The Combined Structural and Kinetic Characterization of a Bacterial Nitrate Monooxygenase from Pseudomonas aeruginosa PAO1 Establishes NMO Class I and II*

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Background: The annotation of >4,900 genes for nitrate monooxygenase, which detoxifies a deadly toxin, is inaccurate due to the lack of functional data.

Results: The protein PA4202 from Pseudomonas aeruginosa is characterized structurally and biochemically as the first bacterial nitrate monooxygenase.

Conclusion: Four conserved motifs are identified in PA4202.

Significance: New classes of enzymes are established based on the functional annotation of PA4202.

Nitrate monooxygenase (NMO) oxidizes the mitochondrial toxin propionate-3-nitrate (P3N) to malonate semialdehyde. The enzyme has been previously characterized biochemically in fungi, but no structural information is available. Based on amino acid similarity, 4,985 genes are annotated in the GenBank™ as NMO. Of these, 4,424 (i.e. 89%) are bacterial genes, including several Pseudomonads that have been shown to use P3N as growth substrate. Here, we have cloned and expressed the gene pa4202 of Pseudomonas aeruginosa PAO1, purified the resulting protein, and characterized it. The enzyme is active on P3N and other alkyl nitrate, but cannot oxidize nitroalkanes.

P3N is the best substrate at pH 7.5 and atmospheric oxygen with $k_{cat}/K_m$ of 1.2 x $10^6$ M$^{-1}$ s$^{-1}$, $k_{cat}$ of 1300 s$^{-1}$, and $K_m$ of 110 μM. Anaerobic reduction of the enzyme with P3N yields a flavosemiquinone, which is formed within 7.5 ms, consistent with this species being a catalytic intermediate. Absorption spectroscopy, mass spectrometry, and x-ray crystallography demonstrate a tightly, non-covalently bound FMN in the active site of the enzyme. Thus, PA4202 is the first NMO identified and characterized in bacteria. The x-ray crystal structure of the enzyme was solved at 1.44 Å, showing a TIM barrel-fold. Four motifs in common with the biochemically characterized NMO from Cyberlindnera saturnus are identified in the structure of bacterial NMO, defining Class I NMO, which includes bacterial, fungal, and two animal NMOs. Notably, the only other NMO from Neurospora crassa for which biochemical evidence is available lacks the four motifs, defining Class II NMO.

Functional annotation of prokaryotic genes based on experimental evidence represents only 0.33% of the microbial genes in COMBREX (COMbination of BRidges to EXperiments) (1, 2). This is primarily due to the rapid progress in the sequencing of entire genomes that supersedes by far the biochemical characterization of the gene products (1, 2). This problem is exacerbated by the limited accuracy of the computational prediction of function based on amino acid sequences (1, 2), mainly due to the paucity of biochemical information available for the reference sequence. Thus, the quality of functional predictions can be significantly improved by establishing rigorous reference standards of proteins with experimentally determined functions and activities.

An emerging biochemical interest related to the functional annotation challenge is the metabolism of nitro toxins, such as propionate-3-nitrate (P3N) and 3-nitropropionate (3-NPA), which at physiological pH exist in equilibrium with a pK_a of 9.1 (3). P3N is a potent inhibitor of succinate dehydrogenase and fumarase in the Krebs cycle (4–6) and is found in some plants and fungi as a defense against herbivores (3, 7). The inhibition of essential metabolic enzymes by P3N immediately halts energy production in poisoned cells, causing neurological disorders (3) and, at sufficiently high doses, death (3). Cases of P3N poisoning have been documented in humans, with mortality rates ~10% (8). P3N neurotoxicity has been conclusively demonstrated in animal models (9, 10), primarily due to the impact of P3N poisoning in the agricultural industry (8, 11–13). 3-NPA, with its conjugate base P3N, is indeed routinely used at low doses for in vivo studies of animal models to investigate the molecular basis of Huntington disease (10, 14). Plants and fungi...
associated with P3N possess detoxifying nitronate monooxygenases (NMO; E.C. 1.13.12.16) as defense from the toxin (3, 15).

NMOs are members of the Group H flavin-dependent monooxygenases (16), and catalyze the oxidation of P3N or other nitronate analogues through a radical mechanism involving a catalytic flavosemiquinone without formation of a canonical C4a-(hydro)peroxoquinone (17). Oxidation of P3N by NMO yields malonic semialdehyde, which is an important metabolite that can be converted to acetyl-CoA, acetate, or 3-hydroxypropionate and enter various catabolic or anabolic pathways (3).

To date the only NMOs that have been characterized biochemically are from Neurospora crassa (Nc-NMO) and Cyberlindnera saturnus (Cs-NMO), which was previously classified as Williopsis saturnus (3). These fungal enzymes contain FMN as cofactor, have fast catalytic turnovers in atmospheric oxygen with $k_{\text{cat}} \geq 430 \text{ s}^{-1}$ and high specificity constants for P3N with $k_{\text{cat}}/K_{\text{M}} \geq 10^6 \text{ M}^{-1} \text{ s}^{-1}$ at pH 7.4 (3). Based on amino acid similarity with the fungal NMOs, 4,985 genes are currently annotated in the GenBankTM as NMO or 2-nitropropane monooxygenases (4,985 genes are currently annotated in the GenBankTM as NMO or 2-nitropropane monooxygenases). One of these genes is expressed in Pseudomonas aeruginosa PAO1 and is highly specific for the metabolic poison P3N. We named the enzyme Pa-NMO (19). Crystal structures of the gene product of pa4202 in the presence and absence of 2-nitropropane are available at 2.3 Å resolution (20). However, biochemical evidence to unequivocally conclude that the product of gene pa4202 is an NMO is lacking, with only a qualitative description of the enzyme being active on 2-nitropropane but no quantitative descriptions of specific activity or kinetic parameters being reported with P3N or other nitronates (20). No experimental data are available on the other two hypothetical NMOs coded by pa0660 and pa1024.

