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Discovery and characterization of a putrescine oxidase from *Rhodococcus erythropolis* NCIMB 11540

Erik W. van Hellemond · Marianne van Dijk · Dominic P. H. M. Heuts · Dick B. Janssen · Marco W. Fraaije

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Abstract A gene encoding a putrescine oxidase (PuO_{Rh}, EC 1.4.3.10) was identified from the genome of *Rhodococcus erythropolis* NCIMB 11540. The gene was cloned in the pBAD vector and overexpressed at high levels in *Escherichia coli*. The purified enzyme was shown to be a soluble dimeric flavoprotein consisting of subunits of 50 kDa and contains non-covalently bound flavin adenine dinucleotide as a cofactor. From all substrates, the highest catalytic efficiency was found with putrescine (K_M=8.2 μM, k_{cat}= 26 s^{-1}). PuO_{Rh} accepts longer polyamines, while short diamines and monoamines strongly inhibit activity. PuO_{Rh} is a reasonably thermostable enzyme with $t_{1/2}$ at 50°C of 2 h. Based on the crystal structure of human monoamine oxidase B, we constructed a model structure of PuO_{Rh}, which hinted to a crucial role of Glu324 for substrate binding. Mutation of this residue resulted in a drastic drop (five orders of magnitude) in catalytic efficiency. Interestingly, the mutant enzyme showed activity with monoamines, which are not accepted by wt-PuO_{Rh}.

Keywords Putrescine oxidase · Flavin · Amine · Activity screening

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E. W. van Hellemond · M. van Dijk · D. P. H. M. Heuts · D. B. Janssen · M. W. Fraaije (✉)
Laboratory of Biochemistry, University of Groningen,
Groningen Biomolecular Sciences and Biotechnology Institute,
Nijenborgh 4, 9747 AG Groningen,
The Netherlands
e-mail: m.w.fraaije@rug.nl

Introduction

Flavoprotein oxidases catalyze the oxidation of a wide range of compounds, while at the same time they reduce oxygen to hydrogen peroxide. They are valuable biocatalysts for the oxidative activation of biomolecules, as they usually selectively oxidize their substrate at a specific position, leaving other positions unaffected. Due to their ability to use molecular oxygen as electron acceptor, no expensive coenzymes like NAD(P)H are needed. This makes oxidases inexpensive and rather straightforward in usage compared to other redox enzymes.

By far, the best studied oxidase is glucose oxidase from *Aspergillus niger*. This enzyme has been applied for decades, mostly for diagnostic applications (Wilson and Turner 1992). Another example is pyranose oxidase (glucose-2-oxidase) from *Peniophora gigantea*, which oxidizes specifically the C2 position of glucose and has been applied for the chemoenzymatic synthesis of rare sugars and sugar-based synthons (Giffhorn et al. 2000). Human monoamine oxidases (MAO-A and MAO-B) are important for the oxidation of neurotransmitters (Abell and Kwan 2001). An engineered mutant of the homologous *A. niger* enzyme (MAO-N) has been applied in an elegant biocatalytic deracemization process to obtain enantiomerically pure chiral amines (Carr et al. 2005; Dunsmore et al. 2006).

So far, the most described oxidases are from eukaryotic origin, and heterologous expression of the recombinant protein in *Escherichia coli* for large scale enzyme production can be problematic. In a search for novel bacterial oxidases, we looked in sequenced genomes and identified genes encoding novel oxidases. By this, we discovered oxidases primarily acting on alditols (Heuts et al. 2007) and phenolic compounds (Jin et al. 2007). These oxidases, both

from actinomycetes, could be highly overexpressed in *E. coli*. Another way of searching for novel bacterial oxidases is to screen unsequenced bacterial genome libraries directly for genes encoding enzyme activity. As actinomycetes appear to be rich in oxidases, we have chosen to explore these organisms for relevant oxidases. Using a plate-based screening protocol for oxidase activity (Alexeeva et al. 2002), we screened a genomic DNA library of the actinomycete *Rhodococcus erythropolis* NCIMB 11540 to find novel oxidases acting on polyols and/or amines.

In this paper, we describe the discovery and characterization of a novel flavin-containing oxidase primarily acting on putrescine. This putrescine oxidase (PuO_{Rh}) shares 67% sequence identity with the enzyme from *Micrococcus rubens* (PuO_{Mr}; Ishizuka et al. 1993). We show that PuO_{Rh} is highly overexpressed in *E. coli* and can be easily purified. Using a structural model, we were able to identify a glutamate residue (Glu324) that is crucial for substrate binding, and we could alter the substrate specificity by protein engineering.

