Role of Residues R184 and W273 in 6-HNA and NADH Binding by 6-Hydroxynicotinate 3-Monooxygenase

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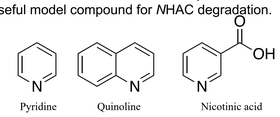
Background and Significance

- *N*-Heterocyclic Aromatic Compounds (*N*HACs) are known pervasive and dangerous environmental contaminants. Nicotinic acid, the subject of this research, is less harmful and serves as a useful model compound for *N*HAC degradation.



- Fortunately, soil bacteria are known to degrade these compounds into safe metabolites. The degradation of nicotinic acid in *Pseudomonas putida* is shown below. A common theme in *N*HAC degradation is hydroxylation prior to ring-opening. In this project, I will be focusing on the second step of this process, a decarboxylative hydroxylation reaction.

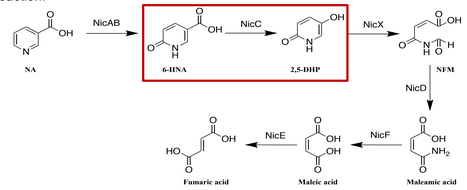


Figure 1. Degradation pathway of nicotinic acid in *Pseudomonas putida*.

Characteristics of NicC

- 6-hydroxynicotinate 3-monooxygenase (NicC) catalyzes this reaction using the oxidation of NADH to NAD^+. NicC is a unique class A flavin monooxygenase (FMO), indicating that it catalyzes the hydroxylation of an aromatic substrate using a catalytic FAD coenzyme.

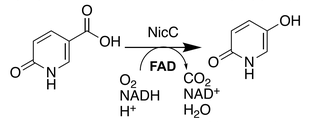


Figure 2. Decarboxylative hydroxylation reaction of 6-HNA to 2,5-DHP catalyzed by NicC.

- Class A FMOs also have a flavin conformation change upon substrate binding from buried within the enzyme ("in") to a 90° turn to be more accessible for reduction ("out").
- This flavin conformation change is not well understood in NicC.

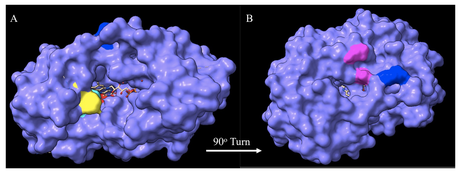
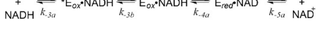


Figure 3. A) Full structure of PpNicC with flavin coenzyme shown in the flavin-binding site. B) Secondary binding tunnel in NicC.

- The binding mechanism of NicC was recently elucidated.[1] The organic substrate 6-HNA binds first to the enzyme, creating a stronger affinity of the enzyme-substrate complex for NADH.
  - This is unique to NicC as other class A FMOs increase the rate of the reduction reaction upon organic substrate binding.
- This allows for the formation of a charge-transfer complex (CTC) the point where the FAD and NADH are aligned just before the hydride transfer of the reduction reaction.

A No 6-HNA



B 6-HNA

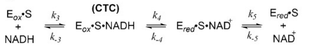


Figure 4. 6-HNA binding is essential for the development of the CTC in NicC.

Hypothesis & Research Objectives

Where is the NADH Binding Site in NicC?
- FAD binding site?
- Secondary binding tunnel?

Residues of Interest:
- Arg184 – FAD Binding Site
- Trp273 – FAD Binding Site
- Arg278 – Exterior of enzyme

Variants to Be Tested:
- R184K
- W273Y
- R278E

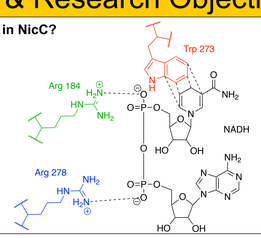


Figure 5. Hypothesized residue interactions with NADH.

PCR-Based Site-Directed Mutagenesis Used to Produce Desired Substitutions

Figure 6. Translated sequencing results for successful W273Y variant.

Stoichiometric Determination for Degree of Uncoupling in Each Variant

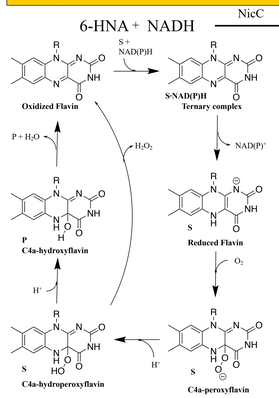


Figure 9. The reduction and oxidation of the flavin coenzyme in NicC. Uncoupling results when H_2O_2 is released from the C4a-hydroperoxyflavin instead of hydroxylating the substrate.

Table 1. Uncoupling percentages for each variant

Variant	% Uncoupling
WT	0 - 10
W273Y	31.5
R278E	42.5
R184K	83.4

SDS-PAGE Used to Confirm Successful Protein Isolation

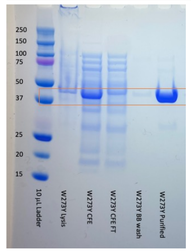


Figure 7. SDS-PAGE gel showing successful purification and isolation of the W273Y variant. Expected size of NicC is 41kDa.

