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Aldoxime Dehydratase Co-existing with Nitrile Hydratase and Amidase in the Iron-Type Nitrile Hydratase-Producer *Rhodococcus* sp. N-771

YASUO KATO,[1] SATOSHI YOSHIDA,[1] SHENG-XUE XIE,[1] AND YASUHISA ASANO[1]*

Biotechnology Research Center, Faculty of Engineering, Toyama Prefectural University, 5180 Kurokawa, Kosugi, Toyama 939-0398, Japan[1]

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We identified an aldoxime dehydratase (Oxd) gene in the 5′-flanking region of the nitrile hydratase–amidase gene cluster in the photoreactive iron-type nitrile hydratase-producer, *Rhodococcus* sp. N-771. The enzyme showed 96.3%, 77.6%, and 30.4% identities with the Oxds of *Rhodococcus globerulus* A-4, *Pseudomonas chlororaphis* B23, and *Bacillus* sp. OxB-1, respectively. The enzyme was expressed in *Escherichia coli* under the control of the *lac*- or T7 promoters in its intact and His_6-tagged forms, purified, and characterized. The enzyme had heme *b* as a prosthetic group, catalyzed a stoichiometric dehydration of aldoxime into nitrile, and exhibited the highest activity at neutral pH and at around 30°C similar to the known Oxd from *Bacillus* sp. OxB-1. The activity was enhanced by reducing agents, such as Na_2S, $Na_2S_2O_4$, 2-mercaptoethanol, and L-cysteine and supplementary additions of electron acceptors such as flavins, sulfite ion, and vitamin K_3. The effect of various chemicals on the enzyme activity was different in the presence and absence of the reducing reagent, Na_2S. The enzyme preferentially acts on aliphatic-type substrates and the substrate specificity of the enzyme coincides with that reported for nitrile hydratase produced by the strain.

[**Key words:** aldoxime dehydratase, nitrile hydratase, amidase, aldoxime–nitrile pathway, *Rhodococcus*]

In nature, two distinct pathways for the degradation of nitriles to carboxylic acids exist (1, 2). Nitrilase (Nit) catalyzes the direct hydrolysis of nitrile to carboxylic acid (3), while nitrile hydratase (NHase) catalyzes the hydration of nitrile to amide (1–6), which is then hydrolyzed to carboxylic acid by amidase (7). NHase in particular has been widely studied because it is used as a biocatalyst in various chemical industries; *i.e.*, the industrial production of acrylamide, nicotinamide, and 5-cyanovaleramide (1–4, 8–11). Despite the importance of the enzyme for industrial use, information about its biological functions is quite limited.

Recently, we have been successful in clarifying the microbial metabolism of aldoximes. We isolated aldoxime-degrading microorganisms and confirmed the metabolism of aldoximes via nitriles into the corresponding carboxylic acids by a combination of a novel aldoxime dehydratase (Oxd; EC 4.2.1.–) and nitrile-degrading enzymes such as NHase and Nit (Fig. 1) (2, 12–14). It has been shown that Oxd, which is widely distributed in microorganisms, co-exists with the nitrile-degrading enzymes and functions to metabolize aldoximes (14, 15). The Oxds are used for the novel enzymatic syntheses of nitrile from aldoxime under mild conditions (13, 16, 17). We purified and characterized Oxd from *Bacillus* sp. strain OxB-1, cloned the gene (*oxd*) and overexpressed that in *Escherichia coli*, and showed that the *oxd* gene is genetically linked with a Nit gene (*nit*) in the genome of the strain (18, 19). On the other hand, however, the possible enzymological and genetic relationships between Oxd and NHase still remain unclear. We have purified Oxd from *Rhodococcus globerulus* A-4 that degrades aldoximes into carboxylic acids via Oxd, NHase, and amidase and the gene of the enzyme was cloned and sequenced (20). We found that the enzyme has 94% identity with a short polypeptide (137 amino acids), which had not been assigned as a protein, coded by the 5′-flanking region of the NHase and amidase gene cluster of *Rhodococcus* sp. N-771: the strain had been isolated based on its ability to degrade acrylonitrile and as a source of an iron-type NHase (21, 22; Watanabe, I., Sato, S., and Takano, T., Japan patent S56-17918). Very recently, an Oxd gene (*oxdA*) was cloned from *Pseudomonas chlororaphis* B23, which had been isolated as an NHase-producer by one of the authors (9), sequenced,

* Corresponding author. e-mail: asano@pu-toyama.ac.jp
phone: +81-(0)766-56-7500 fax: +81-(0)766-56-2498

Abbreviations: C-His_6-tagged OxdRE, His_6-tagged aldoxime dehydratase from *Rhodococcus* sp. N-771 at its C-terminus; DMSO, dimethyl sulfoxide; IPTG, isopropyl-β-D-thiogalactopyranoside; NHase, nitrile hydratase; N-His_6-tagged OxdRE, His_6-tagged aldoxime dehydratase from *Rhodococcus* sp. N-771 at its N-terminus; Nit, nitrilase; ORF, open reading frame; OxdA, aldoxime dehydratase from *Pseudomonas chlororaphis* B23; OxdB, phenylacetaldoxime dehydratase from *Bacillus* sp. OxB-1; OxdRE, aldoxime dehydratase from *Rhodococcus* sp. N-771; OxdRG, alkylaldoxime dehydratase from *Rhodococcus globerulus* A-4; PAOx, phenylacetaldoxime; PyOx, pyridine-3-aldoxime.

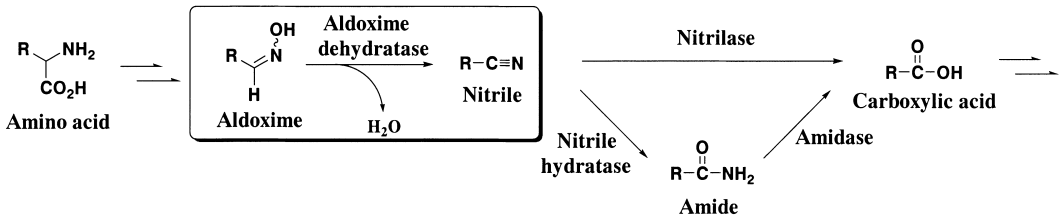

FIG. 1. Aldoxime–nitrile pathway in microorganisms.

and its gene product (OxdA) characterized (23). We are interested in comparing the properties of Oxds from various origins to understand the structure and function of Oxds.

In this report, we cloned and sequenced a gene coding Oxd located in the 5′-flanking region of the NHase and amidase gene cluster in *Rhodococcus* sp. N-771. The enzyme was expressed in *E. coli*, purified, characterized, and its properties were compared with those of known Oxds. We report here the existence and properties of an Oxd genetically linked to NHase and amidase in a strain isolated as a nitrile-degrader.

MATERIALS AND METHODS

Materials DEAE- and Butyl-Toyopearl, and the HPLC column ODS-80Ts were purchased from Tosoh (Tokyo). Standard proteins for the native- and SDS–PAGE were obtained from Dai-ichi Chem. (Tokyo) and Amersham Biosciences (Uppsala, Sweden), respectively. The aldoximes were prepared from their corresponding aldehydes and hydroxylamine according to a previously described method (12, 13, 17). Restriction enzymes and DNA-modifying enzymes were purchased from Takara (Tokyo), Toyobo (Osaka), New England Biolabs. (Beverly, MA, USA), Roche (Mannheim, Germany), and MBI Fermentas (Vilnius, Lithuania) and used according to the manufacturers' protocols. Bacto Tryptone and Bacto yeast extract were from Difco (Detroit, MI, USA). All other chemicals were from commercial sources and used without further purification.

