Functional Annotation of a Presumed Nitronate Monoxygenase Reveals a New Class of NADH:Quinone Reductases

The protein PA1024 from Pseudomonas aeruginosa PAO1 is currently classified as 2-nitropropane dioxygenase, the previous name for nitronate monooxygenase in the GenBank™ and PDB databases, but the enzyme was not kinetically characterized. In this study, PA1024 was purified to high levels, and the enzymatic activity was investigated by spectroscopic and polarographic techniques. Purified PA1024 did not exhibit nitronate monooxygenase activity; however, it displayed NADH:quinone reductase and a small NADH:oxidase activity. The enzyme preferred NADH to NADPH as a reducing substrate. PA1024 could reduce a broad spectrum of quinone substrates via a Ping Pong Bi Bi steady-state kinetic mechanism, generating the corresponding hydroquinones. The reductive half-reaction with NADH showed a $k_{\text{red}}$ value of 24 s⁻¹ and an apparent $K_v$ value estimated in the low micromolar range. The enzyme was not able to reduce the azo dye methyl red, routinely used in the kinetic characterization of azoreductases. Finally, we revisited and modified the existing six conserved motifs of PA1024, which define a new class of NADH:quinone reductases and are present in more than 490 hypothetical proteins in the GenBank™, the vast majority of which are currently misannotated as nitronate monooxygenase.

The discrepancy between the rapid increase in the number of sequenced genomes of prokaryotes and the slower experimental determination of protein function has resulted in the presence of a large number of hypothetical proteins in the databases, with gene function prediction often unreliable (1, 2). One case of an enzyme family consisting mainly of hypothetical proteins is represented by the nitronate monooxygenases (NMOs), which includes >5000 genes in the GenBank™. NMOs are FMN-dependent enzymes that catalyze the detoxification of propionate 3-nitronate (P3N), a metabolic poison produced by plants and fungi as a defense mechanism against herbivores (3). The kinetic mechanism of NMO has been investigated in previous studies on the fungal enzymes from Neurospora crassa (4) and Cyperlindnera saturnus (previously known as Williopsis saturnus) (5). The recent structural and kinetic characterization of the gene product PA4202 from Pseudomonas aeruginosa PAO1 as Pa-NMO identified four motifs that establish class I NMO, with 500 sequences from bacteria, fungi, one insect, and one animal (6). Class I NMO oxidizes the anionic nitronate form of the substrate, whereas class II NMO can oxidize both the neutral and anionic forms of P3N (6). P. aeruginosa PAO1 possesses two other genes coding for hypothetical NMOs, namely $\text{pa}0660$ and $\text{pa}1024$; however, these proteins do not carry the four motifs characteristic of class I NMO nor any of the motif signatures of class II NMO, raising the possibility that they code for enzymes with different function. Although there is no experimental evidence at transcript or protein levels for the gene product $\text{pa}0660$, a crystal structure for the hypothetical protein PA1024 is available for the enzyme in free form and in complex with 2-nitropropane (PDB codes 2GJL and 2GJN) at 2.0 and 2.3 Å resolution, respectively (7). The protein PA1024 is currently classified as 2-nitropropane dioxygenase, a previous name for NMOs, based on the gene function prediction (~32% sequence identity) and a qualitative enzymatic assay performed with 20 mM 2-nitropropane at pH 6.5 (7). Nonetheless, the kinetic parameters with 2-nitropropane were not determined, and the physiological substrate P3N was not tested, because it was unknown at the time of the study. The structural characterization of PA1024 previously highlighted six motifs conserved in other hypothetical proteins similar to PA1024 (7), which are different from the four motifs described for class I NMO (6).

In this study, we have cloned from genomic DNA the gene $\text{pa}1024$, purified the His-tagged recombinant PA1024, and characterized the spectral and kinetic properties of the enzyme. We demonstrated that purified PA1024 does not exhibit NMO activity. The enzyme belongs to an operon that contains two acyl-CoA dehydrogenases, an acyl-CoA hydratase/isomerase, a short chain dehydrogenase, and a porin (all hypothetical). All of these enzymes together imply the operon plays a role in β-oxidation. Based on the genomic context of PA1024, we hypothesized the enzyme oxidizes NAD(P)H to NAD(P)⁺ to maintain a favorable $[\text{NAD(P)H}]/[\text{NAD(P)}⁺]$ ratio, because it has been observed that a low ratio stimulates β-oxidation (8, 9). The connection between the $[\text{NAD(P)H}]/[\text{NAD(P)}⁺]$ ratio and β-oxidation is assumed to be due to the cofactor requirement of NAD⁺ for 3-hydroxyacyl-CoA dehydrogenases, which catalyze
the third step in \(\beta\)-oxidation (10). We also hypothesized that the reduced enzyme is oxidized by quinones because they are prevalent redox agents in nature (11, 12). To investigate this hypothesis, we experimentally tested NADH, NADPH, and various quinones for catalytic activity. Furthermore, we reanalyzed and modified the conserved motifs identified by Ha et al. (7) in the protein sequence of PA1024, and we showed that they are present in more than 490 sequences in the non-redundant protein database. The results reinforce the need for accurate experimental data on select hypothetical proteins to work in concert with computational methods for improved gene function prediction.

**Results**

**Protein Purification**—The gene pa1024 was cloned from the genomic DNA of *P. aeruginosa* PAO1 in the expression vector pET20b(+)**, with the addition of a His tag at the C terminus of the recombinant protein. The recombinant protein PA1024 was expressed in *Escherichia coli* and purified to high yield by affinity chromatography. The presence of 200 mM NaCl in a storage buffer composed of 20 mM Tris-Cl, pH 8.0, 10% v/v glycerol, was necessary for the *in vitro* stability of purified PA1024. SDS-PAGE analysis of the purified protein estimated a high level of purity.

