Published in final edited form as:

Biochemistry. 2010 June 22; 49(24): 5035–5041. doi:10.1021/bi100610e.

Identification of a Hypothetical Protein from *Podospira anserina* as a Nitroalkane Oxidase[†]

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Abstract

The flavoprotein nitroalkane oxidase (NAO) from *Fusarium oxysporum* catalyzes the oxidation of primary and secondary nitroalkanes to their respective aldehydes and ketones. Structurally, the enzyme is a member of the acyl-CoA dehydrogenase superfamily. To date no enzymes other than that from *F. oxysporum* have been annotated as NAOs. To identify additional potential NAOs, the available database was searched for enzymes in which the active site residues Asp402, Arg409, and Ser276 were conserved. Of the several fungal enzymes identified in this fashion, PODANSg2158 from *Podospira anserina* was selected for expression and characterization. The recombinant enzyme is a flavoprotein with activity on nitroalkanes comparable to the *F. oxysporum* NAO, although the substrate specificity is somewhat different. Asp399, Arg406, and Ser273 in PODANSg2158 correspond to the active site triad in *F. oxysporum* NAO. The k_{cat}/K_m -pH profile with nitroethane shows a pK_a of 5.9 that is assigned to Asp399 as the active site base. Mutation of Asp399 to asparagine decreases the k_{cat}/K_m value for nitroethane over two orders of magnitude. The R406K and S373A mutations decrease this kinetic parameter by 64 and 3-fold, respectively. The structure of PODANSg2158 has been determined at a resolution of 2.0 Å, confirming its identification as an NAO.

The flavoenzyme nitroalkane oxidase (NAO) from the soil fungus *Fusarium oxysporum* catalyzes the oxidation of nitroalkanes to the corresponding aldehydes or ketones with the release of nitrite and the consumption of molecular oxygen to yield hydrogen peroxide (Scheme 1) (1,2). NAO is unusual, since it catalyzes substrate oxidation by removing a substrate proton to form a carbanion intermediate (3) (Scheme 2), whereas flavoproteins that oxidize carbon-nitrogen and carbon-oxygen bonds typically catalyze cleavage of the substrate carbon-hydrogen bond by removal of a hydride rather than a proton (4–6). The NAO reaction is initiated by abstraction of the α -proton from the neutral form of the nitroalkane by Asp402, the active site base (7), creating a nucleophilic nitroalkane anion (Scheme 2). The anion then attacks the N5 position of FAD to form an adduct; this eliminates nitrite to generate a cationic, electrophilic flavin imine that can be attacked by hydroxide (8–11). Release of the aldehyde or ketone

[†]This work was supported in part by NIH grant GM 058698 to PFF and Robert A. Welch Foundation grant AQ-1399 to PJH.

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[‡]The atomic coordinates for PODANSg2158 have been deposited with the Protein Data Bank with the file name 3MKH.

product forms reduced FAD (7). In the more typical oxidative half-reaction, the reduced FAD is oxidized by molecular oxygen to form hydrogen peroxide (2). Release of products from the oxidized enzyme limits turnover with primary nitroalkanes, the best substrates (12). With the slow substrate nitroethane (13), formation of the substrate anion is rate-limiting for the reductive half-reaction (12).

The proton transfer reaction between nitroethane and Asp402 catalyzed by *F. oxysporum* NAO provides an excellent opportunity for a study of the basis for enzymatic catalysis because the enzymatic process can be modeled by the reaction of nitroethane and acetate in water (14). Nitroalkanes represent a prototypical system for the study of carbon acidity, in that they exhibit surprisingly slow deprotonation rates compared with their high acidities, the so-called nitroalkane anomaly (15). NAO from *F. oxysporum* catalyzes the ionization of nitroethane 10^9 -fold more effectively than acetate. Computations suggest that there is a slight enhancement of quantum mechanical tunneling of the proton in the active site compared to the non-enzymatic reaction (16).