Materials and methods

Chemicals

Restriction enzymes were from Roche and New England Biolabs. One-shot electrocompetent *E. coli* TOP10 cells and the TOPO TA Cloning Kit were purchased from Invitrogen. Plasmid isolation was performed using the Qiagen DNA purification kit. Oligonucleotides were purchased from Sigma. Nitrocellulose filters (Protran BA85 132 mm, 0.45 μm pore size) were from Schleicher and Schuell BioScience, Dassel (Germany). Deprenyl and rasagiline were a kind gift from Prof. A. Mattevi (University of Pavia, Italy). All other chemicals were of analytical grade. The genomic DNA library from *R. erythropolis* NCIMB 11540 was provided by DSM (Geleen, The Netherlands). Constructs were sequenced at GATC Biotech (Kostanz, Germany).

Plate-based screening method for oxidase activity

A gene library of *R. erythropolis* NCIMB 11540 in pZErO-2 was screened for oxidases using the plate-based oxidase activity screening method adapted from the group of Turner (Alexeeva et al. 2002). This gene library was constructed by partial digestion of genomic DNA from *R. erythropolis* NCIMB 11540 by *Sau*3A1. Fragments of 4–10 kb were isolated, ligated in pZErO-2 (cut with *Bam*HI), and followed by transformation of *E. coli* DH10B. Colonies were collected from plate, stored as glycerol stock, and plasmids were isolated. The total amount of plasmids contained a mean insert size of 6.0 kb and 1% of self-ligated vector molecules.

Electrocompetent *E. coli* TOP10 cells were transformed with the gene library, and the transformed cells were diluted in Luria–Bertani (LB) medium to obtain single colonies on plate. The diluted cell suspensions were plated on nitrocellulose filters. The filters were placed on top of LB agar containing 0.05 mg/ml kanamycin and incubated for 48 h at 30°C. Subsequently, the nitrocellulose filters were transferred to empty petri dishes and stored at −20°C to partially lyse the cells. Each filter was submersed with 50 ml 50 mM sodium phosphate buffer pH 7.5 containing 1% (*w*/*v*) agarose, 2 U/ml Horseradish peroxidase (HRP) and a mixture of 1 mM 4-chloro-1-naphthol, 100 μM cholesterol, 10 mM xylitol, 10 mM sarcosine, 10 mM L-alanine, 10 mM D-glucose, 10 mM D-galactose, 10 mM lactose, and 10 mM D-glucosamine. Plates were incubated at room temperature and regularly checked by visual inspection for color formation in and around colonies. Positives were picked and cultivated overnight in 5 ml liquid LB medium and subjected to another screening cycle to assure that color formation was caused by single clones.

Sequence analysis

To identify unique positive clones, plasmids were isolated and subjected to restriction analysis by *Eco*RI. Inserts were sequenced and open reading frames (ORFs) were identified using the ORF Finder tool of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). A BLAST search (blastp) was performed with the protein sequence of PuO_{Rh} using the BLAST function of the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST).

Cloning and expression of the gene encoding PuO_{Rh}

To overexpress the novel putrescine oxidase, the corresponding gene (puo_{Rh}) was amplified from the fragment of genomic DNA of *R. erythropolis* using the following primers: puo_{Rh}_fw: 5′-GCTC*CATATG*CCTACTCTCCAGAGAGATG (*Nde*I site shown in italics) and puo_{Rh}_rv: 5′-GCTC*AAGCTT***TCA**GGCCTTGCTGCGGGCG (*Hin*dIII site shown in italics). The amplified gene was purified from gel and ligated between the *Nde*I and *Hin*dIII restriction sites of the pBAD/*Myc*-His vector (Invitrogen). The plasmid carrying the puo_{Rh} gene (pBADpuo_{Rh}) was transformed to $CaCl_2$-competent *E. coli* TOP10 cells and spread on LB agar plates containing 50 μg/ml of ampicillin. *E. coli* TOP10 cells containing pBADpuo_{Rh} were tested for overexpression of the protein at 17, 30, and 37°C and at arabinose concentrations of 0, 0.00002, 0.0002, 0.002, 0.02, and 0.2% (*w*/*v*). Cell extracts and cell-free extracts of *E. coli* TOP10 containing

pBAD*puo*$_{Rh}$ were analyzed on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel to find the best conditions for overexpression of soluble PuO_{Rh}.

Enzyme purification

To obtain purified protein, *E. coli* TOP10 containing pBAD*puo*$_{Rh}$ was cultivated for 24 h at 30°C in 1 l of terrific broth medium containing 50 μg/ml ampicillin and 0.02% (*w*/*v*) arabinose. Cells were harvested by centrifugation for 15 min at 6,000 rpm at 4°C. The supernatant was discarded, and the pellet was suspended in 40 ml of 50 mM Tris–HCl pH 7.5 and sonicated for 10 min to break the cells. The cell extract was centrifuged for 30 min at 15,000 rpm at 4°C to remove the broken cells and obtain cell-free extract. From this cell-free extract PuO_{Rh} was purified using a Q-Sepharose anion exchange column. Unbound protein fractions were washed from the column with 50 mM Tris–HCl buffer pH 7.5. PuO_{Rh} was eluted from the column with a 50 mM Tris–HCl buffer pH 7.5 by increasing the KCl concentration. Eluted fractions containing PuO_{Rh} were combined, concentrated using an Amicon filter, and desalted using a HiPrep 26/10 Desalting Column (Amersham Biosciences).