**Spectral Properties**—The UV-visible absorption spectrum of purified PA1024 shows a maxima at 370 and 461 nm (Fig. 1), which are consistent with the presence of FMN as a cofactor, as demonstrated previously in the crystal structure of PA1024 (PDB code 2GJL) (Fig. 2) (7). The flavin cofactor extracted by heat denaturation was released to the bulk solvent, indicative of a non-covalent attachment of FMN to the protein, which is also in agreement with the crystal structure of the enzyme. The molar ratio FMN/enzyme was \(\approx 0.9\), consistent with a 1:1 stoichiometry per monomer of protein. The enzyme-bound flavin emitted light at 545 nm when excited at 461 nm, with an intensity equivalent to \(\approx 10%\) that of an equimolar concentration of FMN in solution (Fig. 1).

**Lack of Nitronate Monoxygenase Activity**—Nitronate monooxygenase activity was tested at pH 7.5 and atmospheric oxygen, i.e. 230 \(\mu\)M, as described previously (5, 6, 13), to determine the validity of the previous classification of PA1024 as an NMO. No enzymatic activity was detected with 1 mM P3N or 3-nitropropionic acid. No enzymatic activity was detected with 20 \(\mu\)M nitroethane, 1-nitropropane, 2-nitropropane, nor the anionic forms ethylnitronate, propyl-1-nitronate, and propyl-2-nitronate. In the case of propyl-2-nitronate and ethylnitronate, velocities of 16 and 5 \(\mu\)M oxygen consumed per min were detected, which would correspond to enzymatic rates of 1 and 0.5 \(s^{-1}\) with 180 \(\mu\)M enzyme. However, the same velocities were detected by incubating propyl-2-nitronate or ethylnitronate in the reaction buffer without PA1024, and they therefore represent non-enzymatic reactions. The activity reported by Ha et al. was thus likely due to the non-enzymatic reaction of propyl-2-nitronate with oxygen.

**Reducing Substrate**—The operon where PA1024 is found suggests that PA1024 could serve to regenerate NAD(P)\(^+\) for use by fatty acid-oxidizing enzyme(s) also found in the operon. To evaluate the reduction of PA1024 by NAD(P)H, the reduction of the FMN cofactor was followed by monitoring the decrease in absorbance at 461 nm under anaerobic conditions at pH 7.0 and 25 °C, with the use of a stopped-flow spectrophotometer. The enzyme was fully reduced with NADH in a biphasic pattern (Fig. 3). The fast phase, which accounts for more than 95% of the total change in absorbance at 461 nm, was assigned to flavin reduction. A slow phase accounting for less than 5% of the total change in absorbance at 461 nm was seen, with a substrate concentration-independent \(k_{obs}\) value of 1 \(s^{-1}\). The latter phase, which was considerably slower than the \(k_{cat}\) value determined with various quinone substrates (see below), could be due to a fraction of the enzyme being damaged. The fast phase of flavin reduction was analyzed with Equation 2, demonstrating that the enzyme was fully saturated with NADH from 90 to 500 \(\mu\)M, with a \(k_{red}\) value of 24 ± 1 \(s^{-1}\). An accurate \(K_d\) value was not determined because it was not possible to lower the NADH below 90 \(\mu\)M to maintain pseudo first-order conditions. However, the observation that the enzyme was fully saturated with 90 \(\mu\)M NADH suggests a \(K_d\) value in the 5 \(\mu\)M range or lower. When the enzyme was anaerobically mixed with 500 \(\mu\)M NADPH, there was slow reduction of the enzyme-bound flavin over 10 min, with an estimated rate constant of 0.0074 ± 0.0006 \(s^{-1}\). Thus, NADH is the preferred reducing substrate of PA1024.

**Oxidizing Substrates**—Quinones were selected as potential oxidizing substrates based on their powerful oxidizing ability and pervasive nature in the cell. Turnover of PA1024 was monitored with a quinone as the oxidizing substrate and NADH as the reducing substrate at pH 7.0 and 25 °C. PA1024 turned over with a number of different quinones, and the apparent steady-state...
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kinetic parameters were determined at a fixed, saturating concentration of 100 μM NADH. As shown in Table 1, benzoquinone and 5,8-dihydroxy-1,4-naphthoquinone were the best substrates for the enzyme based on the $k_{cat}/K_m$ value. Naphthoquinone substrates with hydroxyl groups on the aromatic ring of the naphthoquinone showed the highest $k_{cat}/K_m$ values for the bi-cyclic series. The $k_{cat}/K_m$ values plotted against the one-electron reduction potentials did not show a linear relationship (data not shown).

**Steady-state Kinetic Mechanism with 5-Hydroxy-1,4-naphthoquinone**—The traditional approach to obtain the steady-state kinetic mechanism of varying both NADH and quinone concentrations yielded overlapping lines in a double-reciprocal plot (data not shown), likely due to NADH being saturating at all concentrations used. Consequently, the product inhibition pattern using NAD$^+$ and 5-hydroxy-1,4-naphthoquinone as a substrate was used to establish the steady-state kinetic mechanism of PA1024. 5-Hydroxy-1,4-naphthoquinone was chosen for its occurrence and importance in nature (14), and because of its high $k_{cat}/K_m$ value (Table 1). A double-reciprocal plot of the initial rate of reaction versus the substrate concentration yielded lines converging on the y axis (Fig. 4), consistent with 5-hydroxy-1,4-naphthoquinone and NAD$^+$ binding to the same form of enzyme. This, in turn, is in line with a Ping Pong Bi Bi steady-state kinetic mechanism, as illustrated in Scheme 1. The $K_m$ for NAD$^+$ was determined to be 2.2 mM.