NAO is a structural member of the flavoenzyme acyl-CoA dehydrogenase (ACAD) superfamily (10,17,18), even though the sequences of the ACAD family members are only 13–23% identical to that of NAO (1,17). Both cofactors and the active site bases occupy similar locations in NAO and ACAD, but the substrates access the active site from opposite sides of the two proteins (11,19). Structural and mutational analyses have identified key active site residues in *F. oxysporum* NAO. The nearest residues to Asp402 are Arg409 and Ser276; together the three residues make up a catalytic triad. Mutation of Asp402 to alanine decreases the rate constant for abstraction of the nitroalkane proton by at least 1000-fold (3). The distance between Asp402 and Arg409 is appropriate for an electrostatic interaction, suggesting that Arg409 is involved in correctly positioning the active site base for catalysis. Mutation of Arg409 to lysine decreases the rate constant for proton abstraction by 100-fold, and alters the position of the active site base (20). The side chain hydroxyl of Ser276 forms hydrogen bonds with the carboxylate oxygens of Asp402 (20). Mutation of Ser276 to alanine decreases the rate of the proton abstraction by 100-fold without perturbing the active site structure (11).

When the sequence of *F. oxysporum* nitroalkane oxidase was first determined, its use as a query in a PSI-BLAST (21) search of the available sequence databases identified multiple sequences of acyl-CoA dehydrogenases, establishing the enzyme as related to that family of flavoproteins (17). However, all of the sequences identified in such a search contained glutamate as the active site. Since ACADs utilize a glutamate as the active site base (22), whereas NAO utilizes aspartate (7), all of these enzymes were likely to be true ACADs. The lack of other NAOs rules out the use of sequence analyses to identify residues important for catalysis and to provide insight into the structural basis for the divergent reactions of ACAD and NAO. We report here the identification of several probable NAOs from other fungi and provide kinetic and structural data firmly establishing the hypothetical protein coded by gene PODANSg2158 of *Podospora anserina* as an NAO.

MATERIALS AND METHOD

Materials

FAD, 1-nitroethane, 1-[1,1- $^2\text{H}_2$]nitroethane (99% D), 1-nitropropane, 1-nitrobutane, 1-nitropentane, 1-nitrohexane, dimethyl sulfoxide, sodium pyrophosphate and phenylmethylsulfonyl fluoride were obtained from Sigma-Aldrich (Milwaukee, WI). Dithiothreitol (DTT) was purchased from Inalco Spa (Milan, Italy). Isopropyl-thio-2-D-galactopyranoside (IPTG) was purchased from Research Products International Corp., (Prospect, IL). Kanamycin monosulfate was purchased from Fisher Bioreagents (Fair Lawn, NJ). Lysozyme was obtained from Roche Molecular Biochemicals (Indianapolis, IN);

ethylenediaminetetraacetic acid (EDTA) was obtained from Acros Organics (Morris Plains NJ); potassium phosphate and 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes) were obtained from Fisher Scientific (Fair Lawn, NJ). The nickel-nitrilotriacetic acid (Ni-NTA) column was obtained from Invitrogen (Carlsbad, CA). Ampicillin was from USB Corp. (Cleveland, OH). The pET21b(+) vector was from Novagen (Madison, WI).

The pJ201:20714 plasmid containing an artificial gene coding for PODANSg2158 (protein XP 001905136.1; GeneID 6189407) from *Podospira anserina* 980 with codons optimized for expression in *E. coli* was obtained from DNA 2.0 (Menlo Park, CA). Oligonucleotides were synthesized by the Nucleic Acid Core Facility at the University of Texas Health Science Center at San Antonio. Restriction endonucleases and Taq polymerase were purchased from New England Biologicals (Ipswich, MA). T4 Ligase and dNTP's were obtained from Promega (Madison, WI). Pfu DNA polymerase was obtained from Stratagene (La Jolla, CA). Plasmids were purified using mini- and midi-kits from Qiagen. *E. coli* strain BL21(DE3) from Novagen was used for protein expression, and strain XL10-Gold (Stratagene) was used during DNA cloning protocols. DNA sequencing was carried out with the Applied Biosystems Big Dye Terminator V3.1 kit and analyzed by the Nucleic Acid Core Facility at the University of Texas Health Science Center at San Antonio. The D399E, R40K and S273A mutations were generated with the QuikChange site-directed mutagenesis kit (Stratagene).

