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Crystallography Coupled with Kinetic Analysis Provide Mechanistic Underpinnings of a Nicotine-Degrading Enzyme

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Abstract

Nicotine oxidoreductase (NicA2) is a bacterial flavoenzyme, which catalyzes the first step of nicotine catabolism by oxidizing *S*-nicotine into *N*-methyl-myosmine. Its use has been proposed as a biotherapeutic for nicotine addiction due to its nanomolar substrate binding affinity. The first crystal structure of NicA2 has been reported, establishing NicA2 as a member of the monoamine oxidase (MAO) family. However, substrate specificity and structural determinants of substrate binding/catalysis have not been explored. Herein, analysis of pH-rate profile, single-turnover kinetics and binding data establish that pH does not significantly affect catalytic rate and product release is not rate limiting. The X-ray crystal structure of NicA2 with *S*-nicotine refined to 2.65 Å resolution reveals a hydrophobic binding site with a solvent exclusive cavity. Hydrophobic interactions predominantly orient the substrate, promoting the binding of a deprotonated species and supporting a hydride-transfer mechanism. Notably, NicA2 showed no activity against neurotransmitters oxidized by the two isoforms of human MAO. To further probe the substrate range of NicA2, enzyme activity was evaluated using a series of substrate analogs, indicating that *S*-nicotine is the optimal substrate and substitutions within the pyridyl ring abolish NicA2 activity. Moreover, mutagenesis and kinetic analysis of active-site residues reveal that removal of a hydrogen bond between the pyridyl ring of *S*-nicotine and the hydroxyl group of T381 has a 10-fold effect on K_M , supporting the role of this bond in positioning the catalytically competent form

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Syntheses of compounds, Supporting Figures S1 and S2, Table S1 and S2 (PDF).

Accession Codes

The coordinates for NicA2/*S*-nicotine structure have been deposited in the Protein Data Bank as entry 6C71.

Notes

The authors declare no competing financial interest.

of the substrate. Together, crystallography combined with kinetic analysis provide a deeper understanding of this enzyme's remarkable specificity.

Graphical Abstract

INTRODUCTION

Pseudomonas putida S16 is a gram-negative soil-dwelling bacteria isolated from tobacco fields. While members of the *Pseudomonas* species are known to degrade hydrocarbons and organic solvents, *P. putida* S16 has uniquely evolved not only to tolerate high concentrations of nicotine, but also to degrade it as a primary source of carbon and nitrogen.¹ Nicotine catabolism occurs in *P. putida* via the pyrrolidine (amino-ketone) pathway, which ultimately generates fumaric acid.² From this bacterial strain, of particular interest is the flavoenzyme, nicotine oxidoreductase (NicA2), which catalyzes the first committed step of nicotine degradation by oxidation of the substrate amine into *N*-methyl-myosmine, followed by non-enzymatic hydrolysis into pseudooxynicotine (Figure 1).³ Subsequently, the reduced enzyme is regenerated to its active form by reaction with molecular oxygen, releasing hydrogen peroxide. This unique evolutionary nicotine-degrading adaptation can be harnessed and refined to develop novel biotherapeutics for smoking cessation and nicotine poisoning, along with tools for tobacco waste bioremediation.⁴

Previously, our laboratories have resolved the first crystal structure of NicA2 at 2.2 Å resolution.⁵ These structure and sequence analyses reveal that NicA2 has the conserved FAD-binding domain of the flavoprotein monoamine oxidase (MAO) structural family.⁵ Based on experimental and computational studies of MAO family members, a mechanism is supported involving the directed transfer of hydride from a deprotonated form of the substrate to the flavin.^{6,7} Preceding work conducted by the Fitzpatrick laboratory involved mechanistic studies of L-6-hydroxynicotine oxidase (LHNO), a flavoenzyme structurally related to NicA2 sharing ~30% sequence identity, and established the oxidation of the substrate pyrrolidine carbon-nitrogen bond and transfer of hydride to the flavin.^{8,9} We posit a similar oxidation step and direct hydride-transfer mechanism for NicA2 (Figure 1) based on the structural similarity of NicA2 with LHNO and with multiple amine oxidases in the MAO family. Additionally, analysis of ligand-bound structures of LHNO and other MAO enzymes indicate that the substrate amines bind with the carbon-nitrogen bond oriented proximal to the flavin isoalloxazine ring, consistent with oxidation of this bond, although the

particular amino acid residues lining the active site and the substrate specificities differ significantly among the enzymes.^{10,11}

In this study, our goal was to identify the factors contributing to the mechanistic and substrate binding properties of NicA2 in efforts to improve its biotherapeutic potential. For this, a pH-rate profile was determined, indicating that catalytic rate is not significantly affected by pH, consistent with previous studies stating that the deprotonated form of the substrate is not required for productive binding and catalysis.⁹ To address the low k_{cat} of NicA2, single-turnover kinetics were performed using two methods- detection of the product of nicotine oxidation and of the product H_2O_2 , showing that product release is not rate limiting. Likewise, assessing binding affinity of a product analog revealed that product inhibition is not a significant determinant of k_{cat} . The crystal structure of NicA2 bound with *S*-nicotine was resolved to 2.65 Å resolution. Notably, ligand binding was accompanied by structural changes including puckering of the flavin isoalloxazine ring and closure of a putative substrate entrance tunnel. This provides an active site excluded from bulk solvent and subsequently opens a second tunnel presumably to allow for binding of the co-substrate, oxygen. Analysis of the active site environment demonstrated that substrate orientation is predominantly driven by hydrophobic interactions, consistent with binding of a deprotonated species. Analysis of NicA2 activity with a substrate panel and *S*-nicotine analogs led to the hypothesis that a hydrogen bond formed to the pyridyl nitrogen of *S*-nicotine significantly affects catalysis. This was further tested by mutagenesis of select active-site residues and kinetic analysis thereof, confirming that this hydrogen bond participates in a significant, but not essential role in substrate binding and/or catalysis. Altogether, our studies reveal the determinants of substrate binding and the unique characteristics of the active-site environment of NicA2, which preferentially accepts *S*-nicotine as substrate and promotes a hydride transfer mechanism.

