Novel Heme-Containing Lyase, Phenylacetaldoxime Dehydratase from *Bacillus* sp. Strain OxB-1: Purification, Characterization, and Molecular Cloning of the Gene[†,‡]

Yasuo Kato,[§] Kazuhiro Nakamura,[§] Haruhiko Sakiyama,[§] Stephen G. Mayhew,[||] and Yasuhisa Asano*[,§]

Biotechnology Research Center, Toyama Prefectural University, Kosugi, Toyama 939-0398, Japan, and Department of Biochemistry, University College Dublin, Merville House, Belfield, Dublin 4, Ireland

Received July 12, 1999; Revised Manuscript Received November 2, 1999

ABSTRACT: A novel dehydratase that catalyzes the stoichiometric dehydration of *Z*-phenylacetaldoxime to phenylacetonitrile has been purified 483-fold to homogeneity from a cell-free extract of *Bacillus* sp. strain OxB-1 isolated from soil. It has a M_r of about 40 000 and is composed of a single polypeptide chain with a loosely bound protoheme IX. The enzyme is inactive unless FMN is added to the assay, but low activity is also observed when sulfite replaces FMN. The activity in the presence of FMN is enhanced 5-fold under anaerobic conditions compared to the activity measured in air. The enzyme has maximum activity at pH 7.0 and 30 °C, and it is stable at up to 45 °C at around neutral pH. The aerobically measured activity in the presence of FMN is also enhanced by Fe^{2+}, Sn^{2+}, SO_3^{2-}, and NaN_3. Metal-chelating reagents, carbonyl reagents, electron donors, and ferri- and ferrocyanides strongly inhibit the enzyme with K_i values in the micromolar range. The enzyme is active with arylalkylaldoximes and to a lesser extent with alkylaldoximes. The enzyme prefers the Z-form of phenylacetaldoxime over its E-isomer. On the basis of its substrate specificity, the enzyme has been tentatively named phenylacetaldoxime dehydratase. The gene coding for the enzyme was cloned into plasmid pUC18, and a 1053 base-pair open reading frame that codes for 351 amino acid residues was identified as the *oxd* gene. A nitrilase, which participates in aldoxime metabolism in the organism, was found to be coded by the region just upstream from the *oxd* gene. In addition an open reading frame (*orf2*), whose gene product is similar to bacterial regulatory (DNA-binding) proteins, was found just upstream from the coding region of the nitrilase. These findings provide genetic evidence for a novel gene cluster that is responsible for aldoxime metabolism in this microorganism.

In nature, aldoximes are considered to be intermediates in the biosynthesis of certain biologically active compounds (*1*). Indoleacetaldoxime (IAOx),[1] which is derived from tryptophan, is reported to be a precursor of indoleacetic acid (IAA), a natural auxin of higher plants (*2*). Several aldoximes derived from hydrophobic amino acids are thought to be intermediates of cyanogenic glucosides and glucosinolates (*1*, *3*). It has been proposed that an enzyme dehydrating an aldoxime to nitrile is active in the above biosynthetic pathway, but very little is known about the aldoxime dehydration enzyme; the enzyme has never been purified to homogeneity nor characterized in detail (*2*, *4-8*).

Asano et al. have isolated various nitrile-degrading microorganisms such as *Rhodococcus rhodochrous* (formerly *Arthrobacter* sp.) J-1 and I-9 (K22, AKU 629) (*9*), *Pseudomonas chlororaphis* B23 (*10*), and *Fusarium* sp. (*11*). Asano et al. discovered, purified, characterized, and named nitrile hydratase (EC 4.2.1.84) from *R. rhodochrous* J-1 (*12*−*14*). They have also demonstrated that *P. chlororaphis* B23 accumulates a large amount of amides from nitriles and is suitable for the industrial production of acrylamide from acrylonitrile (*10*, *14*). Despite the importance of the nitrile-hydrolyzing enzymes for industrial use (*10*, *15*, *16*), there has been no report concerning the biosynthesis of nitrile compounds and the physiological function of the enzymes.

We are studying enzymatic dehydration of aldoxime not only to elucidate its mechanism but also to apply the enzyme to organic synthesis since the chemical dehydration of aldoximes usually requires harsh reaction conditions (*17*, *18*). It is also of our research interest to study a possible relationship between the enzyme and nitrile-hydrolyzing enzymes and the function in the microbial nitrile biosynthesis. We screened for various aldoxime-degrading microorganisms from soil and isolated *Bacillus* sp. strain OxB-1 (*19*) and *Rhodococcus* sp. strain YH3−3 (*20*), which degrades *Z*-phenylacetaldoxime (PAOx) and *E*-pyridine-3 aldoxime, respectively. Both of the isolated strains metabolized aldoximes through nitriles into the corresponding carboxylic acid by a combination of aldoxime dehydration and nitrile-hydrolyzing enzymes (*19*, *20*) (Figure 1). The

[†] This work was supported in part by a Grant-in-Aid for "Research for the Future" (JSPS-RFTF 96I00302) from the Japan Society for the Promotion of Science.

[‡] The nucleotide sequence reported in this paper has been submitted to the GenBank/EBI Data Bank with the accession number AB028892.

* To whom correspondence should be addressed. Phone: +81-766-56-7500. Fax: +81-766-56-2498. E-mail: asano@pu-toyama.ac.jp.

[§] Toyama Prefectural University.

[||] University College Dublin.

[1] Abbreviations: PAOx, phenylacetaldoxime; PAN, phenylacetonitrile; IAOx, indoleacetaldoxime; IAA, indoleacetic acid; IAN, indoleacetonitrile; IPTG, isopropyl-1-thio-β-D-galactoside; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; LB, Luria-Bertani; PQQ, pyrroloquinoline quinone; PLP, pyridoxal-5′-phosphate.

10.1021/bi991598u CCC: $19.00
Published on Web 01/04/2000

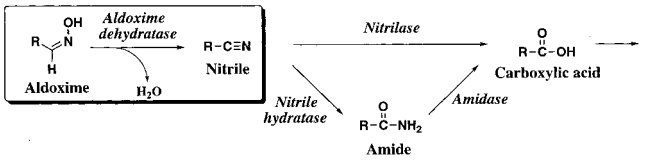

FIGURE 1: Microbial metabolism of aldoxime. *Bacillus* sp. strain OxB-1 metabolizes *Z*-phenylacetaldoxime (R = $PhCH_2$) to form phenylacetonitrile, which is successively hydrolyzed to phenylacetic acid by the action of nitrilase (*19*). On the other hand, *Rhodococcus* sp. strain YH3-3 metabolizes *E*-pyridine-3-aldoxime (R = 3-pyridyl) as follows: the aldoxime is dehydrated to form 3-cyanopyridine, which is converted to nicotinamide by nitrile hydratase, and the nicotinamide is successively hydrolyzed to nicotinic acid by amidase (*20*).

dehydration enzyme from *Rhodococcus* sp. strain YH3-3 was applied to the enzymatic synthesis of nitriles under mild conditions (*21*). A nitrile hydratase responsible for aldoxime metabolism was purified and characterized from the *Rhodococcus* strain, and its properties were compared with the known nitrile hydratases (*22*).

In this paper, we describe the purification and characterization of the novel dehydration enzyme, termed phenylacetaldoxime dehydratase, from the cell-free extract of *Bacillus* sp. strain OxB-1 as well as the cloning and base sequencing of its gene.

