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The mechanism of the flavoprotein L-6-hydroxynicotine oxidase: pH and solvent isotope effects and identification of key active site residues

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Abstract

The flavoenzyme L-6-hydroxynicotine oxidase (LHNO) is a member of the monoamine oxidase family that catalyzes the oxidation of (S)-6-hydroxynicotine to 6-hydroxypseudooxynicotine during microbial catabolism of nicotine. While the enzyme has long been understood to catalyze oxidation of carbon-carbon bond, it has recently been shown to catalyze oxidation of a carbon-nitrogen bond (Fitzpatrick et al., *Biochemistry* 55, 697–703). The effects of pH and mutagenesis of active site residues have now been utilized to study the mechanism and the roles of active site residues. Asn166 and Tyr311 bind the substrate, while Lys287 forms a water-mediated hydrogen bond with the flavin N(5). The N166A and Y311F mutations result in decreases of ~30 and ~4-fold in the k_{cat}/K_m and k_{red} values for (S)-6-hydroxynicotine, respectively, with larger effects on the k_{cat}/K_m value for (S)-6-hydroxynornicotine. The K287M mutation results in a decrease of ~10-fold in these parameters and a 6,000-fold in the k_{cat}/K_m value for oxygen. The shapes of the pH profiles are not altered by the N166A and Y311F mutations. There is no solvent isotope effect on the k_{cat}/K_m value for amines. The results are consistent with a model in which both the charged and neutral forms of the amine can bind, with the former rapidly losing a proton to a hydrogen bond network of water and amino acids in the active site prior to hydride transfer to the flavin.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information Available

The Supporting Information is available free of charge on the ACS Publications website. Table S1 and Figures S1–3. (PDF) pH dependence of chemical shifts for substrates; solvent isotope effects; structure of proposed hydrogen bond network.

The flavoenzyme L-6-hydroxynicotine oxidase (LHNO) catalyzes an early step in the bacterial metabolism of nicotine, the oxidation of (S)-6-hydroxynicotine to 6-hydroxypseudonnicotine¹. There is a great deal of structural information regarding LHNO, including structures of the enzyme with substrates and products^{2, 3}, but there has been little study of the mechanism. While the enzyme was long assumed to catalyze the oxidation of a carbon-carbon bond in its substrate⁴, we recently established that LHNO catalyzes oxidation of a carbon-nitrogen bond (Scheme 1)⁵. Amine oxidation by LHNO is consistent with the protein structure, in that it is a member of the monoamine oxidase (MAO) structural family of flavoproteins³.

Members of the MAO family contain a highly-conserved FAD-binding domain and a divergent substrate-binding domain⁶. The crystal structure of LHNO with (S)-6-hydroxynicotine bound (Figure 1) shows that two residues in the substrate-binding domain, Asn166 and Tyr311, are positioned appropriately to form hydrogen bonds with the substrate. A residue in the flavin domain, Lys287, corresponds to a lysine residue that is conserved throughout the MAO family⁶. We describe here the use of pH effects and site-directed mutagenesis to probe the roles of these residues in substrate specificity and catalysis for LHNO.

Experimental Procedures

Materials

(R,S)-4-Hydroxynicotine was from Princeton Biomolecular Research (Princeton, NJ). (S)-4-Hydroxynornicotine was from Asiba Pharmatech Inc. (Milltown, NJ). Site-directed mutagenesis of pETHLNO⁵ was carried out using the QuikChange protocol (Stratagene). Wild-type and variant LHNOs from *Arthrobacter nitotinovorans* were expressed in *Escherichia coli* and purified as previously described⁵. All three variant enzymes were sufficiently stable for purification and characterization, although the expression of the K287M enzyme was substantially lower.

NMR spectroscopy

¹H-NMR Spectra were collected on a Bruker Avance 600 spectrometer using a 5 mm TXI (¹H/¹³C/¹⁵N) CryoProbe equipped with z-axis pulsed field gradients. To determine the pK_a values for substrates, spectra of 5 mM solutions in H₂O were obtained at intervals of ~0.2 pH units from pH 7 to 12. NMRPipe and SpinWork4.0 were used to analyze the data. The Henderson-Hasselbalch equation was used to calculate the pK_a value from the effect of pH on the chemical shift of the methyl peak of 6-hydroxynicotine or C1' of the pyrrolidine ring of 6-hydroxynornicotine) as a function of pH.