Enzyme activity assay and determination of steady-state kinetic parameters

PuO_{Rh} activity was measured at 25°C using a peroxidase-coupled assay containing 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) as the chromogenic substrate (Childs and Bardsley 1975). The H_2O_2 formed by PuO_{Rh} can be coupled to the Horseradish-peroxidase-mediated oxidation of ABTS resulting in the formation of a green product that can be measured at 420 nm (ε_{420}=35.7 mM^{-1} cm^{-1}). The standard assay mixture with ABTS (HRP-ABTS assay) contained 50 μM putrescine, 50 mM Tris–HCl buffer, pH 8.0, 100 μM ABTS, 5 U of Horseradish peroxidase, 10 μl of enzyme dilution (appropriately diluted), and a fixed amount of substrate. The reaction was started with adding the enzyme solution. Alternatively, 0.1 mM 4-aminoantipyrine (AAP) and 1.0 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid (DCHBS) were used as chromogenic substrates for HRP (Federico et al. 1997; HRP-AAP/DCHBS assay). The oxidation of these substrates results in the formation of a purple product, which can be measured at 515 nm (ε_{515}= 26 mM^{-1} cm^{-1}). For the determination of kinetic parameters, PuO_{Rh} activity was measured at different substrate concentrations at 25°C using the HRP-ABTS enzyme assay. The kinetic parameters of the mutant enzymes (PuO_{Rh} Glu324Ala and PuO_{Rh} Glu324Leu) for putrescine were determined by measuring directly the H_2O_2 production at 240 nm (ε_{420}= 43.6 M^{-1} cm^{-1}). Data were fitted with Origin 7.0 using the Michaelis–Menten equation for enzyme kinetics.

Influence of temperature and pH on enzyme activity and stability

To determine the optimal temperature for PuO_{Rh} activity, the oxidase activity was measured at temperatures between 20 and 60°C. Before adding the enzyme, the assay mixture was equilibrated for 20 min to assure the right temperature. The influence of temperature on the enzyme stability was determined by incubating 1-ml portions of the enzyme at 4, 30, 37, and 50°C. Twenty-microliter samples were taken, placed on ice after which activity was measured. In both experiments, the activity was determined with the HRP-AAP/DCHBS activity assay using 50 μM putrescine and 0.01 μM PuO_{Rh}.

The pH optimum for putrescine oxidase was determined by measuring the activity at different pH values at 25°C. The following buffers were used: 50 mM Pipes buffer (pH 6.4–7.2), 50 mM Tris–HCl (pH 7.4–8.8), and 50 mM Ches buffer (pH 8.7–9.6). The enzyme activity was measured using the HRP-AAP/DCHBS assay.

Inhibition experiments

Rasagiline, deprenyl, and cyclopropylamine were tested to probe whether they were able to inhibit PuO_{Rh} by the formation of a covalent adduct with the flavin adenine dinucleotide (FAD) cofactor, as is the case for monoamine oxidase B (Binda et al. 2005). For 900 μl of a 20 μM PuO_{Rh} solution, an absorbance scan was recorded from 650–300 nm. After the addition of 100 μl of 1 mM inhibitor (final inhibitor concentration, 100 μM), absorbance spectra were recorded regularly in time to see if addition of the inhibitor would cause an alteration of the typical FAD spectrum of PuO_{Rh}. For cyclopropylamine, also a final inhibitor concentration of 1 mM was tested as described above.

Butylamine, aminoethanol, ethylenediamine, 1,3-diaminopropane, and (2-aminoethyl)-trimethylammonium were tested as competitive inhibitors of PuO_{Rh}. For this, the Michaelis constant (K_M) of PuO_{Rh} for putrescine was determined for three different concentrations for each of these amino compounds with the HRP-ABTS assay. Inhibition constants (K_I) for each compound were calculated with the following formula:

$$K_{M,\text{inhibitor}} = K_m \cdot \left(1 + \frac{[I]}{K_I}\right) \quad (1)$$

Where [*I*] represents the inhibitor concentration, $K_{M,\text{inhibitor}}$ the apparent Michaelis constant in presence of the inhibitor and the K_I the inhibition constant.

Analytical methods

All absorbance spectra were recorded in 50 mM Tris–HCl pH 8.0 at 25°C on a Perkin Elmer Lambda Bio40 spectrophotometer. From a cuvet containing 5 μM PuO_{Rh} all oxygen was removed by flushing with argon and an absorbance spectrum was recorded from 650 to 300 nm. After adding 50 μM putrescine, another spectrum was recorded for the reduced enzyme. Reoxidation was monitored by collecting spectra in time after the cuvet was exposed to air. A spectrum of the unfolded enzyme was recorded by adding 0.1% SDS and heating for 5 min at 80°C.