Expression and Purification of PODANSg2158

The DNA coding for PODANSg2158 was subcloned from pJ201:20714 into pET21b(+) using the NcoI and EcoRI sites at the 5' and 3' ends, respectively, to obtain pPODNE. A single colony of *E. coli* strain BL21(DE3) transformed with pPODNE was used to inoculate 25 mL of LB containing 100 µg/mL of ampicillin at 37°C. The starter culture was grown for 10–12 h; 10 mL were used to inoculate 1 liter of LB containing 100 µg/mL ampicillin at 37°C. When the A₆₀₀ value of the culture reached 0.6, IPTG was added to a final concentration of 0.25 mM and the temperature of the culture was lowered to 18 °C. After 14–20 h, cells were harvested by centrifugation at 5,000 g and 4 °C for 30 min. The cells were suspended in ten volumes of 50 mM Hepes, pH 7.0, 1.0 mM EDTA, 0.1 mM FAD, 0.1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 25 µg/mL lysozyme. The suspension was sonicated for 7 min on ice and centrifuged at 12,000 g for 20 min. The extract was loaded onto a Ni-NTA column that had been previously equilibrated with 50 mM Hepes buffer, pH 8.0. The column was eluted with a linear gradient from 0–2 M imidazole (200 mL total) in the same buffer. Fractions with the highest purity as judged by UV-visible absorbance and SDS-PAGE were pooled and concentrated using an Amicon Ultra (Millipore) centrifugal filter device. The mutant enzymes were expressed and purified following the same enzyme protocol. Purified enzymes were stored with 15% glycerol at –80°C.

Extinction coefficient of PODANSg2158

An equal volume of 7 M urea was added to enzyme in 50 mM Hepes, pH 8.0 (8). Any precipitated protein was removed by centrifugation for 10 min at 14000 g, and the visible absorbance spectrum of the supernatant was recorded. The change in absorbance between the enzyme-bound FAD and the free FAD after denaturation was used to calculate the extinction coefficient of the purified enzyme.

Assays

Nitroalkane oxidase activity was routinely measured in 0.5 mM FAD, 50 mM Hepes, pH 8.0, by monitoring the rate of oxygen consumption with a computer-interfaced YSI Model 5300A biological oxygen electrode (YSI Incorporated, Yellow Springs, OH) at 30 °C, as described previously (23). When the pH was varied, 50 mM sodium pyrophosphate was used over the pH ranges pH 5.4–7.0 and 8.0–11.0, and 50 mM potassium phosphate was used between pH

7.0 and 8.0. To vary the concentration of oxygen, oxygen and argon were combined in different ratios with a MaxBlend low flow air/oxygen blender (Maxtec Inc., Salt Lake City, UT), and the assay buffer was equilibrated with the gas mixture. Substrate solutions of nitroalkanes were prepared as stocks in dimethyl sulfoxide to prevent the formation of the anionic substrate. The concentration of active enzymes were determined using an extinction coefficient at 446 nm of $11.9 \text{ mM}^{-1}\text{cm}^{-1}$.

Data Analysis

Steady-state kinetic data were analyzed using the programs KaleidaGraph (Synergy Software, Reading, PA) and IgorPro (WaveMetrics, Inc., Lake Oswego, OR). Steady-state kinetic parameters were determined by fitting the data to the Michaelis-Menten equation. The pH dependence of the k_{cat}/K_M value for nitroethane was determined by fitting initial rate data to eq 1, which applies for pH profiles that show a decrease with unit slope at low pH. Here, Y is the $k_{\text{cat}}/K_{\text{NE}}$ value at a given pH, c is the $k_{\text{cat}}/K_{\text{NE}}$ value in the pH-independent range, and K_1 is the dissociation constant for the ionization of a group that must be unprotonated for binding or catalysis.

$$\log Y = \log \left(\frac{c}{1 + (H/K_1)} \right) \quad (1)$$

Crystallization, structure determination and refinement

Preliminary crystals of PODANSg2158 were grown in the UTHSCSA X-ray Crystallography Core Laboratory from crystallization screen kits (Qiagen Inc., Valencia, CA) with a Phoenix crystallization robot (Art Robbins Instruments, Sunnyvale, CA). Optimized crystals were grown within 1 week by the vapor diffusion method with the protein solution mixed in a 1:1 ratio with a buffer containing 2.5 M magnesium sulfate, 0.1 M 2-(N-morpholino)ethanesulfonic acid buffer, pH 6, and 18% glycerol. The crystals were flash-cooled using liquid nitrogen prior to data collection. Data were collected at the Advanced Photon Source beam line 24-ID-C (Argonne National Laboratory, Argonne, IL) equipped with an ADSC Quantum 315 CCD detector. Data were processed using HKL-2000 (24). Phases were generated by the molecular replacement method in PHASER (25) using the *F. oxysporum* NAO coordinates in Protein Databank entry 2C0U (10) as the search model. Coordinates were refined against the data using PHENIX (26), including simulated annealing, and alternated with manual rebuilding using COOT (27). The coordinates have been deposited in the Protein Data Bank with accession code 3MKH.