Assays

Initial rates of amine oxidation were measured by monitoring oxygen consumption using an oxygen electrode (Model 5300A, Yellow Springs Instruments, Yellow Springs, OH) at 25 °C. The buffer was 0.1 M sodium Hepes at pH 7–8, 0.1 M sodium CHES at pH 8.5–10, and 0.1 M sodium CAPS at pH 10.5–11; all buffers also contained 0.1 M NaCl. For solvent isotope effects, buffers and substrates were made up in D₂O. The concentrations of all the

enzymes were determined using the ϵ_{445} value of $11.3 \text{ mM}^{-1}\text{cm}^{-1}$ for the wild-type enzyme⁸, since there were no significant changes in the visible absorbance spectra of the variants. The use of the flavin absorbance to determine the enzyme concentration meant that only holo-enzyme was included in the enzyme concentration. The concentration of oxygen was varied by bubbling the desired concentration of oxygen ($62 \text{ }\mu\text{M}$ - 1.25 mM) into the oxygen electrode cell for ~ 10 min. Assays typically contained $0.1 \text{ }\mu\text{M}$ enzyme, with the exception of the K287M variant, for which $10 \text{ }\mu\text{M}$ enzyme was used. The steady-state kinetic analyses could be simplified because the enzyme exhibits a ping-pong steady state kinetics profile, with amine oxidation occurring to produce the reduced enzyme prior to the reaction with oxygen and product release⁵. As a result the $k_{\text{cat}}/K_{\text{m}}$ values for the amine and oxygen are independent of the concentration of the other substrate. Values of k_{cat} and $k_{\text{cat}}/K_{\text{O}_2}$ with (S)-6-hydroxynicotine were determined by varying the concentration of oxygen at a saturating concentration of the amine ($1\text{--}4 \text{ mM}$) and fitting the data to the Michaelis-Menten equation. The K_{m} values for (S)-6-hydroxynicotine in Table 2 were calculated from the $k_{\text{cat}}/K_{\text{m}}$ values determined in air-saturated buffer and the k_{cat} values determined by varying the concentration of oxygen. For pH profiles, the $k_{\text{cat}}/K_{\text{m}}$ values for amine substrates and the k_{cat} values were determined by varying the concentration of the amine substrate at $250 \text{ }\mu\text{M}$ oxygen and fitting the data to the Michaelis-Menten equation. The program KaleidaGraph (Synergy Software) was used for fitting of the relevant equations. The errors in the individual values in figures are less than the size of the points.

Stopped-flow experiments were carried out anaerobically using an Applied Photophysics SX-20 MV stopped-flow spectrophotometer. The conditions were $20\text{--}50 \text{ }\mu\text{M}$ LHNO and $60\text{--}2000 \text{ }\mu\text{M}$ substrate in 0.1 M buffer, 0.1 M NaCl, at $25 \text{ }^\circ\text{C}$. Oxygen was removed from the instrument and reagents as previously described.⁹

Results

Substrate pK_{a} values

The steady-state kinetics of LHNO with (S)-6-hydroxynicotine and (S)-6-hydroxynornicotine (Schemes 1 and 2) as substrates have been described previously⁵. Since the pK_{a} values of substrates will contribute to pH profiles, the pK_{a} values of these compounds were determined by NMR spectroscopy (Figure S1). The chemical shifts of 6-hydroxynicotine and 6-hydroxynornicotine from pH 7 to 12 were readily fit by the Henderson-Hasselbalch equation to yield pK_{a} values of 8.60 ± 0.01 and 9.46 ± 0.05 , respectively. These can be assigned to the pyrrolidine nitrogens, since the pyridinone nitrogens will have pK_{a} values less than 5.

