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Structural Analysis Provides Mechanistic Insight into Nicotine Oxidoreductase from *Pseudomonas putida*

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Abstract

The first structure of nicotine oxidoreductase (NicA2) was determined by X-ray crystallography. *Pseudomonas putida* has evolved nicotine-degrading activity to provide a source of carbon and nitrogen. The structure establishes NicA2 as a member of the monoamine oxidase family. Residues 1–50 are disordered and may play a role in localization. The nicotine-binding site proximal to the isoalloxazine ring of flavin shows an unusual composition of the classical aromatic cage (W427 and N462). The active site architecture is consistent with the proposed binding of the deprotonated form of substrate and the flavin-dependent oxidation of the pyrrolidone C-N bond followed by non-enzymatic hydrolysis.

GRAPHICAL ABSTRACT

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Supporting Information. Experimental procedures for protein expression and structure determination. The Supporting Information is available free of charge on the ACS Publications website at <http://pubs.acs.org>.

Tobacco is harmful to human health and directly linked to numerous diseases, including cancer and cardiovascular disease. Among the many chemical constituents of tobacco products is nicotine, the major addictive substance attributed to habitual smoking^{1,2}. In humans, nicotine is metabolized in the liver by cytochrome P450 enzymes³. Roughly 85% of nicotine is metabolized into cotinine, although other metabolites include nicotine N'-oxide, normicotine, nicotine isothemonium ion and nicotine glucuronide^{3,4}. Several tobacco soil bacteria have adapted to using nicotine as their primary source of carbon and nitrogen^{5,6} via a differing catabolic pathway. These bacteria, including the *Arthrobacter* and *Pseudomonas* families, degrade nicotine through an aminoketone pathway (Scheme 1). In *Arthrobacter nicotinovorans*, nicotine catabolism begins with the hydroxylation of L- and D-nicotine by nicotine hydroxylase. In subsequent steps in L-nicotine catabolism, two FAD-dependent enzymes, 6-hydroxy-L-nicotine oxidase (6HLNO) and 6-hydroxy-D-nicotine oxidase (6HDNO), oxidize the pyrrolidone ring of 6-hydroxy-nicotine forming 6-hydroxy-N-methyl-myosmine⁷. Several structures of 6HLNO and 6HDNO are available both in substrate and inhibitor-bound forms^{8,9} and the enzymes mechanisms of action have been characterized^{10,11}. In *Pseudomonas putida*, the flavoprotein nicotine oxidoreductase (NicA2) has been identified as the primary enzyme for bacterial nicotine oxidation^{12,13}. To date, both kinetic and biophysical analysis of NicA2 has been carried out, but no structural characterization is available.

Despite sharing only ~27% sequence identity, NicA2 and 6HLNO catalyze nearly identical reactions involving the oxidation of their amine substrates into N-methyl-myosmine or 6-OH-N-methyl-myosmine, respectively followed by hydrolysis into a pseudooxynicotine product (Scheme 1). Evidence has been provided for the mechanism of oxidation of (S)-6-OH-nicotine catalyzed by 6HLNO as a two-step reaction: the flavin-dependent formation of 6-OH-N-methyl-myosmine followed by hydrolysis to form 6-OH-pseudooxynicotine⁹. A study analyzing the product of 6HLNO by nuclear magnetic resonance and continuous-flow mass spectrometry supports the flavin-dependent oxidation of the C-N bond of the pyrrolidone ring followed by non-enzymatic hydrolysis¹⁰. This mechanism is consistent with that generally accepted for flavin-dependent amine oxidases^{14,15}. Based on sequence identity, flavin dependence, and substrate similarity, we propose a similar mechanism for NicA2.