Analytical methods The native- and SDS–PAGE were carried out as described by Davis (24) and Laemmli (25), respectively, with an electrophoresis model unit made by ATTO (Tokyo). The molecular weight (M_r) of the enzyme was determined as previously described (18).

Bacterial strains, plasmids and culture conditions *Rhodococcus* sp. N-771 (FERM-P4445) was used as the source of the DNA throughout this study. We re-identified the strain and classified it as *Rhodococcus erythropolis* based on an analysis of its fatty acid composition and 16S rRNA sequence similarities. Major fatty acids present were tetradecanoic, pentadecanoic, anteiso-pentadecanoic, ω-7-*cis*-hexadecenoic, hexadecanoic, ω-8-*cis*-heptadecenoic, and ω-9-*cis*-octadecenoic acids; C_{32} to C_{44} mycolic acids of the mycolic acid present. The partial (first 500 bp) sequence of the 16S rRNA showed 99.8% similarity to that of *R. erythropolis* DSM 43188.

The *E. coli* strains, 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)]}, JM109 {*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*)/F′[*traD36 proAB*$^+$ *lacIq lacZ* M15]}, and BL21 Star™(DE3) {F$^-$ *omp*T *hsd*S_B (r_B^-, m_B^-) *gal dcm rne131* (DE3)} were used as hosts. Plasmids pBluescript II SK(−), pUC18, and pRSETB were used as cloning and expression vectors. Recombinant *E. coli* cells were cultured at 20°C to 37°C in Luria–Bertani (LB) medium (1% Bacto Tryptone, 0.5% Bacto yeast extract, and 1% NaCl, pH 7.5) or MMI medium (1.25% Bacto Tryptone, 2.5% Bacto yeast extract, 0.85% NaCl, 20 mM Tris–HCl [pH 7.5] and 0.4% glycerol) containing the appropriate amount of antibiotics. For the induction of the gene under the control of *lac*- or T7 promoters, 1 mM of isopropyl β-D-thiogalactopyranoside (IPTG) was added to the LB medium.

Enzyme assay The Oxd activity was assayed by measuring the formation of nitriles from the corresponding aldoximes. The standard assay solution contained 50 mM potassium phosphate buffer (KPB, pH 7.0), 5 mM aldoxime, 10% (v/v) DMSO, 1 mM Na_2S, and the enzyme solution in a final volume of 0.5 ml. After the reaction was run at 30°C for 10 min during which the reaction proceeded linearly, 0.5 ml of 0.5 M H_3PO_4 was added to stop the reaction. The supernatant obtained by centrifugation (15,000×*g*, 10 min) was assayed by HPLC using an ODS-80Ts column and a mobile phase of aqueous CH_3CN containing 10 mM H_3PO_4 delivered at a flow rate of 1.0 ml/min and monitored at 254 nm or by gas-liquid chromatography (GLC; Shimadzu, Kyoto) equipped with a flame ionization detector with a glass column (2 mm by 2 m) packed with polyethylene glycol (PEG20M, 60–80 mesh; GL-Science, Tokyo). One unit (U) of the enzyme activity is defined as the amount of enzyme that catalyzes the conversion of the substrate to the product at a rate of 1 μmol/min. Mean values from two experiments are shown in the text.

General recombinant DNA techniques The plasmid DNA was isolated by a PI-100 Automatic Plasmid Isolation System (Kurabo, Osaka) or by a plasmid purification kit from Qiagen (Valencia, CA, USA). The other general procedures were performed as described by Sambrook *et al*. (26). The nucleotide sequence was determined by the dideoxy-chain termination method (27) using a SequiTherm EXCEL™II Long-Read™ DNA Sequencing Kit LC (Epicenter Technologies, Madison, WI, USA) or Thermo Sequenase Cycle Sequencing Kit (Amersham, Cleveland, OH, USA) and a 4000L DNA autosequencer (LI-COR, Lincoln, NB, USA). A homology search was performed using the sequence similarity searching programs FASTA (28) and BLAST (29). The ClustalW method was used to align the sequences (30). The *Pwo* polymerase-mediated PCR amplification was carried out in a reaction mixture that contained 25 ng of template DNA, 100 pmol of each primer, and 0.5 U of *Pwo* polymerase in a final volume of 50 μl. Thirty 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.

Cloning of the aldoxime dehydratase gene from *Rhodococcus* sp. N-771 The chromosomal DNA of *Rhodococcus* sp. N-771 was isolated by the method of Saito and Miura (31) and was completely digested with several restriction enzymes. DNA fragments were separated by agarose gel electrophoresis and transferred to a nylon membrane, GeneScreen Plus™ (Dupont, Boston, MA, USA), and the membrane was hybridized with the *oxd* gene of *R. globerulus* A-4 (20) labelled with the digoxigenin (DIG) system (Roche) according to the procedure recommended by the manufacturer. A specific positive signal was detected in the *Cla*I-digested DNA fragment (2.4 kb) with genomic Southern hybridizations. After digestion of the genomic DNAs with *Cla*I, the 2.0–3.0 kb DNA fragments were purified by agarose-gel electrophore-

sis and ligated with *Cla*I-digested and dephosphorylated pBluescript II SK(−) using the T4 ligase. The ligated DNA was used to transform *E. coli* JM109 to construct a sub-genomic library of *Rhodococcus* sp. N-771. Colony hybridization and Southern blot hybridization analysis against the library was carried out with the *oxd* gene as a probe. One positive clone, pOxS-1, carrying a 2.5-kb *Cla*I fragment, was selected for further analysis. The nucleotide sequence of the insert for pOxS-1 has been submitted to the GenBank/EMBL/DDBJ Data Bank with accession no. AB094201.

Construction of plasmid to overexpress Oxd in *E. coli* A 1.1-kb *Hin*dIII-*Xba*I fragment containing the *oxd* gene was amplified using the primers OxS-S2 (5′-ACAAAGCTTAAaggagg ATTAGTA**ATG**GAATCTGCAATCGG-3′; the restriction site, a Shine–Dalgarno sequence, and initiation codon are highlighted by underlining, lower case, and bold letters, respectively) and OxS-A (5′-TTCTCTAGATCAGTGCTCGGCG -3′) and the pOxS-1 DNA as a template, then subjected to enzyme digestion with *Hin*dIII and *Xba*I. The fragment was cloned into *Hin*dIII- and *Xba*I-digested pUC-18 to give pOxS16. A 1.1-kb PCR reaction product amplified with the primers OxS-NS (5′-GGAATTCCATATGGAATCTGCA ATCGG-3′) and OxS-AH (5′-TTCAAGCTTTCAGTGCTCGGCG-3′) from pOxS1 was digested with *Nde*I and *Hin*dIII, and was ligated into *Nde*I- and *Hin*dIII-digested pRSETB, affording pOxS17. The PCR product synthesized with the primers OxS-NS (5′-GGA ATTCCATATGGAATCTGCAATCGG-3′) and OxS-AN (5′-GG AATTCCATATGGTGCTCGGCG-3′) was purified, digested with *Nde*I, and then ligated into the *Nde*I-digested pRSETB to give pOxS18. The purified PCR product amplified with the primers OxS-SNC (5′-GGACCATGGAATCTGCAATCGG-3′) and OxS-AH (5′-TTCAAGCTTTGAGTGCTCGGCG-3′) was digested with *Nco*I and *Hin*dIII, and then ligated into *Nco*I- and *Hin*dIII-digested pRSETB to produce pOxS19.