RESULTS

Identification of NAO orthologs

To identify orthologs of *F. oxysporum* NAO, PSI-BLAST searches of the available sequence databases were carried out. Since the use of aspartate versus glutamate as the active site base readily distinguishes NAO from ACAD, all positive hits containing a glutamate in the position corresponding to Asp402 were discarded. This screen eliminated all plant, bacterial, and vertebrate sequences. However, several fungal proteins fit this initial criterion for NAO orthologs (Figure 1). Their sequence identities to *F. oxysporum* NAO range from 43–86%. All contain an arginine corresponding to Arg409 of the *F. oxysporum* enzyme. All but one contain a serine corresponding to Ser276; the lone exception from *Gibberella zeae* contains a threonine in that position. We selected the hypothetical protein from the fungus *Podospira anserina* 980 coded by gene PODANSg2158 for further study since it has the lowest sequence identity to the *F. oxysporum* enzyme at 43%.

Characterization of PODANSg2158 expressed in *E. coli*

The DNA encoding PODANSg2158 was subcloned directly from pJ201:20714 into pET21b (+) using the NcoRI and EcoRI sites at the 5' and 3' ends, respectively, for expression of the His-tagged enzyme. Approximately 40 mg of pure NAO could be obtained from 2 liters of culture. The visible absorbance spectrum of the purified enzyme (Figure 2) establishes that the enzyme is a flavoprotein. Based on the small change in absorbance of the flavin when the protein is denatured, the extinction coefficient of the enzyme at 446 nm is $11.9 \text{ mM}^{-1} \text{ cm}^{-1}$. The crystal structure of the enzyme shows FAD bound to the active site (see below), establishing this as the form of the cofactor. The purified enzyme is active with nitroethane as the substrate, with a specific activity of $7.5 \mu\text{mol min}^{-1} \text{ mg}^{-1}$. This is comparable to the value of $10.6 \mu\text{mol min}^{-1} \text{ mg}^{-1}$ for *F. oxysporum* NAO (17). Thus, this protein can be classified as a nitroalkane oxidase.

Steady-state kinetic parameters for PODANSg2158

Previous steady-state kinetic analyses of *F. oxysporum* NAO have established the steady-state kinetic mechanism as a modified ping-pong mechanism typical of flavoprotein oxidases (Scheme 3) (2,7,28). Eq 2 gives the appropriate rate equation for such a mechanism. The appropriateness of eq 2 rather than the more complex eq 3 for a sequential mechanism can readily be determined using the fixed ratio method (29). In this approach initial rates are measured at different concentrations of the two substrates, keeping the ratio of their concentrations constant. Curvature in a double reciprocal plot of the resulting data suggests a sequential mechanism, since there will be a term containing the square of the concentration of one substrate in the rate equation (eq 4, $a = [\text{O}_2]/[\text{NE}]$). On the other hand, a linear plot will arise if there is no term in the rate equation containing the concentration of both substrates (eq 5). For PODANSg2158 a double reciprocal plot of initial rate vs. nitroethane concentration is linear (Figure 3), establishing eq 2 as appropriate and yielding a k_{cat} value of 15 s^{-1} . For such a mechanism, the $k_{\text{cat}}/K_{\text{M}}$ values of the nitroalkane and oxygen are independent of the concentration of the other substrate. The $k_{\text{cat}}/K_{\text{M}}$ values for oxygen and nitroethane were determined in separate analyses by varying each at a fixed concentration of the other. These values are given in Table 1. The K_{O_2} value for PODANSg2158 is higher than that for the *F. oxysporum* NAO (2), but within the range found for a number of other flavoprotein oxidases (30–34).