A recent study by Xue *et al.* characterized NicA2 and offered a preliminary biochemical profile. Steady-state kinetic parameters determined using LC-MS, reported a K_m of 44 nM and a k_{cat} of $6.64 \times 10^{-3} \text{ s}^{-1}$ yielding k_{cat}/K_m of $1.53 \times 10^5 \text{ s}^{-1}\text{M}^{-1}$ at ambient temperature¹⁶. The very low value of k_{cat} indicates a slow oxidative half-reaction. This suggests that NicA2 may not be a true oxidase, but instead a dehydrogenase and that the physiological electron acceptor has yet to be determined. The apparent K_m reported is defined as the concentration of L-nicotine needed to obtain half-maximum velocity with the poor second substrate, O₂. Therefore, the K_m for L-nicotine may differ with an optimal oxidizing substrate. Assuming ping-pong kinetics, k_{cat}/K_m will not depend on the second substrate and thus the reported value of $1.53 \times 10^5 \text{ s}^{-1}\text{M}^{-1}$ is valid¹⁶. Given its good catalytic efficiency, NicA2 has been proposed as a candidate in smoking cessation treatment. Improving O₂ reactivity of NicA2 may enhance turnover in therapeutic applications. Elucidating the three-dimensional structure of NicA2 could provide a structural basis for

protein engineering and allow insight into the determinants of substrate specificity and mechanism of catalysis. Herein, we present the first structure (at 2.2 Å) of a nicotine oxidoreductase, NicA2 from *Pseudomonas putida*.

A synthetic gene encoding NicA2, codon optimized for heterologous expression in *E. coli*, was synthesized from the sequence from *Pseudomonas putida* S16 strain (PPS_4081). The gene was cloned into a pET-52b vector for overexpression in *E. coli* BL21(DE3) cells. The gene contains a sequence for a C-terminal polyhistidine tag, allowing for purification (see SI for protein purification and crystallographic procedures). Consistent with previous reports,¹⁶ the protein was shown by size-exclusion chromatography to be a 52,500 Da monomer in solution while spectroscopic analysis and protein quantification yielded one molecule of FAD bound per protein subunit. The overall structure of NicA2 is globular and exhibits the topology initially described for p-hydroxybenzoate hydroxylase (PHBH)¹⁷ (Figure 1). The general fold of NicA2 classifies it as a member of the flavoprotein amine oxidase family, sharing similar secondary structural elements and the FAD-binding domain (Pfam01593). A search for proteins that are structural homologs to NicA2 using the DALI database¹⁸ indicates that the closest matches are monoamine oxidase N (MAO-N) D3 variant from *Aspergillus niger* (rmsd 2.1 Å; 28% identity), 6HLNO from *Arthrobacter nicotinovorans* (rmsd 2.6 Å; 28% identity) and monoamine oxidase-B (MAO-B) from *Homo sapiens* (rmsd 2.9 Å; 22% identity). Notably, multiple sequence alignments indicate that NicA2 contains an additional 50-residue N-terminal segment that is not found in 6HLNO and other MAO family enzymes (Figure S1). For instance, Met1 of 6HLNO aligns with Asp53 in NicA2, while Met1 of MAO-B aligns with Gly50 in NicA2, indicating that this segment is a unique feature of NicA2. However, there is no electron density observed for residues 1–50 and thus these are not represented in the model. A similar observation was reported in MAO-N, in which the first 40 amino acids were truncated to improve crystal packing and quality¹⁹. In NicA2 crystals, an empty volume exists in the lattice that is large enough to accommodate the N-terminus. Although the functional role of the N-terminus, if any, remains to be elucidated, sequence analysis using XtalPred-RF²⁰ predicts a signal sequence (residues 1–16) followed by a transmembrane helix (residues 17–39). Additionally, the servers InterProScan and MyHits Motif Scan identify residues 1–38 as a signal sequence for the twin-arginine translocation pathway, which exports folded proteins across a lipid membrane^{21,22}. NicA2 does not contain the C-terminal helix extension to the flavin-binding domain, which in MAO-B is involved in anchoring the protein to the mitochondrial membrane²³. While this suggests that the N-terminus of NicA2 may be used as an anchor, its cellular localization remains unknown.