Expression of Oxd in *E. coli* The recombinant *E. coli* cells were grown in 3 ml of LB medium at 37°C for 12 h. A 1% aliquot of the grown cells was added into several volumes of LB or MMI media containing 100 μg/ml of ampicillin and incubated with shaking (200 rpm) at 37°C for 3–4 h. When the optical density at 610 nm of the medium reached 0.5–1.0, IPTG was added to a final concentration of 1 mM, and the culture was further incubated at various temperatures. The cells were harvested by centrifugation (3500×*g*, 10 min) at appropriate intervals, suspended in 0.1 M potassium phosphate buffer (KPB, pH 7.0) containing 2 mM DTT, and then used for measuring the Oxd activity.

Purification of the recombinant OxdRE Unless otherwise stated, all purification procedures were performed at 4°C. KPB (pH 7.0) containing 5 mM 2-mercaptoethanol and 1 mM DTT was used throughout the purification.

Purification of an intact OxdRE from E. coli BL21 Star™(DE3)/pOxS17 *E. coli* BL21 Star™(DE3)/pOxS17 was grown in an MMI medium containing 100 μg/ml of ampicillin (1.5 *l* of medium in a 2-*l* Erlenmeyer flask) and incubated with shaking (200 rpm) at 37°C for 3 h. When the optical density of the medium at 610 nm reached 1.0, IPTG to a concentration of 1 mM was added, and the cells were further grown at 25°C for 14 h. The cells were harvested by centrifugation (1630×*g*, 10 min) and washed with saline. Forty g (wet weight) of the cells obtained from 6 *l* of culture were suspended in 160 ml of 100 mM buffer and then disrupted by a model 201 M Insonator (9 kHz; Kubota-shoji, Tokyo) for 10 min at 4°C. After centrifugation, the resulting supernatant was fractionated with solid $(NH_4)_2SO_4$. The precipitate obtained at 30–60% saturation was collected, dissolved and dialyzed against four changes of 20 *l* of the buffer. The dialyzate was put on a DEAE-Toyopearl column (5.0×16 cm), eluted with a linear gradient of 0–0.7 M NaCl in 20 mM buffer, and the active fractions were combined. After the $(NH_4)_2SO_4$ concentration had been adjusted to 20% saturation, the enzyme solution was loaded onto a Butyl-Toyopearl column (2.8×26.4 cm) and eluted by linearly lowering the ionic strength of $(NH_4)_2SO_4$ from 20% saturation to 0% in 5 *l* of the buffer. The active fractions were dialyzed, concentrated, then loaded onto a MonoQ HR10/10 column equipped with the FPLC system (Amersham) and eluted with a linear gradient of 0–1 M NaCl in the buffer. The active fractions were collected, dialyzed and concentrated for further studies.

Purification of C-His$_6$-tagged OxdRE from E. coli BL21 Star™(DE3)/pOxS18 The cells (15.7 g wet weight from 3 *l* of culture) of *E. coli* BL21 Star™(DE3)/pOxS18, grown in MMI medium at 25°C for 22 h after the supplementation of IPTG were obtained by centrifugation and disrupted by ultrasonication for 10 min at 4°C. The supernatant was put onto a 10 ml of Ni-NTA column (Novagen, Madison, WI, USA), and the column was sequentially washed with Binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris–HCl [pH 7.9]) and Wash buffer (60 mM imidazole, 500 mM NaCl, 20 mM Tris–HCl [pH 7.9]). The enzyme eluted with Elute buffer (1 M imidazole, 500 mM NaCl, 20 mM Tris–HCl [pH 7.9]) was collected, fractionated with 30–60% saturation of $(NH_4)_2SO_4$, and dialyzed. After the $(NH_4)_2SO_4$ concentration of the enzyme solution had been adjusted to 20% saturation, it was loaded onto a Butyl-Toyopearl column (2.8×26.4 cm). The active fraction were eluted by linearly lowering the ionic strength of $(NH_4)_2SO_4$ from 20% saturation to 0% in 20 mM buffer and used for further analysis.

Purification of N-His$_6$-tagged OxdRE from E. coli BL21 Star™(DE3)/pOxS19 *E. coli* BL21 Star™(DE3)/pOxS19 was grown as described above and the cells were harvested by centrifugation. The cell-free extract prepared by ultrasonication of the cells (14.5 g wet weight from 3 *l* of culture) was successively passed through the Ni-NTA and Butyl-Toyopearl columns and the purified active fraction was used for further analysis.

RESULTS AND DISCUSSION

Cloning of the aldoxime dehydratase gene from *Rhodococcus* sp. N-771 We found by a BLAST search of the nucleic acid databases that *Rhodococcus* sp. N-771 contains a part of a gene (411 bp) at the 5′-flanking region of the NHase and amidase gene cluster that is homologous to the *oxd* gene of *R. globerulus* A-4 (Fig. 2). To determine whether the gene could code for Oxd, the gene was cloned from the genomic library of *Rhodococcus* sp. N-771. By genomic Southern hybridization using the *oxd* gene of *R. globerulus* A-4 as a probe, specific positive signals were detected in *Bam*HI- (4.4 kbp), *Cla*I- (2.4 kbp), *Nsp*V- (9.5 kbp), *Pvu*I- (4.4 kbp), *Pst*I- (7.0 kbp), and *Kpn*I- (6.5 kbp) digested DNAs of the strain. Using colony hybridization, we screened

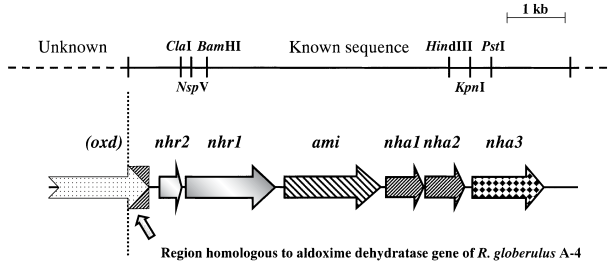

FIG. 2. Gene organization of NHase and amidase operon in *Rhodococcus* sp. N-771. Abbreviations: *nhr2*, NHase regulator 2; *nhr1*, NHase regulator 1; *ami*, amidase; *nha1*, NHase α-subunit; *nha2*, NHase β-subunit; *nha3*, NHase activator.