RESULTS AND DISCUSSION

It should be noted that all *S*-nicotine experiments described herein were performed with commercially available *S*-nicotine, which contains a small (<5%) amount of nicotyrine contaminant (see Methods); nicotyrine is not a substrate for NicA2 and binds weakly compared to substrate ($K_I = 11 \text{ uM}$, data not shown) so that is not expected to significantly affect kinetic results.

NicA2 Activity is Not Significantly Affected by pH.

To investigate the effect of enzyme and substrate protonation state on catalysis, the reaction of NicA2 with *S*-nicotine as substrate was followed across a pH range of 6.0–9.5. The pH-rate measurements were performed in Bis-Tris propane buffer for 10 minutes using LC-MS to detect pseudooxynicotine product (Figure 2). There is a statistically significant but unremarkable increase in activity from pH 7.5 to pH 9.5 of approximately 2-fold. Because the velocity change is not significant, we did not measure the steady-state kinetic parameters, k_{cat} and K_M with respect to pH (although these constants are reported in subsequent sections at pH 7.4).

These data indicate that catalysis or substrate binding is not significantly altered by pH. The pH-rate data do not support a model where the enzyme requires or preferentially binds a deprotonated form of the substrate (the pK_a of nicotine is 8.02).¹² Thus, substrate protonation does not significantly contribute to binding, although a neutral substrate is directly associated with hydride transfer. Such was observed previously in mechanistic studies of LHNO that generated a model in which both forms of the substrate can bind, but the charged form is rapidly deprotonated via a hydrogen bonding network between water and amino acid residues in the active site prior to catalysis.⁹ Our pH-rate profile results are consistent with the interpretation of previous results that the deprotonated form of the substrate is not required for productive binding and catalysis. However, we do not observe residues in the active site to directly deprotonate the substrate or an ordered network of waters in NicA2 (see discussion below of NicA2/*S*-nicotine structure), although such a water network may be not be observable.

Product Release Is Not Rate Limiting.

Alternatively, the pH-rate finding suggests that there may be an additional rate-limiting reaction step included in both k_{cat} and K_M , which is not pH sensitive. Based on our mechanism and the slow rate of turnover (0.006 s^{-1})¹³, we hypothesized that product release may be this slow step. To evaluate this model, we performed single-turnover kinetics experiments, in which $1.5\text{ }\mu\text{M}$ NicA2 was combined with $1.0\text{ }\mu\text{M}$ of *S*-nicotine and the reaction was monitored for 30 minutes using the Amplex Red Assay.¹⁴ This assay is used to detect the release of hydrogen peroxide, which occurs in one-molar equivalence with the release of product (Figure 3). The experiment thus monitors all steps up to and including the second half reaction with oxygen. The single-turnover kinetics data were fit to equation (1), a single exponential function, which generated a rate constant (k_{obs}) of 0.005 s^{-1} (Figure 3; black circles). This is consistent with the k_{cat} previously reported (0.006 s^{-1})¹³ and the k_{cat} determined in this study using the same H_2O_2 detection assay. Because reaction of oxygen with the reduced enzyme can occur either before or after product release, we performed single-turnover kinetics monitoring the degradation of *S*-nicotine using LC-MS (Figure 3; blue circles). Although the observed rate constant for this reaction was slightly higher (0.03 s^{-1}) compared with the rate determined using peroxide detection (0.005 s^{-1}), this is not a remarkable difference. These results are consistent with the interpretation that product release is not the rate-limiting step, as the rate constant for multiple turnovers of enzyme is equivalent to a single turnover rate, which does not rely on product release for detection.

In parallel to the single-turnover kinetic experiments, we sought to determine if product release could be rate limiting by assessing the binding affinity of the imine product. Because the direct product of *S*-nicotine, *N*-methyl-myosmine, is unstable and not readily available, myosmine, a product analog, was used as a surrogate. The active site of NicA2 contains a tryptophan residue, W427, at the substrate binding site (see crystal structure below), which is amenable for monitoring binding by measuring the quenching of tryptophan fluorescence at excitation of 280 nm and emission of 350 nm. As such, binding of myosmine was determined and data were fit to equation (2), from which a K_D of $121.3\text{ }\mu\text{M}$ was calculated (Figure 4).