$$\frac{E}{v_0} = \frac{1}{k_{\text{cat}}} + \frac{K_{\text{O}_2}}{k_{\text{cat}}[\text{O}_2]} + \frac{K_{\text{NE}}}{k_{\text{cat}}[\text{NE}]} \quad (2)$$

$$\frac{E}{v_0} = \frac{1}{k_{\text{cat}}} + \frac{K_{\text{O}_2}}{k_{\text{cat}}[\text{O}_2]} + \frac{K_{\text{NE}}}{k_{\text{cat}}[\text{NE}]} + \frac{K_{\text{NE}}K_{\text{O}_2}}{k_{\text{cat}}[\text{NE}][\text{O}_2]} \quad (3)$$

$$\frac{E}{v_0} = \frac{1}{k_{\text{cat}}} + \frac{K_{\text{O}_2}}{k_{\text{cat}}a[\text{NE}]} + \frac{K_{\text{NE}}}{k_{\text{cat}}[\text{NE}]} + \frac{K_{\text{NE}}K_{\text{O}_2}}{k_{\text{cat}}a[\text{NE}]^2} \quad (4)$$

$$\frac{E}{v_0} = \frac{1}{k_{\text{cat}}} + \frac{K_{\text{O}_2}}{k_{\text{cat}}a[\text{NE}]} + \frac{K_{\text{NE}}}{k_{\text{cat}}[\text{NE}]} \quad (5)$$

F. oxysporum NAO prefers linear nitroalkanes as substrates, with primary nitroalkanes of four or more carbons having the highest activity. To probe the substrate specificity of the *P. anserina* enzyme, the k_{cat}/K_M values for a number of unbranched primary nitroalkanes were determined. As shown in Figure 4 the latter enzyme also prefers longer nitroalkanes, with 1-nitrohexane having the highest activity. However, this enzyme does not show the monotonic increase in the k_{cat}/K_M value with substrate chain length that *F. oxysporum* NAO does.²

pH-Dependence of $k_{\text{cat}}/K_{\text{NE}}$

The effect of pH on the k_{cat}/K_M value with nitroethane as substrate for PODANSg2158 was determined in order to obtain information about amino acid residues important for binding and catalysis in that enzyme. Figure 5 shows that the $k_{\text{cat}}/K_{\text{NE}}$ value is constant at high pH and decreases at low pH, resulting in a pK_a of 5.9 ± 0.1 . In comparison the pK_a for *F. oxysporum* NAO is 6.9 ± 0.1 (12).

Mutagenesis of active site residues

To probe the role of the catalytic triad in PODANSg2158 (Asp399, Arg406 and S273), these residues were replaced with asparagine, lysine and alanine, respectively. The steady state kinetic parameters of the mutant enzymes with nitroethane are listed in Table 1. The largest decrease was observed for the mutation of Asp399, as there is a 400-fold decrease in k_{cat} and ~200-fold decrease in the k_{cat}/K_M for nitroethane. The effect on the k_{cat} value is a more valid measure of the effect on the activity, in that the very low activity of this mutant enzyme makes accurate measurement of a $k_{\text{cat}}/K_{\text{NE}}$ value difficult. Mutagenesis of Arg406 to lysine results in only a 1.7-fold decrease in k_{cat} and a 24-fold decrease in $k_{\text{cat}}/K_{\text{NE}}$. Mutation of the Ser273 to alanine results in only a decrease of ~3-fold for both the k_{cat} and $k_{\text{cat}}/K_{\text{NE}}$ values.

Crystal Structure of PODANSg2158

In order to provide a more complete identification of the *P. anserina* enzyme as an NAO, we determined the three-dimensional structure of the wild-type enzyme by X-ray crystallography. The data collection and refinement are reported in Table 2. The crystallization conditions were different from those used for *F. oxysporum* NAO, yielding crystals that diffracted to 2.0 Å resolution. The protein crystallized in space group $P4_3$, with four subunits in the asymmetric unit, consistent with the tetrameric structure of *F. oxysporum* NAO (10). The structure was determined by molecular replacement using the structure of that enzyme as the search model (10). FAD could be clearly identified in each of the subunits. Figure 6 shows an overlay of the electron density map with the FAD and active site residues. This figure also shows the chain of ordered water molecules that extends from the FAD 2'hydroxyl to the protein surface through a different tunnel than the substrate binding site. A similar water chain is seen the structure of *F. oxysporum* enzyme, where it has been proposed to be involved in accepting the proton from the water molecule that forms the hydroxide required in the mechanism in Scheme 2 (11). Figure 7 depicts an overlay of the carbon backbones for one subunit from each enzyme. The two proteins clearly have the same overall fold; this is confirmed by the RMSD of 0.925 over 376 residues. An overlay of the catalytic triad from *P. anserina* and *F. oxysporum* NAOs, as well as the bound FAD, is shown in Figure 8. The positions of the FAD and the active site residues are conserved in the two enzymes.