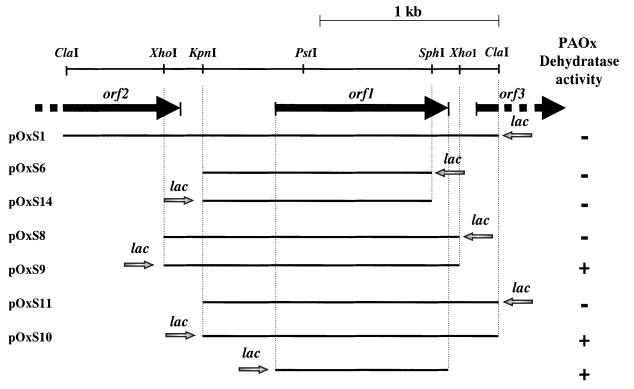

FIG. 3. Restriction map of the insert of pOxS-1 and construction of various plasmids for the expression of OxdRE. The small arrows indicate the direction of transcription from the *lac* promoter. *Z*-PAOx dehydration activities of the cells in each clone are presented on the right.

a genomic library constructed with the enzyme digests of the genomic DNA and pBluescript II SK(−). One positive clone, pOxS-1, carrying a 2.5-kb insert digested with *Cla*I was selected for further analysis. Figure 3 shows a restriction map of the insert of pOxS-1, and the nucleotide sequence of the fragment revealed the existence of a single open reading frame (ORF), *orf1*, comprised of 1059 bp, starting at position 1006 and ending at position 2064, which encoded a predicted protein of 353 amino acids with an M_r of 39,868. A putative ribosome-binding site (AGGGAG) is located 11 bp upstream of the gene. No clear promoter consensus sequence is detectable upstream of the gene. We searched the SWISS-RROT, PIR, PRF, NR-AA, and GENES databases using the BLAST programs. Figure 4 shows the amino acid sequence similarities of ORF1 with the known Oxds. The ORF1 showed identity with the Oxds of *Bacillus* sp. OxB-1 (OxdB) (18) and *R. globerulus* A-4 (OxdRG) (20) at 30.4% and 96.3%, respectively, and with the very recently isolated Oxd from *Pseudomonas chlororaphis* B23 (OxdA) (23) at 77.6%. On the other hand, no homology was seen with the other proteins.

Further sequencing of the flanking regions of the gene in pOxS-1 revealed two other incomplete ORFs; *i.e.*, *orf2* which did not contain a start codon and ended 205 bp upstream of the *orf1* start codon and *orf3* which started 13 bp downstream of *orf1*. The gene product of *orf2* is similar to possible transcriptional regulatory proteins (% identity); the transcriptional regulator (NitR) of the *nit* gene of *R. rhodochrous* J1 (32) (29%; GeneBank accession no. JC6117), the *araC* family regulatory protein (31%; AL939122) and the

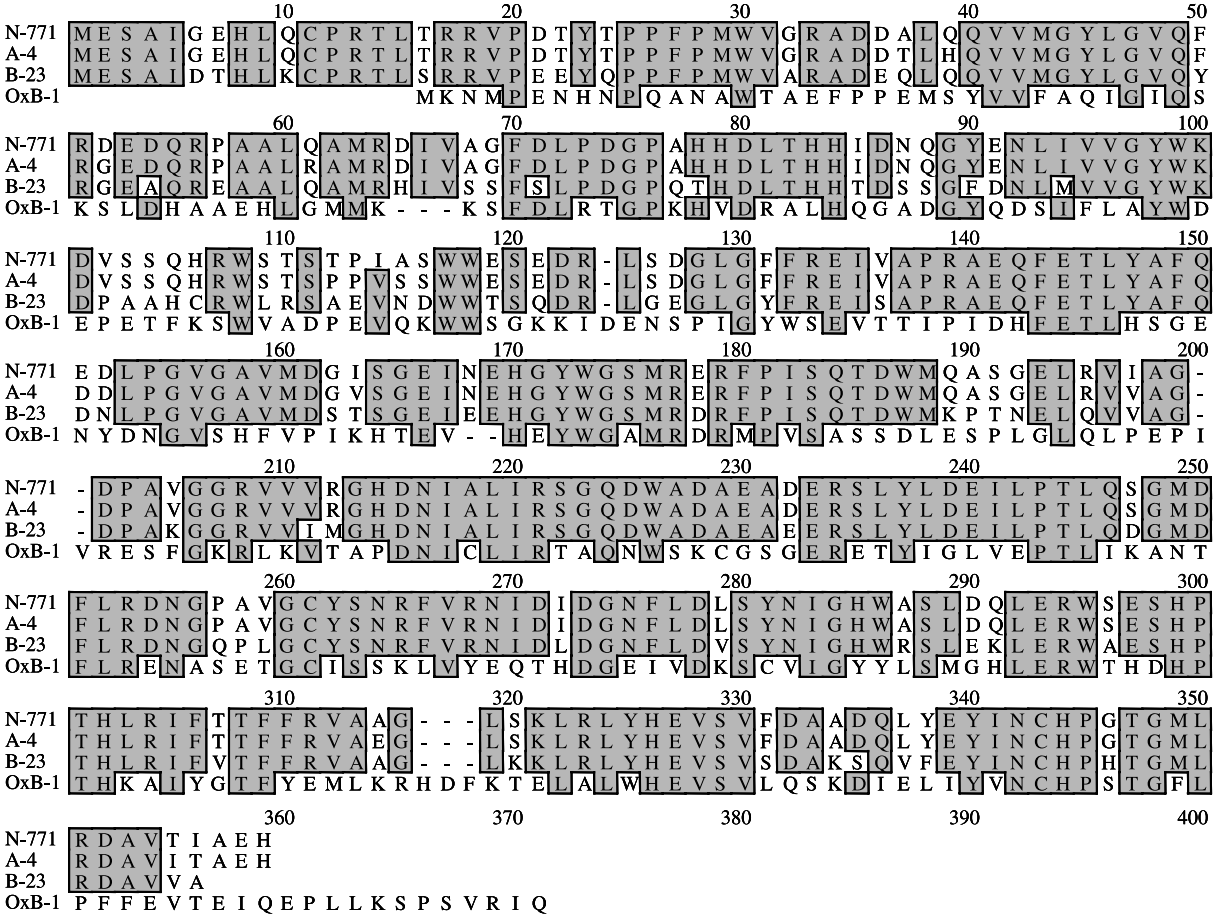

FIG. 4. Amino acid sequence comparison of Oxds. Amino acid sequences of the Oxd from *Rhodococcus* sp. N-771 (N-771), *R. globerulus* A-4 (A-4), *P. chlororaphis* B23 (B23) and *Bacillus* sp. OxB-1 (OxB-1) were aligned by introducing gaps (hyphens) to archive maximum homology. Residues in gray boxes indicate identical sequences.

TABLE 1. Overexpression of OxdRE in the recombinant *E. coli*

Plasmid	Medium	Inducer	Growth temp (°C)	Medium volume (ml)	Cell growth (A^{610})	Total activity (U/*l* cult.)	Fold-increase
WT[a]	YMG[b]	PAOx	30	4	5.63	7.97	1
pOxS9	LB	IPTG	25	4	2.12	4.32	0.54
pOxS10	LB		30	4	3.16	860	108
pOxS16	LB		30	4	3.61	2420	303
	MMI		30	4	5.18	3580	449
			30	8	3.53	4120	517
pOxS17	MMI		25	6	1.97	7860	986
pOxS18	MMI		25	8	1.51	15200	1910
pOxS19	MMI		25	6	2.52	10800	1360

One % of the cells grown in 3 ml of LB medium at 37°C for 12 h were added to LB medium in a test tube containing 100 μg/ml of ampicillin and incubated with shaking (200 rpm) at 37°C for 3–4 h. When optical density at 610 nm reached 0.5–1.0, IPTG was added (final concentration of 1 mM) and further incubation at various temperatures was carried out. *Z*-PAOx dehydration activity was measured as described in Materials and Methods. No activity was seen in the strain harboring pOxS-1, -6, -8, -11, and -14 grown under all cultivation conditions.

[a] Wild-type strain, *Rhodococcus* sp. N-771 (14).