MATERIALS AND METHODS

Materials. ^1H and ^{13}C NMR spectra were recorded with JEOL EX-400 or LA-400 spectrometers (Tokyo, Japan) using tetramethylsilane as an internal standard. HPLC columns ODS-80Ts and G-3000SW, and gels for column chromatography DEAE- and Butyl-Toyopearl 650M were purchased from Tosoh (Tokyo, Japan). Gigapite was from Seikagaku Kogyo (Tokyo, Japan). Standard proteins for high-performance gel filtration chromatography and SDS−polyacrylamide gel electrophoresis (PAGE) were obtained from Oriental Yeast (Tokyo, Japan) and Pharmacia (Uppsala, Sweden), respectively. Restriction enzymes and DNA-modifying enzymes were purchased from Takara (Tokyo, Japan), Toyobo (Osaka, Japan), New England Biolabs. Inc. (Beverly, MA), and MBI Fermentas (Vilnius, Lithuania) and used according to the manufacturers' protocols. Shrimp alkaline phosphatase was from Boehringer Mannheim GmbH (Germany). All other chemicals were from commercial sources and used without further purification.

Analytical Methods. Protein concentration was determined with a Bio-Rad protein assay kit (Hercules, CA) (*23*) with bovine serum albumin as a standard or by measuring the absorbance at 280 nm. Native− and SDS−PAGE was carried out as described by Davis (*24*) and Laemmli (*25*), respectively, with an electrophoresis unit from ATTO (Tokyo, Japan). The molecular weight (M_r) of the enzyme was determined as described previously (*26*). The M_r was also estimated by matrix-assisted laser desorption ionization (MALDI)−time-of-flight (TOF) mass spectrometry on a Voyager DERP instrument (PerSeptive Biosystems, Framingham, MA). Heme staining was carried out as described by Klatt et al. (*27*). The absorption and fluorescence spectra were recorded on a HITACHI U-3210 UV−vis and a HITACHI F-4500 fluorescence spectrophotometers (Tokyo, Japan), respectively. Heme was determined by the pyridine hemochromogen method (*28*) using the molecular extinction coefficient of the α-peak at 557 nm of the hemochromogen of protoheme IX (ϵ = 34.4 mM^{-1} cm^{-1}) (*29*). The iron content of the enzyme was measured with an atomic absorption flame emission spectrophotometer (Nippon Jarrell-Ash, Kyoto, Japan).

Synthesis of Substrates. Aldoximes were synthesized as described (*17*−*20*). *Z*-PAOx was prepared from phenylacetaldehyde as colorless needles in a yield of 38.0%; ^1H NMR ($CDCl_3$) δ (ppm) 9.89 (br, 1H), 7.28−7.32 (m, 2H), 7.20−7.24 (m, 3H), 6.91 (t, 1H, J = 5.4 Hz), 3.74 (d, 2H, J = 4.8 Hz); ^{13}C NMR ($CDCl_3$) δ (ppm) 151.1, 136.9, 129.1, 129.0, 126.9, 32.0.

Bacterial Strains, Plasmids, and Culture Conditions. Bacillus sp. strain OxB-1, isolated from soil and identified in our laboratory (*19*), was used throughout this study. It was cultured in a nutrient medium that consisted of 1.0% meat extract (Kyokuto, Tokyo, Japan), 1.0% Polypepton (Nippon Seiyaku, Tokyo, Japan), and 0.5% NaCl, pH 8.0. A loopful of the strain, grown on a nutrient agar plate at 30 °C for 2−3 days, was inoculated into 5 mL of nutrient medium containing 0.05% *Z*-PAOx and incubated with shaking at 30 °C for 12 h. Five milliliters of the culture was added to 500 mL of an enzyme production medium that consisted of 0.4% sodium malonate, 0.1% *Z*-PAOx, 0.05% yeast extract, 0.2% $(NH_4)_2HPO_4$, 0.2% K_2HPO_4, 0.1% NaCl, 0.02% $MgSO_4 \cdot 7H_2O$, and 1% each of vitamin mixture and trace element solution (*19*), pH 8.0, in a 2-L Sakaguchi flask. The cultivation was carried out at 30 °C for 24 h with shaking and cells were harvested by centrifugation (15000*g*, 1 min). *Escherichia coli* XL1-Blue MRF′ {Δ(*mcrA*)*183*, Δ (*mcrCB-hsdSMR-mrr*)*173 endA1*, *supE44*, *thi-1*, *recA1*, *gyrA46*, *relA1*, *lac*[F′, *proAB*, *lacIqZΔM15*, Tn10(Tet^r)]} (Toyobo) was used as a host strain for gene cloning and in most DNA manipulations. Plasmids pUC18 and pWE15 were used as a cloning vector. Recombinant *E. coli* cells were cultivated at 37 °C in Luria-Bertani (LB) medium [1% Bacto Tryptone (Difco, Detroit, MI), 0.5% Bacto yeast extract (Difco), and 1% NaCl, pH 7.5] containing 100 μg of ampicillin/mL. For an induction of the gene under the control of *lac* promoter, 1 mM of isopropyl-1-thio-β-D-galactoside (IPTG) was added to the LB medium. To select the recombinant cells harboring pUC18 with inserted DNA, 0.01% (wt/vol) 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was added to the LB agar (2%, wt/vol) medium.

Enzyme Assay. The standard assay contained 50 μmol of potassium phosphate buffer, pH 7.0, 125 nmol of FMN, 2.5 μmol of *Z*-PAOx, and the enzyme in a total volume of 500 μL. The mixture without the substrate was incubated for 1 min at 30 °C before starting the reaction by addition of *Z*-PAOx. The reaction was stopped after 10 min by addition of 500 μL of 0.5 M H_3PO_4, and supernatant was obtained by centrifugation (18000*g*, 10 min). The reaction product was determined by HPLC (Waters, MA) at 254 nm, with an ODS-80Ts column (4.6 × 150 mm) at a flow rate of 1.0 mL/min using an elution solvent consisting of 10 mM of H_3PO_4 in 40% CH_3CN (vol/vol). One unit of activity is defined as the amount of enzyme that produces 1 μmol of PAN/min. In an experiment to determine the stoichiometry of the enzyme reaction, the reaction was stopped with an equal volume of 2-propanol.

The substrate specificity was examined in the same assay and using the same analysis procedure. Aliphatic aldoximes

and nitriles were analyzed by gas−liquid chromatography (GLC; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector with a glass column (2 mm by 2 m) packed with poly(ethylene glycol) (PEG20M, 60−80 mesh; GL-Science, Tokyo, Japan).

Assays were also carried out under anaerobic conditions (*30*). The substrate was placed in a sidearm on the cuvette while making the cuvette anaerobic by 5 cycles of evacuation and filling with nitrogen. The cuvette was incubated at 30 °C for 1 min before tipping in the substrate to start the reaction. The reaction was stopped by addition of 500 mM H_3PO_4. The effect of reducing the FMN in the assay mixture before starting the reaction was studied by including EDTA (1 mM) in the assay. After making the cuvette anaerobic, it was exposed to the light from a 250 W slide projector until the FMN was fully reduced, as judged from the absorption spectrum (approximately 30 s).

Spectral Titration of PAOx Dehydratase with FMN and $Na_2S_2O_4$. The effect of FMN on the absorption spectrum of PAOx dehydratase was investigated using double sector cuvettes. The front compartment of the sample and reference cuvettes contained 30 μM PAOx dehydratase in 0.1 M potassium phosphate buffer, pH 7.0. The rear compartments contained 0.1 M potassium phosphate buffer, pH 7.0. FMN was added to the front compartment of the sample cuvette and the rear compartment of the reference cuvette. Buffer was then added to the front compartment of the reference cuvette before recording a difference spectrum. The effect of PAOx dehydratase on the fluorescence of FMN was examined by adding aliquots of a 20 μM solution of enzyme to 2 mL of 1 μM FMN in 0.1 M potassium phosphate buffer, pH 7.0, and 0.3 mM EDTA. The fluorescence of the FMN was measured after each addition using exciting light at 445 nm and emission at 525 nm.