In this study, we expressed and purified the recombinant enzyme code by gene pa4202 from P. aeruginosa PAO1. The biochemical, kinetic, and structural characterization revealed that the product of gene pa4202 is an NMO that is highly specific for the metabolic poison P3N. We named the enzyme Pa-NMO. The crystal structure of Pa-NMO solved to 1.44 ÌÁ resolution allowed us to identify four consensus motifs in the primary structure of the protein that are conserved in Cs-NMO for which biochemical data are available. The consensus motifs are found in 475 sequences annotated as hypothetical NMO belonging to bacteria, fungi, and two animals, establishing a new class of enzymes based on functional annotation of a reference standard protein for which structural-functional information is now available. Notably, Nc-NMO does not belong to this group and, being the only other NMO for which biochemical evidence for enzymatic activity is available, it defines a separate class of NMOs.

**EXPERIMENTAL PROCEDURES**

**Materials—**Plasmid pET21a(+) containing the gene pa4202 from P. aeruginosa PAO1 with a C-terminal histidine tag was prepared in a previous study (18). Escherichia coli strain Rosetta(DE3)pLysS was from Novagen (La Jolla, CA). Isopropyl 1-thio-β-D-galactopyranoside (IPTG) was from Promega, (Madison, WI), nitroalkanes were from Sigma. HiTrap™ Chelating HP 5-ml affinity column and PD-10 desalting columns were from GE Healthcare (Piscataway, NJ). All other reagents were of the highest purity commercially available.

**Purification of Recombinant PA4202 Enzyme (Pa-NMO)—**

E. coli expression strain Rosetta(DE3)pLysS harboring the plasmid pET21a(+) /pa4202 was used to inoculate 1 liter of Terrific Broth medium containing 50 µg/ml of ampicillin and 34 µg/ml of chloramphenicol. When the cell culture incubated at 37 °C with shaking at 160 rpm reached an $A_{600}$ of 1, IPTG was added to a final concentration of 100 µM and the temperature was lowered to 18 °C. After 19 h, the cells were harvested by centrifugation yielding 29 g of wet cell paste, which was resuspended in 120 ml of lysis buffer (10 mM imidazole, 300 mM NaCl, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, 5 mM MgCl₂, 2 mg/ml of lysozyme, 5 µg/ml of DNase, 5 µg/ml of RNase, and 20 mM sodium phosphate, pH 7.4) and subjected to several cycles of sonication. The cell-free extract obtained after centrifugation at 12,000 × g for 20 min was loaded directly onto a HiTrap™ Chelating HP 5-ml affinity column equilibrated with buffer A (10 mM imidazole, 300 mM NaCl, 10% (v/v) glycerol, and 20 mM potassium phosphate, pH 7.4). After washing with 10 column volumes of buffer A at 5 ml/min, Pa-NMO was eluted with 30% buffer B (buffer A + 0.5 M imidazole). The purest fractions based on SDS-PAGE analysis were pooled, dialyzed against 10 mM Tris-Cl, pH 8.0, 10% glycerol, and stored at −20 °C.

**Enzyme Assays—**Protein concentration was determined using the Bradford method with bovine serum albumin as standard (21). UV-visible absorbance was recorded with an Agilent Technologies diode-array spectrophotometer Model HP 8453 PC equipped with a thermostated water bath; SDS-PAGE analysis of protein samples was performed using wide range molecular mass markers (6,500–200,000 Da) from Sigma. The extinction coefficient of the enzyme-bound flavin was calculated in duplicate by passing a fraction of purified Pa-NMO trough a PD-10 desalting column equilibrated in 50 mM potassium phosphate, pH 7.0, before heat denaturation at 100 °C for 30 or 35 min. The protein was removed by centrifugation and the concentration of the extracted FMN was determined from the UV-visible absorption spectrum of the yellow soluble fraction using an $\varepsilon_{450}$ nm of 12,200 M⁻¹ cm⁻¹ for free FMN (22). Cofactor identification was performed by mass spectrometry of the flavin extracted from a desalted sample of Pa-NMO in water treated at 100 °C for 30 min using a Waters Micromass Q-TOF micro (ESI-Q-TOF) in negative ion mode at the Mass Spectrometry Facility of Georgia State University.

