Investigating the Importance of Arg 108 in the Binding of NADH by NicC

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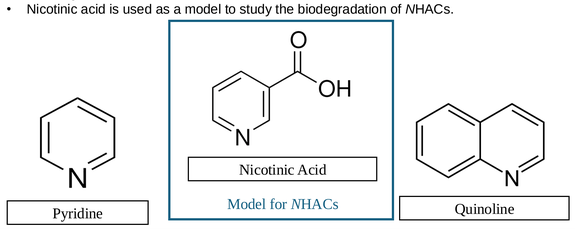
Background and Significance

- *N*HACs are prevalent in nature and are known to contaminate soils and groundwater, posing serious environmental and health risks, as they have been identified as carcinogenic[1] and toxic[2].
- Nicotinic acid is used as a model to study the biodegradation of *N*HACs.



Structure and Mechanism of NicC

- The two potential NADH binding sites in nicC: a larger site associated with FAD binding and a smaller site near the proposed 6-HNA entry channel.

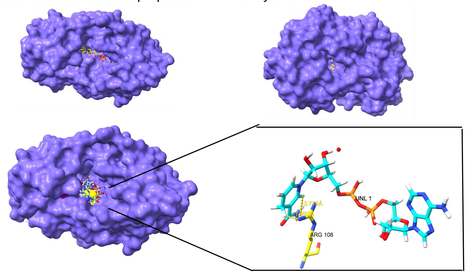


Figure 1. Potential interactions between NADH and key residues within nicC.

- This study hypothesizes that the FAD site heavily contributes to NADH recruitment and alignment. Additionally, the role of a residue within the FAD site is of particular interest, as it may be crucial for stabilizing NADH within the active site, potentially facilitating optimal electron transfer.
- Nicotinic acid's degradation pathway serves as a model for understanding the biodegradation of *N*-heterocyclic aromatic compounds.

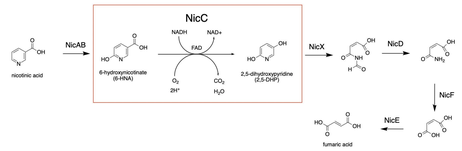


Figure 2. The degradation pathway of nicotinic acid (NA) leads to its conversion into fumaric acid, a central metabolic intermediate. nicC is emphasized as it facilitates the transformation of 6-hydroxynicotinate (6-HNA) to 2,5-dihydroxypyridine (2,5-DHP).

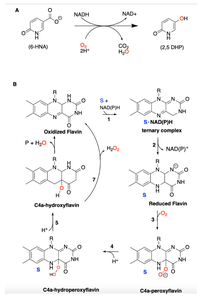


Figure 3. Reaction between flavin and NADH leads to the formation of the C4a-hydroperoxyflavin intermediate which serves as the oxygen atom donor to the nicotinamide ring via an electrophilic aromatic substitution mechanism.

- nicC belongs to the flavin monooxygenase (FMO) Group A class, which is characterized by catalyzing reactions where FAD is reduced by NADH. In nicC's catalytic process, after NADH reduces FAD, the reduced flavin forms an adduct with molecular oxygen, generating a reactive intermediate known as C(4a)-hydroperoxyflavin. This intermediate is key to the hydroxylation and decarboxylation of 6-HNA.

Hypothesis & Research Objectives

- **NADH binds NicC at the FAD binding site.**
- **Arg 108 helps to facilitate this binding.**
- Arg108 is positioned near the FAD-binding region and likely stabilizes the proper orientation of the flavin cofactor.
- Arg108 was subjected to site-directed mutagenesis, which generated the R108K lysine variant. In the absence of this stabilizing interaction, FAD may adopt a less favorable conformation for NADH binding, necessitating a higher concentration of NADH to achieve saturation and reducing the efficiency of hydride transfer.

R108K UV-Vis exhibits a decrease in NADH binding affinity and suggests substrate inhibition

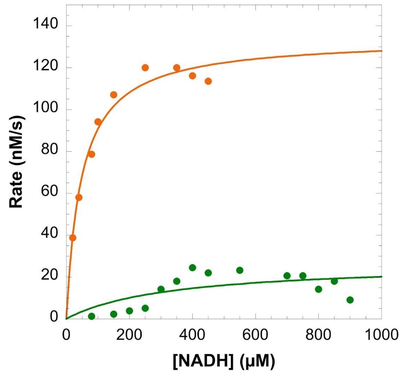


Figure 4. Steady-State Kinetics of the nicC R108K Variant at Two 6-HNA Concentrations by Following the Rate of NADH Oxidation at 340nm. The plot shows the reaction rate (nM/s) as a function of NADH concentration (μM) for the nicC R108K variant, with rates measured at 7500 μM (orange curve) and 500 μM (green curve) 6-HNA. The data were fitted to the Michaelis-Menten equation. The orange curve was repeated to determine whether the observed trend at higher NADH concentrations indicates potential substrate inhibition.

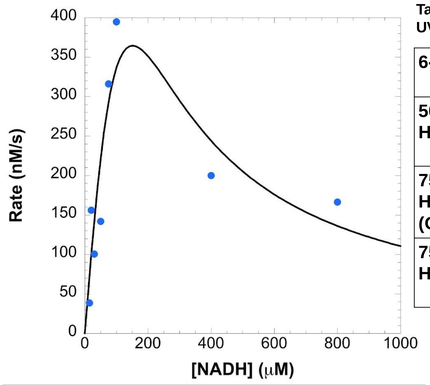


Figure 5. Steady-State Kinetics Using Substrate Inhibition Model of the nicC R108K Variant at 340 nm. The plot depicts the reaction rate (nM/s) as a function of NADH concentration (μM) for the nicC R108K variant in the presence of 7500 μM 6-HNA and 1 μM enzyme. The data exhibit a substrate inhibition trend, where the reaction rate increases at lower NADH concentrations but declines at higher levels. The curve is fitted to a non-linear regression fit to a non-linear regression model. The K_m of the fitted plot was derived to be 42 ± 17 μM, with a k_{cat} of 0.4 s^{-1} and a K_i of 665 ± 430 μM.