In comparison with the low K_M for *S*-nicotine (114 nM; see description of NicA2 steady-state kinetics below), the binding affinity for myosmine ($K_D = 121 \mu\text{M}$), insofar as it mimics that of *N*-methyl-myosmine, shows that product inhibition is not a significant factor. Moreover, k_{off} can be calculated using the measured K_D and assuming k_{on} for product is at the diffusion-controlled limit for a macromolecule and a small molecule ($1 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$). This places a lower limit on the value of k_{off} of 121 s^{-1} which is >20,000 fold greater than the turnover number $k_{\text{cat}} = 0.006 \text{ s}^{-1}$. Therefore, together with the single-turnover kinetics data, we can infer that product release is not the rate-limiting step. Alternatively, oxidation of the reduced flavin complex may be rate limiting if the affinity for oxygen is poor or the oxygen-binding step is slow. This oxidation step can occur pre or post product release as modeled in the canonical kinetic mechanisms of flavoprotein oxidases.¹⁵ Future experiments using stopped-flow are necessary to determine if flavin reoxidation is the rate-limiting step in this mechanism. Additionally, limitation of the reactivity of oxygen could be a result of the accessibility of oxygen to the reduced flavin. Analyses of other flavoenzymes indicate that molecular oxygen diffuses through distinct, transient tunnels into the active site.¹⁶ It is possible that oxygen binding/reactivity in NicA2 may be rate limiting.

Hydrophobic Interactions Mainly Orient Substrate Binding.

In order to delineate the interactions of NicA2 with substrate and determine the criteria underlying substrate specificity, the crystal structure of NicA2 bound with *S*-nicotine was determined at 2.65 Å resolution. The ligand-bound structure was obtained by reducing native NicA2 crystals with sodium dithionite, preventing catalytic turnover and subsequently allowing diffusion of *S*-nicotine into crystals prior to cryo-cooling. Although the overall structure of *S*-nicotine-bound NicA2 is very similar to the native enzyme with an RMSD of 0.6 Å, there were significant conformational changes in both the enzyme and the FAD cofactor. The general positioning of FAD is clearly defined in the electron density maps and consistent with that observed in the native enzyme. However, reduction of FAD and/or substrate binding induced puckering of the isoalloxazine ring towards the pyrrolidine ring of *S*-nicotine, with a bend of 24.0° from a planar conformation (Figure S1). Because FAD was in a reduced state prior to substrate binding, we cannot differentiate the induction of ring puckering with flavin reduction versus substrate binding. However, previous structures of MAO-B show that binding of a weak substrate with FAD in the oxidized state (as indicated by the yellow color of crystals) still results in puckering of the isoalloxazine ring.¹⁷ Indeed this flavin conformational change is commonly observed with ligand binding in amine oxidase flavoproteins.^{18,19} In addition, solvent accessible tunnels were computed for both unliganded and substrate-bound NicA2 using CAVER v3.0.1²⁰ (Figure S2), revealing that ligand binding induced the enclosure of putative substrate entry tunnels (shown in cyan and purple), excluding solvent from the active site. Subsequently, additional tunnels, depicted as tan spheres in Figure S2B, were generated extending from the *si*-face of the flavin isoalloxazine ring, presumably to allow for entry of oxygen during the second half reaction. The substrate was built in well-defined electron density with B-factors consistent with those of surrounding residues. Although the $I/\sigma(I)$ is low in the highest resolution shell (Table S2), comparison of maps made with and without these data showed that the inclusion of the highest resolution shell improved map quality without introducing noise. Notably, the ligand density is nearly identical at either resolution cutoff. *S*-nicotine is positioned at the *re*-face of

the flavin isoalloxazine ring with the pyrrolidine ring nitrogen aligned in parallel with the C4a of FAD (Figure 5A), consistent with the oxidation of the carbon-nitrogen bond and substrate binding within other MAO family members.⁸

Flanking the substrate on opposing sides of the isoalloxazine ring is a pair of conserved residue positions constituting the “aromatic cage” (Figure 5B). In members of the MAO family, these residues occur as a pairwise combination of phenylalanine, tyrosine and tryptophan (e.g. F/Y, Y/Y, W/Y) proposed to facilitate binding of the substrate amine.¹¹ Mutagenesis studies of MAO showed that substitution of these residues had a significant impact on catalysis, decreasing k_{cat}/K_M by 4–40 fold.¹⁷ However, NicA2 contains a noncanonical composition of the aromatic cage containing an asparagine residue (N462) in addition to tryptophan (W427). While N462 does not directly interact with the substrate, the role of this residue in substrate binding and catalysis remains unclear. Rather, substrate orientation is predominantly driven by hydrophobic interactions with W108, T250, W364, L217, Y218 and W427, with the exception of a single 2.9 Å hydrogen bond formed between the pyridine nitrogen of *S*-nicotine and the hydroxyl group of T381 (Figure 5B). Alternatively, the orientation of the pyridine ring could be flipped, such that a hydrogen bond is formed with T250. The electron density cannot be used to distinguish these two ring-flipped orientations. However, the orientation of the side chain of T250 in our structure is not consistent with formation of such a hydrogen bond. T250 would need to adopt a different rotamer orientation upon ring flipping. The choice of rotamer in the current structure reflects the shortest possible hydrogen bond (a hydrogen bond formed with T250 would be 3.5 Å in length).

The hydrophobic nature of the binding site would be expected to lower the pKa of nicotine, favoring the uncharged form. In addition to shape complementarity, the electrostatic properties of the active site are complementary to those of the substrate. Calculation of the electrostatic potential of NicA2 reveals a partially negative, but predominantly neutral electrostatic potential (Figure 6), further supporting activity with the deprotonated form of the substrate and the hydride transfer mechanism. Thus, the distinct character of the active site is amenable for hydride transfer from the deprotonated form of the substrate and subsequent release of the cationic *N*-methyl-myosmine product (Figure 1).