pH Dependence of kinetic parameters

The effects of pH on the steady-state kinetic parameters with (S)-6-hydroxynicotine and (S)-6-hydroxynornicotine as the amine substrate for LHNO were determined over the pH range 7–11; outside this pH range the enzyme lost activity too quickly for reliable assays. The results are shown in Figure 2 and the resulting pK_{a} values are given in Table 1. The $k_{\text{cat}}/K_{\text{amine}}$ and k_{cat} profiles for both substrates were well fit with eq 1, which describes a bell-shaped profile with basic and acidic limbs. The $k_{\text{cat}}/K_{\text{O}_2}$ profiles for both substrates

were well fit with eq 2, which applies when activity only decreases at high pH. The acidic pK_a values seen in the k_{cat} and k_{cat}/K_m profiles for the physiological substrate (S)-6-hydroxynicotine are significantly lower than those in the (S)-6-hydroxynornicotine profiles, to the point where the former are poorly defined. The basic pK_a values in all three profiles for both the amine and oxygen are higher with (S)-6-hydroxynornicotine than with (S)-6-hydroxynicotine. None of the pK_a values in the pH profiles matches those of the substrates, although the higher pK_a values seen with (S)-6-hydroxynornicotine are consistent with the higher pK_a of that substrate.

$$\log Y = \log \frac{c}{1 + H/K_1 + K_2/H} \quad (1)$$

$$\log Y = \log \frac{c}{1 + K_2/H} \quad (2)$$

Solvent isotope effects

The solvent kinetic isotope effect on the k_{cat}/K_m value for the amine substrate was determined with both (S)-hydroxynicotine and (S)-hydroxynornicotine as substrate. For both substrates, the k_{cat}/K_{amine} -pD profiles are similar to the profiles in H_2O , with the pK_2 values being shifted higher (Figure S2). Accordingly, initial rates were determined as a function of the amine concentration for (S)-hydroxynicotine in H_2O at pH 8.0 and in D_2O at pD 8.5 and for (S)-hydroxynornicotine at pH 9.0 in H_2O and pD 9.5 in D_2O . The data were fit to eqs 3 and 4 to determine the isotope effects. Eq 3 applies for discrete isotope effects on both k_{cat} (E_V) and k_{cat}/K_m (E_{VK}) while eq 4 applies for an isotope effect only on k_{cat} . Fitting the data to eq 3 yielded $^{D20}(k_{cat}/K_{amine})$ values within error of 1.0 for both substrates and $^{D20}k_{cat}$ values of 1.6 ± 0.1 and 1.31 ± 0.04 for (S)-hydroxynornicotine and (S)-hydroxynicotine, respectively (Table S1). Fitting the data to eq 4 yielded the same $^{D20}k_{cat}$ values with no change in the X^2 value, consistent with no change in the k_{cat}/K_{amine} value for either substrate in D_2O .

$$v = \frac{k_{cat} A}{K_a (1 + F_i(E_{VK} - 1)) + A (1 + F_i(E_V - 1))} \quad (3)$$

$$v = \frac{k_{cat} A}{K_a + A (1 + F_i(E_V - 1))} \quad (4)$$

Rapid reaction kinetics

The effect of pH on the limiting rate constant for flavin reduction, k_{red} , was determined with (S)-hydroxynornicotine as the amine substrate (Figure 2). The k_{red} value decreases with decreasing pH from a limiting value at high pH; fitting the data to eq 6 yields a pK_a value of

7.7 ± 0.1 . The K_d value was much less sensitive to pH, with an average value of 2.6 ± 1.2 mM from pH 7–9 (data not shown). No indication of an intermediate with a spectrum consistent with a flavin radical was seen at any pH.