DISCUSSION

The results described here identify PODANSg2158 as an NAO. The enzyme is a flavoprotein, and the steady state kinetic parameters in Table 1 establish the enzyme has nitroalkane oxidase

²Longer chain nitroalkanes are poorly soluble in aqueous solutions and therefore were not characterized as substrates.

activity. The identities between the *P. anserina* and *F. oxysporum* NAO are spread through the sequence; more importantly, the active site aspartate (Asp399/402), arginine (Arg406/409) and serine (Ser273/276) are conserved. The very similar three-dimensional structures confirm the assignment. These results suggest that the remaining proteins with sequences in Figure 1 can also be described as nitroalkane oxidases. NAO was previously proposed to be a member of the ACAD superfamily distinct from acyl-CoA dehydrogenases and acyl-CoA oxidases (1, 17), and SCOP (35) classifies NAO as a member of the medium chain acyl-CoA dehydrogenase-like family, despite the low (~25%) sequence identity to other members of the family. The distinct activities of NAO and acyl-CoA dehydrogenases and the very different active site residues suggest that NAO and related enzymes are better classified as a separate family within the superfamily. *F. oxysporum* NAO has been a model system for understanding enzyme-catalyzed carbanion formation (16,36). The availability of NAOs from additional sources expands the utility of the enzyme as a model system.

While PODANSg2158 is clearly an NAO, this enzyme differs from *F. oxysporum* NAO in a number of properties. The latter enzyme prefers linear primary nitroalkanes as substrates (13). There is an increase in the k_{cat}/K_M with each additional methylene group of the substrate up to 1-nitrobutane due to an increase in the forward commitment with increasing chain length and a decrease of 16-fold in the K_d with each additional methylene group (12). PODANSg2158 is similarly more active on longer nitroalkanes, but the increase in the k_{cat}/K_M values suggests a preference for longer chains than is the case for the *F. oxysporum* enzyme. Figure 9 (top) shows the active site of NAO with 1-nitrooctane bound. The substrate binds near the flavin at the end of a tunnel that extends to the protein surface (11). The nitroalkane binding pocket fits 1-nitrooctane well, with a constriction in the tunnel just distal of the terminal carbon of the substrate. PODANSg2158 has a similar tunnel, as shown in Figure 9 (bottom). Comparison of the nitroalkane binding cavities and the tunnels in the two enzymes shows that the tunnel in PODANSg2158 is much more open. This structural difference provides a reasonable explanation for the differences in substrate specificities of the two enzymes.

A key distinction between an NAO and the acyl-CoA dehydrogenases and oxidases that are also in the ACAD superfamily is the use of an aspartate as the active site base in NAO and a glutamate in ACADs. Asp402 is the active site base in *F. oxysporum* NAO, with a pK_a of 6.9 in the free enzyme (3). Mutagenesis of this residue to alanine or asparagine decreases the rate constant for proton abstraction by three orders of magnitude. The structural and kinetic data presented here establish that Asp399 is the active site base in PODANSg2158. The decrease in the k_{cat} of the D399N enzyme of 400-fold provides a lower limit for the effect; with the *F. oxysporum* enzyme, product release is rate-limiting for turnover, so that decreases in the rate-constant for CH bond cleavage are not fully reflected in the k_{cat} value (7). The pK_a of 5.9 in the $k_{\text{cat}}/K_{\text{NE}}$ profile for PODANSg2158 can reasonably be assigned to Asp399. It cannot be established from the present data whether the differences in the pK_a values of the bases in the two enzymes truly differ by one pK unit, since the pK_a value for PODANSg2158 could be perturbed by a significant commitment to catalysis with nitroethane as substrate. In the case of the *F. oxysporum* enzyme, the pK_a of 6.9 seen with nitroethane shifts to lower values with longer chain nitroalkanes due to increasing commitment with increased chain length (12).