[b] The medium contained 1.0% malt extract, 0.4% yeast extract, 0.4% D-glucose and 0.05% *Z*-PAOx (pH 7.2).

DNA-binding regulator protein (28%; T36475) of *Streptomyces coelicolor* A3, the transcription regulator PA4094 of *Pseudomonas aeruginosa* PA01 (25%; C83135), and the helix-turn-helix DNA binding protein of *Streptomyces hygroscopicus* (27%; T30238). Furthermore, it showed a 25% identity with a possible regulatory protein (*orf2*) in an operon involved in the aldoxime-nitrile degrading pathway in *Bacillus* sp. OxB-1 (18). Therefore, although its detailed function is not yet clear, the protein coded by *orf2* could be a regulatory protein in *Rhodococcus* sp. N-771. The protein coded by *orf3* was identical with the N-terminal part of the already known NHase regulator protein of *Rhodococcus* sp. N-771 (Nhr2) (22), and showed 53% identity with that of *R. rhodochrous* J1 (NhlD) (33).

Expression of the aldoxime dehydratase in *E. coli*

To express the *orf1* gene, we constructed several plasmids as shown in Fig. 3. The *E. coli* transformants harboring these plasmids were cultivated under various cultivation conditions. The Oxd activity was measured with *Z*-PAOx as a substrate (Table 1). *E. coli* strains having pOxS-1, 6, 8, 11, and 14 did not show Oxd activity, however, Oxd activity was observed in the strains harboring pOxS-9 and 10 when they were grown with IPTG. This suggests that the enzyme was actively formed when it was expressed under the control of the *lac* promoter in *E. coli*. We next examined the stoichiometry of the enzyme reaction. To the cell-free extract prepared by ultrasonication of the cells of *E. coli* JM109/pOxS10, 5 mM *Z*-PAOx was added and the mixture incubated at 30°C. After 20 min of incubation, almost the same amount (4.95 mM) of PAN was formed in the reaction mixture, suggesting that the enzyme catalyzed a stoichiometric dehydration reaction of *Z*-PAOx to form PAN; it is evident that the *orf1* codes for Oxd. Therefore, we changed the name of *orf1* to *oxd* and the protein encoded by the gene to OxdRE.

Since the expression level of OxdRE was not high in the recombinant *E. coli* (~1000 U/*l* of culture), we constructed the pOxS16 overexpression plasmid by placing the ribosome-binding site (AGGAGG) 9 bp upstream of the gene and in-frame with the *lac* promoter in the pUC vector. Using the *E. coli* JM109 strain transformed by pOxS16, we examined various cultivation conditions to overproduce the enzyme. As seen in the overexpression of the *Bacillus* enzyme (19), high Oxd activity was detected when the relevant transformant was grown at 25–30°C in a high medium volume. We also found that the enzyme activity was increased several fold by culturing the strain in an MMI medium further supplemented with yeast extract, glycerol and Tris–HCl buffer in LB medium (Table 1). We also expressed the gene under the control of the strong T7 promoter. Furthermore, His_6-tagged OxdRE was prepared and its properties compared with those of non-His_6-tagged OxdRE, in order to confirm the effect of purification steps on the enzyme properties. Using an expression vector pRSETB, plasmids pOxS17, 18, and 19 were also constructed to express OxdRE under the control of the T7 promoter in its intact form, C-His_6-tagged, and N-His_6-tagged forms, respectively. Using the *E. coli* BL21 Star™(DE3) strains harboring pOxS17, 18, and 19, we examined various cultivation conditions for overexpressing the enzyme. As shown in Table 2, low activity was observed when the strains were grown at 20°C and 30°C, whereas, a remarkably high activity was seen when they were grown at 25°C in high medium volumes. Under the optimized conditions, the enzyme activity reached over 15,000 U/*l* of culture, which is over 1900 times higher than that of the wild-type strain (Table 1). We examined the time course of the enzyme production by the recombinant *E. coli* strains using 3/4 volumes of MMI medium in a 2-*l* Erlenmeyer flask. The Oxd activity gradually increased in association with cell growth (14–24 h of cultivation) and then decreased when the cell growth reached the stationary phase. The cell-free extract was prepared and isolated into the soluble and insoluble fractions by centrifugation and subjected to SDS–PAGE analysis. Bands of almost the same density corresponding to OxdRE were seen both in the soluble and insoluble fractions, indicating that half of the OxdRE was expressed as an inclusion body under the cultivation conditions. These results show that Oxds are highly expressed by the T7 promotor under the conditions used but correct folding of all of the enzyme expressed does not occur.

TABLE 2. Overexpression of the OxdREs in *E. coli* BL21 Star™(DE3)/pOxS17, 18 and 19

Plasmid	Medium volume (ml)	Growth temperature (°C)					
		20		25		30	
		CG[a]	Act[b]	CG[a]	Act[b]	CG[a]	Act[b]
pOxS17	2	2.16	403	2.45	1660	4.52	1630
	4	2.39	780	2.15	2360	3.37	1420
	6	2.04	806	1.97	7860	2.73	1460
	8	1.74	770	2.31	1700	3.01	1880
pOxS18	2	0.44	N.D.	1.36	3400	2.25	591
	4	0.89	784	2.02	1200	2.39	2980
	6	0.75	N.D.	1.61	8710	2.43	4930
	8	0.89	940	1.51	15200	2.53	1850
pOxS19	2	4.17	505	4.84	4330	5.07	N.D.
	4	3.04	1140	3.24	8640	3.81	817
	6	2.85	2750	2.52	10800	2.85	1670
	8	2.08	681	2.06	6550	2.28	1320

One % of the cells grown in 3 ml of LB medium at 37°C for 12 h were added to various volumes of MMI medium containing 100 μg/ml of ampicillin and incubated with shaking (200 rpm) at 37°C for 3 h. When the optical density of the medium at 610 nm reached 0.5–1.0, 1 mM of IPTG was added and further cultivation at various temperatures was carried out.

[a] Cell growth (A^{610}).

[b] *Z*-PAOx dehydration activity (U/*l* culture).

N.D., Not detected.

TABLE 3. Summary of purification of intact OxdRE from *E. coli* BL21 Star™(DE3)/pOxS17

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Cell-free extract	2470	914	0.37	100
$(NH_4)_2SO_4$ (30–60%)	869	417	0.48	45.6
DEAE-Toyopearl	227	325	1.43	35.6
Butyl-Toyopearl	36.1	105	2.91	11.5
Superdex 200	11.3	37.6	3.33	4.11
Gigapite	6.01	21.1	3.51	2.31

Purification of OxdREs from the recombinant *E. coli*

The intact OxdRE was first purified to homogeneity from the cells of the recombinant *E. coli* harboring pOxS17 by $(NH_4)_2SO_4$ fractionation and several column chromatographies. The total enzyme activity decreased from 18,500 U to 920 U by sonication and centrifugation. Nearly 80% of the enzyme activity was also lost when the cells were disrupted by glass beads (ϕ0.1 mm) with the Mini-BeadBeater (model 3110BX; BioSpec Products, Artlesville, OK, USA). It is not clear whether the loss of the activity was due to the conformational changing occurred during cell disruption or by the elimination of some unknown cofactor from the enzyme molecule. Further work is needed to compare the characteristics of the enzyme by detailed spectrophotometric analysis before and after disruption of the cells. Using the purification procedures listed in Table 3, the enzyme was purified 9.5-fold with a yield of 2.3% from the cell-free extract. The specific activity of the purified recombinant OxdRE for *Z*-PAOx was 3.51 (U/mg), which is about 2/5 that of the *Bacillus* enzyme (8.55 U/mg).