**Quinone Mode of Reduction**—To assess whether the reduction of the quinones catalyzed by PA1024 occurs through a single- or two-electron transfer from the enzyme-bound flavin, the obligatory one-electron transferring protein Cyt $c$ was employed as a reporter. A single-electron flux, as defined by Iyanagi (15), is the coupled Cyt $c$ reduction rate ($v$) divided by the doubled NADH oxidation rate ($2v$). The single-electron flux can have a value of 1 or lower, depending upon whether the reaction of PA1024 with the quinone occurs via a single- or a two-electron transfer. As shown in Fig. 5, all quinones tested had single-electron fluxes considerably lower than unity, ruling out a pure single-electron reduction of the quinone being catalyzed by PA1024.

In an independent experiment, the initial rates of the coupled Cyt $c$ reduction were measured in the absence and presence of...
SOD, because in the latter case a roughly 50% decrease in the rate of reaction would be expected if quinone reduction by PA1024 occurred through a two-electron reaction. As illustrated in Table 2, in all cases except 2,6-dimethoxy-1,4-benzoquinone, the initial rates for the coupled Cyt c reduction catalyzed by PA1024 were equal to or lower than 50% in the presence of SOD, further ruling out a single-electron reaction. The turnover number for the product NADH was monitored with the azo dye methyl red at a saturating NADH concentration at pH 7.0, 25 °C. No significant enzymatic reduction of methyl red was observed over 10 min, indicating PA1024 is not an azoreductase.

Lack of Azoreductase Activity—To determine whether PA1024 could perform azoreductase reactions, which is a common activity shared among most quinone reductases, turnover of PA1024 was monitored with the azo dye methyl red at a saturating NADH concentration at pH 7.0, 25 °C. No significant enzymatic reduction of methyl red was observed over 10 min, indicating PA1024 is not an azoreductase.

Bioinformatics—In a previous study, Ha et al. (7) identified six consensus motifs in the amino acid sequence of PA1024 and annotated PA1024 as 2-nitropropane dioxygenase, the former official name of NMO. That study was carried out before the crystal structure of Pa-NMO (PA4202) was available (6), which established class I NMO. A reanalysis of the conserved motifs of PA1024 based on the comparison of the active site residues of the two enzymes, PA1024 and Pa-NMO, in addition to a comprehensive PHI-BLAST search was carried out here, identifying six motifs originally reported (Table 3). The PHI-BLAST search of the six consensus motifs of PA1024 recognized ~500 (erroneously annotated) hypothetical proteins that share these motifs, the bulk of which comes from bacterial sources and are currently annotated as NMOs. The complete list of the genes and the PHI-BLAST search parameters can be found in supplemental Table S1.

FIGURE 5. Single electron flux for quinone reduction by PA1024. Values are expressed as the ratio of coupled Cyt c reduction rate divided by the doubled NADH oxidation rate. The dashed line represents the theoretical limit for a one-electron quinone reductase. Experiments were performed in 20 mM potassium phosphate, 200 mM NaCl, pH 7.0, at 25 °C. The concentration of each quinone was saturating (if possible), and NADH was held at a fixed, saturating concentration of 100 μM. BQ, benzoquinone; NQ, naphthoquinone.

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The current classification of PA1024 is based on a 2006 study (7), but the enzyme does not carry the consensus motifs characteristic of the recently defined class I and class II NMOs (6). The biochemical and kinetic characterization of PA1024 presented herein shows that the enzyme indeed is not an NMO, as indicated by the lack of oxygen consumption with propionate-3-nitrate and other nitroates. The enzyme, instead, uses NADH and quinones as reducing and oxidizing substrates, respectively. Thus, PA1024 should be reclassified as an NADH: quinone reductase (EC 1.6.99.5), along with ~500 genes that contain the consensus motifs identified in PA1024.

PA1024 efficiently uses NADH as a reducing substrate; the enzyme was fully reduced by NADH with a kcat value of 24 s⁻¹ and a Kd value estimated in the low micromolar range (Fig. 3). PA1024 displayed a marked preference for NADH over NADPH, as indicated by the fact that flavin reduction with 500 μM NADPH is ~3,500 times slower than with an equivalent concentration of NADH. Preference for NADH as a substrate was previously reported for some flavin-dependent monooxygenases (16), FMN-dependent quinone reductases in bacteria, such as tryptophan (W) repressor-binding protein (WrbA) (17), and the FMN-dependent azoreductases AzOA (18), AcpD (19), and AzoR (20). In the case of PA1024, the structural determinants for NADH specificity are currently unknown, and future crystallographic studies of the enzyme in complex with the product NAD⁺ will be required for their elucidation.

PA1024 catalyzes the reduction of various quinone substrates as shown in Table 1. The enzyme prefers benzoquinone, but naphthoquinones carrying hydroxyl groups in the 5- or 8-positions are also good substrates, as indicated by the large kcat/Km values determined with these substrates. Addition of methyl groups on the benzoquinone ring or methoxy groups in the 2-position results in a significant decrease in the kcat/Km value, possibly due to increased steric constraints for the formation of a catalytically competent enzyme-substrate complex. Interestingly, PA1024 does not react when the hydroxyl group is in the 2-position of the naphthoquinone substrate. Non-reactivity with 2-hydroxy-1,4-naphthoquinone is in stark contrast to Enterococcus cloacae nitroreductase, which shows a marked preference for this substrate (21).