Structure-Activity Relations Provides Insight into Substrate Specificity.

To probe the substrate specificity of NicA2, we determined enzyme activity with a panel of compounds containing common amine substrates of MAO and related oxidase enzymes, including serotonin, dopamine and norepinephrine. In addition to neurotransmitters, this library consisted of nicotine metabolites and common neuroactive drugs. NicA2 activity was assessed using the Amplex Red Assay kit (see Methods). The rates for each substrate were generated using saturating substrate concentrations, measuring the slope of the change in fluorescence intensity over time and normalized by setting the activity with *S*-nicotine as 100% (Figure 7). Examination of NicA2 activity revealed that these compounds did not act as substrates. Unlike MAO, NicA2 does not have a broad substrate range although both enzymes have similar hydrophobic character and active site volume including FAD at ~350 Å³ and ~400 Å³ for NicA2 and MAO-A (PDB ID 2Z5X), respectively.²³ The substrate

specificity of MAO family members is primarily driven by differences in residues lining the substrate cavity, affecting not only steric accommodations but also substrate entry.²³

To provide a more detailed insight into the specificity of NicA2, analogs of *S*-nicotine were obtained commercially or synthesized as described (Supporting Information). As with the substrate panel, the rates for each compound were determined using concentration 200x K_M for the natural substrate, *S*-nicotine, by measuring fluorescence change over time and normalizing activity with *S*-nicotine as 100% (Figure 8). Comparison of percent activity with alterations in substrate chemical structure provided insight into the specificity of NicA2. The enzyme is stereospecific with poor activity with *R*-nicotine as substrate. Unlike 6-hydroxy-nicotine oxidase, for which there are two analogs that each are selective for the D or L enantiomer, no enzyme has been identified that takes the opposite enantiomer of the NicA2 nicotine substrate.^{24,25}

Overall, substitutions made in the pyrrolidyl ring decreased activity >50%, while those in the pyridyl ring abolished NicA2 activity altogether. Loss of the methyl group on the pyrrolidine ring in **1** (nornicotine) decreased activity by only 50%. Similarly, previous studies of LHNO with the substrate analog *S*-6-hydroxynornicotine indicate that the pyrrolidyl methyl group does not significantly impact specificity.⁹ Interchanging the pyrrolidine ring with a piperidine ring further decreased activity to 20% of control in **4**. Substituting the pyrrolidine ring with imidazolidine (**3**) or oxazolidine (**5**) also reduced NicA2 activity. Presumably, introduction of the nitrogen or oxygen increases the electronegativity of the pyrrolidine ring in correlation with decreased enzyme activity, which would be less favorable for hydride transfer. Nicotine analogs with alterations in the pyridyl ring resulted in no detectable activity as substrates for NicA2, with the exception of **6**. Loss of aromaticity in the pyridyl ring by substitution with a piperidyl ring as in **8** eliminated enzyme activity regardless of the presence of the pyridyl nitrogen, such as in the cyclohexyl group of **7**. Moreover, altering the position of the pyridyl nitrogen from N8 to N9 in **9** and introducing an additional nitrogen by replacing pyridine with pyrimidine in **10** both abolished NicA2 activity as well. This may be the consequence of a disruption of the hydrogen bond between the pyridyl nitrogen and T381 observed in the X-ray crystal structure (Figure 5B). However, **6**, which lacks the nitrogen in the pyridyl ring, retained 50% activity as substrate, most likely due to additional hydrophobic interactions aiding substrate binding.

To probe structure/activity further, steady-state kinetic parameters with **6** as substrate for NicA2 revealed a 3-fold increase in K_M with no significant alteration in k_{cat} (Table 1), indicating that loss of the nitrogen on the pyridyl ring does not greatly affect substrate affinity, or alternatively that K_M does not reflect substrate affinity (if a slow step is included in K_M).

To provide a better estimate of the contribution of this hydrogen bond interaction to catalysis, active-site residues were subjected to site-directed mutagenesis and kinetic parameters were determined with *S*-nicotine as substrate using the Amplex Red assay and KinTek Explorer for data fitting using global simulation (see Methods). As described above, the crystal structure shows two residues (T381 and T250) in the active site positioned within

an appropriate distance to form a hydrogen bond with the pyridyl nitrogen of *S*-nicotine (each in one of two possible flipped orientations). Replacing the hydroxyl group of T381 with a methyl group via a mutation to valine resulted in a 2-fold increase in K_M (Table 1). However, flexibility in the pyridyl ring would also allow the formation of a hydrogen bond with T250, provided that a different rotamer orientation was available, *vide supra*. Consequently, a double mutation (T250V/T381V) was generated and subsequent kinetic analysis exhibited over a 10-fold increase in K_M (Table 1). In all instances, k_{cat} was not significantly altered, although overall catalytic efficiency decreased by 5-fold, which can be attributed to the loss in binding affinity. The >10-fold change in K_M is consistent with the energetic contribution of a hydrogen bond (approximately 2.5 kJ/mol) such that loss of this bond resulted in a decrease in affinity for the substrate, assuming K_D is similar to K_M . Alternatively, this decrease could be the result of a change in another slow step, which is included in K_M . In addition, we have determined the Michaelis constants for the T250V/T381V variant using **6** as substrate, which replaces the pyridyl N with C and thus cannot benefit from the hydrogen bond interactions. As expected, the k_{cat} and K_M values for T250V/T381V against this substrate do not differ from those for *S*-nicotine; any new van der Waals interactions do not compensate for the loss of the hydrogen bond. Overall, the hydrogen bond interaction to T381 plays a significant, but not essential, role in substrate binding and/or activity.