Modeling and mutant construction

Based on the structure of MAO-B in complex with rasagiline (PDB/1S2Q; Binda et al. 2004b), a model of PuO_{Rh} was made using the CHPmodels 2.0 Server. Mutants were constructed by Quick Change PCR. PuO_{Rh} Glu324Ala was made by a substitution of the codon GAG for GCG using the following primers: puo_{Rh}Glu324Ala_fw, 5′-CGAGGTAGTGCAGGCGGTGTACGACAACACC, and puo_{Rh}Glu324Ala_rv, 5′-GGTGTT GTCGTACAC CGCCTGCACTACCTCG (mutated codon underlined). For construction of PuO_{Rh} Glu324, the codon GAG was replaced by CTG using the following primers: puo_{Rh}-Glu324Leu_fw, 5′-CGAGGTAGTGCAGCTGGTGTAC GACAACACC, and puo_{Rh}Glu324Leu_rv, 5′- GGTG TTGTCGTACACCAGCTGCACTACCTCG (mutated codon underlined). Both mutants were expressed in *E. coli* TOP 10 and cultivated and purified in the same way as wild-type PuO_{Rh}.

Nucleotide sequence accession number

The nucleotide sequence of the PuO_{Rh} encoding gene (puo_{Rh}) has been submitted to GenBank under accession number EU240877.

Results

Identification of a novel putrescine oxidase

To identify novel oxidases acting on alcohols and/or amines, a genomic library from the actinomycete *R. erythropolis* NCIMB 11540 was screened using a plate-based oxidase assay. Positive clones, expressing oxidase activity, could be identified by their purple color formation. From 55,000 transformants, 5 were observed to produce a purple color around the colony. Retransformation to *E. coli* TOP10 cells and repeated screening on plate resulted in three positive clones, which showed oxidase activity. Restriction analysis with *Eco*RI and *Pvu*I showed a different insert for each clone indicating that these were independent clones. Sequencing of one of these clones revealed an inserted fragment of genomic DNA of 5,311 bp. On this fragment, an ORF was identified which shares 67% sequence identity (at the amino acid level) with putrescine oxidase (EC 1.4.3.10) from *M. rubens*, a FAD-containing amine oxidase that catalyzes the oxidative deamination of putrescine (Ishizuka et al. 1993). When we tested the cell extract of the clone containing the above-mentioned insert of 5,311 bp, we found that it was indeed active with putrescine. The other two positive clones probably contained the same gene, as for both clones by using the primers puo_{Rh}_fw and puo_{Rh}_rv a PCR product was seen on gel.

Gene cloning, overexpression, and protein purification

For overexpression, the PuO_{Rh} encoding gene was cloned into the pBAD/*Myc*-His A vector behind the araBAD promotor (without His-tag), and the plasmid carrying the *puo* gene was transformed to *E. coli* TOP10. Several temperatures (17, 30, and 37°C) and inducing conditions (0–0.2% *L*-(+)-arabinose) were tested to find the optimal conditions for overexpression. The gene was well expressed at arabinose concentrations of 0.02 and 0.2% as judged by a dominant protein band of ±54 kDa on SDS-PAGE. At 17 and 30°C, the protein was mainly present in the soluble fractions while at 37°C the unsolube fractions contained most of the overexpressed protein. The overexpressed protein did not show any fluorescence under UV-light upon SDS-PAGE, which is an indication that this protein does not contain a covalently histidyl-bound flavin cofactor (Fraaije et al. 1997).

PuO_{Rh} was purified from a 1-l culture containing 0.02% arabinose that was grown for 24 h at 30°C. Due to the high level of overexpression (200 mg), PuO_{Rh} could be easily purified in one-step by anion exchange chromotography. SDS-PAGE analysis of the purified protein revealed that, besides the major protein band present at around ~54 kDa, there is a minor band corresponding to a protein mass which is approximately 3 kDa larger (Fig. 1, lane A). It was concluded that this must be the product of the same gene with a His-tag extension, as this minor protein band could be removed by an additional purification step using a Ni-agarose column. (Fig. 1, lane B). Although in the plasmid pBADpuo_{Rh} the stop codon (TGA) was properly introduced, it is known that in some cases, this stop codon can be translated (MacBeath and Kast 1998). With the constructed plasmid, this would indeed result in a 3 kDa larger protein.

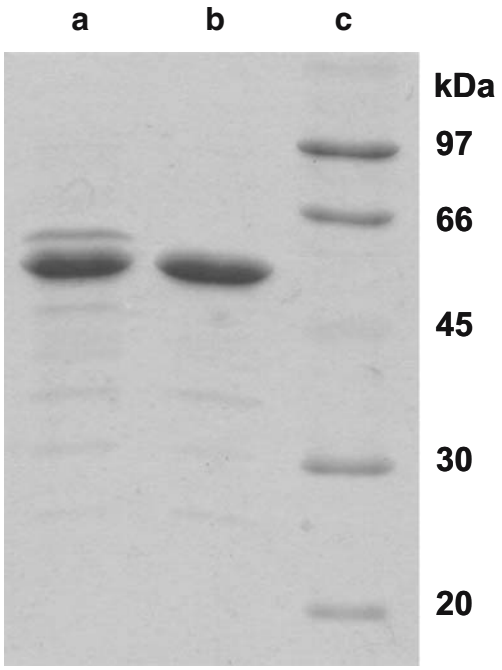


Fig. 1 SDS-PAGE gel of purified recombinant PuO_{Rh}. *Lane A* Purified PuO_{Rh}. *Lane B* Purified PuO_{Rh} after removal of His-tagged PuO_{Rh}. *Lane C* Low molecular weight marker