The NicA2 monomer consists of two domains including a substrate-binding domain and an FAD-binding domain. The central core constituting the FAD-binding domain contains three layers, a central parallel β -sheet of five strands (comprised of β - α - β units) and an antiparallel β -sheet of four strands, together forming a $\beta\beta\alpha$ sandwich (SCOP_51904). A similar core domain exists in the FAD-linked reductase family, including human MAO-B¹⁴. FAD co-purifies in a 1:1 complex with NicA2, and the UV-vis spectrum of the protein solution and yellow color of the crystals indicate that FAD is oxidized. As in 6HLNO, FAD is non-covalently bound in the central core as demonstrated by the electron density, which is not contiguous with any enzyme residue (Figure 2). Additionally, NicA2 does not contain a

homologous residue to the conserved Cys397 or His72 that form a covalent linkage to the isoalloxazine ring of FAD in MAO-B and 6HDNO, respectively^{23,24}.

The isoalloxazine ring is oriented in a bent conformation with an angle of 30.8° about the N(5)–N(10) axis. Similar conformations are observed in other MAO members, indicating that steric constraints on the isoalloxazine ring prevent it from being planar¹⁵. The FAD molecule is positioned in a predominantly hydrophobic environment with the exception of several hydrogen-bond interactions (Figure S2). Numerous water networks connect the flavin to side chains or backbone of the protein. The O2-N3-O4 locus of the isoalloxazine ring is in contact with the backbone of Trp108, Thr248 and the side chain of Gln113 via bridging water molecules. The pyrophosphate of FAD binds to the amides of non-polar residues 62–65 in a conformation termed the “PP-loop,” observed in other amine oxidases^{24,25}. The ribose hydroxyls are stabilized by hydrogen bonds to the side chain of Arg85 and Glu83. Notably, mutation of the corresponding residue in MAO-B resulted in a loss of FAD incorporation, highlighting its significance in FAD binding and protein function²⁶. The adenine ring interactions with the protein include hydrogen bonds to the amide of Ala84, backbone of Val279 and a water molecule bridging to Thr312. Several of these residues oriented along the flavin are retained among amine oxidases. These include residues Gly62, Gly65, Gly89, Arg91, Val279, which are highly conserved in NicA2 and throughout the MAO family, while others such as Phe63, Ala84, Arg85, Trp108, Gln113, Thr307, Thr312, Phe422, Glu454, Ile463 and Asp464 are conservatively replaced (Figure S1). Altogether, NicA2 binds FAD in a manner consistent with that of most amine oxidases, conserving the geometry of the isoalloxazine ring and numerous binding interactions within the FAD-binding domain.

Despite overall high structural similarities, the substrate-binding domain of flavin amine oxidases exhibits a high degree of structural variability. Comparative analysis of NicA2 to its homologs via superposition of the separate subdomains indicate that while the FAD domain is highly conserved (rmsd values between 0.78 – 1.25 Å), the substrate-binding domain is much more divergent (rmsd values 13.2 – 19.1 Å). Additionally, if the entire structures are overlaid, the rmsd increases consistent with differing positions of the subdomains with respect to one another. The putative substrate-binding domain of NicA2 consists of two subdomains, which exhibit distinct topology. The first subdomain I (residues 152 – 249) is composed of seven alpha helices arranged in a helical bundle. Subdomain II (residues 137 – 150 and 339 – 415) forms seven antiparallel beta sheets twisted around an alpha helix in a “hot dog”-like fold. Between the two subdomains, the isoalloxazine ring of FAD is positioned with the *re*-face oriented toward a cavity, putatively for interaction with the substrate amine in catalysis. The presumed substrate-binding site consists of a large cavity and a hydrophobic channel extending from the substrate-binding domain to the protein surface with a volume of 2,700 Å³.