When the pyridyl group is replaced with a phenol (**12**) and a hydroxyl group is introduced on the pyridyl ring (**11**) forming *S*-6-hydroxynicotine, NicA2 activity is not detectable. Importantly, this signifies that NicA2 is not an ortholog of LHNO. LHNO contains a tyrosine residue (Y311) in the active site, which forms a hydrogen bond with the hydroxyl group of *S*-6-hydroxynicotine.²⁵ In NicA2, the residue in the homologous position is tryptophan (W364). While the active site of NicA2 can sterically accommodate *S*-6-hydroxynicotine, lack of the appropriate orientation of the substrate prevents productive binding and/or catalysis.

The binding affinity of inactive *S*-nicotine analogs was evaluated in order to determine if compounds could act as competitive inhibitors and if the loss of activity is attributable to lack of proper substrate orientation or interference with the chemical mechanism. The binding of analogs was determined by measuring the change in tryptophan fluorescence across a concentration range from 0.1 to 100 mM as described above. Unlike the binding of myosmine, **7**, **9**, **10** and **12** bound with millimolar affinity (50% binding at >5 mM compound), such that we were unable to accurately determine the binding constant, K_D . Therefore, these compounds bind NicA2, but with very poor affinity and are not substrates. Taken together, these subtle changes in the substituents of the substrate significantly impact or eliminated enzyme binding and/or activity, presumably by altering the electrostatics important for substrate binding or the mechanism of hydride transfer.

Conclusion.

The results of this study provide detailed insights into the features underlying the specificities of substrate binding and the architecture of the active site in NicA2. By analysis of the pH-rate profile, we have shown that the substrate protonation state does not

significantly contribute to enzyme activity. The reaction rate-limiting step is not pH dependent. Analysis of single-turnover kinetics and binding affinity of a product analog has shown that product release is not the rate-limiting step. We posit the rate-limiting step to be reoxidation of the reduced flavin, although future experiments are necessary to confirm this hypothesis. Importantly, the crystal structure of NicA2 bound with *S*-nicotine revealed a hydrophobic active site primed for hydride transfer and illustrated the mode of binding in an enclosed substrate cavity. These conclusions are consistent with those previously published in mechanistic and structural studies of LHNO and other MAO family members.^{9,23,26}

In addition, analysis of NicA2 activity using a substrate panel screen revealed no activity with common neurotransmitters, nicotine metabolites and psychiatric drugs. This implies that NicA2 is a promising therapeutic candidate, as has been proposed,²⁷ because a lack of activity with neurotransmitters and high specificity for *S*-nicotine would diminish any potential off-target effects. Evaluation of NicA2 activity with *S*-nicotine analogs demonstrated that subtle alterations in substituents of the pyrrolidyl and pyridyl rings significantly affected enzyme activity. Notably, these data demonstrates that for the pyridyl class of substrate analogs, substituents exhibit a major role in binding. Further kinetic analysis of **6** and active site variants revealed that the hydrogen bond interaction with the pyridyl nitrogen contributes to substrate affinity or affects a rate-limiting step. Overall, these results demonstrate a narrow substrate range for NicA2, a strong preference for *S*-nicotine, and elucidate the determinants of binding.

NicA2 has an unusually high affinity for *S*-nicotine with K_M of 114 nM. Although the catalytic rate for NicA2 is slow (0.006 s^{-1}), *S*-nicotine remains the best substrate compared with other biogenic amines and byproducts of tobacco – nornicotine, anatabine and anabasine. However, this slow rate raises the possibility of alternative electron acceptors to oxygen. In *P. putida*, NicA2 catalyzes the first committed step of nicotine catabolism. Alternatively, other enzymes downstream in this pathway may have a similar catalytic rate, reducing the evolutionary pressure to increase this reaction despite nicotine being a major nutrient source. Moreover, an additional enzyme in this pathway, NicA1, may compensate for this first metabolic step. Despite their common nomenclature, there is very low sequence similarity (~12%) between these two enzymes, and they are not encoded in the same operon.³ While the catalytic rate of NicA1 is unknown, the *P. putida* bacterium is selectively dependent on NicA2 for growth on nicotine-enriched media, but not NicA1.³ Future analysis is necessary to unravel the evolutionary adaptation of NicA2 for high nicotine specificity, but slow turnover.

METHODS

Materials.

Chemicals, biochemicals and buffers were purchased from Sigma-Aldrich Chemical Co. and Thermo Fisher Scientific. Anatabine (**2**), nicotyrine, and nicotine-*N*-oxide were purchased from Cayman Chemical. *R*-nicotine and 6-hydroxy-*L*-nicotine (**11**) were purchased from Toronto Research Chemicals. All other commercially available compounds were purchased from Sigma-Aldrich, including nornicotine (**1**), cotinine, acetylcholine, nicotinamide, choline, glutamate, nicotinamide adenine dinucleotide (NAD), varenicline, cytosine, γ -

aminobutyric acid (GABA), mecamylamine, dopamine, serotonin, anabasine (**4**), and myosmine. Syntheses of **3**, **5**, **6**, **8**, **9**, **10**, and **12** are detailed in the Supporting Information. *S*-nicotine purchased from Sigma-Aldrich is sold as 99% pure. Upon further analysis by LC-MS, this stock solution was found to be contaminated with ~5% nicotine.