The enzymatic activity of the purified Pa-NMO with nitronates was determined with the method of the initial rates (23)
following oxygen consumption with a Hansatech Instruments computer-interfaced Oxy-32 oxygen-monitoring system. The method of the initial rates ensures to prevent conversion between the nitronate form and the nitroalkane form of the substrate. Pa-NMO was prepared by desalting chromatography through a PD-10 column equilibrated with 50 mM potassium phosphate, pH 7.5, 10% (v/v) glycerol just prior to kinetic analyses. Stock solutions of nitronates and nitroalkanes were prepared as previously described (24, 25). Enzymatic assays were carried out at atmospheric oxygen and 30 °C (i.e. 0.23 mM oxygen) in 50 mM potassium phosphate, pH 7.5, with the initial rates normalized for the enzyme-bound flavin using the experimentally determined ε443 nm = 12,500 M⁻¹ cm⁻¹. Enzyme concentrations ranged from 1.4 to 8.8 nM; substrates were in the 0.02 to 20 mM range. Because the second-order rate constants for protonation of the nitronates are in the 15–75 M⁻¹ s⁻¹ range (26, 27), enzymatic activity assays were started with the addition of the nitronate to the reaction mixture to ensure that a negligible amount of neutral species, i.e. nitroalkane, was present during the time required to acquire initial rates of reactions (typically ~30 s).

In the case of the steady-state kinetic experiment performed at 30 °C and pH 7.5 the methods of the initial rates following oxygen consumption were applied as described for the enzymatic assays above, with the enzyme concentration ranging from 1 to 3.5 nM, the P3N concentration from 0.03 to 0.8 mM, and oxygen concentration from 15 to 156 μM. No differences in the initial rates were observed when different concentrations of enzyme were used with the same concentrations of substrate and oxygen.

Time-resolved absorbance spectroscopy of the anaerobic reduction of Pa-NMO with P3N was performed with an SF-61DX2 Hi-Tech KinetAsyst high-performance stopped-flow spectrophotometer, thermostated at 30 °C, equipped with a photo-diode array detector. The stopped-flow instrument was made anaerobic by overnight incubation of glucose (5 mM)/Na-KOH and diluted in water were subjected to 25 cycles of degassing by applying vacuum and flushing with argon. The syringes containing the buffer (50 mM potassium phosphate, 10% (v/v) glycerol, pH 7.5) or the substrate P3N prepared in water/KOH and diluted in water were flushed for 30 min with argon before mounting onto the stopped-flow spectrophotometer. To ensure complete removal of traces of oxygen, glucose (2 mM) and glucose oxidase (0.5 μM) were present in the buffer, enzyme, and substrate solutions. The concentrations of enzyme and P3N after mixing were 15 μM and 1 mM, respectively. Spectra acquisition was set every 1.5 ms.

Data Analysis—The kinetic parameters for the enzymatic assays carried out at atmospheric oxygen with P3N or different nitronates were obtained from fitting the experimental points to the Michaelis-Menten equation for one substrate using Kaleidagraph software (Synergy Software, Reading, PA). Steady-state kinetic data were fit with Enzfitter software (Biosoft, Cambridge, UK) to Equation 1, which represents a ternary complex mechanism in which the second substrate, i.e. oxygen, reacts with the reduced enzyme before release of the product of P3N oxidation. kcat is the first-order rate constant for enzyme turnover at saturating concentrations of both substrates, Ka and Kb are the Michaelis constants for P3N and oxygen, respectively, Kia is a kinetic constant that accounts for the intersecting line pattern in the double reciprocal plot, e is the concentration of enzyme, and k cat/K b is the initial velocity.

$$\frac{V_0}{e} = \frac{k_{cat}[P3N][O_2]}{K_a[O_2] + K_b[P3N] + [P3N][O_2] + K_bK_a} \quad (\text{Eq. 1})$$

Protein Crystallization and Structure Determination—Recombinant, purified Pa-NMO was crystallized by the vapor diffusion hanging drop method at room temperature. The composition of the reservoir solution was 14% (w/v) PEG 5000 monomethylether and 0.1 M Na-HEPES at pH 7.0. A 2-μl drop of this solution mixed with 2 μl of protein solution (14 mg ml⁻¹ in 50 mM Tris-Cl, pH 8.0, 100 mM NaCl, 20 mM 2-nitropropane) was equilibrated against 1000 μl of reservoir solution and crystals grew in 7–10 days. Single crystals were cryo-cooled with 22% (v/v) glycerol as cryoprotectant. X-ray data were collected at 100 K on beamline BM-22 of the Southeast Regional Collaborative Access Team (SER-CAT) at the Advanced Photon Source, Argonne National Laboratory. The x-ray data were integrated and scaled using HKL-2000 (28). Molecular replacement was performed with Phaser (29) in the CCP4 suite of programs (30), using as initial model the main chain atoms of nitroalkane oxidase from Streptomyces anachromogenes (PDB code 3BW2) (31). Refinement of the crystal structure was carried out with REFMAC5 (32) and manual adjustment and rebuilding was performed with Coot (33). No electron density that could be modeled with the ligand 2-nitropropane was observed. The crystal structure was deposited as PDB entry 4Q4K. Protein structures were superimposed on Cn atoms by using SUPERPOSE of the CCP4 suite (34). Figures of the structures were generated with PyMol and CCP4mg (35). The detection of tunnels to the active site was performed with the software CAVER (36). The number of approximating balls was set at 12, the minimum probe radius was 1.0 Å, the shell depth 4 Å, and the starting point was set on the N5 atom of the flavin cofactor with a maximum distance of 3 Å and a desired radius of 5 Å.