PA1024 reduces quinones to the hydroquinone form through a two-electron mechanism. Two lines of evidence support this conclusion. First, single-electron fluxes considerably

3 The product of this substrate may reduce Cyt c primarily through the hydroquinone form of the product, and the autoxidation products are a minor reduction pathway; thus, SOD exerts less of an inhibitory effect.
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TABLE 2
Rate constants for the coupled Cyt c reduction by PA1024 with various quinones as substrates in the presence or absence of SOD
ND indicates enzymatic activity was not detected. The kinetic parameters were determined with 100 μM NADH in 20 mM potassium phosphate, 200 mM NaCl, pH 7.0, at 25 °C, at a saturating (if possible) concentration of the quinone substrate with or without the addition of superoxide dismutase.

| Electron acceptor | Coupled Cyt c reduction | Coupled Cyt c reduction + SOD |
|-------------------|-------------------------|-----------------------------|
| 1,4-Naphthoquinones |                         |                             |
| 2-Methyl-5-hydroxy- | 20.0 ± 1.5              | 7.5 ± 0.3                   |
| 5-Hydroxy-         | 22.1 ± 1.1              | 11.1 ± 1.2                 |
| 2-Methyl-          | 13.0 ± 0.3              | 4.6 ± 0.1                  |
| 5,8-Dihydroxy-     | 14.5 ± 0.3              | 7.0 ± 0.3                  |
| 2-Methoxy-         | 4.6 ± 0.2               | 2.1 ± 0.1                  |
| 1,4-Benzoquinones  |                         |                             |
| Benzoquinone       | 0.9 ± 0.1               | ND                          |
| 2,6-Dimethoxy-     | 9.3 ± 0.9               | 6.7 ± 0.2                  |
| 2-Methyl-5,6-dimethoxy- | 3.8 ± 0.2         | 1.4 ± 0.1                  |
| 2,3,5,6-Tetramethyl- | 0.9 ± 0.2              | ND                          |

TABLE 3
Conserved motifs in the protein sequence of PA1024
The numbering of the residues refers to the protein sequence of PA1024; the parentheses identify alternative residues that can be present in that position; h represents a position occupied by a hydrophobic residue; and X represents a position where any amino acid is accepted. Motifs were identified in a previous study by Ha et al. (7) and modified in this study. The modifications to the consensus sequence are underlined.

| Motif | Consensus sequence |
|-------|--------------------|
| I     | 17P/hOGGNGQ(WY)h20 |
| II    | 66T/hXPWGSXNTdP27 |
| III   | 121hHKC(T/V)hRHAa11 |
| IV    | 144[IV(M)DGFECHPGEXDXh25] |
| V     | 177hASGhAXXh(G/S)(L/V)hATA(V/v)hALGAXXhAGMTRI284 |
| VI    | 220hEXh(T/S)hXAHhARKXhNT/S)hAR(V/I)h241 |

smaller than 1 were obtained with a number of quinone sub-
strates when the obligatory one-electron acceptor Cyt c was used in a coupled enzymatic assay (Fig. 5). In this context, a single-electron flux is the ratio of the rate constant for Cyt c reduction to the doubled rate constant for NADH oxidation (15). The data rule out the alternative possibility of a single-electron reduction of the quinones by the enzyme-bound flavin of PA1024, because if this were the case single-electron fluxes of 1 would have been observed. The different single-electron fluxes determined with the various quinone substrates tested can be reconciled with their autoxidation rates, which were previously shown for naphthohydroquinones to follow the order of 5-hydroxy > 2-methoxy > 2-methyl > unsubstituted hydroquinone (22). In essence, hydroquinones that are more prone to non-enzymatic autoxidation can indirectly reduce Cyt c at faster rates through the products of autoxidation. The effect of SOD on the enzymatic activity of the enzyme in the Cyt c-coupled assay provides independent evidence for quinone reduction to the hydroquinone rather than the semiquinone state. With all quinone substrates tested except 2,6-dimethoxy-1,4-benzoquinone, the presence of SOD decreased the initial rates for the coupled Cyt c reduction catalyzed by PA1024 by 50% or more (Table 2). In the presence of Cyt c, if a semiquinone radical were produced in the quinone reduction catalyzed by PA1024 it would redox cycle without reducing oxygen and bypass superoxide formation (23). This property explains the ability to significantly inhibit the coupled Cyt c reduction by a two-electron reductase with SOD, but not a one-electron reductase, and the latter phenomenon has been observed in studies with ferric reductase B from Paracoccus denitrificans (23). Additional evidence of a two-electron reduction comes from the $k_{cat}/K_m$ values, which did not show a linear relationship with the one-electron reduction potentials and were sensitive to the structure of the substrate. The last two features are common among most two-electron quinone reductases (21, 24).

The two-electron reduction of quinones by PA1024 occurs through a Ping Pong Bi Bi steady-state kinetic mechanism, as demonstrated by the competitive inhibition pattern observed between NAD$^+$ and the quinone substrate (Fig. 4). NAD$^+$ must first leave the reduced enzyme before the quinone can bind and the naphthoquinone and NAD$^+$ compete for the reduced free enzyme (Scheme 1). All known two-electron flavin-dependent NAD(P)H:quinone reductases operate via a Ping Pong Bi Bi mechanism (25). This is likely due to the requirement for both reducing and oxidizing substrates to alternately position close to the flavin for the transfer of a hydride in the reductive and oxidative half-reactions catalyzed by NADH:quinone reductases (26).