The effect of $Na_2S_2O_4$ on the visible absorption spectrum of PAOx dehydratase was examined by using an anaerobic cuvette that allowed incremental additions of $Na_2S_2O_4$ (*30*).

Purification of PAOx Dehydratase. All purification procedures were performed at 4 °C. Potassium phosphate buffer, pH 7.0, containing 5 mM 2-mercaptoethanol was used throughout the purification. The washed cells of *Bacillus* sp. (59.4 g wet weight obtained from 30 L of culture) were suspended in 300 mL of the 100 mM buffer containing 1 mM of dithiothreitol. The cells were disrupted for 10 min by a Kubota-shoji 9 kHz sonic oscillator (Tokyo, Japan) and centrifuged (18800*g*, 20 min) to remove the disrupted cells. The pellet formed between 40 and 70% ammonium sulfate saturation was dissolved in the 10 mM buffer and dialyzed against five changes of 10 L of the same buffer. The enzyme solution was put on a DEAE-Toyopearl column (4.2 × 30 cm) equilibrated with the 10 mM buffer, and the enzyme was eluted with the 100 mM buffer. The active fractions were collected and then brought to 30% ammonium sulfate saturation. The enzyme solution was placed on a Butyl-Toyopearl column (2.5 × 30 cm) which had been equilibrated with the 10 mM buffer containing 30% saturated ammonium sulfate. The active fractions that were eluted with a linear gradient of ammonium sulfate (30 to 0% saturation) in the 10 mM buffer were combined and dialyzed. The enzyme solution was put on a Q-Sepharose 35/100 column (Pharmacia) equilibrated with the 10 mM buffer. The column was eluted with a linear gradient of the 10 to 100 mM buffer containing 200 mM NaCl (1 L each) at a flow rate of 10 mL/min in a Bio-Pilot System (Pharmacia). The active fractions were collected, dialyzed, and concentrated to about 10 mL with an Amicon membrane filter apparatus (Amicon, Beverly, MA) equipped with a YM-30 membrane. The enzyme solution was loaded on a Gigapite column (2 × 15 cm) equilibrated with the 10 mM buffer. The enzyme was eluted with the 10 mM buffer, and the active fractions were collected.

Preparation of Internal Peptides and Amino Acid Sequencing. The purified PAOx dehydratase was subjected to SDS−PAGE and stained with Coomassie Brilliant Blue R-250. Five pieces of the gel that contained the stained enzyme were excised with a razor blade and digested with lysyl-endopeptidase (Wako Pure Chem., Osaka, Japan). Digestion was carried out with 100 mM Tris-HCl, pH 9.0, in the presence of 0.1% SDS at 37 °C for 12 h. Proteolytic peptides were isolated by HPLC on the ODS-80Ts column using a linear gradient of CH_3CN (20 to 70% for 80 min) containing 0.1% TFA at a flow rate of 0.5 mL/min. To determine the amino acid sequences, the native enzyme and the isolated internal peptides were covalently bound to Sequelone-arylamine and -diisothiocyanate membranes and then analyzed with a Prosequencer 6625 automatic protein sequencer (Millipore, MA) as described previously (*25*).

General Recombinant DNA Techniques. Plasmid DNA was isolated by an automatic plasmid isolation system PI-100 (Kurabo, Osaka, Japan) or by a plasmid purification kit from Qiagen (Valencia, CA). Transformation of *E. coli* with plasmid DNA by electroporation was carried out under standard conditions, using a BTX ECM 600 electroporation system (Biotechnologies and Experimental Research Inc., San Diego, CA). Other general procedures were performed as described by Sambrook et al. (*31*).

Cloning of the oxd Gene. Several degenerate primers were synthesized on the basis of the determined *N*-terminal and internal peptide fragments of the enzyme. Chromosomal DNA of *Bacillus* sp. strain OxB-1, isolated by the method of Saito and Miura (*32*), was amplified by the polymerase chain reaction (PCR) using a thermal cycler (Perkin-Elmer Cetus Instruments, Norwalk, CT). Reaction mixtures contained 1 μg of chromosomal DNA, 100 pmol of each primer, and *Taq* DNA polymerase (Boehringer) in a volume of 50 μL. Thirty-five thermal cycles were employed, each consisting of 95 °C for 1 min, 50 °C for 1.5 min, and 72 °C for 2.5 min. All combinations of sense and antisense primers were tested for PCR amplification. Only the combination of the sense primer I [5′-GAYMGRGCNYTNYAYCARGGNGC-NGAYGG-3′] and antisense primer II [5′-RTCNACDATYTC-NCCRTCRTGNGTYTGYTCRTANAC-3′], which were prepared based upon the internal sequence DRAL(H/Y)QGADG and VYEQTHDGEIVD, respectively, amplified a 600 bp fragment. Degenerate positions are indicated by Y for C or T, R for A or G, M for A or C, D for A, G, or, T, and N for all bases. The gel-purified PCR-product was cloned into the *Sma*I site of pUC18 replicative form DNA and designated as pOxD-1. The PCR product was purified from the plasmid and was further used as a radiolabeled probe to clone the full-length PAOx dehydratase gene.

Chromosomal DNA was partially digested with *Sau*3AI and size fractionated on a sucrose density gradient (10 to 40%) in a Beckman L-70 ultracentrifuge (Beckman Instru-

ments, Palo Alto, CA) at 100000*g* for 16 h. Fragments in the molecular size of around 40 kbp were collected and ligated with a cosmid vector pWE15, which had been digested and dephosphorylated with *Bam*HI and alkaline phosphatase, respectively. The ligated DNA was packaged in vitro with a Gigapack Plus Packaging Kit (STRATAGENE, La Jolla, CA) and transduced into *E. coli* XL1-Blue MRF′ to construct a genomic library of *Bacillus* sp. strain OxB-1. A total of 500 transformants were replicated onto sterilized nitrocellulose filter (Advantec, Tokyo, Japan) and grown overnight at 37 °C. Colonies were lysed by the alkaline method (*31*). Colony hybridization and Southern blot hybridization analysis utilized the [α-^{32}P]dCTP-labeled 600 bp DNA fragment as a probe. Filters were washed twice with a buffer containing 0.3 × SSC (45 mM NaCl and 4.5 mM sodium citrate) and 0.2% SDS for 30 min at 65 °C. Recombinant cosmids from positive clones were isolated, analyzed, and used for subcloning the PAOx dehydratase gene into the pUC18.

Base Sequence Analysis. The clone, pOxD-5C, carrying the 4.5 kb fragment was selected and used as sequencing template. The nucleotide sequence was terminated by the dideoxy-chain termination method (*33*) with SequiTherm EXCELII Long-Read DNA Sequencing Kit LC (Epicenter Technologies, Madison, WI) or Thermo Sequenase Cycle Sequencing Kit (Amersham, Cleveland, OH) and a 4000L DNA autosequencer (LI-COR, Lincoln, NB). A homology search was performed by the sequence similarity searching programs FASTA (*34*) and BLAST (*35*). The ClustalW method was used to align the sequences (*36*).

