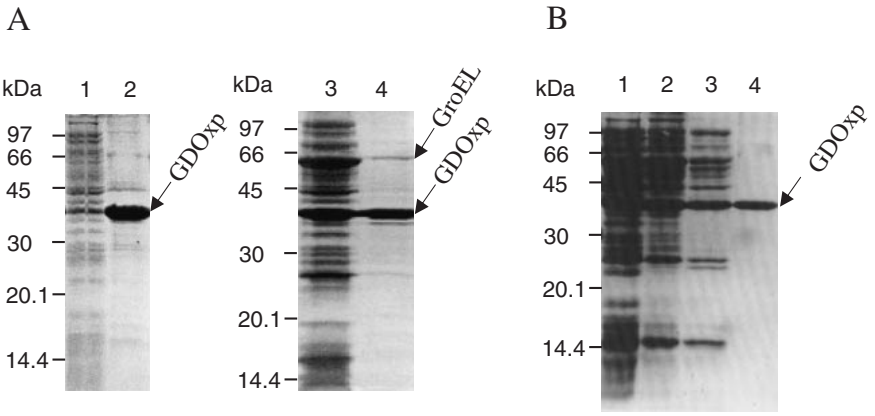


**Fig. 2.** SDS–PAGE Analyses of GDOxp.

Electrophoresis was performed with 15% polyacrylamide gel in the presence of SDS, and the gel was stained with Coomassie brilliant blue. A, Lanes 1 and 2 show soluble and insoluble fractions obtained from *E. coli* BL21 (DE3) cells transformed with pET25b-GDOxp, respectively. Lanes 3 and 4 are soluble and insoluble fractions from *E. coli* BL21 (DE3) cells harboring both pET25b-GDOxp and pSTV28-GroES/EL, respectively. Phosphorylase b (97,000), albmin (66,000), ovalbumin (45,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and alpha-lactalbumin (14,400) were used as molecular standard markers. GroEL and GDOxp protein bands are indicated by arrows. B, Comparison of the purity of GDOxp by SDS–PAGE. Lane 1, total soluble proteins prepared from *E. coli* BL21 (DE3) cells transformed by pET25b-GDOxp/pSTV28-GroES/EL. Lane 2, after ammonium fractionation. Lane 3, after purification by anion exchange column (HitrapQ) chromatography. Lane 4, after gel filtration column (Superdex200pg) chromatography.

through three purification steps to apparent homogeneity by SDS–PAGE analysis (Fig. 2B). Finally, 5.6 mg of GDOxp was purified from 1 liter of culture. The specific activity of the purified enzyme was 102 units/mg (pH 7.5). The molecular mass of GDOxp was estimated to be 40 kDa on SDS–PAGE, comparable to the value calculated from its deduced amino acid sequence, 39.3 kDa. When GDOxp was analyzed by gel filtration chromatography, it eluted at a position corresponding to a molecular weight of 146 kDa, suggesting that GDOxp formed a homotetramer. It has been reported that GDOs require ferrous ion for their catalytic activity.[6] The ferrous ion content was measured and the amount of ferrous ion bound to 1 mol subunit of GDOxp was determined to be 0.98 mol. This result suggests that only one of the cupin domains in GDOxp, probably the first one, was functional as an active ligand for ferrous ion.

*Kinetic properties*

Initial reaction rates were measured at different concentrations of gentisate (3.3 μM–0.5 mM). Under these conditions, the enzyme followed Michaelis–Menten kinetics. $K_m$ and $k_{cat}$ values were determined to be $18.6 \pm 1.6$ μM and $5{,}430 \pm 330\ min^{-1} \cdot site^{-1}$ respectively. The $K_m$ and $k_{cat}$ values of GDO from *P. acidovorans* and *Comamonas testosteroni* for gentisate have been reported to be 74 μM and 19,300 $min^{-1}\ site^{-1}$, and 85 μM and 22,000 $min^{-1}\ site^{-1}$, respectively.[12] *X. polyaromaticivorans* 127W is capable of degrading PAHs and HACs under ELO conditions. The cells maintained 23.5% of dibenzothiophene degrading activity even under an oxygen condition of 6.7 μM (0.2 ppm) as compared to the aerobic condition, 218.8 μM (6.5 ppm).[7] The $K_m$ and $K_{cat}$ values of GDOxp for oxygen were determined to be $19.2 \pm 2.4$ μM and $4{,}420 \pm 230\ min^{-1}\ site^{-1}$ respectively. The values for GDO from *P. acidovorans* and *C. testosteroni* for oxygen are 55 μM and 19,700 $min^{-1}\ site^{-1}$ and 96 μM and 38,500 $min^{-1}\ site^{-1}$ respectively.[12] We do not know whether GDOxp is a rate-limiting enzyme in the degradation pathway, but lower $K_m$ values of GDOxp against oxygen than other GDOs would help the cells to metabolize PAHs and HACs effectively under ELO conditions.