$$k_{\text{obs}} = S \cdot k_{\text{red}} / (K_d + S) \quad (5)$$

$$\log Y = \log \frac{c}{1 + H/K_1} \quad (6)$$

Active site variants

To determine the contribution of active site residues to catalysis, the kinetic parameters of the N166A, K287M, and Y311F enzymes were determined. The N166A and Y311F enzymes retained sufficient activity that the kinetic parameters for both (S)-6-hydroxynicotine and oxygen could be determined, as well as the apparent kinetic parameters in air-saturated buffer with (S)-6-hydroxynornicotine. The steady-state kinetic parameters at pH 8 for these variants are summarized in Table 2. The N166A enzyme showed larger decreases in the k_{cat} and the k_{cat}/K_m values for both the amine and oxygen than the Y311F enzyme, and for both variants the effect on the k_{cat}/K_m value for (S)-6-hydroxynicotine was greater than the effect on the k_{cat}/K_m value for oxygen. With (S)-6-hydroxynornicotine as substrate for these two variants, the decreases in the k_{cat}/K_m value were about twice those with the native substrate, but otherwise the effects of the mutations were qualitatively similar.

In contrast to the results with the N166A and Y311F variants, no activity could be detected in preliminary assays with the K287M variant. Only by using a high concentration of enzyme (10 μM) and oxygen-saturated buffer was it possible to obtain reliable initial rates when the concentration of the amine was varied. Consequently, it was only possible to obtain apparent k_{cat} and K_{amine} values, although the $k_{\text{cat}}/K_{\text{amine}}$ value is independent of the concentration of oxygen. This value decreased ~14-fold as a result of the mutation. When the activity of the enzyme was determined as a function of the concentration of oxygen at a high concentration of (S)-6-hydroxynicotine, the initial rate increased directly with the oxygen concentration, with no indication of saturation at 1.2 mM oxygen, the highest concentration attainable. The k_{cat}/K_m value for oxygen for this variant could be calculated from this concentration dependence; this value is 5600-fold lower than the value for the wild-type enzyme.

To determine more directly the effect of these mutations on the rate constant for amine oxidation, the kinetics of flavin reduction by (S)-6-hydroxynicotine were examined for the variants. For all three enzymes the decrease in the k_{red} value was comparable to that in the k_{cat}/K_m value, with the N166A mutation having the largest effect (Table 3). In contrast the apparent K_d values were relatively unaffected.

Discussion

While the mechanism by which flavoproteins oxidize amines has been the subject of substantial controversy, the results of mechanistic studies of flavin amine oxidases in the MAO and D-amino acid oxidase structural families are all well accommodated by a mechanism involving direct transfer of a hydride from the neutral amine to the flavin (Scheme 3)^{10, 11}. This conclusion is supported by recent computational studies^{12, 13} We previously established that LHNO catalyzes oxidation of a carbon-nitrogen bond, as expected from its structural similarity to MAO⁵. The present results extend that study and identify residues that are critical for substrate binding and catalysis.

The decrease below a pK_a value of 7.7 in the k_{red} value for (S)-6-hydroxynornicotine as a substrate for LHNO (Figure 3) is most consistent with hydride transfer to the flavin in this enzyme requiring a neutral nitrogen in the enzyme-substrate complex. If the charged form of the substrate does bind, it must rapidly lose an amine proton prior to oxidation. The lack of a solvent isotope effect on the k_{cat}/K_m for either (S)-6-hydroxynicotine or (S)-6-hydroxynornicotine establishes that no proton is in flight during the transition state for amine oxidation.