While the active site triad of an aspartate interacting with an arginine and a serine is maintained in PODANSg2158, the effects of mutating the arginine and serine are less in PODANSg2158 than in *F. oxysporum* NAO. In the latter enzyme, the R409K and S276A mutations decrease the $k_{\text{cat}}/K_{\text{NE}}$ value by 64-fold and 21-fold, respectively (11,20), compared to decreases of 24-fold and 3-fold observed here. A significant forward commitment to catalysis could decrease the effect of mutating an active site residue, in addition to perturbing the pK_a value seen in the $k_{\text{cat}}/K_{\text{NE}}$ -pH profile, since CH bond cleavage would no longer be fully rate-limiting for nitroethane oxidation.

In summary, the data presented here establish that PODANSg2158, designated as a hypothetical protein in the sequence database, is better classified as a nitroalkane oxidase. They also suggest that the presence of the active site aspartate, arginine, and serine discriminates between NAOs and true ACADs.

Acknowledgments

This work is based upon research conducted at the Northeastern Collaborative Access Team beam lines of the Advanced Photon Source, supported by award RR-15301 from the National Center for Research Resources at the National Institute of Health. Use of the Advanced Photon Source is supported by the U.S. Department of Energy, Office of Basic Energy Sciences, under contract No. W-31-109-ENG-38. We thank Dr. Jonathan P. Schuermann at the Advanced Photon Source for collecting the X-ray diffraction data. Support for the X-ray Crystallography Core Laboratory by the UTHSCSA Executive Research Committee and the San Antonio Cancer Institute is also gratefully acknowledged.

Abbreviations

NAO	nitroalkane oxidase
Ni-NTA	nickel-nitrilotriacetic acid
IPTG	isopropyl-thio-2-D-galactopyranoside
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
Hepes	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
ACAD	acyl-CoA dehydrogenase

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PODANSg2158	FGA F DGS-- AVLVGAM GVGL	VVL P I FDGGNVGI RRRHLLQQ
TSTA 069890	ENS F GMS-- AALVGAM SAGI	VCL S L FDGGNVGV RRRQLQQ
PMAA 028790	ENS F GMS-- AALVGAM GVGI	ICL S L FDGGNVGV RRRQLQR
FG02379.1	EMA F SFT-- AALVGAM ACGI	TVL S L FDGGNVGV RRRQYER
SNOG 15929	ERT F GTS-- AALVGAM SVGV	ACL P L FDGGNVGV RRRQIEE
CHGG 01268	FGA F DAS-- AVLVGAM GVGL	MVL P I FDGGNVGI RRRHLLQQ
BC1G 11641	SGS F DCS-- AVLVGAM SVSV	IVL P I FDGGNVGI RRRHMQE
SS1G 09730	SRA F DFS-- AALVGAM SVSV	IVL P I FDGGNI GI RRRHMQE
NFIA 030710	LHS F EIS-- AMLVGAM GVGV	LVL P I FDGGNVGI RRRALQD
AN9162.2	TH T F E IS E T A MLVGAMGVGI	LVL P I FDGGNVGM RRRALQE
NAO	ETA F AMS-- AALVGAM AI G T	MCY P L FDGGNI GL RRRQ MQR
	276	402 409

Figure 1.

Alignment of active site residues of *F. oxysporum* NAO with likely orthologs: PODANSg2158, *Podospora anserina* DSM 980 hypothetical protein; TSTA 069890, *Talaromyces stipitatus* putative acyl-CoA dehydrogenase; PMAA 028790, *Penicillium marneffe* electron transport oxidoreductase; FG02379.1 *Gibberella zeae* PH-1 hypothetical protein; SNOG 15929, *Phaeosphaeria nodorum* SN15 hypothetical protein; CHGG 01268 *Chaetomium globosum* CBS 148.51 hypothetical protein; BC1G 11641, *Botryotinia fuckeliana* B05.10 hypothetical protein; SS1G 09730, *Sclerotinia sclerotiorum* 1980 hypothetical protein; NFIA 030710, *Neosartorya fischeri* putative acyl-CoA dehydrogenase; AN9162.2, *Aspergillus nidulans* hypothetical protein; NAO, *Fusarium oxysporum* nitroalkane oxidase. Conserved residues are in bold and the active site catalytic triad Ser276, Asp402, and Arg409 in NAO are in bold and underlined.