*Substrate specificity*

The oxygenation activity of GDOxp against gentisate and salicylate substitutes was examined spectrophotometrically. Substrates of 1-hydroxy-2-naphthoate dioxygenase (HNDO), 1-hydroxy-2-naphthoate, and 1,4-dihydroxy-2-naphthoate were also tested for the enzyme assay because GDOxp shared significant amino acid sequence similarity (33% identical) with HNDO. Among the compounds tested, gentisate and 1,4-dihydroxy-2-naphthoate were the most preferred, and 5-aminosalicylate was a partly preferred substrate for GDOxp (Table 1). GDOs from *P. testosteroni* and *P. acidovorans* also have been found to be active against 1,4-dihydroxy-2-naphthoate, but only in part (0.3–11% active).[1] In contrast to these GDOs, GDOxp was fully active against 1,4-dihydroxy-2-naphthoate. GDO from *P. alcaligenes* NCIB9867, the closest relative of GDOxp, showed even higher activity (127%) against 3-methylgentisate than gentisate, but the availability of 1,4-dihydroxy-2-naphthoate to the enzyme is unknown.[18] It should also be noted that HNDO has been reported to be inactive against gentisate.[11] The $K_m$ and $k_{cat}$ values of GDOxp for 1-hydroxy-2-naphthoate were determined to be $20.9 \pm 3.6$ μM and $33.9 \pm 2.6\ min^{-1}\ site^{-1}$ respectively. These values suggest that the low activity of GDOxp for 1-hydroxy-2-naphthoate was due to a reduction in the turnover rate rather than to poor affinity of the enzyme for the bulky hydroxyl naphthoate. The activity of GDOxp against gentisate was reduced to 18.5% in the presence of equimolar (0.33 mM) of 1-hydroxy 2-naphthoic acid, and 38% by salicylate, respectively. Comparable $K_m$ values for GDOxp against 1-hydroxy-2-naphthoate and gentisate indicate that the structure of the substrate binding site of GDOxp is more flexible than other GDOs, flexible enough to accept both mono- and polycyclic aromatic hydrocarbons as substrates. A unique salicylate dioxygenase (AAQ91293) has been reported from *Ps. salicylatoxidans* that is active against salicylate variants. It showed highest activity against 1-hydroxy-2-naphthoate.[19] The enzyme showed 19% and 18% activity against salicylate and gentisate respectively as compared with its activity against 1-hydroxy-2-naphthoate. Salicylate dioxygenase from *Ps. salicylatoxidans* and GDOxp shared 33% amino acid sequence identity, suggesting evolutional distance.

**Table 1.** Relative Activity of GDOxp against Several Substrates

| Substrate | relative activity (%) |
|---|---|
| Gentisate | 100[a] |
| Catechol | 0 |
| Benzoate | 0 |
| 5-Methylsalicylate | 0 |
| 5-Chlorosalicylate | 0 |
| 5-Aminosalicylate | 5 |
| Homogentisate | 0 |
| 1-Hydroxy 2-naphthoate | 2.9 |
| 1,4-Dihydroxy2-naphthoate | 102.8 |

[a]The activity against gentisate was considered to be 100%.

*Stability of GDOxp*

GDOxp lost its activity after being kept in buffers below pH 6.5 or above 8.0 for 12 h in the absence of ferrous ion and cysteine. In order to shed light on this unusual instability of GDOxp against pH, GDOxp, which had been dialyzed against 10 mM phosphate buffer (pH 9.0) for 12 h, was analyzed as to its structure by gel filtration chromatography. It was found that most of the protein existed as inactive monomers (39 kDa), and that these monomer proteins did not contain ferrous