Expression of the oxd Gene in E. coli. To obtain the entire genes without excessive flanking parts and to change the start codons from TTG to ATG, PCR amplification was carried out in a 50 μL reaction mixture containing 100 pmol of each primer, 25 ng of pOxD-5C, and 2.5 units of *Pwo* DNA polymerase (Boehringer) at 94 °C for 1 min, 60 °C for 2 min, and 72 °C for 3 min for a total of 30 cycles. The primers contained a Shine-Dalgarno sequence (lowercase letters), ATG initiation codon (bold letters), and restriction site (underlined sequence). The sense and antisense primers had the respective sequences 5′-GAACACTGCAGAAaggaggATTAGTA**ATG**AAAAATATGCCGG-3′ and 5′-AGTTTCAAGCTTTCACTGGATCCTG-3′, respectively. The amplified PCR product was digested with the required restriction enzymes, separated, and purified by agarose gel electrophoresis. The amplified DNA, which contained the complete coding sequence of the *oxd* gene, was inserted downstream from the *lac* promoter in pUC18 and the constructed plasmid was designated pOxD-Oxd and then used to transform *E. coli* XL1-Blue MRF′ cells.

RESULTS AND DISCUSSION

Purification of PAOx Dehydratase. The enzyme was purified 483-fold with a 47% yield from the cell-free extract of *Bacillus* sp. strain OxB-1 grown in the enzyme production medium containing 0.1% of *Z*-PAOx. To the best of our knowledge, this is the first report of the purification and characterization of an enzyme that dehydrates aldoxime. A summary of the purification procedure for the enzyme is shown in Table 1. The enzyme was judged to be homogeneous by SDS−PAGE and HPLC on a G-3000SW column,

Table 1: Purification of Phenylacetaldoxime Dehydratase from *Bacillus* sp. Strain OxB-1

step	total protein (mg)	total activity (units)	specific activity (units/mg)	yield (%)
cell-free extract	8360	148	0.0177	100
ammonium sulfate	3690	154	0.0417	104
DEAE-Toyopearl	338	140	0.414	94.6
Butyl-Toyopearl	35.0	132	3.77	89.1
Q-Sepharose 35/100	21.1	122	5.76	82.2
Gigapite	8.14	69.6	8.55	47.0

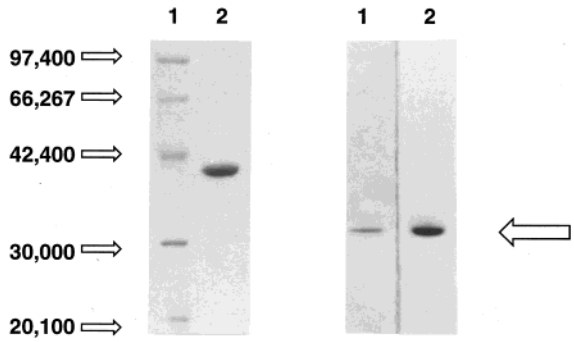

FIGURE 2: (A) SDS− and (B) native−polyacrylamide slab gel electrophoresis of purified phenylacetaldoxime dehydratase. (A) Lane 1, standards: phosphorylase (M_r = 97 400), bovine serum albumin (66 267), aldolase (42 400), carbonic anhydrase (30 000), and soybean trypsin inhibitor (20 000). Lane 2, purified phenylacetaldoxime dehydratase (10 μg). The enzyme was stained with Coomassie Brilliant Blue. (B) The enzyme was stained with Coomassie Brilliant Blue (lane 1) and with 3,3′-dimethoxybenzidine/H_2O_2 (heme staining, lane 2).

as each of these procedures yielded a single band or a single peak. The M_r of the subunit was calculated to be 40 000 by comparing the mobility on SDS−PAGE to that of standard proteins (Figure 2A). The M_r of the native enzyme was 42 000 according to gel filtration chromatography, indicating that the native enzyme is a monomer. The enzyme showed two molecular ion peaks with M_r of 39 900 and 40 100 on MALDI-TOF mass spectrometry. Ampholytic isoelectricfocusing with the Phast System (Pharmacia) yielded only one band and its pI value was 3.04.

Optical Spectrum and Prosthetic Group. Figure 3A shows the absorption spectrum of the enzyme. It has maxima at 280, 356, and 423 nm and smaller absorption bands around 520−580 nm, suggesting that the enzyme contains heme. The A_{423}/A_{280} absorption ratio is 1.55. The absorption spectrum is not affected by the addition of the reducing reagents $Na_2S_2O_4$, 2-mercaptoethanol, dithiothreitol, cysteine, ascorbic acid, and $NaBH_4$, or the oxidizing reagents $KMnO_4$ and ferricyanide. Dithionite ion ($S_2O_4^{2-}$) reacts rapidly with molecular oxygen, and therefore, experiments with $Na_2S_2O_4$ were carried out anaerobically, as described in Materials and Methods. The absorption spectrum of the enzyme is also not changed by addition of *Z*-PAOx and PAN. The ability of the enzyme to react with the common heme ligands such as CO and KCN was investigated but no spectral shifts were seen. To examine whether the heme constitutes an integral part of the purified enzyme, heme-staining was carried out after native PAGE (Figure 2B). The gel was stained with 3,3-dimethoxybenzidine/H_2O_2 (*27*) and Coomassie Brilliant Blue for heme and protein, respectively. Both methods gave

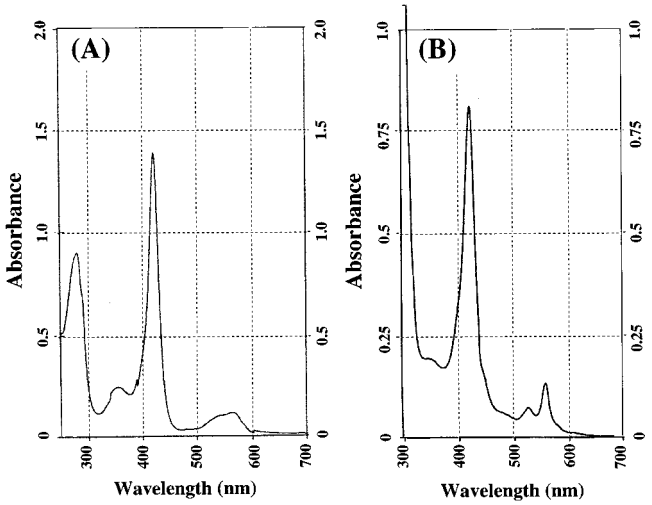

FIGURE 3: Absorption spectra of phenylacetaldoxime dehydratase. (A) Absorption spectrum of the enzyme (0.9 mg/mL) in 10 mM potassium phosphate buffer, pH 7.0. (B) Absorption spectrum of pyridine hemochromogen of the enzyme, prepared by mixing the enzyme solution (1.75 mg/mL) in 10 mM potassium phosphate buffer with 3 vol of pyridine containing 0.1 N KOH.

a single band, and the positions of the bands were the same.

To examine the properties of the heme in the enzyme, the pyridine hemochrome was prepared by mixing the enzyme with alkaline-pyridine. As shown in Figure 3B, the absorption maxima of the hemochromogen occur at 418 (Soret), 524 (β-band), and 556 (α-band), and are characteristic of the reduced pyridine hemochrome of protoheme IX (*29*). Treatment of the hemochromogen with various reducing reagents showed no change in the spectrum. On the other hand, the addition of 1 mM ferricyanide caused the immediate disappearance of the α- and β-bands of the hemochromogen, and the absorption maximum of the Soret band shifted to 398 nm with a decrease of its intensity, suggesting that the hemochromogen was converted to the oxidized form with some release of the heme molecule. The absorption spectra of the hemochromogen with the common heme ligands were also determined. With CO, the Soret peak decreased and a new shoulder at 430 nm appeared with simultaneous disappearance of the α- and β-bands. On the other hand, no significant change in the spectrum was observed with 1 mM KCN. On the basis of the results, we infer that the heme iron is present in the ferrous state. This conclusion is supported by the observation that the absorption spectrum of the enzyme is not affected by anaerobic treatment with $Na_2S_2O_4$.