Spectral and molecular properties of PuO_{Rh}

PuO_{Rh} shows a typical flavoprotein spectrum with absorbance maxima at 377 and 459 nm (Fig. 2). In the presence of 5% trichloroacetic acid, PuO_{Rh} precipitated and was visible as a white pellet after centrifugation. The supernatant contained a yellow color, and its absorbance spectrum with maxima at 375 and 450 nm was typical for that of free FAD. This shows that the FAD cofactor in PuO_{Rh}, like in PuO_{Mr}, is not covalently bound. At room temperature, the addition of 0.1% SDS did not unfold PuO_{Rh}, indicating this flavoprotein is rather robust. When 50 μM putrescine was mixed with PuO_{Rh} under anaerobic conditions, the FAD cofactor was completely reduced (Fig. 2, dotted line). Heating the same sample for 5 min at 80°C completely unfolded the enzyme as can be seen from the resulting free FAD UV/VIS spectrum (Fig. 2, dashed line). The reduced enzyme could be rapidly and fully reoxidized by addition of oxygen, indicating that the enzyme is a true oxidase. From the difference in absorbance between protein-bound FAD and free FAD, the molar extinction coefficient for PuO_{Rh} (ε_{459}) was calculated, 11.0 mM^{-1} cm^{-1}. Based on the protein concentration as determined by Wadell's method (Wolf 1983) and the FAD concentration (A_{459}), a protein/FAD ratio of 0.5 was calculated. This is consistent with what has been described earlier for PuO_{Mr} (Desa 1972). Incubation of PuO_{Rh} with additional FAD did not yield an increase of FAD incorporation or oxidase activity. Gel filtration experiments revealed that PuO_{Rh} is mainly present as a dimer of ±100 kDa. This corresponds well with the theoretical monomeric mass of 49,375 Da.

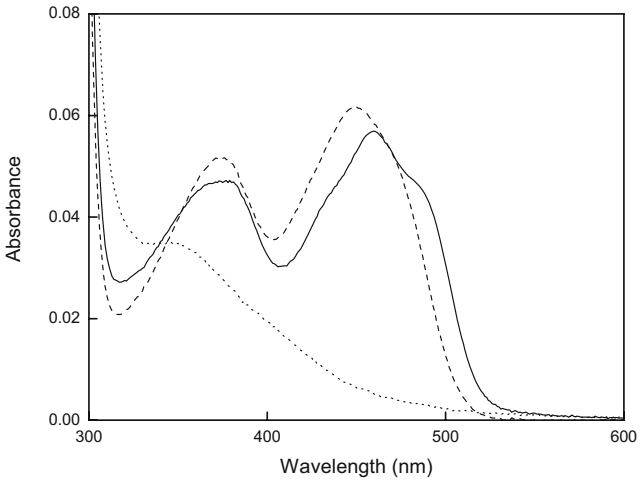


Fig. 2 Spectral properties of PuO_{Rh}. Absorbance spectra of oxidized native (*solid line*) and unfolded (*dashed line*) PuO_{Rh} are shown. After addition of 50 μM putrescine under anaerobic conditions, the native enzyme is fully reduced (*dotted line*)

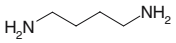
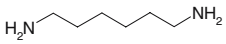
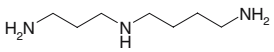
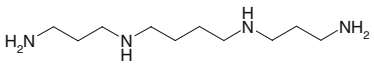
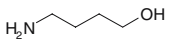
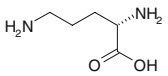
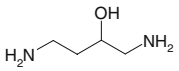
Temperature and pH dependence of activity and stability

To investigate the effect of temperature on enzyme activity and stability, both were determined at different temperatures. The optimal temperature for enzyme activity was found to be 30°C. Temperature stability experiments revealed that PuO_{Rh} is a reasonable thermostable enzyme. At 50°C, half of the activity was lost after 2 h, while at 37°C, such a degree of inactivation was reached only after 1 day. PuO_{Rh} has a rather sharp pH optimum for activity with putrescine with a peak around pH 8. Below pH 6.4, no significant activity can be detected.