A BlastP (37) analysis of the protein sequence of Pa-NMO was carried out against a non-redundant protein sequence database. The conserved motifs were designed manually based on the BlastP multiple sequence alignment of the results of the query. The multiple alignment in Fig. 7 was created with Clustal Omega (38) and Jalview 2.8 (39) and the neighbor joining tree in Fig. 8 was generated with Jalview 2.8 (39).

Expression of Recombinant NMO from Burkholderia phytofirmans—The genes encoding hypothetical NMOs bphyt_6745 and bphyt_4144 from B. phytofirmans PsJN were cloned in the vector pET21a(+) in a previous study (18). E. coli expression strain Rosetta(DE3)pLysS harboring this plasmid was used to inoculate 100 ml of Luria-Bertani medium containing 50 μg/ml of ampicillin and 34 μg/ml of chloramphenicol. When the cell culture incubated with shaking at 37 °C reached an A600 of 0.6, IPTG was added to a final concentration of 50 μM and the temp...
RESULTS

Purification of Recombinant Pa-NMO—Pa-NMO was expressed in the E. coli strain Rosetta(DE3)pLysS and purified to a high level as judged from SDS-PAGE analysis (Fig. 1). The purified enzyme showed a specific activity of 542 min\(^{-1}\) mol of O\(_2\) min\(^{-1}\) mg\(^{-1}\) with 1 mM P3N as substrate at pH 7.5 and 30 °C in atmospheric oxygen (i.e., 0.23 mM).

Cofactor Content—The UV-visible absorption spectrum of purified Pa-NMO showed maxima at 370 and 443 nm, which are characteristic of flavin-containing enzymes (Fig. 2). The identification of the cofactor after extraction from the enzyme was carried out by ESI mass spectrometric analysis in negative ion mode (Fig. 2) indicating that FMN is present in the enzyme. The FMN cofactor is non-covalently bound to the protein as indicated by its complete release to bulk solvent after heat denaturation of the purified enzyme. An extinction coefficient of 12,500 M\(^{-1}\) cm\(^{-1}\) was calculated for the enzyme-bound FMN, consistent with an FMN/enzyme stoichiometry of ~0.4 (21).

Substrate Specificity—The enzymatic activity of purified Pa-NMO was measured with various nitronates or nitroalkanes by monitoring oxygen consumption at pH 7.5 and 30 °C. Oxygen consumption was seen with nitronates, but not with any of the nitroalkanes tested (i.e., 3-NPA, nitroethane, 1-nitropropane, or 3-NPA) as substrate as high as 20 mM. Ethylnitronate and propyl-2-nitronate, although with \(K_m\) values that were 60- to 400 times lower than with P3N (Table 1), Ethylnitronate and propyl-1-nitronate had \(K_m\) values of ~5 mM, which were 50-fold larger than with P3N, whereas propyl-2-nitronate, butyl-1-nitronate, and pentyl-1-nitronate could not saturate the enzyme up to 20 mM, consistent with even larger \(K_m\) values (Table 1).

Steady-state Kinetic Mechanism—The steady-state mechanism of Pa-NMO was determined at 30 °C and pH 7.5 by varying the concentrations of both P3N and oxygen. Fig. 4, panel A, reports the plot of the initial rates of reaction versus P3N concentration at different oxygen concentrations, whereas Fig. 4,
panel B, shows the double reciprocal plot of the initial rates of reaction and P3N concentration. The different slopes and y-intercept in the double reciprocal plot are consistent with a ternary complex mechanism, in which the second substrate oxygen reacts before the release of the first reaction product from the enzyme-bound anionic flavosemiquinone, in agreement with values typically observed for other anionic flavosemiquinones (17, 24, 25). Despite the molar excess of P3N the flavosemiquinone persisted anaerobically for at least 20 min, indicating that the 2-electron reduced hydroquinone or an N5-flavin adduct of the type seen with nitroalkane oxidase (41) are not reaction intermediates in the normal catalytic pathway of Pa-NMO.

**Structure of Pa-NMO**—The crystal structure of Pa-NMO was solved by molecular replacement in the space group P21 and refined to 1.44-Å resolution with an R factor of 20.3. The crystallographic data and refinement statistics are presented in Table 2. The protein crystallized with a homodimer in the asymmetric unit (Fig. 5A) with each monomer consisting of 351 well defined residues. No electron density was observed for the histidine tag at the C terminus. Each monomer consists of an FMN-binding domain (residues 1–71, 112–249, and 333–351) and a substrate-binding domain (residues 72–111 and 250–332) as highlighted in Fig. 5. The N-terminal and C-terminal residues are positioned on the same side of the FMN-binding domain facing each other at 16.3 and 18.7 Å distance in chain A and B, respectively. The dimer interface includes eight contacts well defined by loops (Fig. 5). A DALI (42) search of the Protein Data Bank database highlights how the overall folding resembles the structure of nitroalkane oxidase from *Streptomyces ansochro-

![FIGURE 4. Steady-state kinetics of Pa-NMO with P3N as substrate at 30 °C and pH 7.5. Panel A, plot of the initial rates of reaction versus [P3N] at oxygen concentration of 15 (empty circle), 34 (solid square), 87 (solid triangle), and 156 μM (solid circle). Panel B, double reciprocal plot of the initial rates of reaction versus [P3N] at oxygen concentration of 15 (empty circle), 34 (solid square), 87 (solid triangle), and 156 μM (solid circle). The lines represent the linear fit of the data to Equation 1.