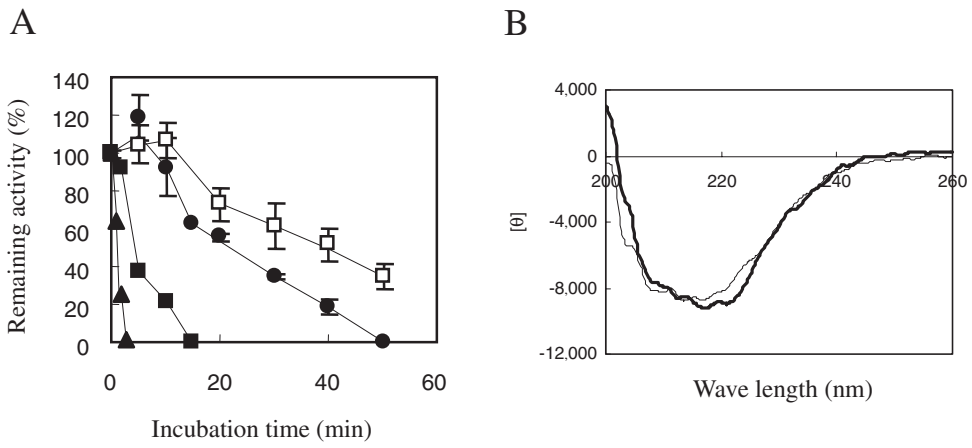


**Fig. 3.** Heat Treatment of GDOxp.

A, Reduction in the activity after heat treatment is shown. GDOxp (4.3 μM) in 10 mM MOPS buffer (pH 7.2) containing 10% glycerol was incubated at 46 °C (closed circle), 50 °C (closed square), or 55 °C (closed triangle) for appropriate times. A portion of the incubation sample was taken at the indicated times and the activity was assayed at 30 °C. GDOxp incubated at 50 °C in the presence of 20 μM ferrous ion is also shown (open square). B, CD spectra of GDOxp with no incubation (thick line) and after incubation for 1 h at 50 °C (thin line) were measured using J-720W.

ion (0.06 mol/mol subunit). GDOxp was also heat-unstable without ferrous ion (Fig. 3A). It lost almost all its activity after incubation at 50 °C for 15 min. However, far-UV CD spectra suggested that there was no significant change in the secondary structure of GDOxp even after incubation at 50 °C over 1 h (Fig. 3B). When the $\theta$ value at 218 nm was monitored while increasing the temperature at a rate of 1 °C/min, a dramatic irreversible change was observed between 70 and 80 °C. These results suggest that the backbone structure of GDOxp, including the cupin domains, is rather heat-stable.

*Role of ferrous ion in GDO activity and structure formation*

apo-GDOxp was prepared by a treatment of purified GDOxp (holo-GDOxp) with 10 mM EDTA and dialysis against buffer A to remove ferrous ion and EDTA. When apo-GDOxp was analyzed by gel filtration chromatography, most of the protein eluted at a position corresponding to inactive monomer protein without binding to ferrous ion (0.05 mol/mol subunit).

It was also found that inactive apo-GDOxp could be reactivated in the buffer containing ferrous ion. The initial reaction rates of apo-GDOxp under different ferrous ion concentrations exhibited sigmoidal curves (Fig. 4, Hill coefficient = 5.0). These results suggest that ferrous ion acted as a cooperative effector to form an active tetramer holo-enzyme. In order to test this possibility, the monomer apo-GDOxp was purified by gel filtration chromatography, concentrated to 0.65 mg in 2 ml buffer, and applied to the gel filtration column again with and without the addition of 80 μM ferrous ion. When ferrous ion was added to the fraction, about 90% of GDOxp was eluted as higher molecular weight species corresponding to a tetramer. Moreover, 68% of

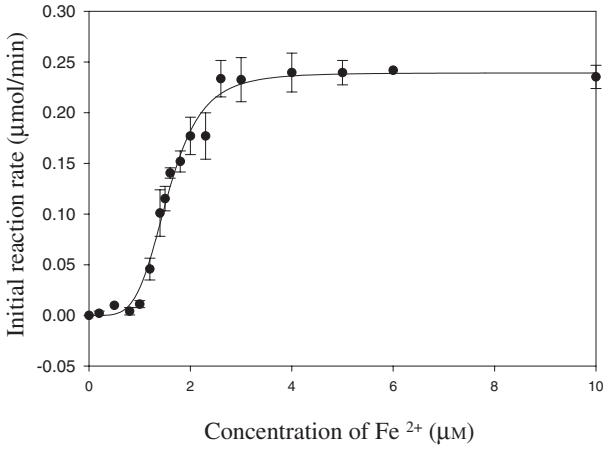


**Fig. 4.** Effect of Ferrous Ion on the Reactivation of Monomer Apo-GDOxp.

The activity of each 30.4 nM monomer apo-GDOxp was measured at different ferrous ion concentrations. The curve was fitted to the Hill equation.[13)]

the full activity was restored in this fraction, and the tetramer GDOxp contained 0.95 mole ferrous ion/mole subunit. The sample with no addition of ferrous ion was eluted again at the position of the monomer species. No reconstitution was observed with the addition of 2 mM cysteine or other divalent cations such as $Zn^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Mn^{2+}$, and $Ca^{2+}$. GDOxp was completely inactivated by 2 mM of ferric ion, and the addition of 1 mM copper ions formed a visible precipitate. These results indicate that ferrous ion specifically induces tetramerization and activation of monomer apo-GDOxp.