Protein Expression and Purification.

Expression and purification of His₆-tagged nicotine oxidoreductase (NicA2) from *Pseudomonas putida* S16 was produced using two different methods for separate sets of experiments. For the pH-rate profile and substrate specificity screen, the protein was purified by the previously published procedure.¹³ For single-turnover kinetics, steady-state kinetics, tryptophan fluorescence assays and crystallization of NicA2, protein was purified by a modification of a previously published method.⁵ Briefly, a construct containing the *nicA2* (PPS_4081) gene with a 3' 6x His tag was inserted into a pET-52b(+) vector (Genewiz). Recombinant NicA2 was transformed into *E. coli* BL21(DE3) competent cells (New England Biolabs). Cells were grown at 37 °C in LB broth until the appropriate OD_{600nm} of 0.6–0.8 was reached. Expression was induced with the addition of 1 mM IPTG, and the temperature was lowered to 16 °C overnight. Subsequently, the cell pellet was resuspended in 50 mL of lysis buffer [50 mM Tris pH 8.2, 500 mM NaCl, 10 mM Imidazole, 1 mM DTT] supplemented with 600 µL of 10 mg mL⁻¹ DNase I and one EDTA-free protease inhibitor tablet (Sigma-Aldrich). The clarified cell lysate was applied to a Ni Sepharose column (GE Healthcare Life Sciences) using lysis buffer to bind and a gradient of elution buffer [50 mM Tris pH 8.2, 500 mM NaCl, 500 mM Imidazole, 1 mM DTT] to elute the enzyme. NicA2 was further purified by size exclusion chromatography (Superdex 200 10/300 GL, GE Healthcare Life Sciences), dialyzed overnight into 1X phosphate-buffered saline (PBS) pH 7.4 and stored at 4 °C.

Generation of NicA2 Variants.

NicA2 variants (T381V and T250V/T381V) were constructed using the Q5 site-directed mutagenesis kit (New England Biolabs) using the pET-52b(+) plasmid as template. Primers were designed using NEBase Changer (New England Biolabs) and are listed in Table S1 of Supplementary Information. For generation of the double mutant (T381V/T250V), the pET-52b(+) plasmid containing T381V mutation was used as template. Incorporation of mutations was confirmed by DNA sequencing analysis using Genewiz. Expression and purification of NicA2 variants were performed in the same manner as the wild-type enzyme prepared for kinetics analysis.

pH-rate profile.

Initial pH rate experiments were performed testing the activity of NicA2 in Bis-Tris propane buffer across a pH range of 6.0–9.5 at 22 °C. Stock solutions of NicA2 (5 µM) and *S*-nicotine (0.5 mM) were prepared in Bis-Tris propane buffer with varying pH (6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5). Equal volumes of enzyme and substrate were mixed with a final concentration of 100 nM NicA2 and 5 µM *S*-nicotine. The reaction proceeded for 10 minutes, followed by acid quenching using a 1:10 dilution of 20% (v/v) TFA in water. The pseudooxynicotine product was detected by LC-MS using an Agilent 1260 Infinity liquid

chromatography system with 6230 quadrupole mass spectrometry as previously described.¹³ For standard curve generation, 31.25, 62.5, 125, 250, 500, 1000 and 2000 nM *S*-nicotine and 400 nM NicA2 was incubated for 2 h for complete oxidation of nicotine. Data is presented on a linear scale as enzyme activity in nM sec⁻¹ against pH (Figure 2).

Steady-state Kinetics.

For steady-state kinetics experiments, reactions were carried out in 50 mM sodium phosphate buffer pH 7.4 at 22 °C. Enzyme activity was determined using the Amplex Red assay kit (Thermo Fisher Scientific) to measure hydrogen peroxide produced during the NicA2 reaction.¹⁴ Each cycle of enzyme turnover generates one molecule of hydrogen peroxide in a 1:1 stoichiometric equivalence of product. Twenty-five microliters of *S*-nicotine or **6** were diluted in 50 mM sodium phosphate buffer pH 7.4 and mixed with 25 µL of NicA2 or NicA2 variants to obtain final concentrations of 310, 625, 1250, 2500, 5000, and 10,000 nM *S*-nicotine/**6** and 200 nM NicA2/NicA2 variants, along with 0.1 U mL⁻¹ horseradish peroxidase and 50 µM Amplex Red in 100 µL final volume. The reaction was monitored continuously for 40 minutes, and the fluorescent product was read every 5 minutes at 490 nm excitation and 585 nm emission using a Spectromax M5 microplate reader (Molecular Devices). The resulting data were fit globally by simulation to a single kinetic model using KinTek Explorer, Version 6.3 (KinTek Corporation).^{28,29} A model with three in-line equilibrium steps (E+S=ES=EP=E+P) was applied and substrate-binding rates were fixed at the diffusion limit, such that only the computation of k_{cat} and K_{M} were fitted. The steady-state kinetic parameters are presented in Table 1.

As a standard curve, varying concentrations of hydrogen peroxide (39, 78, 156, 312, 625, 1250, 2500 nM), 0.1 U mL⁻¹ horseradish peroxidase and 50 µM Amplex Red were combined in a 100 µL reaction and incubated for 40 minutes.

Single-Turnover Kinetics.