The heme prosthetic group was extracted from the enzyme by HCl/acetone-treatment (*29*), and the extract then concentrated in a stream of argon. The spectrum of the pyridine hemochromogen in the extract was identical with that of the hemochromogen shown in Figure 3B. The extractability of the chromophore with acid-acetone and the shape of its pyridine hemochromogen spectrum suggests that the chromophore is protoheme IX.

Quantitative analysis for iron using atomic absorption spectroscopy revealed that the enzyme contains 0.35 g of atom iron/mol. The heme content of the enzyme, determined from the spectrum of its pyridine hemochromogen, was calculated to be 0.35 mol/mol. A similar value was determined for the iron in the HCl/acetone extracted pyridine hemochromogen. On the basis of these results, it is concluded that the enzyme contains iron and heme in the ratio of 1:1 and that all of the iron is probably present in the heme molecule. However, the heme content of the preparation is not equimolar with the protein, suggesting that the enzyme loses heme during the purification procedure. It was reported that a part of the heme in P-450 BM3 is similarly lost during purification and that the purified enzyme contained 0.55 mol of heme/mol of enzyme (*37*). After 4 months storage of PAOx dehydratase at 4 °C, the heme content was found to decrease below 15% in proportion to the activity loss (data not shown), indicating that the heme and the enzyme activity are lost in parallel. On the basis of these observations, it is concluded that heme is necessary for the enzyme activity. Addition of Fe^{2+} to the purified enzyme or the pyridine hemochromogen caused no change in the spectrum, suggesting that the external addition of Fe^{2+} does not reconstitute the apo-protoporphyrin IX in the enzyme preparation. The observation that the ferricyanide treatment causes the loss of intensity of the Soret band suggests that the heme is lost from the enzyme by its oxidation and the stability of the heme in the enzyme depends on its oxidation state. Further work is in progress to find conditions to protect the heme of PAOx dehydratase during the enzyme purification.

Table 2: Effect of Anaerobic Conditions, Oxidation State of FMN, and Addition of SO_3^{2-} on the Reaction[a]

condition	oxidation state of FMN	addition	specific activity[b] (units/mg)	K_m[c] (μM)	V_{max}[c] (units/mg)
aerobic	oxidized	none	5.4	30.0	5.85
aerobic	—[d]	none	ND[e]	NM[f]	
anaerobic	oxidized	none	28.5	30.8	32.3
anaerobic	—	none	ND	NM	
anaerobic	reduced[g]	none	ND	NM	
anaerobic	reduced[h]	none	2.67	NM	
aerobic	oxidized	Na_2SO_3	20.4	NM	
anaerobic	oxidized	Na_2SO_3	12.2	NM	
aerobic	—	Na_2SO_3	11.3	308	19.2
anaerobic	—	Na_2SO_3	0.74	NM	

[a] Enzyme activities were measured as described in Materials and Methods. FMN was added to 0.25 mM as indicated; sodium sulfite was added to 0.5 mM as indicated. [b] The specific activity is the activity with 0.25 mM FMN and/or 0.5 mM sulfite as indicated in columns 2 and 3. [c] Values for the apparent K_m and apparent V_{max} were obtained from Lineweaver−Burk plots by varying the concentrations of FMN or Na_2SO_3. The values given in lines 1 and 3 refer to FMN while those in line 9 refer to Na_2SO_3. [d] A dash indicates that FMN omitted from assay. [e] ND indicates that no activity was detected. [f] NM indicates that a measurement was not made. [g] The FMN was photoreduced with 1.5 mM EDTA in the assay. [h] The FMN was reduced with 0.5 mM $Na_2S_2O_4$ in the assay.

Effect of FMN on the Enzyme Activity. It was observed that the activity is lost during the purification procedure and that the activity is restored by addition of FMN to the assay. Most of the activity loss occurs during the dialysis of the enzyme after the salt fractionation, and the final preparation is completely devoid of activity if FMN is omitted from the assay (Table 2). FMN was therefore present in the standard enzyme assay. By measuring the enzyme activity with different FMN concentrations in the assay, the apparent K_m for FMN was estimated to be 30.8 μM. The result of this kinetic experiment suggests that the enzyme interacts rather weakly with FMN. However, attempts to obtain direct evidence for a complex of FMN and enzyme, as described

Table 3: Kinetic Parameters for Various Aldoximes of Phenylacetaldoxime Dehydratase

aldoxime[a]	K_m (mM)	V_{max} (units/mg)	V_{max}/K_m (units/mg/mM)	relative activity (%)[b]
Z-phenylacetaldoxime	0.872	19.5	22.4	100
Z-3-phenylpropionaldoxime	1.36	14.3	10.5	62.9
E/*Z*-4-phenylbutyraldoxime	5.24	3.35	0.639	6.79
Z-*p*-chlorophenylacetaldoxime	1.24	1.62	1.31	7.35
Z-*p*-methoxyphenylacetaldoxime	3.08	2.37	0.769	6.84
E/*Z*-indoleacetaldoxime	2.40	5.42	2.26	18.1
Z-naphthoacetaldoxime	0.846	0.867	1.02	4.48
E/*Z*-propionaldoxime	4.32	3.28	0.759	8.2
E/*Z*-*n*-butyraldoxime	11.1	9.49	0.855	10.9
E/*Z*-*n*-valeraldoxime	2.42	12.6	5.21	39.3
E/*Z*-isovaleraldoxime	3.58	7.72	2.16	20.2
E/*Z*-*n*-capronaldoxime	6.12	32.3	5.28	57.1
E/*Z*-isocapronaldoxime	2.98	10.1	3.39	29.4

[a] The following aldoximes were inert as substrates: arylalkyl-aldoximes, such as *E*/*Z*-2-phenylpropionaldoxime, *E*/*Z*-cinnamaldehyde oxime, *E*/*Z*-*p*-hydroxyphenylacetaldoxime, *Z*-*p*-toluacetaldoxime, *E*/*Z*-thiophene-2-acetaldoxime, *E*/*Z*-diphenylacetaldoxime, and *E*/*Z*-mandelaldoxime; aryl-aldoximes such as *E*-benzaldoxime, *E*-*p*-chlorobenzaldoxime, *E*-*p*-tolualdoxime, *E*-1-naphthoaldoxime, *E*-furfurylaldoxime, *E*-thiophene-2-carboxaldoxime, *E*-anisaldoxime, *E*-indole-3-carboxaldehyde oxime, *E*-quinoline-2-carboxaldehyde oxime, *E*-terephthalaldehyde oxime, *E*-isophthalaldehyde oxime, and *E*-pyrazinecarboxaldoxime; alkyl-aloximes, such as *E*/*Z*-isobutyraldoxime, *Z*-crotonaldoxime, *E*/*Z*-methacrylaldoxime, *E*/*Z*-cyclohexanecarboxaldehyde oxime; and *Z*-phenylacetaldoxime derivatives, such as *E*/*Z*-*O*-methyl phenylacetaldoxime, *E*/*Z*-*O*-benzyl phenylacetaldoxime, *E*-phenylacetaldehyde hydrazone, *E*/*Z*-*O*-acetyl-phenylacetaldoxime, *E*/*Z*-phenylacetone oxime, and *E*/*Z*-acetophenone oxime. [b] Measured at 1 mM concentration of the substrate.

in Materials and Methods, were not successful. Addition of enzyme to FMN caused no change in the fluorescence of the flavin, and the optical spectrum of a mixture of FMN and enzyme was the same as the sum of the spectra of the two components. Similarly, there is no evidence that the enzyme as isolated contains FMN because neither the native enzyme nor the extract of the enzyme produced by heat treatment of a 30 μM solution at 95 °C for 10 min shows fluorescence typical of that of FMN. Hence, the precise nature of the FMN requirement is unclear.

