Assessing substrate specificity in 6-hydroxynicotinic acid 3-monooxygenase: the effects of 5-hydroxypicolinic acid and 6-chloro-5-hydroxypicolinic acid as NicC substrates

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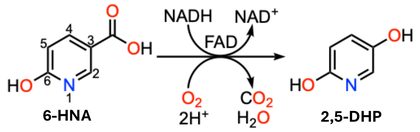
Substrate Specificity in Flavin Monooxygenases

- 6-Hydroxynicotinic acid 3-monooxygenase (NicC) is a Class A Flavin Monooxygenase (FMO) that catalyzes the *ipso* decarboxylative hydroxylation of 6-hydroxynicotinic acid (6-HNA).



- NicC *exclusively* catalyzes the *ipso* decarboxylative hydroxylation for all viable substrates.

- Substrate interacts with the active site of NicC primarily through His47, Tyr214, and Arg184. His47 and Tyr214 play a crucial role in substrate catalysis by ionizing the substrate to enhance the conformational protein dynamics and favor the substrate to undergo an electrophilic aromatic substitution mechanism. The C(4)a-hydroperoxyflavin intermediate either hydroxylates the substrate or degrades to release hydrogen peroxide. Thus, proper conformational dynamics are critical to ensure that NADH oxidization results in product formation **(Fig. 4, Reaction Cycle)**.

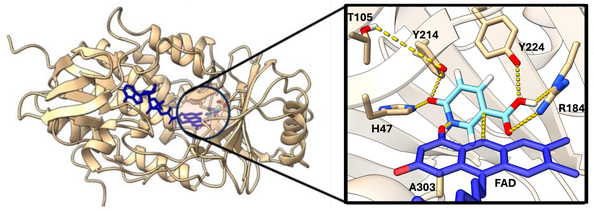
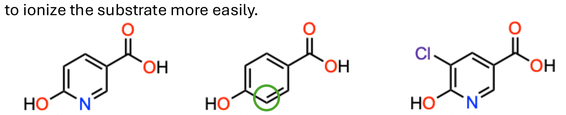


Figure 1 | Binding of 6-HNA to the NicC$_{WT}$ active site. 6-HNA (light blue) is observed interacting with nearby important residues (tan) as well as FAD (dark blue).

- NicC is highly substrate specific for 6-HNA, but it does weakly catalyze reactions with other mono-aromatic substrates like 4-hydroxybenzoic acid (4-HBA).

- NicC also strongly catalyzes the reaction with 5-chloro-6-hydroxynicotinic acid (5-Cl-6-HNA), as the chlorine adjacent to the C6 hydroxyl moiety is postulated to improve catalysis by lowering the pK_a to enable the enzyme to ionize the substrate more easily.

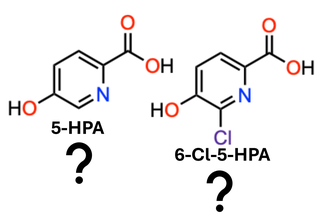


$k_{cat}/K_M = 4.2 \times 10^4\ (M^{-1}s^{-1})$ $k_{cat}/K_M = 1.2 \times 10^2\ (M^{-1}s^{-1})$ $k_{cat}/K_M = 5.6 \times 10^5\ (M^{-1}s^{-1})$

Figure 2 | Known Substrate Analogues of NicC with k_{cat}/K_M values.

Objective, Hypothesis, and Significance of Project

Objective: Determine whether 5-hydroxypicolinic acid (5-HPA) – the isomer of 6-HNA – and 6-Cl-5-HPA – the chlorinated version of 5-HPA – are viable NicC substrates.



Hypothesis: Both 5-HPA and 6-Cl-5-HPA will be viable NicC substrates due to the similarities in chemical structure of good NicC substrates.

Significance: This project will enhance the mechanistic understanding of how Class A FMOs act on substate analogues and provide essential background research to build an enzymatic model that can bioremediate hazardous environmental contaminants with similar chemical identity to the pyridine derivates that are currently NicC substrates, or other *N*-heterocyclic aromatic compounds.