*Limited chymotryptic digestion of GDOxp*

In order to analyze the domain structure of GDOxp, it

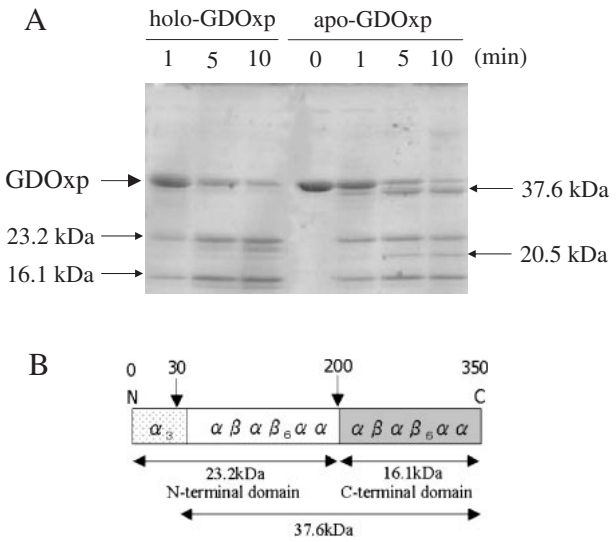


**Fig. 5.** Analysis of Domain Structure of GDOxp.

A, SDS–PAGE analysis of chymotryptic digestion at different treament times. The reaction was performed at 37 °C in 10 mM MOPS (pH 7.2) containing 2.5 mM DTT for 0 to 10 min at a ratio of chymotrypsin/GDOxp = 1:100 (w/w). Samples were boiled for 1 min to stop the reaction at the indicated times, and analyzed on 15% SDS–polyacrylamide gel. Arrows indicate 23.2 kDa and 16.1 kDa products corresponding to N-terminal and C-terminal cupin domains respectively. B, The position of cleavage by chymotrypsin is shown schematically. The 23.2 kDa and 16.1 kDa proteins were produced by cleavage between Y199 and G200. The 36.7 kDa protein was truncated at N-terminal 30 amino acids.

was partially digested with chymotrypsin. Peptide bonds between domains are generally more susceptible to proteolytic digestion than those within a domain, and limited proteolysis is a useful method to disconnect domains from one another. The time course of chymotrypsin digestion of GDOxp is shown in Fig. 5. Chymotrypsin digestion generated two major bands at 23.2 kDa and 16.1 kDa, suggesting that there are two structural domains in GDOxp. The N-terminal sequence of each product was determined to be $S^2$FAKA (amino acid redidues at 2 to 6) for the 23.2 kDa protein, and $G^{200}$ANLM (residues at 200 to 204) for the 16.1 kDa protein. This clearly indicates that a peptide bond between $Y^{199}$ and $G^{200}$ was cleaved by chymotrypsin. The first methionine, $M^1$, would have been processed in *E. coli* cells. The two-domain structure of GDOxp is consistent with a structure prediction that GDOxp is a member of the bicupin family with a flexible loop structure from 179–229 (Fig. 1). The C-terminal domain containing putative motif 3 and motif 4 should form a rigid structure like the N-terminal domain containing motif 1 and motif 2.

In order to obtain further insight into the subunit structure of GDOxp, chymotryptic digestion products from apo-GDOxp and holo-GDOxp were analyzed comparatively. When apo-GDOxp was digested with chymotrypsin, 37.6 kDa and 20.5 kDa protein bands were specifically observed. The N-terminal amino acid sequence of 37.6 kDa protein was determined to be $T^{30}$VLNNI, while that of 20.5 kDa protein was not determined. Because apo-GDOxp consists mainly of monomer protein, a hypothesis is that the flanking region of this sequence is one of the interfaces of the tetramer structure of GDOxp. We constructed another expression plasmid, pET25b-dGDOxp, for the production of truncated GDOxp in the N-terminal peptide (from $S^2$ to $W^{29}$) region. Heterologously produced dGDOxp formed monomers, although it contained 1.2 mol ferrous ion/mol protein. Monomeric dGDOxp showed no activity against gentisate. These results suggest that the N-terminal region is important for tetramerization, and also that the tetramerization of GDOxp is essential to form an active structure.

In this study, we found that GDOxp was fully functional against 1,4-dihydroxy-2-naphthoate as well as gentisate. Further experiments are necessary to demonstrate that GDOxp is a bi-functional enzyme in the PAHs and HACs degradation pathways of strain 127W. Monomer and tetramer structures were reversible. GDOxp formed an active tetramer structure by binding with ferrous ion in a cooperative manner.

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