### Table 2

| Data collection | Monoclinic P 21 |
|-----------------|-----------------|
| Crystal class and space group |  |
| Number of molecules per asymmetric unit | 2 |
| Unit cell parameters (Å, deg) |  |
| a = 70.12, b = 54.52, c = 88.51, β = 96.01 |
| Wavelength (Å) | 1.00 |
| Resolution (Å) | 37.6–1.44 (1.48–1.44)* |
| Total observations | 1,193,755 |
| Unique reflections | 123,114 |
| Rmerge (%) | 5.1 (41.5) |
| Complementarity (%) | 94.9 (75.5) |
| Redundancy | 4.5 (3.5) |

### Refinement

| Number of reflections Rwork/Rfree | 108,415/5,780 |
| Number of atoms | 5,911 |
| Number of solvent molecules | 593 |
| Isotropic B factors (Å²) | 25.85 |
| Protein (main chain) | 23.11 |
| Protein (side chain) | 27.44 |
| FAD | 23.33 |
| Solvents | 32.77 |
| R.m.s. deviation | 0.017 |
| Bond length (Å) | 1.99 |
| Ramachandran plot results |  |
| Residues in most favored regions (%) | 540 (93.3%) |
| Residues in additionally allowed regions (%) | 37 (6.4%) |
| Residues in generously allowed regions (%) | 2 (0.3%) |
| Residues in disallowed regions (%) | 0 (0.0%) |

* Values in parentheses are given for the highest resolution shell.
Bioinformatics Analysis of Pa-NMO—Most of the residues highlighted by the analysis of the crystal structure of Pa-NMO, such as Met\textsuperscript{20}, Asn\textsuperscript{69}, Phe\textsuperscript{71}, Tyr\textsuperscript{109}, His\textsuperscript{133}, His\textsuperscript{183}, Tyr\textsuperscript{299}, Tyr\textsuperscript{303}, and Lys\textsuperscript{307}, are conserved in the protein sequence of NMO (Fig. 7 and 8) from C. saturnus (25) and in pno\textsubscript{A} from Pseudomonas sp. JS189 (18), for which the physiological role was established to be the metabolism of P3N (18). This observation led to the identification of four conserved motifs (Table 3 and Fig. 9). A protein BLAST search was performed and 475 sequences annotated as hypothetical NMO belonging to bacteria, fungi, and two animals, i.e. Ceratitis capitata and Pantholops hodgsonii, possess all of the four conserved motifs identified in this study. Motif I, which contains the conserved Met\textsuperscript{20} that makes contact with the re face of FMN, provides protein main chain atoms that extend from the re face of the FMN to the entrance to the active site. The conserved active site residue His\textsuperscript{133} is present in Motif II, which is located near the N1 atom of the FMN cofactor. Motif III represents a β strand followed by a loop in the active site of the enzyme and contains the fully conserved His\textsuperscript{183}. Motif IV identifies an α helix delimiting the entrance to the active site and carrying the side chains of the conserved residues Tyr\textsuperscript{299}, Tyr\textsuperscript{303}, and Lys\textsuperscript{307}. The protein sequences of NMO from C. saturnus and pno\textsubscript{A} from Pseudomonas sp. JS189, which were previously established to be involved in P3N detoxification, carry the motifs identified in this study. We selected two additional hypothetical NMOs carrying motifs I to IV, namely the gene products bphyt\textsubscript{4144} and bphyt\textsubscript{6745} from B. phytofirmans PsJN to experimentally demonstrate that the genes identified based on the bioinformatics analysis were able to oxidize P3N. The cell-free extracts of the recombinant proteins expressed in E. coli have a specific activity with P3N of 12 and 5.5 units/mg, respectively. In contrast no oxygen consumption was detected with 1 mm nitroethane, 1-nitropropane, or 3-NPA. The cell-free extract of the control samples without addition of IPTG showed no activity with P3N. For comparison, the cell-free extract of Pa-NMO obtained using the same procedure had a specific activity of 27 units/mg with P3N. The motifs I to IV identified in the sequence of Pa-NMO are not present in the sequence of PA0660 and PA1024, the other two putative NMOs in P. aeruginosa PAO1.

DISCUSSION

The product of the gene pa4202 of P. aeruginosa PAO1 has been recombinantly expressed in E. coli, purified to a high level, and characterized in its biochemical, kinetic, and structural properties. This approach has established the first biochemical and kinetic characterization of a bacterial NMO and the first determination of the three-dimensional structure of NMO using x-ray crystallography. The concomitant availability of biochemical and structural information on Pa-NMO has allowed us to identify conserved motifs that define a new class
of NMO enzymes. These NMOs are primarily present in bacteria and fungi, but are also found in two animals.

The **pa**4202 **Gene Encodes for NMO**—Pa-NMO is a flavoprotein and contains FMN non-covalently bound, as indicated by the mass spectrometric analysis of the extracted cofactor showing an \( m/z \) ratio of 455.1 in negative ion mode. No peaks were seen in the 600–1000 \( m/z \) region of the mass spectrum, consistent with absence of FAD in the enzyme. The non-covalent binding of the flavin to the protein was further confirmed by x-ray crystallography. These findings are in keeping with all the biochemical data available on various NMOs, showing that they all utilize FMN, but not FAD, as cofactor (3, 17, 24, 25).