With benzoquinones, flavin reduction is fully rate-limiting for the overall turnover of the enzyme. Evidence for this conclusion comes from the comparison of the limiting rate constant for flavin reduction ($k_{cat}$) and the $k_{cat}$ value that define turnover at saturating concentrations of both substrates, showing similar values of 24–28 s$^{-1}$. In contrast, with all naphthoquinones used, flavin reduction is partially rate-limiting for the overall turnover of the enzyme, as indicated by the $K_{red}$ value being significantly larger than the $k_{cat}$ values. This may reflect a slower rate constant for the release of the product of the reaction for the bulkier naphthoquinones compared with the smaller benzoquinones.

The $K_m$ value for NAD$^+$ of ~2 mM is at least 400 times larger than the $K_m$ value for NADH. This substantial difference is consistent with an unfavorable interaction of the positively charged NAD$^+$ in the active site of the enzyme as compared with the neutral NADH. The crystallographic structure of PA1024 (PDB 2GJL) shows the presence of the side chain of Lys-124 at ~4 Å from the N1-C2 locus of the flavin (Fig. 2B), allowing us to speculate that this residue may be responsible for NAD$^+$ binding being weaker than NADH due to electrostatic repulsion.

PA1024 has a small NADH oxidase activity, with a turnover rate of ~1 s$^{-1}$ at saturating NADH concentration and atmospheric oxygen. This indicates that the NADH oxidase activity
is likely not the primary function of PA1024. Slow oxidase activity has been observed in the case of NADH:quinone oxidoreductases (27), chromate reductase (28), flavin-dependent monooxygenases (uncoupling) (16), and old yellow enzyme (29, 30). In contrast to PA1024, FAD-dependent NAD(P)H:quinone oxidoreductase 1 (NQO1) and the quinone reductase Lot6p are not oxidized by molecular oxygen (25). In PA1024, the presence of a positive charge near the N1-C2 locus of the flavin, i.e. on the side chain of Lys-124, may contribute to the small oxidase activity observed, as in the case of several other flavin-dependent oxidases (31).

The UV-visible absorption spectrum of purified PA1024 displays the characteristic flavin signature, but the low-energy band of the cofactor at 461 nm is significantly red-shifted compared with free FMN (450 nm) and Pa-NMO (443 nm) (6). A large red shift in the low-energy peak is a feature usually indicative of a more polar protein environment around a flavin cofactor (32, 33). Accordingly, in the crystal structure of PA1024 (7), there is a polar Thr-75 located 3.8 Å from the N3 atom of FMN, although in the same position in Pa-NMO (PDB 4Q4K), there is the hydrophobic Phe-71 at 3.6 Å from N3 atom of FMN (6). Furthermore, the flavin in PA1024 is exposed to the solvent (Fig. 2B), which could contribute to additional hydrogen bond interactions with water. The fluorescence emission peak of PA1024 upon exciting the enzyme-bound flavin at 461 nm demonstrates a significant red shift to 543 nm and is only 10 times less fluorescent than FMN free in solution (Fig. 1, inset).

Enzymes with NAD(P)H:quinone reductase activity were recently reported from bacteria with the ability to detoxify azo dyes from the environment (34–36). Convergent evolution of azoreductases and quinone reductases has been proposed based on similar reaction mechanisms involving the flavin-mediated reduction of an organic molecule with NAD(P)H and substrate scope (37, 38). The lack of enzymatic activity with the azo dye methyl red indicates that PA1024 is not an azoreductase. Although there are several azoreductases from P. aeruginosa capable of reducing quinones (38), WrbA is the only other two-electron NAD(P)H:quinone reductase identified to date that does not possess azoreductase activity (39) beside PA1024.

PA1024 is a novel NADH:quinone reductase as illustrated below. The enzyme shares little overall sequence similarity to the well characterized human NQO1 and NAD(P)H:quinone oxidoreductase 2 (NQO2), with only 20 and 19% identity in its amino acid sequence, respectively. As shown in Fig. 6, which compares the active sites of the three enzymes, there are prominent differences in the active site frameworks of the three enzymes. A major difference is the presence of His-152 above the flavin of PA1024 and Ser-288 near the C7/C8 methyls of the flavin, both of which are absent in the NQOs. Also, Lys-124 is located near the N1 of PA1024, as opposed to Thr-148 in the NQOs. Notably, a Met-23 is positioned behind the re face of the flavin in PA1024, whereas a hydrophilic residue is present in NQO1 and NQO2. Similarities include the presence of a tryptophan below the flavin and hydrophilic residues near the N1-C2(O) locus of the flavin. Besides the active site, PA1024 displays several differences with respect to mammalian NQOs. First, PA1024 contains FMN, whereas the mammalian NQOs use FAD (7, 25). Second, PA1024 has a clear preference for NADH, whereas NQO1 can utilize both NADH and NADPH with similar efficacy, and NQO2 prefers modified derivatives of NADH, such as dihydronicotinamide riboside (40). Third, PA1024 is unable to reduce azo dyes, which are substrates of NQO1 and NQO2 (41–44).

Comparison of PA1024 with other known NADH:quinone reductases indicates that the enzyme is distinct in sequence and structure from those enzymes as well. The overall fold of PA1024 is a TIM barrel, distinguishing it from the flavodoxin-like fold of most NAD(P)H:quinone reductases characterized to date (25). As mentioned earlier, very few FMN-dependent quinone reductases specific for NADH have been identified. Other FMN-dependent quinone reductases, which however utilize both NADH and NADPH, have also been identified in bacteria, fungi, and plants, including Methanothermobacter marburgensis NQO (45), Lot6p from Saccharomyces cerevisiae (46), YhdA (47), FQR1 (48), and NQR (49), among others. It is worth noting that other flavin-dependent enzymes with known functions have been shown to exhibit NAD(P)H:quinone reductase activity, for example bacterial nitroreductases and glucose oxidase (50). Thus, the possibility that the primary function of PA1024 may be different from the NADH:quinone reductase activity characterized in this study cannot be excluded.