To identify key active-site residues, the liganded complex of 6HLNO was superimposed with NicA2 to dock the 6-hydroxy-L-nicotine substrate into the active site of NicA2 (Figure S3). In 6HLNO, the pyridyl ring of 6-hydroxy-L-nicotine is positioned via hydrogen-bonding interactions to Asn166 and Tyr311. The side chains of Tyr59, Leu198, and Phe326 form a hydrophobic cage around the pyridine moiety. A charge relay system between

His187, Tyr407, a water molecule and Ser197 position Tyr407 in proximity to the substrate amine⁹. In NicA2, there are no residues within hydrogen-bonding distance to position the pyridine moiety of the substrate. This suggests that there may be a conformational change upon substrate binding that repositions these residues, or alternatively, hydrophobic interactions alone orient the bound substrate. The hydrophobic cage consisting of Tyr59, Leu198, Phe326 in 6HLNO is replaced by residues Trp108, Thr250 and Thr381 in NicA2, respectively. Conservation of the hydrophobic character of the active site is consistent with binding of a deprotonated form of the L-nicotine substrate and subsequent release of the cationic N-methyl-myosmine product. Overall, key features of the active site of NicA2 demonstrate a unique cavity apt for nicotine binding.

Another significant feature of the active site are two residues, Trp427 and Asn462, which flank the isoalloxazine ring of flavin opposite one another (Figure S3A). Trp427 and Asn462 are structural homologs of residues that form the “aromatic cage”, a highly conserved region among flavin-dependent amine oxidases. Detailed structural studies of MAO-A and MAO-B have revealed insight into the role of the aromatic cage and the mechanisms of oxidative catalysis^{27,28}. In MAO-B, two tyrosine residues are positioned so that the substrate amine binds between them to react with the isoalloxazine ring. Notably, site-directed mutagenesis of Tyr435 in MAO-B drastically decreased catalytic function, as reflected in k_{cat}/K_m by ~4–40 fold depending on substrate²⁸. While the overall structure was unchanged, the k_{cat}/K_m varied drastically in the order of substitution Y>F>H>W²⁸. From these results, the aromatic cage is proposed to play a positioning role in substrate binding and to increase the nucleophilicity of the substrate amine. In NicA2, Tyr435 is replaced by Asn462. With the presence of this polar residue, the NicA2 active site resembles that of dehydrogenases. Asn462 in NicA2 is similarly positioned to Asn732 in cellobiose dehydrogenase from *P. chrysosporin* with respect to the isoalloxazine ring²⁹ (although it is not in the homologous position in a structural superposition). Cellobiose dehydrogenase utilizes quinones as redox partners. Together, the low observed k_{cat} and the structural analysis suggest that NicA2 may prefer an alternative redox partner, such as a quinone. Future studies will clarify the role of the NicA2 “aromatic cage” in substrate specificity and catalysis and identify redox partners alternative to molecular oxygen.

The structural analysis of NicA2 demonstrates conservation of the amine oxidase fold with similarities in the FAD binding domain architecture, while depicting clear and unusual differences in the substrate-binding domains. The FAD molecule was bound by similar, mostly hydrophobic, interactions as those of other amine oxidases. However, the residues forming the active site and the aromatic cage are unique. The X-ray structure of NicA2 now allows the targeting of specific mutations within the putative active site to further probe catalytic function and aid in biotherapeutic development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

NicA2	nicotine oxidoreductase
6HLNO	6-hydroxy-L-nicotine oxidase
6HDNO	6-hydroxy-D-nicotine oxidase
MAO	monoamine oxidase

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Figure 1. Ribbon diagram of NicA2 colored by secondary structure (helices in green, sheets in purple and loops in cyan). FD represents the FAD-binding domain; SD-I and SD-II represent the substrate-binding subdomains. The FAD cofactor is depicted as yellow sticks.

Figure 2. Stereoview of the noncovalently bound FAD co-factor. Unbiased electron density corresponding to FAD before refinement with model is shown as an omit (*F_o-F_c*) map at a 2σ contour level. Residues homologous to the aromatic cage are depicted as green sticks.

Scheme 1.

Aminoketone pathway of L-nicotine degradation in *P. putida* via NicA2 and *A. nicotinovorans* via 6HLNO.