The flavin requirement appears to be specific for FMN because no activity was observed when riboflavin or FAD was substituted for FMN. Similarly, other enzyme cofactors including pyrroloquinoline quinone (PQQ), pyridoxal-5′-phosphate (PLP), and NAD(P)(H) neither substituted for FMN nor enhanced the activity in the presence of FMN. The role of FMN in the reaction is not clear because the overall reaction does not involve oxidation−reduction. However, it has been observed that the activity in the presence of oxidized form of FMN increases by over 5-fold when the assay is carried out under anaerobic conditions as compared with the activity measured in air (Table 2). It also appears that the flavin is required in the oxidized form because, when it is photoreduced in the presence of EDTA (*37*), the enzyme is inactive (Table 2). It is possible that the flavin is transiently reduced during the catalytic cycle of the enzyme reaction. If this occurs, a large fraction of the reducing equivalents transferred to the flavin might be lost under aerobic conditions by a subsequent chemical oxidation of the reduced flavin by oxygen. Use of $S_2O_4^{2-}$ to reduce the FMN in the assay also causes the activity to decrease, but in this case, the activity is not completely lost. It seemed possible that the sulfite ion (SO_3^{2-}) produced by oxidation of $S_2O_4^{2-}$ could affect the reaction. Tests in which Na_2SO_3 was used in place of FMN showed that activity is observed under these conditions, although with a smaller value for V_{max} compared with the activity with anaerobic FMN (Table 2). The activity in the presence of SO_3^{2-} is decreased when the assay is carried out anaerobically. This decrease, which is most marked when the assay is done in the absence of FMN, is not understood. The fact that SO_3^{2-} can replace FMN suggests that SO_3^{2-} can act as an alternative electron acceptor during catalysis.

Stoichiometry of the Dehydration Reaction of Z-PAOx. The stoichiometry of the enzyme reaction was examined with a reaction mixture containing 100 μmol of potassium phosphate buffer (pH 7.0), 0.25 μmol of FMN, 5 μmol of *Z*-PAOx, and 1 unit of the purified enzyme in a total volume of 1 mL. After an incubation at 30 °C, the reaction mixture was sampled at appropriate intervals and added to an equal volume of 2-propanol to stop the reaction, and the amount of *Z*-PAOx and PAN in the mixture was determined with HPLC. PAN was observed as the only product, and the amount of *Z*-PAOx consumed and PAN formed were equal at any reaction period. The result shows that the enzyme catalyzes the stoichiometric dehydration reaction of *Z*-PAOx into PAN. The enzyme did not catalyze a hydration reaction of PAN under any conditions tested. This is not too surprising because the free energy of *Z*-PAOx is calculated to be more than 2.21 kcal/mol greater than that of PAN (calculations made with the program SPARTAN 4.1; Wave function Inc., Irvine, CA), showing that the enzymatic dehydration reaction is thermodynamically more favorable.

Substrate Specificity and Kinetic Properties. To determine the substrate specificity, various aldoximes were synthesized and used in place of *Z*-PAOx (1 mM) under the standard assay conditions. As shown in Table 3, the enzyme is active against several *Z*-aryl-acetaldoximes and *E*/*Z*-alkyl-aldoximes to give the corresponding nitriles. The enzyme is also active toward arylalkyl-aldoximes such as 3-phenylpropionaldoxime and 4-phenylbutyraldoxime. However, phenylacetaldoximes having a substituent group at an α-site of an oxime group, such as *E*/*Z*-2-phenylpropionaldoxime and *E*/*Z*-mandelaldoxime, were inert as substrates. Other compounds that are not accepted as substrates include aryl-aldoximes; unsaturated alkyl-aldoximes; PAOx derivatives *O*-methyl-, *O*-benzyl-, and *O*-acetyl-PAOx; phenylacetaldehyde hydrazone; and ketoximes, phenylacetone oxime, and acetophenone oxime. Values for V_{max} and K_m were determined from Hanes−Woolf plots of the kinetic data (Table 3). The calculated K_m values

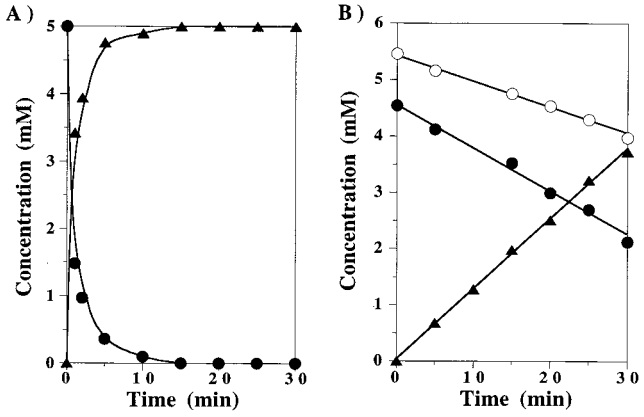

FIGURE 4: Conversion of (A) *Z*- and (B) *E*/*Z*-phenylacetaldoxime by phenylacetaldoxime dehydratase. A reaction mixture (500 μL) containing (A) 5 mM of *Z*-phenylacetaldoxime and (B) 10 mM of *E*/*Z*-phenylacetaldoxime (*E*/*Z* = 6/4), 100 mM of potassium phosphate buffer, pH 7.0, and 1.0 unit of the enzyme was incubated at 30 °C. Symbols: *Z*-phenylacetaldoxime (●); *E*-phenylacetaldoxime (○); phenylacetonitrile (▲).

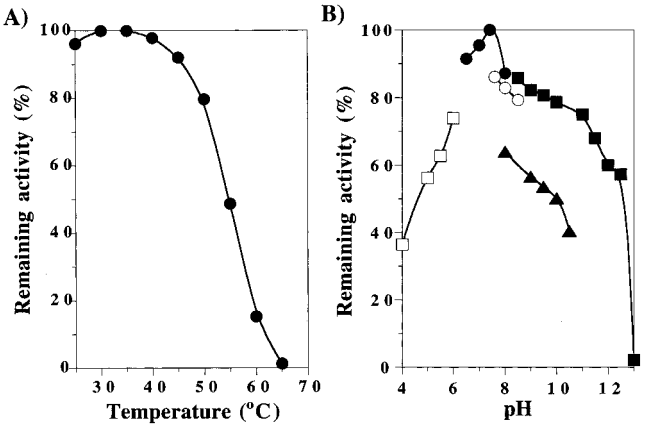

FIGURE 5: The effect of (A) temperature and (B) pH on the stability of phenylacetaldoxime dehydratase. The enzyme activity was measured (A) after preincubation for 15 min at various temperatures in 50 mM potassium phosphate buffer, pH 7.0, and (B) after preincubation at 30 °C for 15 min in the following buffers (100 mM): CH_3CO_2Na-CH_3CO_2H (□), potassium phosphate (●), Tris-HCl (○), NH_4Cl-NH_4OH (▲), and glycine-NaCl-NaOH (■). The activity remaining is expressed relative to that of untreated enzyme.

for arylalkyl-aldoximes were lower than those for alkyl-aldoximes, whereas the V_{max} values for the latter were greater than those of the arylalkylaldoximes.