The best substrate of Pa-NMO is P3N with steady-state kinetic parameters determined in atmospheric oxygen that compare well with values previously determined for the two fungal NMOs from *N. crassa* and *C. saturnus* previously characterized, with \( k_{\text{cat}}/K_{\text{m}} \) and \( k_{\text{cat}}/K_{\text{m}} \) values \( \geq 10^6 \text{ M}^{-1} \text{s}^{-1} \) and \( \geq 450 \text{ s}^{-1} \), respectively, at pH 7.5 and 30 °C (15). The steady-state kinetic mechanism of Pa-NMO was established to be a ternary complex mechanism, similarly to *C. saturnus* NMO (25). As for the case of the *C. saturnus* enzyme, bacterial Pa-NMO can also effectively oxidize other primary and secondary nitronates, although with \( k_{\text{cat}}/K_{\text{m}} \) values 60–400 times lower than P3N, and cannot oxidize nitroalkanes (24).

The oxidation of P3N by Pa-NMO is extremely fast and results in the formation of an enzyme-bound flavosemiquinone. Evidence for this conclusion comes from the anaerobic mixing of the enzyme with P3N in a stopped-flow spectrophotometer, immediately yielding within 7.5 ms a flavin species with maxima at 364, 401, and 480 nm that are typical of anionic flavosemiquinones. This was previously observed with Cs-NMO, for which formation of an anionic flavosemiquinone was also too fast to be monitored in a stopped-flow spectrophotometer (25).

**FMN-binding Site**—The crystal structure of Pa-NMO was solved to 1.44 Å resolution and is the first structural analysis of an NMO enzyme, as no crystal structure is available for the well characterized fungal enzymes from *N. crassa* and *C. saturnus* (25, 43). The FMN-binding domain displays a TIM barrel-fold, which is found in other 15 FMN-dependent proteins (44). The phosphate group of the FMN is deeply buried in this domain and interacts via hydrogen bonds with the N atoms of the main chains of Gly181, Gly218, Gly239, and with the hydroxyl group of Thr240 (Scheme 1). In a similar way the ribityl moiety is held in

![FIGURE 6. The active site of Pa-NMO from different views. Panel A, the FMN cofactor is shown as yellow sticks in two different orientations. The omit map contoured at 0.5 e/Å³ is shown in blue. Panel B, FMN cofactor is represented as yellow sticks and the side-chains of active site residues as green sticks. Panel C, one different view of the active site is shown as electrostatic surface with blue indicating areas of positive electrostatic potential, red areas of negative electrostatic potential, and white areas of neutral electrostatic potential. Panel D, the same view of the active site of panel C is shown with the residues lining the entrance to the active site highlighted as green sticks and the tunnel to the active site computed by CAVER is shown as gray surface.](image-url)
place only by non-ionic interactions with the main and side
chain atoms of Gln\textsuperscript{176}, Gly\textsuperscript{181}, and Thr\textsuperscript{240}. The positioning of the ribityl and phosphate moieties of the cofactor via non-ionic interactions is conserved in 5 FMN-containing TIM barrel proteins, namely dihydroorotate dehydrogenase (PDB code 1DOR), glutamate synthase (PDB code 1LM1), PA1024 (PDB code 1P0N), isopenthenyl-diphosphate/H\textsuperscript{9004}-isomerase (PDB code 1P0N), and dihydropyrimidine dehydrogenase (PDB code 1H7W). Remarkably, in the other 10 FMN-containing TIM barrel proteins the binding of the phosphate group of the cofactor involves one or two salt bridges with arginine residues. In the case of mandelate dehydrogenase (PDB code 1HUV), glycolate oxidase (PDB code 1GOX), lactate oxidase (PDB code 2DU2), and flavocytochrome \textit{b\textsubscript{2}} (PDB code 1FCB) the phosphate group of the cofactor interacts with one or two arginine residues via salt bridges.

**FIGURE 7.** Multiple sequence alignment of protein sequences annotated as NMO enzymes from both prokaryotic and eukaryotic sources. NMO Class I is represented by sequences 1 to 7 including the biochemically characterized Pa-NMO (i.e. PA4202) and Cc-NMO (i.e. C. saturnus) and the P3N-NMO from Pseudomonas sp. JS189 (Ps. JS189). The numbering of the residues is according to the Pa-NMO protein sequence. Motifs I to IV identified are boxed in yellow and the conserved active site residues identified in the crystal structure of Pa-NMO are marked with a red star. Sequences 8, 9, and 10 belong to PA0660 and PA1024 and Nc-NMO from \textit{N. crassa}, respectively. The sequence identifier used is: PA4202, \textit{P. aeruginosa} PAO1 (NP_252891.1); Ps.JS189, \textit{Pseudomonas} sp. JS189 (ACX83564.1); C. saturnus (AAAS4484.1); Klebsiella pneumoniae (WP_004179795.1); C. capitata (XP_00432609.1); Z. rouxii, Zygosaccharomyces rouxii (XP_210029863.1); P. hodgsonii (XP_005969806.1); PA0660, \textit{P. aeruginosa} PAO1 (NP_249351.1); PA1024, \textit{P. aeruginosa} PAO1 (NP_249715.1); \textit{N. crassa} OR74A (XP_957588.1).
phate group establishes ionic bonds with the side chains of two arginine residues, whereas in the case of Old Yellow Enzyme (PDB code 1OYA), tRNA dihydrouridine synthase 2 (PDB code 3B0U), histamine dehydrogenase (PDB code 3K30), trimethylamine dehydrogenase (PDB code 2TMD), 12-oxophytodienoate reductase (PDB code 1ICS), and 2,4-dienoyl-CoA-reductase (PDB code 1PS9) only one arginine side chain contacts the phosphate group.