For hydrogen peroxide detection, reactions were carried out in 50 mM sodium phosphate buffer pH 7.4 at 22 °C, and activity was monitored using the Amplex Red assay (Thermo Fisher Scientific) to measure hydrogen peroxide production. Equal volumes of NicA2 and *S*-nicotine were mixed to produce final concentrations of 1.5 µM and 1.0 µM, respectively, in a 100 µL reaction along with 0.1 U mL⁻¹ horseradish peroxidase and 50 µM Amplex Red. Each time point (0, 0.5, 1, 5, 10, 15, 20, 25 minutes) was achieved by terminating the reaction with the addition of 20 µL Stop Reagent (Thermo Fisher Scientific). After the final time point, the fluorescent product was read at 490 nm excitation and 585 nm emission using a Spectromax M5 microplate reader (Molecular Devices). Data were fit to a single exponential function (GraphPad Prism version 7.0c) to determine the first-order rate constant (k_{obs}) as indicated by equation 1.

$$\text{Product} = A_0(1 - e^{-k_{\text{obs}}t}) \quad (1)$$

For a standard curve, varying concentrations of hydrogen peroxide (39, 78, 156, 312, 625, 1250, 2500 nM), 0.1 U mL⁻¹ horseradish peroxidase and 50 µM Amplex Red were combined in a 100 µL reaction and incubated for 30 minutes.

For detection of *S*-nicotine degradation, reactions were carried out in 50 mM HEPES buffer pH 7.4 at 22 °C and monitored using LC-MS. Similarly, equal volumes of NicA2 and *S*-nicotine were mixed to produce final concentrations of 1.5 µM and 1.0 µM, respectively, in a 100 µL reaction. Each time point was determined by quenching the reaction using a 1:10 dilution of 20% (v/v) TFA/H₂O (containing 2 µM nicotine methyl D-3 as an internal standard) and flash freezing in liquid N₂. The assay was analyzed using LC-MS as previously reported¹³ and data were fit to equation 1. For data analysis, the ratio of a blank sample containing only *S*-nicotine, (no enzyme control) and the last time point for full conversion of *S*-nicotine to product was used to determine the amount of *S*-nicotine degraded.

Substrate Specificity Screen.

All compounds were examined at a final concentration of 10 µM with 100 nM NicA2 at 22 °C. Stock solutions (3X) of each compound and NicA2 were prepared in 50 mM sodium phosphate pH 7.4. For the assay, 50 µL of each compound stock, 50 µL of NicA2 and 50 µL of Amplex Red working solution were mixed per manual instructions (Thermo Fisher Scientific). The reaction was monitored for 20 min using a SpectraMax M2 microplate reader (Molecular Devices) with an excitation and emission of 571 nm and 585 nm, respectively. The slope of each curve was calculated as a reaction rate, and the rate for each compound was normalized using activity with *S*-nicotine as 100%.

Tryptophan Fluorescence Assay.

Compound binding was assessed by monitoring quenching of tryptophan fluorescence using a Spectromax M5 microplate reader (Molecular Devices) at 22 °C. Enzyme at a concentration of 4 µM in PBS pH 7.4 was mixed with equal amounts of compound diluted in the same buffer at a final concentration range of 1 µM to 100 mM. Fluorescence of tryptophan was monitored at 350 nm following excitation at 280 nm. The percent change in fluorescence due to compound binding was plotted against compound concentration. The data were fit to a one-site specific binding function in GraphPad Prism version 7.0c to determine the K_D and data were fit to equation 2:

$$\Delta F / F_0 \times 100 = (\Delta F / F_{\max} \times 100) \times [C] / K_D + [C] \quad (2)$$

where ($F/F_0 \times 100$) represents the percent change in fluorescence intensity upon addition of compound [C] relative to the initial fluorescence value (F_0), and ($F/F_{\max} \times 100$) is the maximum percent quenching of the fluorescence intensity that occurs upon saturation of the substrate-binding site.³⁰

Crystallization and Ligand-Soaking Experiments.

Crystals of NicA2 were grown under previous conditions using the hanging-drop vapor diffusion method.⁵ Initial crystallization conditions were optimized using the Hampton Research Additive screen by mixing protein and well solution in a 1:2 ratio. The condition comprising of 0.3 M NaCl, 0.1 M Bis-Tris propane pH 9.0, 15% (v/v) PEG 10,000 produced rod-shaped crystals with dimensions of 0.2 mm × 0.3 mm × 0.8 mm. A liganded structure of NicA2 with the substrate *S*-nicotine was prepared by first soaking the enzyme crystals in a solution of mother liquor and 50 mM sodium dithionite for 5–10 minutes. Upon reduction of the FAD cofactor, a color change from yellow to clear was observed in the crystals. Crystals were then transferred to a solution of 30% (v/v) ethylene glycol, mother liquor and 5 mM *S*-nicotine and allowed to soak for approximately 20 minutes. Immediately following this soak, crystals were harvested and flash-frozen in liquid N₂ at 100 K.

Data Collection and Structure Refinement.