The previous analysis of the substrate specificity of LHNO established that the pyridyl nitrogen and oxygen play key roles in substrate binding. The k_{cat}/K_m and k_{red} values for (S)-nicotine, which lacks the oxygen, and (S)-4-(1-methyl pyrrolidine-2-yl) phenol (Scheme 2), which lacks the pyridyl nitrogen, are both ~1,000-fold lower than the value with (S)-6-hydroxynicotine at pH 8⁵. However, the predominant tautomer of (S)-6-hydroxynicotine in solution is the pyridinone (Scheme 1), while the predominant tautomers of (S)-nicotine and (S)-4-(1-methyl pyrrolidine-2-yl) phenol contain pyridyl and phenyl rings, respectively. Consequently, the effects of mutating the active site residues that interact with the nitrogen and oxygen in the pyridinone, Asn166 and Tyr311, provide better estimates of the contributions of these interactions to catalysis and specificity than the kinetic parameters for the alternate substrates. The Y311F mutation, which eliminates a hydrogen bond with the substrate oxygen, decreases both the k_{cat}/K_m value and the k_{red} value with (6)-hydroxynicotine by ~4-fold, consistent with a contribution of ~1 kcal/mol to binding and catalysis from a hydrogen bond in which the tyrosine hydroxyl is the donor and the substrate oxygen the acceptor. The effects of the N166A mutation are greater than those of the Y311F mutation, with the k_{red}/K_m and k_{red} values for (6)-hydroxynicotine decreasing by two orders of magnitude. This can be attributed to the loss of two hydrogen bonds with the substrate. Overall, the effects of mutating Tyr311 and Asn166 establish that these two residues are important for productive binding of the substrate. For both variants the effect on the k_{cat}/K_m value for (S)-6-hydroxynornicotine is larger than the case with the physiological substrate. This may be due to the increased mobility of the substrate in the active site when the contributions of both the methyl group and the oxygen to proper positioning for catalysis are lost. In addition, the relative change in the K_{cat}/K_m value correlates with the change in the k_{red} value, while the K_d value is essentially unchanged. This is consistent with the full contribution of these two residues only being expressed in the transition state for hydride transfer, with a much smaller contribution to substrate binding to form the Michaelis complex.

The K287M mutation has a much larger effect on the kinetics of oxidation of the reduced flavin than on amine oxidation. The k_{red} and $k_{\text{cat}}/K_{\text{m}}$ values with (S)-6-hydroxynicotine both decrease an order of magnitude for this protein, whereas the $k_{\text{cat}}/K_{\text{m}}$ value for oxygen is down ~6,000-fold. This lysine residue is conserved throughout the MAO structural family¹⁴, where it forms a water-mediated hydrogen bond with the flavin N5 (Figure 1). The effects of mutating this residue to methionine vary greatly in different members of the MAO family. In tryptophan 2-monooxygenase¹⁴ and maize polyamine oxidase¹⁵, the mutation results in a decrease of 3–6 orders of magnitude in the $k_{\text{cat}}/K_{\text{m}}$ value for the amine, but the $k_{\text{cat}}/K_{\text{O}_2}$ value for the latter enzyme only decreases 30-fold¹⁶. In the case of mouse polyamine oxidase, the mutation has no effect on the $k_{\text{cat}}/K_{\text{m}}$ value for the slow substrate spermine, but decreases the $k_{\text{cat}}/K_{\text{O}_2}$ value 25-fold¹⁷. In this case pH and solvent isotope effects are consistent with the charged lysine side chain acting as a proton donor during flavin oxidation. The variability of the results suggests that this residue plays two critical roles in this enzyme family. First, the water-mediated hydrogen bond to the lysine is important in properly positioning the cofactor with respect to the substrate; the rate constant for hydride transfer will be very sensitive to the distance between the substrate C2 and the flavin N5¹⁸, so that any increase in the distance over which the hydride must be transferred could have a dramatic effect on the rate constant for amine oxidation. The magnitude of the effect would vary depending upon how much the active site restricts movement of the isoalloxazine ring. Second, the lysine serves as a positive charge and proton donor during flavin oxidation.

Among these three active-site residues, both Tyr311 and Lys287 could have pK_{a} values between 7 and 11 and thus contribute to the pH profiles. The Y311F mutation does not change the shape of the $k_{\text{cat}}/K_{\text{m}}$ profile for either (S)-6-hydroxynicotine or (S)-6-hydroxynornicotine. In addition, the pK_{a} values derived from those profiles match those from the same pH profiles for the N166A protein. Thus, Tyr311 is unlikely to be a source of one of the pK_{a} values seen in the pH profiles. In contrast, as discussed below, it is likely that Lys287 contributes to the pH profiles.