Substrate specificity

Several alcohols, amino alcohols, and amines were tested as substrate, and if they turned out to be converted by PuO_{Rh}, the steady-state kinetic parameters were determined at pH 8.0. Enzyme activity was found with several aliphatic diamines, amino alcohols, and polyamines. From Table 1, it is clear that putrescine is by far the best substrate. With aromatic amines and amino alcohols, no activity was observed. An increase in carbon chain length of the diamine from 4 to 6 C-atoms resulted in a more than 1,000-fold drop in catalytic efficiency (k_{cat}/K_M). For diamines containing 3 or 2 C-atoms and for *n*-butylamine, no activity could be detected. These amines were found to strongly inhibit the enzyme (Table 2). Apparently, they bind efficiently but are not converted. Clearly, a minimum of two amino groups is required to be a substrate for PuO_{Rh}, and these amino

Table 1 Steady-state kinetic parameters of PuO_{Rh} at pH 8.0

Substrate		K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	relative activity (%)	
					PuO_{Rh}	PuO_{Mr}[a]
Putrescine		8.2 ± 0.5	26.4 ± 0.5	3200	100	100
Cadaverine		18 ± 3	3.9 ± 0.2	220	14	8.8
1,6-Hexanediamine		170 ± 20	0.37 ± 0.02	2.2	1.4	1.0
Spermidine		100 ± 20	1.4 ± 0.1	14	5.3	19.0
Spermine		470 ± 80	0.18 ± 0.01	0.38	0.7	0.1
4-Amino-1-butanol		2000 ± 300	0.17 ± 0.01	0.09	0.6	ND
L-Ornithine		330 ± 20	0.0198 ± 0.0001	0.06	0.08	ND
2-Hydroxyputrescine		65 ± 3	7.5 ± 0.1	120	28	ND

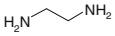
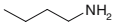
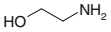
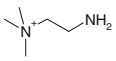
ND Not determined
[a] Values of putrescine oxidase from *Micrococcus rubens* (Okada et al. 1980)

groups should be preferably 4 C-atoms apart. Polyamines like spermine and spermidine were also converted by PuO_{Rh} but are poor substrates. Compared to putrescine, 4-amino-1-butanol is also a poor substrate, while 1,4-butanediol is not accepted at all. This again indicates that two amino groups are essential for efficient catalysis, and it confirms that PuO_{Rh} is a true amine oxidase. Inhibition studies showed that aminoethanol is a very effective competitive inhibitor of PuO_{Rh} (K_I=1.8 μM). Apparently, this amino alcohol binds very strongly in the active site, while it cannot be oxidized. When we tested 2-hydroxyputrescine, which contains an aminoethanol moiety, we found that this compound is readily oxidized. L-Ornithine was found to be a very poor substrate. Apparently, the presence of a negatively charged and relative bulky carboxylic acid group at the C1 position prevents efficient amine oxidation. Taken together, it can be concluded that PuO_{Rh} has a narrow substrate specificity and is very selective for putrescine.

Model structure of PuO_{Rh} reveals a key role for Glu324 in substrate binding

PuO_{Rh} shares 32% sequence identity with human monoamine oxidase B (MAO-B), which is a 59 kDa FAD-containing protein involved in the oxidation of neurotransmitters and other arylalkylamines like benzylamine and phenylethylamine (Shih et al. 1999). Based on the structure of MAO-B (Binda et al. 2004b), it was possible to construct a model for PuO_{Rh}. The C-terminal membrane anchor of MAO-B is absent in the resulting model structure of PuO_{Rh}. This could explain why PuO_{Rh} is well expressed in the cytosol as a soluble protein in contrast to MAO-B, which is membrane associated (Binda et al. 2004a). In the model, the active site of the PuO_{Rh} exhibits a similar architecture as that of MAO-B (Fig. 3). The catalytically important "aromatic cage," present in flavin-containing amine oxidases (Li et al. 2006), is formed by His432 and Tyr395. The other two key amino acid residues, Lys296 and Trp385, involved in non-covalent FAD binding

Table 2 Inhibition constants of competitive inhibitors of PuO_{Rh}

Inhibitor		K_I (μM)	
		PuO_{Rh}	PuO_{Mr}[a]
Ethylenediamine		660	950
1,3-Diaminopropane		64	120
n-Butylamine		120	460
Aminoethanol		1.8	3000
(2-Aminoethyl)-trimethylammonium		5800	ND

[a] Values of putrescine oxidase from *Micrococcus rubens* (Swain and Desa 1976)

(Binda et al. 2002b) can also be found in the model of PuO_{Rh}. However, there are also some striking differences. In MAO-B, FAD is covalently attached to Cys397 at the C8α position of the flavin. PuO_{Rh} has an alanine residue at this position (Ala394), which precludes covalent binding of FAD. Furthermore, the active site of PuO_{Rh} seems to be narrower than that of MAO-B due to the presence of Trp60 and Met173 in PuO_{Rh} (Tyr60 and Cys172 in MAO-B). The model of PuO_{Rh} also shows a glutamic acid residue (Glu324) pointing towards the N5 of the FAD, while MAO-B contains a tyrosine at this position (Tyr326). The presence of such a negatively charged amino acid in the active site has already been predicted for PuO_{Mr} several decades ago by Swain and Desa. They suggested the presence of a negatively charged carboxyl group in the active site, as PuO_{Mr} was found to be irreversibly inactivated by carbodiimides (Swain and Desa 1976). The presence and position of Glu324 can well explain the difference in substrate specificity between MAO-B and PuO_{Rh}. PuO_{Rh} prefers short aliphatic diamines, while MAO-B is active with a range of aromatic monoamines. Probably, Glu324 is involved in binding the protonated amino group of the diamine substrate, while the other amino group is positioned in the "aromatic cage" near the N5 of the flavin. Such a mode of binding for putrescine is consistent with our PuO_{Rh} model. To confirm this hypothesis, we mutated Glu324 into an alanine (PuO_{Rh} Glu324Ala) and a leucine residue (PuO_{Rh} Glu324Leu) and found that the catalytic efficiency for putrescine drops, respectively, 100,000 and 200,000-fold (Table 3).