Kinetic Analysis of Each Variant

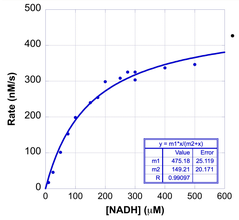


Figure 8. Sample data plot fit to Michaelis-Menten Equation.

- Initial rate steady-state kinetic analysis of each of the three variants was done. Two separate plots of each variant were done as a function of the two substrates:
  - 6-HNA
  - NADH

Effect of Each Variant on Catalysis as a Function of 6-HNA Concentration

Table 2. R278E constants as a function of 6-HNA

R278E	Result	Comparison to WT
K_M^{6-HNA}	270 +/- 30 µM	2.3x Increase
k_{cat}	3.94 +/- 0.10 s^{-1}	No difference
k_{cat}/K_M^{6-HNA}	1.5 × 10^4 $M^{-1}s^{-1}$	2.8x decrease

Table 3. W273Y constants as a function of 6-HNA

W273Y	Result	Comparison to WT
K_M^{6-HNA}	1700 +/- 320 µM	14.4x Increase
k_{cat}	9.4 +/- 0.9 s^{-1}	1.9x Increase
k_{cat}/K_M^{6-HNA}	5.5 × 10^3 $M^{-1}s^{-1}$	7.6x Decrease

Table 4. R184K constants as a function of 6-HNA

R184K	Result	Comparison to WT
K_M^{6-HNA}	1900+/- 290 µM	16.1x Increase
k_{cat}	0.56 +/- 0.03 s^{-1}	8.9x Decrease
k_{cat}/K_M^{6-HNA}	3.0 × 10^2 $M^{-1}s^{-1}$	140x Decrease

Conclusions

- R278E shows a small 2-fold increase in K_M^{6-HNA}
  - Given this is a drastic mutation, it is unlikely that R278 has any role in 6-HNA binding.
- W273Y has a 14-fold increase in K_M^{6-HNA}
  - This is a conservative substitution; it is likely that W273 has a role in 6-HNA binding in NicC.
  - Increase in k_{cat} likely due to experimental error fitting data to Michaelis-Menten Equation.
- R184K displays a 16-fold increase in K_M^{6-HNA}
  - Like W273Y, this is a conservative substitution.
  - Suggests that R184 has a role in binding 6-HNA.

Evidence for Substrate Inhibition by 6-HNA in Variants R184K and W273Y

Oversaturated 6-HNA Concentration Decreases Initial Rate for R184K

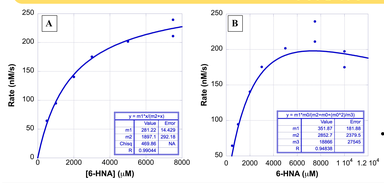


Figure 10. Same plot shown including higher 6-HNA concentrations in B. A) Fit to Michaelis-Menten equation. B) Fit to uncompetitive inhibition equation. Higher concentration of 6-HNA decrease initial rate in R184K when approaching K_i of 6-HNA in WT enzyme (15mM).

- Likely indicates that 6-HNA acts as its own inhibitor at high concentrations.

Saturating W273Y in 6-HNA Leads to Increase in K_M^{NADH}

Table 5. Unsaturated 6-HNA levels in W73Y lead to a decrease in K_M^{NADH}

Kinetic Constant	Result	Comparison to WT	Comparison to Saturated 6-HNA (7500 µM)
K_M^{NADH}	14 +/- 3 µM	2x Increase	11x Decrease
V/E – compare to k_{cat}	0.50 +/- 0.03 s^{-1}	10x Decrease	10x Decrease
$(V/E)/K_M^{NADH}$	(3.6 +/- 0.2) × 10^4 $M^{-1}s^{-1}$	17x Decrease	No Effect

- Saturating W273Y in 6-HNA leads to about a 10-fold increase in K_M^{NADH}
  - Likely due to higher 6-HNA concentrations blocking access to FAD coenzyme in NicC.

Variants R278E and W273Y Have Modest Impact on NADH Binding

Table 6. R278E displays a 7-fold increase and W273Y has a 19-fold increase in K_M^{NADH} compared to WT NicC. Neither variant influenced catalytic turnover.

R278E	Result	Comparison to WT
K_M^{NADH}	57 +/- 15 µM	7x Increase
k_{cat}	3.44 +/- 0.26 s^{-1}	No Effect
k_{cat}/K_M^{NADH}	(6.0 +/- 0.2) × 10^4 $M^{-1}s^{-1}$	10.3x Decrease

W273Y	Result	Comparison to WT
K_M^{NADH}	150 +/- 20 µM	18.5x Increase
k_{cat}	4.80 +/- 0.3 s^{-1}	No Effect
k_{cat}/K_M^{NADH}	(3.2 +/- 0.1) × 10^4 $M^{-1}s^{-1}$	19x Decrease

- Given the drastic residue substitution of a positively charged arginine to glutamate in R278E, it is unlikely the residue has a role in NADH binding as the 7-fold increase is not that large for a disruptive substitution.
- The more conservative W273Y variant had a large impact on NADH binding, increasing K_M^{NADH} 19-fold compared to WT NicC. It is likely that this residue has some role in NADH binding.
- Neither variant influenced k_{cat} which indicates they did not impact the catalytic turnover.