NicC$_{WT}$ Weakly Catalyzes the *Ipso* Decarboxylative Hydroxylation of 5-HPA to 2,5-DHP

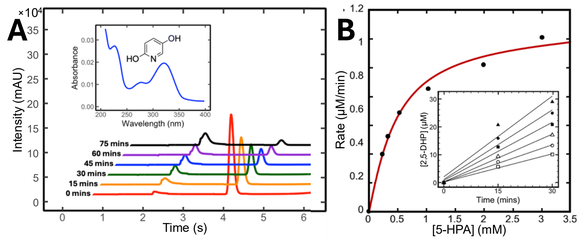
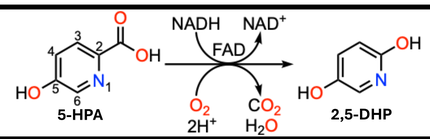


Figure 3 | *Ipso* Decarboxylative Hydroxylation of 5-HPA to 2,5-DHP by NicC$_{WT}$. A) Chromatogram showing the formation of 2,5-DHP (2 mins) and disappearance of NADH (4 mins). **B)** Michaelis-Menten fit using the initial rate data with resulting parameters shown in Table 1.

Table 1 | Kinetic Constants with NicC$_{WT}$

Substrates	K_M (μM)	k_{cat} (s^{-1})	k_{cat} / K_M ($M^{-1}s^{-1}$)	Coupling
6-HNA	118 ± 12	5.1 ± 0.1	(4.2 ± 0.8) × 10^4	90%
4-HBA	600 ± 100	0.074 ± 0.005	(1.2 ± 0.3) × 10^2	52%
5-HPA	503 ± 70	0.039 ± 0.002	(7.8 ± 1.1) × 10^1	10%
6-Cl-5-HPA*	---	---	---	72%