The distance between Glu324 and the N5 of the isoalloxazine ring of the flavin cofactor (9 Å) may explain the specificity for the specific chain length of the substrate.

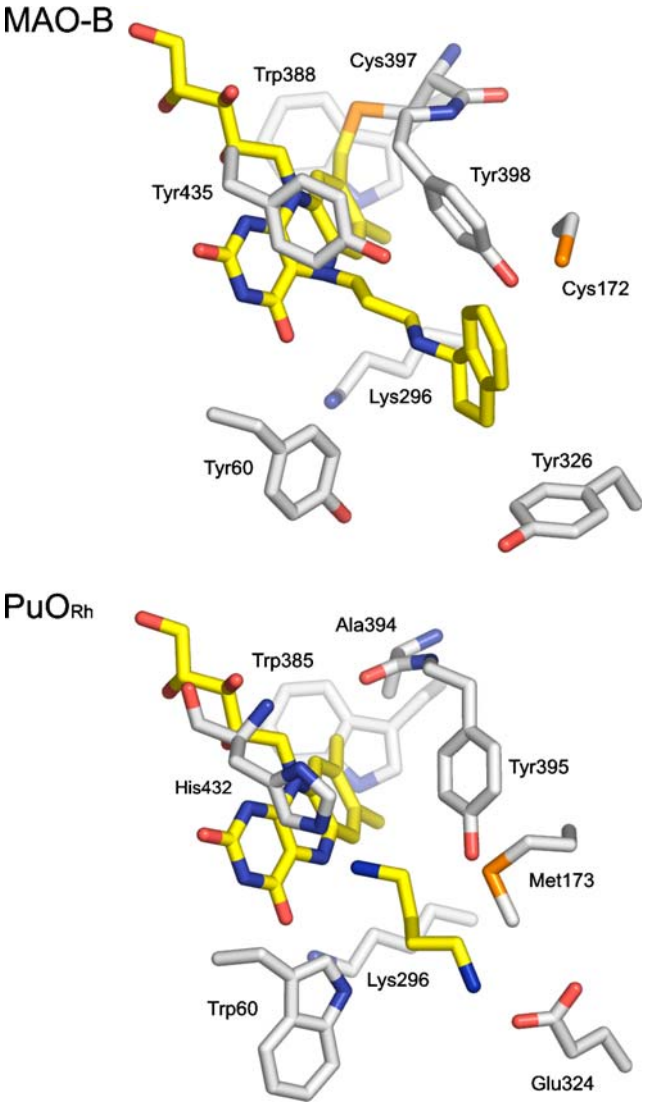


Fig. 3 Active site residues in the structure of MAO-B and in the model of PuO_{Rh}. In MAO-B, the inhibitor rasagiline is covalently bound to the flavin. In the model of PuO_{Rh}, the substrate putrescine is modeled in the active site

To be a substrate, a minimal carbon chain length of 4 C-atoms is required. Shorter diamines can bind to Glu324 but cannot reach the flavin and therefore are strong competitive inhibitors of PuO_{Rh} (Table 2). In case of longer diamines or polyamines, the position of the amino group that is oxidized

Table 3 Steady-state kinetic parameters for putrescine of wild-type PuO_{Rh} and of the mutants Glu324Ala and Glu324Leu at pH 9.0

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
PuO_{Rh} WT	3.5±0.6	20.7±1.1	5900
PuO_{Rh} Glu324Ala	110,000±20,000	6.1±0.4	0.05
PuO_{Rh} Glu324Leu	110,000±20,000	3.2±0.2	0.03

is less favorable compared to the case of putrescine resulting in a decrease in catalytic efficiency. Aliphatic monoamines like *n*-butylamine will bind to Glu324 via the amine moiety but cannot be oxidized and therefore are also inhibitors of PuO_{Rh}. Initial substrate screening using 96-wells plates for the mutants PuO_{Rh} Glu324Ala and PuO_{Rh} Glu324Leu revealed that both showed some activity with aromatic amines like phenyl-*1*-butylamine and with aliphatic monoamines (e.g., k_{cat}/K_M for *n*-butylamine=0.001 s^{-1} mM^{-1} at pH 9.0 for PuO_{Rh} Glu324Leu). Unfortunately, accurate determination of the kinetic parameters of the mutant enzymes is not straightforward due to their low substrate affinity, which requires the use of high concentrations of amines, causing interference with the peroxidase-based assay.