The isoalloxazine ring of the cofactor exposes its si face to the active site. On the re face, the isoalloxazine ring makes contact with the main chain Cα atom of Met20. Interestingly, the N5 atom of FMN is not in contact with any residue of the protein, and is involved only in a hydrogen bond with the solvent, as already observed in lactate oxidase (PDB code 2DU2). This is different from the other 14 FMN-binding TIM barrel proteins, where the N5 atom is located around 3 Å from either amide main chain atoms of the protein or from the side chain atoms of a cysteine, threonine, or lysine residue. In all the 15 FMN-binding TIM barrel proteins the N1 atom of FMN is located 3.0 Å from an arginine or lysine side chain, which is likely positively charged. This interaction is not observed in the crystal structure of Pa-NMO, where the closest contact of the N1 atom is the side chain of His133 at a distance of 4.0 Å, which may provide a positive charge as well. A similar interaction is observed in 12-oxophytodienoate reductase, where the flavin N1 atom is located 3.0 Å from the side chain of Arg239 and 3.5 Å from the side chain of His190. The N3 atom of FMN is 3 Å from the side chain of Asn69. A similar interaction is present in the other 15 FMN-binding TIM barrel proteins where the polar side chain is from glutamine, glutamate, asparagine, threonine, or serine.

**Active Site Residue His133**—In proximity of the N1 atom of FMN there is His133, which may be important for the stabilization of the catalytic anionic flavosemiquinone of Pa-NMO through an ionic interaction of its side chain with the negatively charged N1 atom of the flavin. This histidine is conserved in the fungal NMO from *C. saturnus* (25), which was previously characterized mechanistically with P3N or ethylnitronate as substrate (25). In that enzyme, pH profiles of the steady-state kinetic parameters showed that $k_{cat}/K_m$ and $k_{cat}$ were 2 orders of magnitude larger at acidic pH values than alkaline values with ethylnitronate (25), consistent with catalysis being different.
enhanced in the presence of a positive charge in the active site. No relevant ionizations in fungal NMO were seen in the $k_{\text{cat}}/K_m$ and $k_{\text{cat}}$ pH profiles with P3N, however, similar solvent viscosity effects on the steady-state kinetic parameters suggested that the same enzyme species were present in turnover with ethyl-nitronate and P3N (25).

Active Site Residue His$^{183}$—His$^{183}$ is fully conserved among all the genes currently annotated as NMO in GenBank$^{\text{TM}}$ (i.e. >4,900). In the structure of Pa-NMO, His$^{183}$ points to the N5 atom of the FMN, but it is not sufficiently close to establish an interaction being at almost 6 Å. The corresponding residue His$^{196}$ in Nc-NMO was previously mutated to asparagine, establishing it as the base that deprotonates nitroethane to ethyl-nitronate for the oxidation reaction catalyzed by the enzyme (45). Such a role, however, cannot be envisioned for Pa-NMO, or for Cs-NMO, because the latter catalyze the oxidative denitrification of P3N and other nitronates, but are not active with neutral nitroalkanes (this study and Ref. 24). As illustrated below, Motif III is the only one present in Nc-NMO among the four identified in this study in Pa-NMO, suggesting that the two enzymes may have different active site topologies and (slightly) different catalytic strategies. Nonetheless, the reaction pathway for oxidative denitrification of ethyl-nitronate in Nc-NMO was unaltered upon replacing His$^{196}$ with asparagine (45), suggesting that the fully conserved histidine is not required for the formation of the catalytically obligatory flavosemiquinone. A possible role for His$^{183}$ in Pa-NMO may be in facilitating binding the P3N substrate, which has a double negative charge, through either electrostatic or hydrogen bond interactions.

Other Conserved Active Site Residues—Tyr$^{199}$, Phe$^{134}$, Tyr$^{299}$, and Tyr$^{303}$ are also conserved in the fungal Cs-NMO and other 475 putative NMOs. These residues constitute the walls of the enzyme active site, suggesting important roles in either substrate binding or catalysis. Lys$^{307}$ is conserved among the residues that delimitate access to the active site, probably because it contributes electrostatic attraction for the doubly negatively charged substrate.