R184K Has a Drastic Impact on NADH Binding with No Effect in Catalytic Turnover

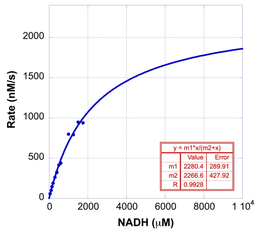


Figure 11. Plot of initial rates of the activity of R184K fit to the Michaelis-Menten Equation.

Table 7. R184K has a K_M^{NADH} value that is almost 300-fold greater than that of WT NicC while displaying no effect in the catalytic turnover.

R184K	Result	Comparison to WT
K_M^{NADH}	2300 +/- 430 µM	284x Increase
k_{cat}	4.56 +/- 0.58 s^{-1}	No Effect
k_{cat}/K_M^{NADH}	(2.0 +/- 0.2) × 10^3 $M^{-1}s^{-1}$	310x Decrease

- R184K significantly impeded the ability of NADH to bind to NicC but allowed the reaction to proceed at a normal rate.

These data suggest that residue R184 is likely a key residue in NADH binding by NicC.

Development of a Novel Hypothesis for NADH Binding and Flavin Conformation Dynamics in NicC

- The results from this study lead to a new hypothesis that could explain how variants R184K and W273Y may have similar effects on 6-HNA binding, but R184K has a much more drastic effect on NADH binding:
  - R184 and W273 form a pi-cation interaction, holding FAD in its "in" conformation when 6-HNA is not bound.
  - R184 electrostatically interacts with the carboxylate group on carbon 3 of 6-HNA upon its binding.
  - The R184 interaction with 6-HNA disrupts the pi-cation interaction of R184 and W273, allowing FAD to swing to its "out" conformation
  - The FAD conformational change allows NADH to access the FAD coenzyme, increasing NADH binding once 6-HNA is bound by NicC.

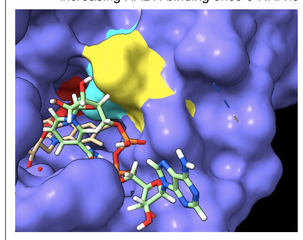


Figure 12. Hypothesized NADH binding site in NicC. The FAD coenzyme is still in its "in" position in this figure, and thus is not exact in how NADH would bind. However, this figure shows how the NADH molecule may be oriented in relation to R184 (cyan) and W273 (yellow).

- This hypothesis is supported by a recent crystal structure of a substrate analog of 6-HNA bound in the active site of the H47Q NicC variant.[2]
  - Those authors noticed that R184 was disordered in structure but would be in position to interact with carboxylate group on carbon 3, although bound substrate was missing this group.

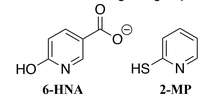


Figure 13. Difference between NicC substrate (6-HNA) and substrate bound in crystal structure (2-MP). Notably absent is the carboxyl group on 2-MP which is hypothesized to interact with R184.

- This hypothesis is further strengthened by examining the FAD conformation change in a well-studied class A FMO (PHBH) compared to NicC.

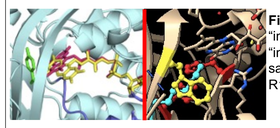


Figure 14. Left: Flavin conformation dynamics from "in" (red) to "out" (yellow) in PHBH.[3] Right: Flavin "in" position for NicC. If this flavin were to make the same move as in PHBH, it would move toward R184 (cyan) and W273 (yellow).

Future Research

- Use stopped-flow transient state kinetics to determine the effects of variants R184K and W273Y on the actual dissociation constant (K_d) as a function of 6-HNA and NADH
- Confirm the sequential binding mechanism of WT PpNicC is maintained in the variants
- Determine if the CTC is formed in the R184K variant to allow for the reduction between NADH and FAD

Acknowledgements

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References

1. Perkins, S. W., Hlaing, M. Z., Hicks, K. A., Rajakovich, L. J., and Snider, M. J. (2023) Mechanism of the Multistep Catalytic Cycle of 6-Hydroxynicotinate 3-Monooxygenase Revealed by Global Kinetic Analysis. Biochemistry. 62, 1553–1567
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3. Westphal, A. H., Tischler, D., Heinke, F., Hofmann, S., Gröning, J. A. D., Labudde, D., and van Berkel, W. J. H. (2018) Pyridine Nucleotide Coenzyme Specificity of p-Hydroxybenzoate Hydroxylase and Related Flavoprotein Monooxygenases. Front. Microbiol. [online] https://www.frontiersin.org/articles/10.3389/fmicb.2018.03050 (Accessed November 15, 2023)