In the chemical dehydration reaction of aldoximes, only *Z*-aldoxime is dehydrated into nitrile whereas *E*-aldoxime is dehydrated via an isomerization to the Z-isomer (*17*, *18*). To examine the effect of geometry of the oxime group in PAOx on the reaction, the enzyme reaction was carried out for both isomers of PAOx. The selective synthesis of *E*-PAOx was not successful, and therefore, this isomer was prepared by heating *Z*-PAOx for 90 min at 80 °C. The heat treatment provided a mixture of the two isomers (*E*/*Z* = 6/4). As shown in Figure 4, both of the isomers are consumed in a time-dependent manner, but the consumption rate of *E*-PAOx is less than that of the Z-isomer, suggesting that *E*-PAOx acts not only as a substrate but also an inhibitor of the reaction. No increase in the concentration of *Z*-PAOx in the reaction mixture was observed when *E*/*Z*-PAOx was used as the substrate. There would be two mechanisms to explain this observation. One is that the enzyme accepts the E- and Z-forms of PAOx without isomerization of the geometry of the aldoxime during the enzyme reaction. The other is that the enzyme reaction occurs with the isomerization as a rate-limiting step. The latter mechanism would account for the apparent inhibitory effect of *E*-PAOx on the net rate of PAN formation.

On the basis of the investigations on the substrate specificity, we have tentatively named the enzyme "phenylacetaldoxime dehydratase (EC 4.2.1.-)".

Effect of Temperature and pH on the Enzyme Activity and Stability. The temperature and pH stabilities of the enzyme were determined by incubation of the enzyme for 15 min at various temperatures in 50 mM of potassium phosphate buffer, pH 7.0, or at various pH values at 30 °C, respectively. Aliquots of enzyme were used to measure the remaining activity. As shown in Figure 5, the enzyme was found to be stable during 15 min at 45 °C and at pH values in the range 6.5−8.0 when maintained at 30 °C. The standard time used in the fixed-time assay of enzyme activity was 10 min. Within the limitations imposed by this assay, the apparent pH optimum for the enzyme activity was found to occur sharply at around pH 7, and the apparent maximum activity at this pH occurred at 30 °C (data not shown). An Arrhenius plot of the rates at different temperatures showed that the activation energy for the overall reaction is 28.2 kcal/mol.

Inhibitors and Activators of the Enzyme. The enzyme activity in the presence of various metal ions (at 1 mM) was measured. The activity is completely inhibited by the heavy metal ions Cu^{2+}, Cu^{+}, Ag^{+}, or Hg^{2+}. The activity is increased 2.5- and 3.4-fold by the addition of Fe^{2+} and Sn^{2+}, respectively, while Fe^{3+} and Sn^{4+} were found to have no effect. Alkaline metal ions, and other divalent and trivalent cations, and the reducing reagents D-penicillamine, reduced glutathione, cysteamine, cysteine, L-ascorbic acid, and Na_2S are similarly without effect.

We measured the enzyme activity in the presence of several compounds at various concentrations and compared with that of a control (Supporting Information, Table S1). Metal-chelating reagents, especially Tiron and 8-hydroxyquinoline, sulfhydryl reagents, and D-cycloserine inhibit the enzyme. The inhibition by Tiron and 8-hydroxyquinoline was found to be uncompetitive with respect to *Z*-PAOx (K_i = 250 and 516 μM for Tiron and 8-hydroxyquinoline, respectively). The carbonyl reagents NH_2OH and phenylhydrazine strongly inhibit the enzyme, whereas, hydrazine does not inhibit the activity even at 1 mM. For reasons which are not clear, azide was found to activate the enzyme. The inhibition by NH_2OH was found to be uncompetitive (K_i = 1.7 μM), and inhibition by KCN and phenyl hydrazine is competitive (K_i = 2.82 mM and 152 μM, respectively). It is interesting that phenylhydrazine competitively inhibits the enzyme but other typical carbonyl reagents, such as hydrazine and NaN_3, do not. Atebrin, an inhibitor for certain flavoenzymes, does not inhibit the enzyme. The enzyme is competitively inhibited by the reaction product, PAN, with a K_i value of 805 μM, but no inhibitory effect was seen with alkyl-nitriles. The enzyme activity is also inhibited by various aromatic-ring-containing oximes, which are inert as substrates, including *E*-benzaldoxime, *E*-pyridine-2-aldoxime, *E*/*Z*-2-phenylpropionaldoxime, and *E*/*Z*-phenylacetoneoxime, and the remaining activities of the enzyme after incubation at 30 °C for 10

min with 1 mM of these aldoximes were found to be 35.5, 61.4, 56.7, and 52.5%, respectively. *E*-Benzaldoxime inhibits the activity in a competitive manner ($K_i = 1.2$ mM). On the other hand, no inhibition was observed with alkyl-aldoximes. However, while these aromatic compounds are inhibitors with *Z*-PAOx as a substrate, they do not inhibit the reaction when *n*-butyraldoxime is used as a substrate (data not shown). It is possible that they will bind but do not interfere with the substrate binding when the substrate is *n*-butyraldoxime.

The effects of further electron donors and acceptors on the enzyme activity were also investigated (Supporting Information, Table S2). The activity is strongly inhibited by electron donors such as hydroquinone and tetramethylphenylenediamine in an uncompetitive manner with K_i values of 2.48 μM and 22.5 nM, respectively. Ferricyanide competitively inhibits the enzyme ($K_i = 5.77$ μM), while ferrocyanide acts as a noncompetitive inhibitor ($K_i = 11.8$ μM). On the basis of these results, it is speculated that the ferrous heme in the enzyme is oxidized to the ferric form when the substrate binds to form the enzyme−substrate complex and that the complex is very labile to reduction or nucleophilic reagents.

Cloning and Sequence Analysis of the oxd Gene. Purified PAOx dehydratase was digested with lysyl-endopeptidase by an in-gel digestion method, and several peptides were obtained by HPLC separation. The amino acid sequences determined for the native enzyme and for internal peptides were as follows: native enzyme, KNMPENHNPQAN-; internal peptides, LVYEQTHDEGIVDK, XXVADPEVQK, TELALWHEVSVLQSK, and XXDRAL(H/Y)QGADGYQ (X indicates an unidentified amino acid). On the basis of these sequences, various degenerate oligonucleotides were prepared. PCR with the primers I and II, shown in Materials and Methods, and chromosomal DNA of *Bacillus* sp. strain OxB-1 as the template yielded an amplified band of 600 bp. This was the only band that was consistently amplified under a variety of conditions. The amplified DNA was then cloned into pUC18 in *E. coli*. Nucleotide sequencing of the 600 bp fragment showed the presence of an open reading frame (ORF) continuing over the entire sequence. We investigated colony hybridization with the fragment as a probe against the established cosmid library of the strain: eight specific positive recombinant clones were obtained from about 500 transformants. One clone, pOxD-2, containing 41 kbp of the *Bacillus* DNA, was selected for further analysis. Restriction mapping of pOxD-2, subcloning into the vector pUC18, and subsequent Southern blot hybridization analysis yielded 4.5 kbp *Xba*I fragment.