Figure 2. UV-visible absorbance spectrum of PODANSg2158. Conditions: 50 mM Hepes at pH 8.0 and 25°C.

Figure 3. Double reciprocal plot of the initial velocity vs. the concentration of nitroethane at a fixed ratio of nitroethane to oxygen of 2 at pH 8.0, 30°C.

Figure 4. Nitroalkane substrate specificities for *F. oxysporum* NAO and PODANSg2158. Conditions for PODANSg2158: 50mM Hepes, pH 8.0 at 30°C (empty bars). Data for *F. oxysporum* NAO from Gadda et. al. (12) (filled bars).

Figure 5. k_{cat}/K_M -pH profile for PODANSg2158 with nitroethane as substrate. The line is from a fit of the data to eq 3.

Figure 6.

The PODANSg2158 active site superimposed on σ_A -weighted electron density with coefficients $2mFo-DFc$ contoured at 1.2σ . The FAD isoalloxazine ring, the catalytic triad consisting of residues S273, D399, and R406, and a chain of hydrogen-bonded water molecules running from the active site to the bulk solvent are shown.

Figure 7. Superposition of subunit A of the *F. oxysporum* NAO structure [PDB code 2C12 (10), pink] and subunit A of the PODANSg2158 structure (cyan). The respective flavins are shown in stick representation with the same color scheme.

Figure 8. Overlay of the active sites from PODANSg2158 (carbon atoms colored in pink) and *F. oxysporum* NAO (carbon atoms colored in cyan). The active site of PODANSg2158 was overlaid with that of *F. oxysporum* enzyme (PDB code 2C12) (10) by superimposing the 4 carbons on the central ring in the FADs.

Figure 9.

Active site entrances in *F. oxysporum* nitroalkane oxidase [PDB code 2C12 (10), top] and *P. anserina* PODANSg2158 (bottom). The placement of the 1-nitrooctane (yellow in both panels) is based on the superposition of the backbone atoms of these structures with the backbone atoms of PDB code 2D9E (11).

Scheme 1.

Scheme 2.

Scheme 3.

Table 1

Steady-state kinetic parameters for wild-type and mutant PODANSg2158 with nitroethane as substrate *

Kinetic parameter	Wild-Type	D399N	R406K	S273A
k_{cat} (s^{-1})	15 ± 1 (9.3 ± 0.7^a)	0.037 ± 0.009^a	9.0 ± 0.5^a	5.0 ± 0.3^a
K_{NE} (mM)	13 ± 4	10 ± 8^a	25 ± 4^a	24 ± 4^a
$k_{\text{cat}}/K_{\text{NE}}$ ($\text{mM}^{-1}\text{s}^{-1}$) ^a	0.74 ± 0.20	0.004 ± 0.002	0.37 ± 0.04	0.21 ± 0.02
K_{O_2} (mM) ^b	0.39 ± 0.08	-	-	-
$k_{\text{cat}}/K_{\text{O}_2}$ ($\text{mM}^{-1}\text{s}^{-1}$) ^b	38 ± 5	-	-	-

* Conditions: 50 mM Hepes, pH 8.0, 30°C.

^a Determined by varying the concentration of nitroethane at 247 μM oxygen.^b Determined by varying the concentration of oxygen at 50 mM nitroethane.

Table 2

Data collection and refinement statistics.

Data collection	
Space group	$P4_3$
Cell dimensions	
a, b, c (Å)	137.5, 137.5, 131.3
α, β, γ (°)	90, 90, 90
Wavelength	0.97949
Resolution (Å)	50 - 2.0
R_{sym}^*	0.089 (0.589)
$I/\sigma I$	17.7 (3.3)
Completeness (%)	99.7 (100)
Redundancy	6.2 (6.1)
Refinement	
Resolution (Å)	44.9 - 2.0
No. reflections	152,266
$R_{\text{work}}/R_{\text{free}}$	0.176/0.209
No. atoms	
Protein	12660
Ligand	224
Solvent	1431
R.m.s. deviations [§]	
Bond lengths (Å)	0.011
Bond angles (°)	1.217

* Values in parentheses are for the highest resolution shell.

[§] R.m.s. deviations are from idealized values for protein.