Discussion

In an attempt to discover novel bacterial oxidases with biocatalytic potential, a putrescine oxidase from *R. erythropolis* NCIMB 11540 was identified. This putrescine oxidase was discovered by using a plate-based screening method for oxidase activity, which has been used before to screen for improved mutants during directed evolution experiments (Alexeeva et al. 2002). Our study shows that this method is also valuable for the discovery of novel bacterial oxidases of unsequenced genomes. Although the physiological substrate of the discovered putrescine oxidase, 1,4-diaminobutane, was not present during screening, clones expressing this oxidase could still be detected based on their oxidase activity. As polyamines like putrescine, spermine, and spermidine are present in most living cells, including *E. coli* (Tabor and Tabor 1984), this resulted in the formation of hydrogen peroxide and detection of positive clones.

The physiological role of putrescine oxidase in *R. erythropolis* is probably related to polyamine degradation (Large 1992), which is supported by the presence of a neighboring gene encoding a putative aldehyde dehydrogenase on the sequenced DNA fragment (data not shown). PuO_{Rh} displays 67% sequence identity with PuO_{Mr}, which is the only bacterial putrescine oxidase that has been characterized so far. A BLAST search with the amino acid sequence of PuO_{Rh} resulted in a high number of homologous (putative) flavin-containing amine oxidases, which are widely distributed among most kingdoms of life. The seven closest homologs (sequence identity >65%) can all be found in actinomycetes. These sequences all contain the active site glutamate (Glu324), involved in substrate binding, the alanine residue (Ala394), which excludes covalent FAD binding, and the residues, which limit the size of the active site cavity (Trp60 and Met173). Most likely, they represent a clade of orthologous putrescine oxidases, which are not active with monoamines, within the family of (putative) flavin-containing amine oxidases (Supplementary information, ESM 1).

PuO_{Rh} shares some properties with PuO_{Mr}. Both enzymes are soluble dimeric proteins of approximately 100 kDa. Interestingly, both contain only 1 mol of non-covalently bound FAD per mole of dimer, which is rather unique among flavoproteins. The substrate specificity of PuO_{Rh} is very narrow, like PuO_{Mr}, the enzyme is very specific for putrescine. Polyamines are also accepted, but aliphatic monoamines are not converted at all. In general, PuO_{Rh} appears to have a higher affinity for its substrates than PuO_{Mr} (K_m for putrescine=8.2 vs 38 μM).

The narrow substrate specificity of PuO_{Mr} for di- and polyamines was already rationalized by the suggestion of the presence of an ‘anionic point’ in the active site. This anionic point, likely due to a carboxylate function, binds one positively charged amino group of the substrate, while another amino group is oxidized (Swain and Desa 1976; Okada et al. 1979). Based on sequence alignment between PuO_{Rh} and PuO_{Mr} and a structural model of PuO_{Rh}, we can now identify Glu344 as the anionic point in PuO_{Mr}. The presence of a carboxylate function as a manner to bind a positively charged amino group is not restricted to putrescine oxidase. In polyamine oxidase (PAO), a negatively charged ‘carboxylate ring’ can be found at one side of its substrate tunnel to guide polyamine substrate molecules into the active site (Binda et al. 1999). In addition, in PAO, two glutamate residues (Glu62 and Glu170) are present in the active site opposite to the N5 of the flavin. However, these residues have been suggested to be protonated (Binda et al. 1999). Moreover, in PAO, Glu62 and Glu170 are in close proximity to a substrate secondary amine group, which will be oxidized, while in PuO_{Rh}, Glu324 interacts with the (primary) amine group, which will not be oxidized. The amine group to be oxidized in PuO_{Rh} is placed between His432 and Tyr395, near the N5 of the isoalloxazine ring. This structural feature resembles the “aromatic cage” found in other flavin-containing amine oxidases, like MAO-A (De Colibus et al. 2005), MAO-B (Binda et al. 2002a), and PAO (Binda et al. 1999). For MAO-B, this “aromatic cage” has been shown to play a steric role in substrate binding and in flavin accessibility and helps to increase the substrate amine nucleophilicity (Li et al. 2006).

Polyamines like putrescine, cadaverine, spermine, and spermidine are related to cell growth and differentiation processes. Increased levels in body fluids and tissue occur in cancer patients, and therefore, polyamines represent important cancer markers (Casero and Marton 2007). Polyamines are also used to monitor food freshness, as an increased level can be found in spoiled food due to amino acid degradation (Bardócz 1995). Due to efficient heterologous expression and its catalytic properties, PuO_{Rh} may

develop as a valuable diagnostic enzyme for the detection of low amounts of putrescine and polyamines. Moreover, based on the structural model, enzyme redesign may also allow creation of mutants that can be used for synthetic purposes.

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