As shown in Fig. 5A (dotted line), the absorption maximum of the Oxd enzyme was at 421 with a shoulder at 549 nm and the enzyme showed a positive band by heme staining (34) at the same position as the band stained by Coomassie Brilliant Blue on a native-PAGE gel, indicating that the enzyme contains heme. The spectrum did not

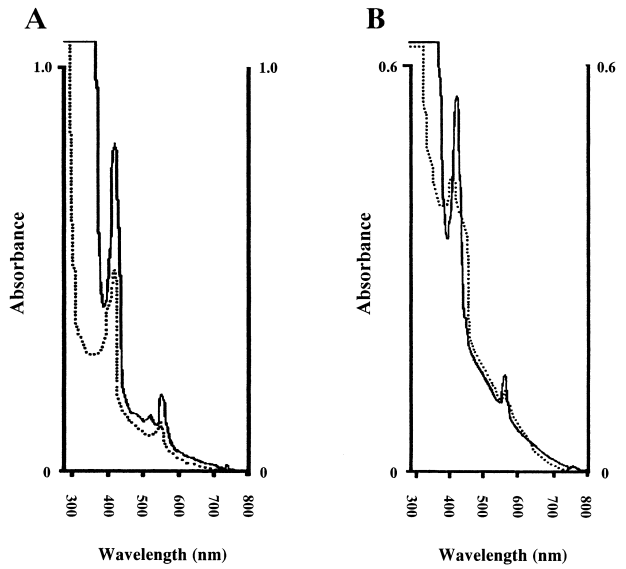

FIG. 5. Absorption spectra of OxdRE from *Rhodococcus* sp. N-771. (A) Absorption spectra of the enzyme (0.9 mg/ml) in 10 mM potassium phosphate buffer, pH 7.0 (dotted line) and of the pyridine hemochromogen of the enzyme, prepared by mixing the enzyme solution (1.75 mg/ml) in 20 mM potassium phosphate buffer with three volumes of pyridine containing 0.1 N KOH. (B) Absorption spectra of the pyridine hemochromogen of the heme prosthetic group extracted by acid-acetone. Solid and dotted lines represent results obtained in the presence and absence of $Na_2S_2O_4$, respectively.

show any changes following the addition of $Na_2S_2O_4$ or $K_3[Fe(CN)_6]$. In the presence of CO, the Soret peak, the α-band, and the β-band decreased and a new shoulder at 430 nm appeared. On the other hand, no significant change in the spectrum was observed in the presence of KCN or various aldoximes and nitriles. The pyridine hemochrome was prepared by mixing the enzyme with alkaline-pyridine in order to determine the properties of the heme in the enzyme. The absorption peaks of the hemochromogen occur-

TABLE 4. Comparison of the properties of Oxds purified from *E. coli* BL21 Star™(DE3)/pOxS17, 18, and 19, *Bacillus* sp. OxB-1 (OxdB), *P. chlororaphis* B23 (OxdA) and *R. globerulus* A-4 (OxdRG)

Property	*Bacillus* sp. OxB-1 (OxdB)	*R. globerulus* A-4 (OxdRG)	*P. chlororaphis* B23 (OxdA)	OxdRE purified from *E. coli* harboring		
				pOxS17	pOxS18	pOx19
Molecular weight (M_r)						
Native	42000	80000	76400	80000	80000[a]	80000[b]
Sequence	40150	39891	40127	39868	45370[a]	44794[b]
Number of subunits	1	2	2	2	2	2
Specific activity for *Z*-PAOx (U/mg)	8.55	2.71	N.D.	3.51	3.92	3.89
Heme content (%)	33	37	69	38	43	32
Optimum pH (KPB)	7.0	8.0	5.5[c]	8.0	8.0	8.0
Temp (°C)	30	30	45[c]	30	30	30
Stability pH	6.5–8.0	6.0–9.5	6.0–8.0	6.0–9.5	6.5–11.5	6.5–10.5
Temp (°C)	<45	<40	<40	<40	<45	<40

[a] C-$(His)_6$-tagged.
[b] N-$(His)_6$-tagged.
[c] The enzyme activity was measured with *n*-butyraldoxime as a substrate.
N.D., Not determined.

red at 419 (Soret), 524 (α-band), and 556 (β-band) (Fig. 5A, solid line), which are characteristic of the reduced pyridine hemochrome of protoheme IX (35). The addition of a few crystals of $Na_2S_2O_4$ caused an increase in each band of the hemochromogen without any change in their absorbance wavelength. The heme prosthetic group could be extracted from the enzyme by HCl/acetone-treatment (36) and the extract then concentrated *in vacuo*. The pyridine hemochrome of the extracted heme showed absorption peaks at 397 and 554 nm which represents the oxidized-type spectrum of protoheme IX (Fig. 5B, dotted line) due to oxidation during heme extraction. An identical spectrum was observed in Fig. 5A following $Na_2S_2O_4$ reduction (Fig. 5B, solid line). These results suggest that the heme in OxdRE is heme *b* and its iron is present in a reduced form. The heme content of the enzyme was calculated to be 0.39 mol heme/mol enzyme determined from the spectrum of its pyridine hemochromogen. The value is quite close to that of OxdB, which is 0.33 (18). It is unclear whether the heme was lost during the purification or the enzyme was originally produced with a low heme content.

The C-His_6-tagged OxdRE was purified using an Ni-NTA column followed by $(NH_4)_2SO_4$ fractionation and a Butyl-Toyopearl column from the cells of *E. coli* BL21 Star™(DE3)/pOxS18. Although its activity also decreased to 1/30 by sonication compared with that in the cells, the enzyme was efficiently purified by the Ni-NTA column. The enzyme was precipitated during overnight storage of the eluted fractions from the column that contained 1 M imidazole. The precipitated enzyme had no Oxd activity nor a heme prosthetic group indicating that the heme was released from the enzyme molecule during storage in the solution. Therefore, we immediately precipitated the enzyme with $(NH_4)_2SO_4$ after passage through the Ni-NTA column and used it for further purification. The enzyme was purified 7.1-fold with a yield of 6.7% from the cell-free extract and the specific activity of the purified enzyme for *Z*-PAOx was 3.92 (U/mg). The N-His_6-tagged OxdRE was also purified from the cells of *E. coli* BL21 Star™(DE3)/pOxS19 in the same manner described above. The enzyme was purified 4.2-fold with a yield of 3.7% from the cell-free extract and the specific activity of the purified enzyme for *Z*-PAOx was 3.89 (U/mg). Both the His_6-tagged OxdREs showed a similar absorption spectrum to that of the intact OxdRE.

Table 4 summarizes some properties of the OxdREs. The effects of pH and temperature on the enzyme activity and stability were carried out in several 0.1-M buffers at various pHs: AcOH–AcONa, pH 3.5–6.0; KPB, pH 6.0–8.5; Tris–HCl, pH 7.5–9.0; ethanolamine–HCl, pH 8.5–11.0; NH_4Cl/NH_4OH, pH 8.0–10.5; and glycine–NaCl–NaOH, pH 8.5–13.0 or at various temperatures between 20°C and 80°C, in 0.1 M KPB (pH 8.0), using *Z*-PAOx as the substrate. The purified OxdREs showed a single band on SDS–PAGE in agreement with its M_r deduced from the gene sequence. The native M_r of the enzymes is estimated to be about 80,000 according to the results of gel filtration chromatography with G-3000 SW indicating that OxdREs exist as dimeric enzymes unlike OxdB which is a monomer. The OxdREs exhibited similar properties to each other even with or without the His_6-tagging of the enzyme molecules.