NMO Class I—The present study identified four motifs in the sequence of Pa-NMO that are conserved in 475 sequences of putative NMOs, including the biochemically characterized Cs-NMO, as illustrated in Fig. 7 showing multiple sequence alignment with Clustal Omega (38) of select protein sequences with the four conserved motifs highlighted in yellow (sequences 1 to 7). We define as NMO Class I the protein sequences containing motifs I to IV. Most of the class I NMOs functionally annotated belong to bacteria and fungi (i.e. 473), and only 2 are from animals. The fact that class I NMOs are so widespread in bacteria and fungi suggests their importance in conserved pathways, such as the detoxification of the toxin P3N and possibly physiological roles still not determined. C. capitata, which is commonly known as the Mediterranean fruit fly or medfly, is considered one of the most economically damaging agricultural pests due to its herbivorous diet, genetic variability, and ability to invade different territories (46). The Tibetan antelope, P. hodgsonii, has instead adapted to highly inhospitable environments that are characterized by low partial pressure of oxygen and high ultraviolet radiation (47). In the Tibetan region, plants of the genus Astragalus, which is extremely rich in P3N (7, 48), are known to become widespread during the frequent droughts. It is attractive to speculate that both organisms may have acquired the NMO function as an adaptation to the presence of the P3N toxin in the diet. A similar case was previously described for the detoxification of pyrrolizidine alkaloids in the host plants by the flavin-dependent monoxygenase (FMO) of the insect Tyria jacobaea (49).

Interestingly, only the equivalent of Met$^{20}$, Asn$^{69}$, and His$^{183}$ in Pa-NMO were conserved in the other hypothetical NMOs of P. aeruginosa PAO1 coded by genes pa0660 and pa1024, whereas motifs I to IV identified in this study are remarkably absent. A BLAST protein search highlighted more than 500 sequences of hypothetical NMOs from bacteria and fungi characterized by the PA1024 motifs identified in the structural study by Ha et al. (20). We suggest that the protein sequences displaying the motifs identified in PA1024, which at this stage is not confirmed to possess NMO function, should be grouped in a class different from that of Pa-NMO. Indeed, no kinetic parameters or specific activity with 2-nitropropane or other nitroalkanes or nitronates are available for PA1024 (20). Thus, it is a priority to kinetically characterize PA1024 and assess if this enzyme possesses characteristic NMO features, namely a $k_{\text{cat}}/K_m$ value for the physiological substrate P3N of $\sim 10^4 \text{M}^{-1} \text{s}^{-1}$, the presence of FMN as cofactor, and a flavosemiquinone catalytic intermediate (3).

NMO Class II—Nc-NMO from N. crassa contains only a few conserved residues identified in NMO Class I, such as Met$^{20}$, Asn$^{69}$, and His$^{183}$, and only parts of motifs I and III (Fig. 7). For this reason, we classify this enzyme as a member of NMO Class II, which defines a much smaller group than Class I with only 10 hypothetical NMOs from fungi other than Nc-NMO, such as from Neurospora tetrasperma FGSC 2508 (EGO54545.1) or from Togninia minima UCRA7 (EOO00344.1). Biochemical characterization of Nc-NMO has indeed established the enzyme to be highly specific for nitronates, with a marked preference for P3N in regard to $k_{\text{cat}}/K_m$ and $k_{\text{cat}}$ values (43). However, this enzyme has the unique ability to be able to utilize nitroalkanes as substrates, setting it aside from both bacterial Pa-NMO and fungal Cs-NMO for which only nitronates can be used as substrates (this study and Ref. 24).

In summary, we have characterized biochemically, kinetically, and structurally the protein encoded by gene pa4202 of P. aeruginosa PAO1 and demonstrated that it is an NMO. The enzyme contains non-covalently associated FMN, has a TIM barrel-fold, marked preference for the toxin P3N as substrate compared with other aliphatic nitronates, is not active on nitroalkanes, and is reduced to the flavosemiquinone upon anaerobic incubation with the substrate. This is the first instance in which a bacterial NMO has been characterized in its biochemical properties and in which the crystallographic structure of an NMO has been reported. The structural-functional approach has allowed us to establish rigorously a functional annotation of NMO genes thereby overcoming the inaccurate prediction of function based solely on amino acid sequence similarities. More than 450 putative NMOs from bacteria, fungi, and two animals are grouped in NMO Class I, which contains four consensus motifs identified in Pa-NMO. Lacking the consensus motifs of Pa-NMO, the only other NMO for
PA4202 Is a Nitronate Monooxygenase

which biochemical evidence of function is available, Nc-NMO, is grouped with few other proteins in NMO Class II. A considerable number of annotated proteins in various microorganisms, i.e. >500, shares the consensus sequences identified in PA1024 by Ha et al. (20). However, there is no biochemical or kinetic evidence yet that the enzyme is an NMO. Furthermore, no experimental data are available for PA0660, the third putative NMO of P. aeruginosa PAO1. Thus, it is imperative to kinetically characterize these proteins to establish whether they are NMO or have different functions. Future studies should also address the physiology of NMO in P. aeruginosa, to establish whether the enzyme is used to detoxify the toxin P3N or in other processes, such as for example, virulence or biofilm formation, besides as a growth substrate. Interestingly, Pa-NMO is conserved in all 12 P. aeruginosa genomes available in the Pseudomonas genome database (50) and even in all fluorescent pseudomonads (46 orthologues in total) (50), suggesting an important biological function. The availability of the structure of NMO sets the stage for future investigations by using site-directed mutagenesis aimed at structural-functional analyses of this unusual Class H flavin-dependent monooxygenase that can address fundamental questions on how oxygen is incorporated in the product of P3N oxidation, or what structural and biochemical determinants allow for the stabilization of a catalytic flavoseminiquinone in catalysis.

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