The six consensus motifs identified in PA1024 contain critical differences to Pa-NMO that discriminate the reactivity and substrate preference between the two enzymes. Motif I and V may aid in binding the cofactor and possibly modulating the redox potential. Motif II is likely related to the folding of the TIM barrel domain, yet a key difference is the presence of Thr-75 in PA1024 as opposed to Phe-71 in Pa-NMO. Motif III...
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is also a part of the TIM barrel fold because all of the residues are not located near the active site, with the exception of the Lys-124, which is unique to the PA1024-like class of quinone reductases. Motif IV is located on the surface above the active site cavity and may play a role in substrate preference. Motif VI is located in the putative substrate binding domain and is completely absent in Pa-NMO. Most importantly, the last parts of motif II, motif III, and motif VI of PA1024 are the most helpful in identifying significant differences in the two classes of enzymes represented by PA1024 and Pa-NMO.

NAD(P)H:quinone oxidoreductases have been implicated in the detoxification of quinones within intracellular pools, thereby exerting an antioxidant protective role. This is due to the fact that upon a two-electron reduction of quinones to the hydroquinones, the highly reactive semiquinones are bypassed, which in turn lowers free radical formation and consequent lipid peroxidation, formation of protein adducts, and DNA modifications (51–53). This could be the case for PA1024. However, when one considers that the operon containing pa1024 includes hypothetical acyl-CoA dehydrogenases, a short chain dehydrogenase, an acyl-CoA hydratase/isomerase, and a porin, an alternative speculation is that PA1024 may be required to maintain an appropriate [NAD+/NADH] ratio for the catabolism of fatty acids in P. aeruginosa PAO1. A link between a low [NAD+/NADH] ratio and the stimulation of β-oxidation was observed previously (8, 9). Furthermore, participation of an NADH:acceptor oxidoreductase as a physiological partner of a butyryl-CoA dehydrogenase was recently proposed in Syntrophomonas wolfei, although the enzyme was not identified (54).

In summary, the results presented in this study remedy the annotations of close to 500, mostly bacterial hypothetical proteins in the databases, and they define a new class of FMN-dependent quinone reductases with a TIM-barrel fold. There appears to be a widening gap between the benchtop and desktop that if left unchecked could potentially cause confusion, raising the necessity for computational biochemists and experimentalists to cooperate more closely to enhance gene function prediction. The results presented herein also highlight the structural diversity among quinone reductases and their deeply rooted evolutionary differences in nicotinamide preference and azoreductase activity. The biological significance of an NADH:quinone reductase activity is possibly more complex than previously considered, and the various effects of quinones and quinone reductases on the cellular setting have yet to be fully unraveled.

Experimental Procedures

Materials—The enzymes XhoI, Ndel, DpnI, calf intestinal alkaline phosphatase, and T4 DNA ligase were from New England Biolabs (Ipswich, MA); Pfu DNA polymerase was from Stratagene (La Jolla, CA), and oligonucleotides were from Sigma Genosys (The Woodlands, TX). E. coli strain DH5α was purchased from Life Technologies, Inc. E. coli strain Rosetta(DE3)pLysS and the expression vector pET20b(+) were from Novagen (Madison, WI); QIAprep spin miniprep kit, QIAquick PCR purification kit, and QIAquick gel extraction kit were from Qiagen (Valencia, CA). The genomic DNA of P. aeruginosa PAO1 was a kind gift from Dr. Jim Spain, Georgia Institute of Technology, Atlanta, GA. HiTrap™ chelating HP 5-ml affinity column was from GE Healthcare, and isopropyl 1-thio-β-D-galactopyranoside was from Promega (Madison, WI). Cytochrome c from bovine heart, superoxide dismutase, nitroalkanes, and quinones were from Sigma. All other reagents used were of the highest purity commercially available.

Cloning—The gene pa1024 was amplified from the genomic DNA of P. aeruginosa PAO1 by PCR in the presence of 5% DMSO. The PCR protocol included an initial denaturation step at 95 °C, 20 cycles of denaturation for 45 s at 95 °C, annealing for 30 s at 56 °C (with the annealing temperature progressively decreasing by 0.2 °C at each cycle), extension for 3 min at 72 °C, and a final step for 10 min at 72 °C.

The pa1024 gene amplified by PCR was purified by agarose gel extraction with the QIAquick gel extraction kit. The amplimer and the expression vector pET20b(+) were subjected to double digestion with NdeI and XhoI and purified with the QIAquick PCR purification kit. After a dephosphorylation step with 0.5 units of calf intestinal alkaline phosphatase for 30 min at 37 °C, the dephosphorylated vector was purified with the QIAquick PCR purification kit and ligated to the insert with incubation for 15 h at 16 °C with T4 DNA ligase. A volume of 5 µl of the ligation mixtures was used to transform E. coli strain DH5α. The resulting colonies grown at 37 °C on Luria-Bertani agar plates containing 50 µg/ml ampicillin were screened for the presence of the desired insert by DNA sequencing at the Cell, Protein, and DNA Core Facility at Georgia State University. The DNA sequencing confirmed the correct insertion of the gene in the plasmid vector and the absence of undesired mutations.