Further characterization of intact OxdRE

Effect of reducing reagents on the enzyme activity We further characterized OxdRE using the intact enzyme. The enzyme activity was markedly enhanced by adding reducing reagents to the assay solution. It was enhanced 4.5-, 5.4-, 2.3-, 13.8-, 1.8-, 3.5-, 2.4-, and 2.3-fold by 1 mM of the reducing reagents Na_2S, Na_2SO_3, $Na_2S_2O_4$, $Na_2S_2O_5$, 2-mercaptoethanol, thioglycerol, L-cysteine and cysteamine, respectively. Due to the ease of handling, 1 mM Na_2S was then added to the standard assay mixture. The activity was not increased by the additions of $NaHSO_3$, Na_2SO_4, $NaHSO_4$ or $Na_2S_2O_7$. No further enhancement of the activity was seen by the reducing reagents if Na_2S was present in the assay solution. We also examined the effect of the reducing reagents on OxdB activity and found that, like OxdRE, the enzyme activity was also increased (2–6-fold) by the presence of 1 mM concentrations of the reagents. We previously speculated that OxdB binds to the aldoxime substrate in the ferrous state of its heme (18). The results presented here enable us to propose that the reductants act to reduce the heme iron of Oxds to its ferrous state, although their actual role in terms of the enzyme activity is still unclear. The OxdRE activity was not increased when the assay was carried out under anaerobic conditions, under which the OxdB

activity was over increased 5-fold (18).

Effect of several chemicals on the enzyme activity We examined the effect of several cofactors on the enzyme activity measured with or without Na_2S. The activity was 7.2-, 3.0-, and 3.4-fold increased by 1 mM FMN, FAD, and riboflavin, respectively, both in the presence and absence of Na_2S. It was also shown that OxdB required some electron acceptors, such as FMN and Na_2SO_3, for the enzyme activity and this result coincides with previous observations. Ascorbic acid and pyridoxal-5′-phosphate (PLP) enhanced the activity 6.2- and 8.0-fold in the presence of Na_2S, respectively, although their roles in the enzyme activity are not clear. The activity was slightly inhibited by glutathione regardless of the presence of Na_2S. The following coenzymes did not affect the reaction: pantothenate, phosphoenolpyruvate, NaF, pyridoxal, dehydroascorbate, glutathione, glutathione disulfide, NAD(P)(H), glucose-6-phosphate, glutathione disulfide, dehydroascorbic acid, CoA-SH, AXP, GXP, and IXP.

The enzyme activity was measured in the presence of various metal ions and chemical compounds with or without Na_2S. The enzyme activity was increased 23.5- and 24.3-fold by the addition of 1 mM Fe^{2+} and Fe^{3+}, respectively, however, the enhancement by the iron ions decreased 3.4- and 1.9-fold, respectively, when Na_2S was omitted from the assay mixture. It is possible to say that the oxidized iron ion is first converted to its reduced form by the Na_2S, which also acts as a reducing reagent as well as the Fe^{2+} ion. Increased enzyme activity was also seen following the addition of the divalent cations Zn^{2+}, Sn^{2+} and Co^{2+}, which enhanced the activity 2.5-, 3.6- and 14-fold, respectively, only in the presence of Na_2S. Their effect on the enzyme activity is not understood at the present time. The enzyme was slightly sensitive to heavy metal ions, such as Cu^{+}, Cu^{2+}, As^{3+}, Cd^{2+} and Hg^{2+}, regardless of the presence of Na_2S. The following metal ions did not inhibit the enzyme activity under any reaction conditions: Li^{+}, B^{4+}, Na^{+}, Mg^{2+}, K^{+}, Ca^{2+}, Mn^{2+}, Ti^{2+}, Cr^{3+}, Rb^{+}, Mo^{6+}, Ag^{+}, Cs^{+}, Ba^{2+} and Pb^{2+}.

EDTA, Tiron, and D-cycloserine (0.1 mM each) inhibited 45%, 55%, and 35% of the activity, respectively, only when Na_2S was omitted from the assay, while they enhanced the activity 2.1-, 7.2-, and 1.6-fold, respectively, in the presence of Na_2S. Phenylhydrazine (0.1 mM) inhibited the activity although hydrazine did not. The enzyme activity was also inhibited by aromatic ring containing-compounds which are inert as substrates, such as *E*-benzaldoxime, phenylacetone and acetophenone, as also observed for OxdB. The residual OxdRE activity after incubating the enzyme at 30°C for 10 min with 0.5 mM of the aldoximes was 20.5%, 5.8% and 28.6%, respectively. It is reasonable to assume that phenylhydrazine might inhibit the reaction by interferring with the binding of the substrate via their aromatic ring. These observations were also seen for OxdB (18). The enzyme was slightly sensitive to electron donors, such as *o*-phenylenediamine, guaiacol and pyrogallol, only when Na_2S was absent from the assay solution: these compounds (0.1–1 mM) inhibited 20–30% of the activity. The activity was strongly increased by the addition of electron acceptors such as vitamin K_3 and duroquinone: it increased 23- and 3.2-fold in the presence of Na_2S, respectively, and to 8.7- and 6.7-fold in the absence of Na_2S, respectively. The result coincides with the observation for OxdB that electron acceptors, such as FMN and Na_2SO_3, increase the enzyme activity (18). Ferricyanide increased the activity 9-fold in the presence of Na_2S whereas ferrocyanide did not. The following compounds did not inhibit the enzyme activity: 8-hydroxyquinoline, NH_2OH, EGTA, *o*-phenanthroline, NaF, phenylmethanesulfonyl fluoride (PMSF), *p*-chloromercuribenzoic acid (PCMB), iodoacetic acid, avidin, *N*-ethylmaleimide, 5,5′-dithiobis (2-nitrobenzoic acid), diphenylhydantoin, bipyridyl, barbital, pepstatin A, tetramethylphenylenediamine, hydroquinone, trimethylhydroquinone, 3,3′-dimethoxybenzidine, 1-aminobenzotriazole, 2,6-dichlorophenolindophenol, benzylviologen, methylviologen and phenazinemethosulfate (PMS).