$k_{\text{cat}}/K_{\text{m}}$ pH profiles reflect the protonation states of the free enzyme and substrate required for productive binding and catalysis. If only the neutral form of a substrate can bind productively, its pK_{a} value will be seen in the $k_{\text{cat}}/K_{\text{m}}$ profile. For neither (S)-6-hydroxynicotine nor (S)-6-hydroxynornicotine does a pK_{a} value in this profile match the pK_{a} value of the amine (Table 1). The finding that the value of the acidic pK_{a} in the $k_{\text{cat}}/K_{\text{m}}$ profile is significantly lower with (S)-6-hydroxynicotine as the substrate than with (S)-6-hydroxynornicotine suggests that the substrate does contribute to this pK_{a} . Frequently, pK_{a} values in $k_{\text{cat}}/K_{\text{m}}$ -pH profiles for physiological substrates are perturbed from their intrinsic values due to a high commitment to catalysis, i. e., the substrate reacts more quickly than it dissociates¹⁹. This phenomenon provides a reasonable explanation for some of the difference between the macroscopic pK_{a} values seen in the $k_{\text{cat}}/K_{\text{m}}$ pH profiles and the intrinsic pK_{a} values of the substrates. When enzymes exhibit perturbed pK_{a} values due to substantial commitments, it is frequently possible to use slower substrates or slower protein variants to decrease the rate constant for the chemical step and lower or eliminate the commitment to catalysis. In the case of LHNO, the acidic pK_{a} value in the $k_{\text{cat}}/K_{\text{m}}$ pH profiles of the N166A and Y311F proteins is indeed higher with both substrates, as expected for a decrease in the forward commitment. Moreover, this pK_{a} value is the same within error

for both protein variants for a specific substrate, with values of ~7.8 with (S)-6-hydroxynicotine and ~8.2 with (S)-6-hydroxynornicotine. This agreement suggests that these pK_a values are unperturbed by commitments and instead reflect the intrinsic pK_a values. Making this assumption allows one to calculate the values of the commitments as ~30 for (S)-6-hydroxynicotine and 2.6 ± 2.0 for (S)-6-hydroxynornicotine^{19, 20}.

The pK_a values seen in the N166A and Y311F variants still do not match the intrinsic pK_a values for the substrates. This suggests that both the protonated and the unprotonated forms of substrates can bind productively to LHNO. The only residue in the active site of LHNO with the potential to act as a base to accept a proton from the substrate is Tyr407; the lack of a significant decrease in activity for the Y407F enzyme⁵ rules out this residue as the sole source of the pK_a . In the structure of the complex of LHNO with (S)-6-hydroxynicotine (Figure S3), the pyrrolidine nitrogen is 3.2 Å from a water molecule²¹. This water molecule participates in a hydrogen bond network that includes additional water molecules and the side chain hydroxyls of Tyr407 and Ser197. The discrepancy between the pK_a values of the substrates and the acidic pK_a values in the k_{cat}/K_m pH profiles of the variants can be rationalized if the charged form of the amine substrate can bind, with this hydrogen bond network then rapidly accepting a proton from the nitrogen. Thus, the pK_a values of ~7.7 and ~8.2 do not reflect the ionization properties of a single residue, but rather the macroscopic pK_a values of the multiprotonic network of water molecules, the substrate, and active site residues. This pK_a includes the ionization properties of the substrate, so that it is lower with 6-hydroxynicotine than with 6-hydroxynornicotine as substrate. Only when an additional proton is added to this network is the activity lost, in that the positively charged substrate cannot bind when the network also contains a positive charge.

There is precedent for a lack of an effect of the amine protonation state on binding of amines to flavin amine oxidases, even though hydride transfer involves the neutral amine. Tryptophan 2-monooxygenase is a member of the MAO family that catalyzes the oxidation of the α -carbon-nitrogen bond in L-amino acids²². The k_{cat}/K_m pH profiles with tryptophan, methionine and phenylalanine as substrates for the enzyme can all be fit to a model involving two acidic pK_a values and one basic pK_a value²³. The highest and lowest pK_a values are ~5.0 and ~9.9 with all three substrates, with the intermediate pK_a matching the pK_a of the amino acid substrate. With phenylalanine and tryptophan as substrates, the protonation state of the amino group does not have a significant effect on the k_{cat}/K_m value, whereas with the slower substrate methionine the intermediate pK_a is associated with an ~8-fold decrease at lower pH. Thus, the substrate pK_a is not seen in the pH profile with the physiological substrate and is difficult to detect even with slower substrates, consistent with the enzyme being able to productively bind the amino acid substrate irrespective of the protonation state of its amino group. The structure of tryptophan 2-monooxygenase with indoleacetamide bound shows a network of water molecules but no obvious base to accept a proton from the substrate¹⁴. When the active site residue Arg98, which binds the substrate carboxylate, is mutated to lysine or alanine, the pK_a of the substrate is clearly seen in the k_{cat}/K_m pH profile for tryptophan and is associated with a 10–100-fold decrease in activity²⁴.