Recombinant Expression and Purification—E. coli expression strain Rosetta(DE3)pLysS transformed with the construct pET20b(+)/pa1024 was used to inoculate 90 ml of Terrific Broth containing 50 µg/ml ampicillin and 34 µg/ml chloramphenicol, which was incubated at 37 °C for 15 h. One aliquot of 8 ml of this culture was used to inoculate 1 liter of Terrific Broth containing 50 µg/ml ampicillin and 34 µg/ml chloramphenicol, which was incubated at 37 °C until it reached an optical density at 600 nm of 0.8. Isopropyl 1-thio-β-D-galactopyranoside was then added to a final concentration of 200 µM, and the culture was incubated at 18 °C for 20 h. The wet cell paste of 8.5 g, recovered by centrifugation, was resuspended in 40 ml of lysis buffer containing 10 mM imidazole, 300 mM NaCl, 10% v/v glycerol, 1 mM phenylmethylsulfonyl fluoride, 5 mM MgCl₂, 2 mg/ml lysozyme, 5 µg/ml DNase, 5 µg/ml RNase, and 20 mM sodium phosphate, pH 7.4. The resuspended cells were subjected to several cycles of sonication. The cell-free extract obtained after centrifugation at 12,000 × g for 20 min was loaded onto a HiTrap™ chelating HP 5-ml affinity column equilibrated with 10 mM imidazole, 300 mM NaCl, 10% v/v glycerol, and 20 mM sodium phosphate, pH 7.4. After washing with 10 column volumes of equilibrating buffer, the column was treated with four intermediate steps at 50, 100, 150, and 250 mM imidazole in equilibration buffer to remove possible contaminants. PA1024 was eluted with 500 mM imidazole in equilibration buffer. The purest fractions based on SDS-PAGE analysis
were pooled, dialyzed against 20 mM Tris-Cl, pH 8.0, 200 mM NaCl, 10% glycerol, and stored at −20 °C.

Spectroscopic Studies—UV-visible absorbance was recorded with an Agilent Technologies diode-array spectrophotometer model HP 8453 PC (Santa Clara, CA) equipped with a thermostated water bath in 20 mM Tris-Cl, pH 8.0, 200 mM NaCl, plus 10% glycerol at 25 °C. The extinction coefficient of purified PA1024 was determined by extracting the FMN cofactor by heat denaturation of the enzyme. After removing the denatured protein by centrifugation, the concentration of free FMN was determined spectroscopically by using \( \epsilon_{350} = 12,500 \text{ M}^{-1} \text{ cm}^{-1} \) (55). The concentration of flavin-bound active enzyme was determined by using the experimentally determined extinction coefficient \( \epsilon_{450} \) of 12,400 \text{ M}^{-1} \text{ cm}^{-1} \) (this study). The total protein concentration was determined using the Bradford method with bovine serum albumin as standard (56). Initial rates of enzymatic reaction were normalized for the concentration of enzyme-bound flavin.

Fluorescence emission spectra were recorded in 20 mM Tris-Cl, pH 8.0, 200 mM NaCl, 10% glycerol, at 25 °C, with a Shimadzu model RF-3301 PC spectrophuorometer (Kyoto, Japan) using a 1-cm path length quartz cuvette. All fluorescence spectra were corrected with the corresponding blanks for Rayleigh and Raman scatterings. For flavin fluorescence, the sample at an FMN concentration of 3.2 \( \mu \text{M} \) was excited at 461 nm (447 nm for free FMN), and the emission scan was determined from 480 to 680 nm. Free FMN was obtained by boiling the native enzyme followed by centrifugation.

Enzymatic Assays—Reduction of the enzyme-bound flavin with NAD(P)H was carried out anaerobically with an SF-61DX2 Hi-Tech KinetAssyst high performance stopped-flow spectrophotometer (Bradford-on-Avon, UK), thermostated at 25 °C. Anaerobiosis of the instrument was obtained by overnight incubation with glucose (5 mM)/glucose oxidase (1 U/ml) in sodium pyrophosphate, pH 6.0. The enzyme was passed through a desalting PD-10 column equilibrated with 20 mM potassium phosphate, pH 7.0, 200 mM NaCl, transferred in a tonometer, and subjected to 20 cycles of degassing by applying vacuum and flushing with argon. The syringes containing 20 mM potassium phosphate, pH 7.0, 200 mM NaCl, as buffer, or the substrate NAD(P)H dissolved in buffer were flushed for 30 min with ultrapure argon before mounting onto the stopped-flow spectrophotometer. To ensure complete removal of traces of oxygen, glucose (2 mM) and glucose oxidase (0.5 \( \mu \text{M} \)) were present in the buffer, enzyme, and substrate solutions. The concentration of the substrate NAD(P)H was determined spectrophotometrically at 340 nm with the extinction coefficient 6,220 \text{ M}^{-1} \text{ cm}^{-1} \) (57). The concentration of the enzyme after mixing was 15 \( \mu \text{M} \) and of NAD(P)H ranged from 90 to 500 \( \mu \text{M} \) to maintain pseudo first-order conditions.

Turnover of PA1024 with NADH was monitored with a suitable quinone in 20 mM potassium phosphate, 200 mM NaCl, pH 7.0, at 25 °C. NADH was kept at a constant saturating concentration of 100 \( \mu \text{M} \). Stock solutions of quinones were prepared in 100% ethanol, except 2,6-dimethoxy-1,4-benzoquinone, which was dissolved in DMSO. The final ethanol or DMSO concentration in all reaction mixtures was kept fixed at 1% to minimize possible effects on enzymatic activity. Reaction rates were measured by following NADH consumption at 340 nm, using \( \epsilon_{340} = 6,220 \text{ M}^{-1} \text{ cm}^{-1} \) (58). In the case of 2-methyl-1,4-naphthoquinone, 343 nm was followed instead, because its oxidized and reduced forms are isosbestic at this wavelength. The coupled Cyt c reduction was monitored by measuring the increase of absorbance at 550 nm using an \( \epsilon_{550} = 29,500 \text{ M}^{-1} \text{ cm}^{-1} \) in 20 mM potassium phosphate, 200 mM NaCl, pH 7.0, at 25 °C. The final concentrations of NADH and Cyt c were saturating at 100 and 8.4 \( \mu \text{M} \), respectively. For the experiments that compared the NADH oxidation versus the coupled Cyt c reduction, assays were done in triplicate in the presence and absence of 330 units of superoxide dismutase (SOD). Control reactions were run in the absence of the enzyme for all experiments.