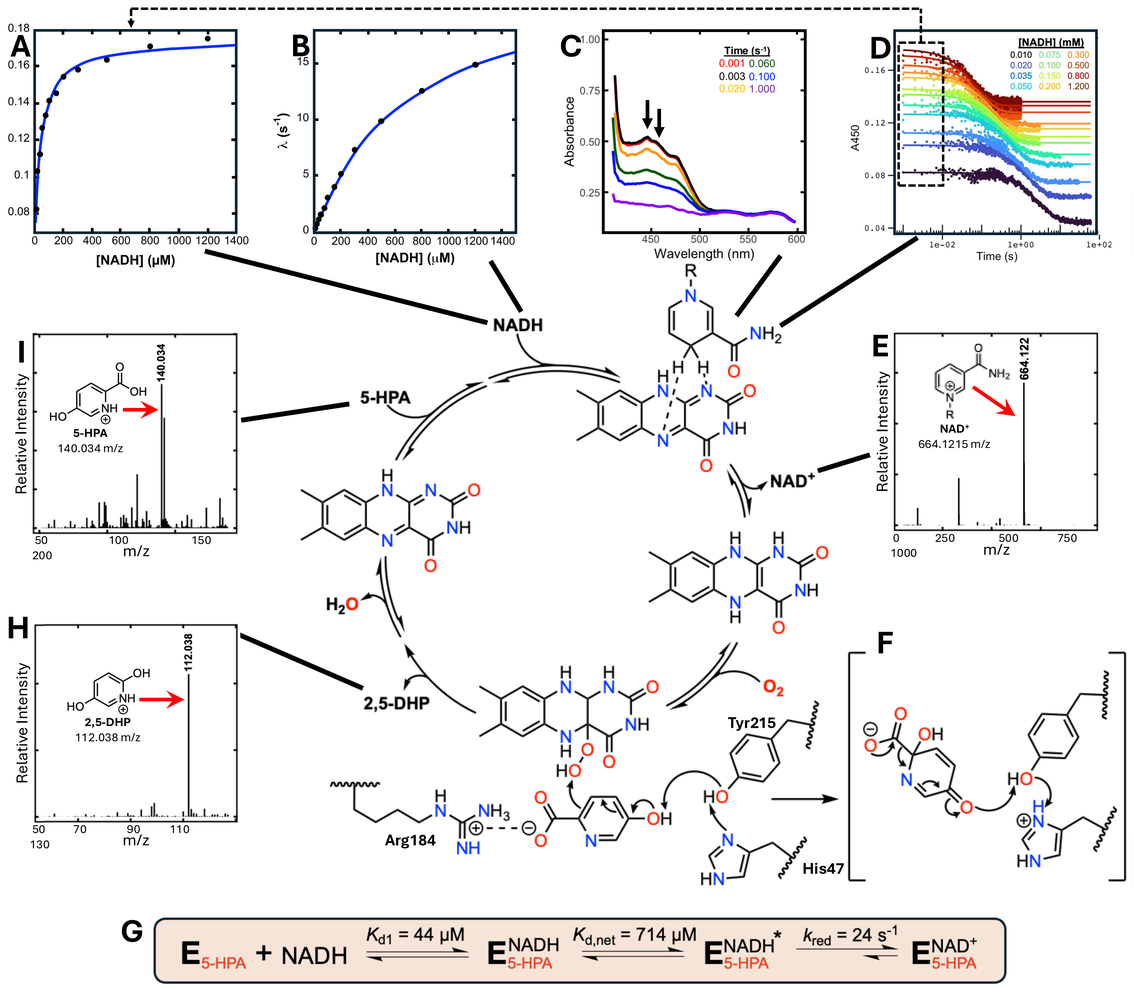


Figure 4 | Reduction Kinetics of NicC$_{WT}$ Saturated with 5-HPA and Reaction Components by LC-QTOF-MS. A) Initial rapid tight binding of NADH. **B)** Weaker NADH equilibrium binding after initial binding. **C)** Disappearance of the FAD 450 nm signal as reduction proceeds. **D)** A450 trace fit by a single exponential showing the reduction kinetics of NicC. **E)** Mass spectrum showing the formation of NAD^+ in the reaction mixture. **F)** Decarboxylative mechanism of NicC with 5-HPA as a substrate. **G)** Reaction scheme with the resulting transient-state reduction kinetic constants and dissociation constants. **H)** Mass spectrum showing the formation of 2,5-DHP during reaction of 5-HPA with NicC$_{WT}$. **I)** Mass spectrum showing the presence of 5-HPA in the reaction mixture. **Reaction Cycle)** Flavin cycle and proposed hydroxylation mechanism of 5-HPA.

Conclusions

- **5-HPA is a weak NicC substrate** that promotes a highly uncoupled reaction. NicC$_{WT}$ saturated with 5-HPA initially enables rapid and tight NADH binding, but prevents NADH to bind in a conformation that enables efficient hydride transfer, likely due to an abnormal FAD conformation not favoring substrate hydroxylation.

- **6-Cl-5-HPA may be a strong NicC substrate** that leads to a more coupled reaction. Two novel products were observed by their unique UV spectra (**Fig. 4**), but could not be properly identified by mass spectrometry. One product is postulated to be a dimer of the other product.

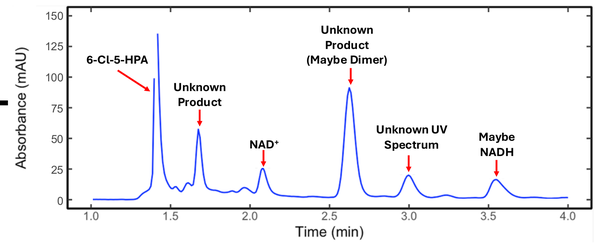


Figure 5 | Reaction of 6-Cl-5-HPA with NicC$_{WT}$. The wavelength of the chromatogram is at 340 nm. Peak 5 did not have a UV spectrum associated with it, and peak 6 had the 340 nm peak, but no 260 nm peak.

Future Directions

- Perform steady-state kinetic analysis of 6-Cl-5-HPA with NicC$_{WT}$ to determine its catalytic parameters to compare with 5-HPA.

- Perform steady-state kinetic analyses of 5-HPA with NicC variants (H47Q, Y225N, T105H) to assess the roles of active site residues in relation to other Class A FMOs. Other Class A FMOs catalyze the *ipso* decarboxylative hydroxylation of 4-HBA, 4HB1H, and 5-HPA, HpaM. Since 4-HBA and 5-HPA are weak NicC substrates, changing residues in NicC to match the other Class A FMOs may enhance how the substrate interacts and reacts with NicC.

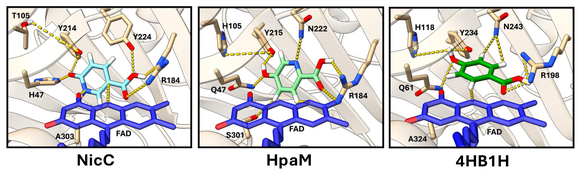


Figure 6 | Active Site Comparisons Between Class A Flavin Monooxygenases that Catalyze *Ipso* Decarboxylative Hydroxylation Reactions. NicC is shown to have the most distinct active site, but retains Tyr214 and Arg184 across all enzymes. HpaM catalyzes the *Ipso* Decarboxylative Hydroxylation for 5-HPA as it's primary substrate. 4HB1H catalyzes the *Ipso* Decarboxylative Hydroxylation for 4-HBA as it's primary substrate. Both enzymes have Gln47 in place of His47 in NicC. NicC also has Thr105, while HpaM and 4HB1H have His105 equivalents instead, which may take over the role of His47 in substrate ionization.

References

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