Substrate specificity of the enzyme To determine the substrate specificity of the purified enzyme, various aldoximes were synthesized (13, 17, 18) and used in the enzymatic dehydration reaction. As shown in Table 5, the enzyme was active toward various arylalkyl- and alkyl-aldoximes, and to a lesser extent to aryl-aldoximes, converting them to the corresponding nitriles. Since *Z*-PyOx was not

TABLE 5. Substrate specificity of OxdRE from *Rhodococcus* sp. N-771

Substrate	Relative activity (%)	
	with Na_2S	without Na_2S
Arylalkylaldoxime		
Z-Phenylacetaldoxime	100	100
E/*Z*-2-Phenylpropionaldoxime	49.8	232
Z-3-Phenylpropionaldoxime	69.6	174
E/*Z*-4-Phenylbutyraldoxime	29.1	44.1
E/*Z*-Indoleacetaldoxime	41.7	15.2
E/*Z*-Mandelaldoxime	11.7	9.57
E/*Z*-Cinnamaldehyde oxime	4.28	7.80
Z-*p*-Chlorophenylacetaldoxime	3.39	6.71
Z-Naphthoacetaldoxime	2.71	4.76
E-Thiophene-2-acetaldoxime	26.3	5.00
Arylaldoxime		
E-Thiophene-2-carboxaldoxime	3.37	2.50
E-Benzaldoxime	1.92	5.61
E-*p*-Chlorobenzaldoxime	0.331	0.366
E-Furfurylaldoxime	0.504	0.549
E-*p*-Tolualdoxime	0.488	3.48
E-Pyridine-3-aldoxime	0.535	4.21
Alkylaldoxime		
E/*Z*-Cyclohexanecarboxaldehyde oxime	157	610
E/*Z*-Propionaldoxime	87.7	59.4
E/*Z*-*n*-Butyraldoxime	100	184
E/*Z*-Isobutyraldoxime	89.6	67.1
E/*Z*-*n*-Valeraldoxime	68.3	87.8
E/*Z*-Isovaleraldoxime	120	242
E/*Z*-*n*-Capronaldoxime	85.5	61.0
E/*Z*-Isocapronaldoxime	48.7	86.6

The enzyme activity for several aldoximes was measured in the standard assay solution in the presence and absence of Na_2S. The enzyme activity for *Z*-phenylacetaldoxime dehydration activity was taken as 100%.

[a] The following compounds were inert as substrates: *E*/*Z*-diphenylacetaldoxime, *E*/*Z*-*p*-hydroxyphenylacetaldoxime, *E*-1-naphthoaldoxime, *E*-anisaldoxime, *E*-quinoline-2-carboxaldehyde oxime, *E*-terephthalaldehyde oxime, *E*-isophthalaldehyde oxime, *E*-pyrazinecarboxaldoxime, *E*-indole-3-carboxaldehyde oxime, *Z*-crotonaldoxime, *E*/*Z*-methacrylaldoxime, *E*/*Z*-*O*-benzyl PAOx, *E*-PAOx hydrazone, *E*/*Z*-*O*-acetyl-PAOx, *E*/*Z*-phenylacetone oxime and *E*/*Z*-acetophenone oxime.

TABLE 6. Kinetic parameters of OxdRE from *Rhodococcus* sp. N-771

Aldoxime	With Na_2S			Without Na_2S		
	K_m (mM)	V_{max} (units/mg)	V_{max}/K_m (units/mg/mM)	K_m (mM)	V_{max} (units/mg)	V_{max}/K_m (units/mg/mM)
Arylalkylaldoxime						
Z-Phenylacetaldoxime	5.37	5.41	1.01	3.22	1.56	0.48
E/*Z*-2-Phenylpropionaldoxime	10.0	7.93	0.79	2.56	4.03	1.57
Z-3-Phenylpropionaldoxime	5.88	4.59	0.78	4.08	2.17	0.53
Alkylaldoxime						
E/*Z*-Cyclohexanecarboxaldehyde oxime	0.99	4.76	4.84	1.25	7.41	5.93
E/*Z*-Propionaldoxime	2.17	5.78	2.66	1.85	4.31	2.32
E/*Z*-*n*-Butyraldoxime	2.64	6.02	2.28	4.34	3.71	0.85
E/*Z*-Isobutyraldoxime	1.41	8.33	5.91	0.76	5.55	7.30
E/*Z*-*n*-Valeraldoxime	1.13	4.59	4.06	1.41	2.59	1.84
E/*Z*-Isovaleraldoxime	2.43	5.71	2.35	6.66	4.27	0.64

accepted as a substrate for the enzyme, it was shown that the enzyme does not prefer arylaldoximes regardless of the geometry of the aldoxime. Interestingly, the relative activity for some aldoximes, such as *E*/*Z*-2-PPOx, *E*-*p*-tolualdoxime, *E*-PyOx and *E*/*Z*-cyclohexanecarboxaldehyde oxime, was increased markedly compared to that for *Z*-PAOx when Na_2S was omitted from the assay, while that for *E*/*Z*-indoleacetaldoxime and *E*-thiophene-2-acetaldoxime was decreased. The results imply that Na_2S acts not only as a reductant but also changes the substrate specificity of the enzyme. The enzyme also acted on *E*/*Z*-2-PPOx, *E*/*Z*-mandelaldoxime, *E*/*Z*-cyclohexanecarboxaldehyde oxime and *E*/*Z*-isobutyraldoxime, which had a substitution at the aldoxime α-position, which dose not occur in OxdB. These results indicated that it may be possible to produce optically active nitriles from the aldoximes. We examined the time course of the dehydration reaction of *E*/*Z*-mandelaldoxime and the mandelonitrile produced was analyzed by HPLC at 254 nm, with a Chiralcel OJ-H column (Daicel Chem., Tokyo) at a flow rate of 1 ml/min using an elution solvent of 10% 2-propanol in hexane (retention times for (*R*)- and (*S*)-mandelonitrile were 15.2 and 19.5 min, respectively) to measure the enantiomeric ratio of the nitrile. However, the synthesized mandelonitrile was racemic at all conversion rates, showing that the enzyme does not recognize the stereochemistry at the α-position of the aldoxime.

The values for V_{max} and K_m were determined from Lineweaver–Burk plots of the kinetic data in the presence and absence of Na_2S (Table 6). The K_m values for the alkylaldoximes were several times lower than those for arylalkylaldoximes whereas the V_{max} values for both types of substrates were similar. These results are different from those for OxdB, which preferentially acts on the arylalkylaldoximes rather than the alkylaldoximes (18). The V_{max}/K_m values measured for *Z*-PAOx and *E*/*Z*-2-PPOx were different with or without Na_2S which coincides with the results shown in Table 5. Further work on the effect of the reducing reagents on the enzyme characteristics, such as enzyme function, structure of the enzyme and substrate specificity, has yet to be carried out.

It has been shown that NHase encoded by the genes (*nha1* and *nha2*) present in the 3′-flanking region of the *oxd* gene preferentially acts on aliphatic nitriles (21, 22). Here we showed, not only genetic, but also enzymological evidence for the existence of Oxd and alkylaldoxime metabolism in the iron-type NHase-producer, *Rhodococcus* sp. N-771, which had been isolated based on its ability to degrade acrylonitrile (Watanabe, I., Sato, S., and Takano, T., Japan Patent S56-17918). Very recently, the Oxd gene (*oxdA*) was also cloned from *P. chlororaphis* B23, which had been isolated as an NHase-producer (9), sequenced, and its gene product (OxdA) characterized (23). The *oxdA* gene was shown to be clustered with the genes for NHase/amidase in the genome of the strain. OxdA also catalyzed the dehydration reaction of alkylaldoxime although quite a few types of substrates were used in the analysis of its substrate specificity. OxdRE showed similar properties to those of OxdA, *e.g.*, M_r, subunit structure, and temperature and pH stabilities. However, some differences were seen in the heme content and absorption spectra of the enzymes. It is possible that these differences are due to the different expression systems and purification procedures used, although the actual reasons for the differences are not clear at the present time. These results allow us to claim the existence of an aldoxime–nitrile pathway both in Nit- and NHase-producers. The results also show that the pathway can be found not only in aldoxime-degraders but also in nitrile-degraders. Thus, it will be of interest to elucidate the pathway enzymologically and genetically and determine whether it is also present in other microorganisms.

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