NADH oxidase activity was monitored on an Oxy-32 oxygen-monitoring system at atmospheric oxygen in 20 mM potassium phosphate, 200 mM NaCl, pH 7.0, at 25 °C, by following the initial rate of oxygen consumption. Final concentrations of NADH and the enzyme were 100 and 0.14 \( \mu \text{M} \), respectively. To test whether hydrogen peroxide was produced during enzymatic turnover, the rate of oxygen consumption was measured at a fixed substrate concentration in the presence and absence of 170 units of catalase.

The NMO activity assay was performed as described previously (5, 6, 13, 59), following the initial rate of oxygen consumption with a Hansatech Instruments computer-interfaced Oxy-32 oxygen-monitoring system at atmospheric oxygen, i.e. 230 \( \mu \text{M} \) oxygen, at 30 °C. Stock solutions of nitro-nates and nitroalkanes were prepared as described previously (5, 13). Enzyme concentration was 180 \( \text{nM} \), and substrate concentration was 1 mM for P3N or 3-nitropropanoic acid and 20 \( \text{mM} \) for 2-nitropropane, propyl-2-nitronate, nitroethane, or ethynitronate. A positive control for nitrate monoxygenase activity was performed in parallel with purified Pa-NMO to a final concentration of 1.4 and 1 mM P3N as described previously (6).

The azoreductase activity of PA1024 was tested, as described previously (19, 60), by monitoring the reduction of methyl red at 430 nm, using \( \epsilon_{430} = 23,360 \text{ M}^{-1} \text{ cm}^{-1} \) in 20 mM potassium phosphate, 200 mM NaCl, pH 7.0, at 25 °C. The final concentrations of NADH, methyl red, and enzyme were 100, 25, and 0.20 \( \mu \text{M} \). The control reaction run in the absence of the enzyme was negligible, consistent with the non-enzymatic reaction between NADH and methyl red occurring only at low pH (61).

Product Inhibition of PA1024 with NAD+—Product inhibition of PA1024 was carried out using NAD+ as the inhibitor with NADH and 5-hydroxy-1,4-naphthoquinone as substrates in 20 mM potassium phosphate, 200 mM NaCl, pH 7.0, at 25 °C. The final concentration of NADH was held fixed at 100 \( \mu \text{M} \), whereas 5-hydroxy-1,4-naphthoquinone concentrations ranged from 2 to 17 \( \mu \text{M} \). The concentration of NAD+ was kept fixed at 0.4, 1.2, and 3 mM, and a set in the absence of NAD+ was included.

Data Analysis—The steady-state kinetic parameters for the enzymatic assays were obtained from the fitting of the experimental points to the Michaelis-Menten equation for one substrate using KaleidaGraph software (Synergy Software, Reading, PA). Double-reciprocal plots were constructed for
product inhibition patterns using KaleidaGraph, and global analysis was carried out using EnzFitter software ( Biosoft, Cambridge, UK). Stopped-flow traces were fit with the software KinetAssyst 3 (TgK-Scientific, Bradford-on-Avon, UK) to Equation 1, which represents a double-exponential process. A represents the absorbance at 461 nm at time t; B1 and B2 are the amplitudes of the decrease in absorbance; kobs,1 and kobs,2 represent the observed rate constants for the change in absorbance; and C is an offset value accounting for the nonzero absorbance of the enzyme-bound reduced flavin at infinite time. 

\[
A = B_1^{-k_{obs,1}} + B_2^{-k_{obs,2}} + C \tag{Eq. 1}
\]

Concentration dependence of the observed rate constants for flavin reduction was analyzed with Equation 2, where S represents the concentration of organic substrate; kred is the rate constant for flavin reduction at saturating substrate concentration, and Kd is the apparent dissociation constant for substrate binding.

\[
k_{obs} = \frac{k_{iso} S}{K_d + S} \tag{Eq. 2}
\]

Bioinformatic Analysis—The analysis of the protein sequence of PA1024 was performed with BLASTp (62), selecting the non-redundant protein sequence database. Multiple sequence alignments were also created with Clustal Omega (63) and Jalview (64). To find hypothetical proteins sharing the six motifs of PA1024 was performed with BLASTp (62), selecting the non-redundant protein sequence database. Multiple sequence alignments were also created with Clustal Omega (63) and Jalview (64). To find hypothetical proteins sharing the six motifs of PA1024, the PHI- BLAST (65) feature was utilized. The modifications to the conserved motifs were designed manually based on the multiple sequence alignment generated by BLASTp, analysis of potential critical residues, and multiple PHI-BLAST reiterations of varying patterns.

Author Contributions—G. G. designed and directed the study. G. G., F. S., and J. B. conceived the experiments. F. S. conducted the cloning, expression, purification, and bioinformatics analysis. F. S. and J. B. carried out the stopped-flow experiments. J. B. conducted the enzymatic assays and spectroscopy experiments. G. G., F. S., and J. B. wrote the manuscript.

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