The acidic pK_a values in the k_{cat} -pH profiles of HLNO with (S)-6-hydroxynicotine and (S)-6-hydroxynornicotine can be attributed to the pK_a of the enzyme-bound substrate. The effects of pH on k_{cat} values reflect the pH dependence of first order steps, including chemical steps, product release, and conformational changes¹⁹. In the kinetic mechanism for LNHO the first order steps are amine oxidation and product release⁵. With (S)-6-hydroxynornicotine as substrate the k_{cat} value at pH 8 is about half the k_{red} value, so that reduction is close to rate-limiting, and the pH dependence of the two parameters should be similar. The acidic pK_a values for the k_{cat} and k_{red} pH profiles are indeed the same within error, 7.7–7.8. (LNHO is not sufficiently stable above pH 9 for stopped-flow analysis.) With (S)-6-hydroxynicotine the acidic pK_a value is ~1.5 units lower than that for (S)-6-hydroxynornicotine. This can be attributed to the lower pK_a of this substrate and to the larger difference (~7-fold) between the k_{red} and k_{cat} values.

All of the pH profiles exhibit a decrease in activity at high pH. The basic pK_a in the k_{cat}/K_m pH profiles can be assigned to Lys287 in the free enzyme with a pK_a of ~10.6. For both substrates the k_{cat} profiles exhibit a basic pK_a of 11–11.5; the precision of this value is limited by the instability of the enzyme above pH 11. This pK_a can be attributed to Lys287 in the enzyme-substrate complex; the lack of change in the value rules out Tyr311 as responsible for this pK_a . The pK_a seen in the k_{cat}/K_{O_2} profiles can be similarly assigned to Lys287 in the reduced enzyme-product complex based on the effects of mutating this residue and the precedent with mouse polyamine oxidase.

The present results and our earlier study of LNHO place the enzymatic reaction catalyzed by the enzyme firmly in the monoamine oxidase family. The effects of mutating Lys287, a residue in the flavin domain, establish the key role of this residue in modulating the reactivity of the flavin with both the amine substrate and with oxygen. The residues in the substrate binding domain, Asn166 and Tyr311, properly position the substrate for oxidation. Contradictory conclusions regarding the protonation state of the amine required for productive binding of the amine substrate by the monoamine oxidase family have been obtained by different laboratories^{25–28}. The present results suggest that the ability of a specific family member to bind the nonreactive protonated substrate productively can depend on bulk properties of the entire active site rather than the presence or absence of a specific active-site base and thus can vary substantially among family members.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

LHNO	L-6-hydroxynicotine oxidase
MAO	monoamine oxidase

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Figure 1. Active site of LHNO with (S)-6-hydroxynicotine (HNA) bound. The figure was drawn with Chimera⁷ and is based on PDB file 3NG7. Distances are given in Ångstroms.

Figure 2.
pH-Rate profiles for wild-type (filled circles), N166A (filled squares) and Y311F (open circles) LHNO with (S)-6-hydroxynicotine (left) or (S)-6-hydroxynornicotine (right) as substrate. The lines are from fits of the data to eq 1 or 2.

Figure 3. pH- k_{red} profile for (S)-6-hydroxynornicotine at 25 °C. The rate constants were determined by mixing varying concentrations of the amine anaerobically with LHNO at the indicated pH and fitting the observed first-order rate constants to eq 5. The line is from a fit to eq 6.

Scheme 1.

The reaction catalyzed by LHNO. The second step is nonenzymatic.

(S)-6-HO-nornicotine

(S)-4-(1-methyl
pyrrolidine-2-yl)phenol

Scheme 2.