The nucleotide sequence of the fragment revealed an ORF that probably initiates at the unique ^{2958}TTG start codon preceded by a potential ribosome-binding site (^{2943}AGGAG). The same initiation codon is rarely used by *E. coli* genes. However, Gram-positive bacteria use it much more frequently: about 17% of the total initiation codons examined for many genes of Gram-positive bacteria are TTG (*39*). Translation of the ORF showed that it encodes a protein predicted to have 351 amino acids, with an amino acid sequence identical to those obtained by amino acid sequencing of the four peptides prepared from purified PAOx dehydratase (Supporting Information, Figure S1). Since the N-terminal sequence of the wild-type enzyme was determined to be KNMPENHNPQAN, the residue (probably *N*-formyl methionine) coded by the initiation codon TTG was removed during processing. The M_r of the encoded PAOx dehydratase from N-terminal lysine was calculated to be 40 018, in agreement with the value of 40 000 determined by SDS−PAGE and MALDI-TOF mass spectrometry of the purified wild-type enzyme. The ORF thus corresponds to the structural gene, *oxd*, of the enzyme. A search of the databases (GeneBank, EMBL, DDBJ, and Protein Data Bank) using the programs BLAST and FASTA revealed no significant homology between PAOx dehydratase and other proteins, including hemoproteins and flavoproteins. It is suggested that the enzyme is a new type of hemoprotein that has a novel structure and function.

Analysis of the Genes in the Region Flanking the oxd Gene. Further sequencing of the flanking regions of the *oxd* gene revealed two other ORFs: *orf1* and *orf2*. *orf1* consists of 1017 bp starting with the initiation codon, ^{1875}TTG, that is located 65 bp upstream from the *oxd* start codon. A probable ribosome-binding site (^{1862}AGGAG) is present 10 bases upstream from the putative start codon. *orf2* starts 314 bp upstream from the gene described above, but in the opposite orientation. This ORF, 675 bp, is also initiated with a TTG codon and it is preceded by a potential ribosome-binding site (AGGAG), which is present 9 bases upstream of the start codon.

Computer alignments using the databases showed that the deduced amino acid sequence of *orf1* has high similarity with nitrilases from the bacteria *Comamonas testosteroni* (*40*), *Rhodococcus rhodochrous* J-1 and I-9 (K22, AKU 629) (*41*, *42*), and *Alcaligenes faecalis* JM3 (*43*). Furthermore, the gene is also similar to nitrilase-like proteins in mammals, viruses, insects, and higher plants. We have purified the nitrilase that is responsible for aldoxime metabolism from the cell-free extract of *Bacillus* sp. strain OxB-1, which had been grown in the PAOx dehydratase-production medium (Asano et al, unpublished data). The N-terminal amino acid sequence of this nitrilase (SNYPKYRVAAVQA-) is identical with that of deduced amino acid sequence of *orf1*. The subunit M_r of the nitrilase is 38 000, in agreement with the M_r of the protein encoded by *orf1* (37 650). We conclude that the *orf1* encodes the structural gene for the nitrilase, which participates in aldoxime metabolism in this organism, and we have therefore assigned it as *nit* gene.

The gene product of *orf2* is similar to the following transcriptional regulatory (DNA-binding) proteins; NitR, which regulates nitrilase in *R. rhodochrous* J-1 (*44*), ORF1, a transcriptional regulator homologue in isoquinoline 1-oxidoreductase genes from *Pseudomonas diminuta* 7 (*45*), and *maoB* gene product, a transcriptional activator of monoamine oxidase in *E. coli* (*46*). Therefore, although its detailed function is not yet clear, the protein coded by *orf2* could be a regulatory protein in *Bacillus* sp. OxB-1.

The coexistence of the structural genes for PAOx dehydratase and nitrilase genes together with that of the regulatory protein, is of advantage to express both enzymes, suggesting that the enzymes are related and exist in a single metabolic pathway of PAOx. It will be interesting to determine whether the gene for aldoxime dehydratase in other microorganisms is also linked to genes coding for nitrile-degrading enzymes (*9*−*14*).

Expression of the oxd Gene in E. coli. To express recombinant PAOx dehydratase in *E. coli*, we amplified the gene by PCR and constructed an expression plasmid. Since the TTG initiation codon for this gene might not act well in Gram-negative bacteria, it was replaced by ATG in order to increase the expression level of the enzyme in *E. coli*. The complete *oxd* gene amplified by PCR from pOxD-5C, with a putative Shine-Dalgarno sequence (GGAG), and an initiation codon (ATG), was inserted into pUC18 so that the enzyme was produced under the control of *lac* promoter. The resulted plasmid was introduced into *E. coli* XL1-Blue MRF′ cells. Base sequencing confirmed the nucleotide sequence of the complete amplified gene and showed that no error had been introduced during PCR. A protein comigrating with the purified PAOx dehydratase was produced specifically upon derepression of the promoter (data not shown). However, the enzyme activity in the cell-free extract of the recombinant *E. coli* was quite low (<10 units/L culture). It was shown that the polypeptide is well expressed under the control of the *lac* promoter, but most of the enzyme is produced as inclusion bodies. In contrast, nitrilase was expressed in the soluble form and comprised more than 50% of the extractable cellular protein (data not shown). This suggests either that an important cofactor for PAOx dehydratase may be lacking in *E. coli* or that the intracellular conditions are not as optimal for folding and/or activation as in *Bacillus* sp. strain OxB-1. Alternative expression systems to overproduce recombinant PAOx dehydratase are also currently under investigation.

Comparison of PAOx Dehydratase with Other Lyases. PAOx dehydratase acts on various arylalkyl- and alkylaldoximes but not on unsaturated alkyl-, substituted alkyl-, nor aryl-aldoximes. In addition, the enzyme is not activated by dehydroascorbic acid, ascorbic acid, nor PLP. IAOx hydro-lyase (EC 4.2.1.29), which catalyzes a specific dehydration reaction of IAOx to form indoleacetonitrile (IAN), was partially purified from *Gibberella fujikuroi* (*2*, *8*). The partially purified IAOx hydro-lyase is specific for IAOx and is activated by these compounds, although its characterization is insufficient (*5*).

Chorismate synthase is known to catalyze an overall anti-1, 4-elimination reaction of phosphate from 5-enolpyruvylshikimate-3-phosphate to chorismate (*47*). The synthase requires reduced FMN for its activity, and the activity is increased by additions of various reducing reagents. Unlike chorismate synthase, PAOx dehydratase does not seem to require reduced FMN.

The NAD(P)H-dependent dehydration of alkyl- and aryl-aldoximes catalyzed by cytochromes P_{450} under anaerobic conditions (ferrous state) has been reported (*48*, *49*). Although PAOx dehydratase binds the substrate with the heme molecule in the ferrous form, the enzyme does not require NAD(P)H for the reaction. PAOx dehydratase accepts both geometrical isomers of aldoximes, while cytochrome P450 catalyzes the dehydration reaction of only *Z*-aldoximes. We are interested in clarifying the difference in the reaction mechanisms between the two enzymes.

The enzyme can be categorized in a group of enzymes that requires flavin and yet catalyzes a reaction involving no net oxidation or reduction, e.g., mandelonitrile lyase (*50*), chorismate synthase (*51*), acetolactate synthase (*52*), and tartronate-semialdehyde synthase (*53*). In these enzymes, the mechanistic role for the flavin is not yet clear. In addition, there has been no report of a heme-containing lyase which requires FMN for its activity although most of the lyases identified to date require cofactors such as PLP, coenzyme A, iron-sulfur cluster, ATP, PQQ, and vitamin B_{12} (*54*). PAOx dehydratase is the first example of such a lyase.

ACKNOWLEDGMENT

The part of the anaerobic reaction and spectral titration of the enzyme was carried out at the Department of Biochemistry, University College of Dublin, Dublin, Ireland.

SUPPORTING INFORMATION AVAILABLE

Effects of various compounds (Table S1) and electron donors/acceptors (Table S2) on PAOx dehydratase activity, and nucleotide sequence and predicted amino acid sequence of the *oxd* gene (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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BI991598U