Diffraction data were collected on beamline 9–2 at the Stanford Synchrotron Light Source at the SLAC National Accelerator Laboratory (Menlo Park, CA). Data were collected using a Dectris Pilatus 6M PAD detector at 100 K under an N₂ gas stream and processed using HKL2000.³¹ Ligand-soaked crystals of NicA2 belonged to the space group $P2_12_12_1$ with four molecules in the asymmetric unit and unit cell dimensions: $a = 86.76 \text{ \AA}$, $b = 135.10 \text{ \AA}$, $c = 167.73 \text{ \AA}$. Data collection and refinement statistics are summarized in Table S2 of Supplementary Information. The overall completeness originally calculated for unmerged data was 95.5%; however, during data reduction, some reflections were rejected and the completeness for the merged data was amended to 92%. The structure of NicA2 bound with *S*-nicotine was determined at 2.65 Å resolution using a single chain of the selenomethionine-incorporated native enzyme (5TTK) as a molecular replacement search model in PHENIX Phaser-MR.³² Iterative rounds of refinement in PHENIX Refine³³ using individual B-factors, translation-libration-screw (TLS) parameters and addition of waters, along with manual model building in Coot³⁴ were used to generate the final model. Ligands were placed using PHENIX LigandFit³⁵ with CC > 0.75 and validated using omit ($F_o - F_c$) and Polder electron density maps in PHENIX.³⁶ All images were produced using PyMOL.³⁷ Solvent accessible tunnels were calculated using CAVER PyMOL plugin v3.0.1 with minimum probe radius set to 0.85 Å.²⁰ Calculation of volume of active site cavities in NicA2 was performed using VOIDOO.³⁸ Electrostatic potential surface was calculated using the APBS web server²¹ and generated using UCSF Chimera.²²

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Proposed mechanism of direct hydride transfer for the NicA2-catalyzed oxidation of *S*-nicotine to *N*-methyl-myosmine.

Figure 2.

Activity of NicA2 determined across pH range. Stock solutions were prepared in Bis-Tris propane buffer with various pH (6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5). Enzyme and substrate were combined at a final concentration of 100 nM NicA2 and 5 μ M *S*-nicotine. The reaction proceeded for 10 minutes at 22 °C and then was quenched using 20% (v/v) TFA (1:10). Product was detected using LC-MS.

Figure 3.

Single-turnover kinetics generated using 1.5 μM NicA2 and 1.0 μM *S*-nicotine. Black circles correspond to the reaction determined using the Amplex Red assay for hydrogen peroxide detection. The formation of product was correlated with a 1:1 stoichiometric equivalence of hydrogen peroxide generation. The observed rate constant for the exponential function, k_{obs} , was calculated as $5.06 \times 10^{-3} \text{ s}^{-1}$ using equation 1. Blue circles indicate the reaction as determined using LC-MS monitoring the degradation of *S*-nicotine over time, for which the observed rate constant was calculated as $3.02 \times 10^{-2} \text{ s}^{-1}$. The error bars denote standard deviation from the mean.

Figure 4. Binding of myosmine to NicA2 monitored using the fluorescence of tryptophan. The percent change in fluorescence is shown with increasing myosmine concentrations ranging from 0.1 mM to 100 mM. Equation 2 was used to determine the binding constant, K_D , of 121.3 μ M. Inset depicts chemical structure of myosmine. The error bars denote standard deviation from the mean.

Figure 5.

(A) Structure of NicA2 modeled as ribbon diagram colored by secondary structure: helices in green, sheets in purple, loops in cyan. The FAD cofactor and the substrate, *S*-nicotine, are shown as stick models in yellow and magenta, respectively. The inset outlined in grey depicts *S*-nicotine, highlighting the well-defined electron density of the ligand with the ($F_o - F_c$) omit electron density before refinement with ligand contoured at 3.0σ (grey wire frame). (B) Stereoview of the enzyme active site with *S*-nicotine (magenta) bound. Residue side chains are shown as stick models in green and isoalloxazine ring of FAD in yellow. Hydrophobic interactions are indicated by grey dashed lines, and hydrogen bonds are shown as cyan dashes. All distances are within 3.0–4.0 Å.

Figure 6.

Clipped cross-section of the molecular surface of NicA2 depicting the active site colored by electrostatic potential displayed as a gradient from red (negative) to blue (positive).

Electrostatic potentials were calculated using the APBS web server²¹ and figure was generated using UCSF Chimera.²² *S*-nicotine and FAD are shown as sticks colored magenta and yellow, respectively.

Figure 7.

Activity of NicA2 with nicotine metabolites, neurotransmitters and common drugs determined using the Amplex Red assay with 10 μ M compound and 100 nM NicA2. The rate for each compound was normalized by setting activity with *S*-nicotine as substrate to 100%.

Figure 8.

Structure and activity of nicotine analogs. Compounds are numbered 1–12 and the corresponding percent activity is depicted below. N/A indicates compounds that were inactive. All compounds were examined at concentrations of 10 μ M with 100 nM NicA2 using the Amplex Red assay. The rate for each compound was normalized by setting the activity with S-nicotine to 100%.

Table 1.Steady-State Kinetic Constants for NicA2 Variants Using *S*-Nicotine and 6 As Substrates^a

	K_M (10^{-9} M)	k_{cat} (10^{-3} s ⁻¹)	k_{cat}/K_M (s ⁻¹ M ⁻¹)
Substrate: <i>S</i>-nicotine			
Wild-type	114 ± 45	6.11 ± 2.2	5.35×10^4
T381V	317 ± 81	11.0 ± 4.0	3.47×10^4
T250V/T381V	2520 ± 220	25.9 ± 7.7	1.03×10^4
Substrate: 6			
Wild-type	318 ± 7.5	7.11 ± 0.10	2.23×10^4
T250V/T381V	1820 ± 570	26.2 ± 11	1.44×10^4

^aThe steady-state kinetic constants were measured in 50 mM sodium phosphate buffer pH 7.4 at 22 °C using the Amplex Red assay as described in Methods.