Alternative substrates for 6-hydroxynicotine oxidase.

Scheme 3.
Mechanism of amine oxidation by the monoamine oxidase family of flavoproteins.

Table 1

pH Dependence of Steady State Kinetic Parameters for LHNO

Variant	Kinetic parameter	eq	pK ₁	pK ₂
wild-type	(S)-6-hydroxynicotine (pK _a 8.6)			
	k _{cat}	1	7.0 ± 0.4	10.6 ± 0.2
	k _{cat} /K _{amine}	1	6.2 ± 0.4	9.7 ± 0.1
		2		9.9 ± 0.1
	k _{cat} /K _{O2}	2		10.6 ± 0.1
N166A	k _{cat} /K _{amine}	1	7.7 ± 0.3	10.5 ± 0.3
	k _{cat}	1	8.0 ± 0.1	11.0 ± 0.2
Y311F	k _{cat} /K _{amine}	1	7.8 ± 0.2	10.8 ± 0.4
	k _{cat}	1	8.0 ± 0.1	11.2 ± 0.4
wild-type	(S)-6-hydroxynornicotine (pK _a 9.5)			
	k _{cat}	1	7.8 ± 0.1	11.6 ± 0.2
	k _{cat} /K _{amine}	1	7.7 ± 0.2	10.2 ± 0.2
	k _{cat} /K _{O2}	2		11.1 ± 0.2
N166A	k _{cat} /K _{amine}	1	8.1 ± 0.1	10.9 ± 0.2
	k _{cat}	1	8.0 ± 0.1	11.0 ± 0.2
Y311F	k _{cat} /K _{amine}	1	8.4 ± 0.2	10.7 ± 0.3
	k _{cat}	1	8.1 ± 0.1	11.5 ± 0.2

Table 2Steady state kinetic parameters for site-specific variants of LHNO^{*}

Variant	k_{cat} , s ⁻¹	k_{cat}/K_{amine} , mM ⁻¹ s ⁻¹	k_{cat}/K_{O_2} , mM ⁻¹ s ⁻¹	K_{amine} , mM	K_{O_2} , mM
(S)-6-hydroxynicotine					
Wild-type ^a	78 ± 10	600 ± 260	270 ± 60	0.13 ± 0.03	0.29 ± 0.10
N166A	4.8 ± 0.3	14 ± 3.0	24 ± 3	0.34 ± 0.08	0.20 ± 0.03
Y311F	20.9 ± 0.8	103 ± 4	110 ± 10	0.20 ± 0.01	0.19 ± 0.02
K287M ^b	0.26 ± 0.05	24 ± 15	0.048 ± 0.005	0.011 ± 0.009	>> 1 mM
(S)-6-hydroxynicotine ^c					
Wild-type ^a	16 ± 1	370 ± 50	150 ± 10	0.064 ± 0.014	0.46 ± 0.07
N166A	2.5 ± 0.1	2.0 ± 0.2	-	1.2 ± 0.2	-
Y311F	9.1 ± 0.5	8.6 ± 1.0	-	1.1 ± 0.2	-

^{*} Conditions: 0.1 M Hepes (pH 8), 0.1 M NaCl, at 25 ° C.^a The values for the wild-type enzyme are from reference 5.^b Determined by varying the concentration of the amine with 1.2 mM oxygen.^c The kinetic parameters for (S)-6-hydroxynicotine were determined by varying the concentration of the amine in air-saturated buffer, so that the k_{cat} and K_M values should be considered apparent values.

Table 3Rapid reaction kinetic parameters with (S)-6-hydroxynicotine for site-specific variants of LHNO^a

Variant	$k_{\text{red}}, \text{s}^{-1}$	K_{d}, mM
wild-type ^b	450 ± 50	0.10 ± 0.05
N166A	3.0 ± 0.1	0.31 ± 0.05
K287M	40 ± 2	0.061 ± 0.010
Y311F	92 ± 5	0.096 ± 0.018

^aConditions: 0.1 M Hepes (pH 8), 0.1 M NaCl, at 25 °C.^bFrom reference 5.