Table 1. Kinetic Parameters Derived from Michaelis-Menten Fits of UV-Vis Kinetics.

6-HNA (μM)	K_m (μM)	V_{max} (nM/s)	k_{cat} (s^{-1})
500 μM 6-HNA (Green)	335 ± 320	27 ± 10	0.03 ± 0.01
7500 μM 6-HNA (Orange)	50 ± 6	135 ± 5	0.13 ± 0.05
7500 μM 6-HNA (Blue)	42 ± 17	400 ± 0	665 ± 430

- Data in **Table 1** demonstrate a clear dependence of enzyme activity on 6-HNA concentration
- As the higher concentration (7500 μM) yields a greater maximum velocity (Vmax) and lower apparent K_m, indicative of more efficient catalysis under saturating substrate conditions.
- The dataset at 500 μM 6-HNA (green curve) exhibits significantly lower enzymatic activity, reflected by a much lower V_{max} and a higher K_m, suggesting that substrate availability is limiting enzyme function at this concentration.

R108K ITC reinforces a decrease in NADH binding affinity

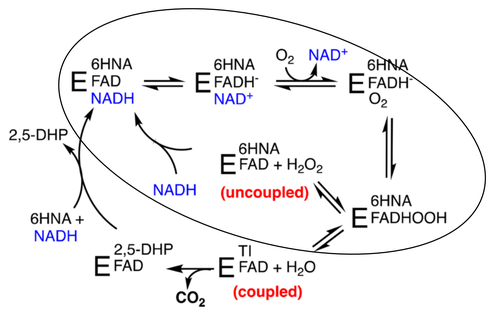


Figure 6. Schematic representation of NicC catalyzed reaction[3].

- The R108K variant exhibited 53% coupling and 47% uncoupling.
- This helped to elucidate findings from ITC steady-state kinetics.

Table 2. Steady-State Kinetic Parameters for nicC R108K at Varying 6-HNA Concentrations.

6-HNA (μM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m	[Enzyme] μM
100	(5.3 ± 0.4) × 10^{-2}	(1.2 ± 0.1) × 10^{-1}	(4.4 ± 0.2) × 10^{-2}	**4.5**
250	(5.3 ± 0.3) × 10^{-2}	(5.8 ± 1) × 10^{-2}	(9.1 ± 0.8) × 10^{-2}	**4**
500	(8.4 ± 0.5) × 10^{-2}	(5.7 ± 0.9) × 10^{-2}	0.15 ± 0.02	**3.5**
1000	(1.3 ± 0.7) × 10^{-2}	(5.4 ± 0.9) × 10^{-2}	0.23 ± 0.05	**3**
2500	(1.1 ± 0.1) × 10^{-2}	(3.9 ± 1) × 10^{-2}	0.28 ± 0.08	**2.5**
5000	(1.9 ± 0.1) × 10^{-2}	(3.9 ± 0.8) × 10^{-2}	0.5 ± 0.2	**2**
7500	(3.9 ± 0.3) × 10^{-2}	(5.2 ± 1) × 10^{-2}	0.74 ± 0.5	**2**
10000	(3.4 ± 0.1) × 10^{-2}	(4.2 ± 0.5) × 10^{-2}	0.81 ± 0.6	**2**
12500	(7.9 ± 0.3) × 10^{-2}	(1.1 ± 0.1) × 10^{-1}	0.71 ± 0.5	**1.5**
15000	(3.5 ± 0.3) × 10^{-2}	(5.1 ± 1) × 10^{-2}	0.69 ± 0.5	**1**

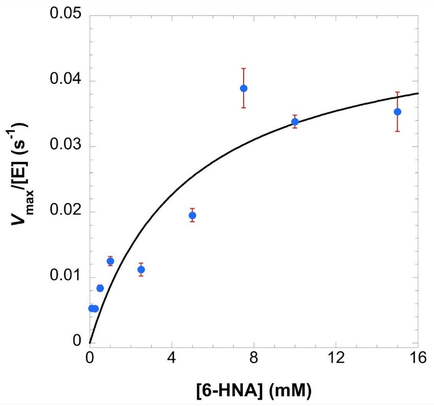


Figure 7. Figure 18. Re-Plot Showing Dependence of V_{max} on [6-HNA] for R108K nicC.

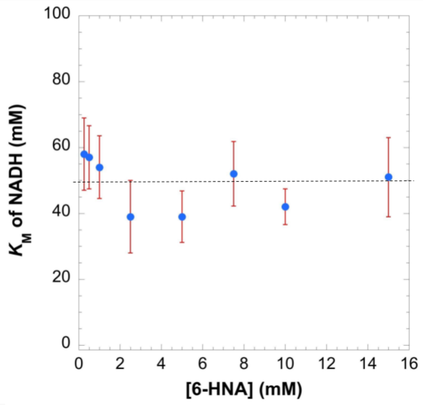


Figure 8. Re-Plot of Negligible Effect of 6-HNA on R108K nicC K_m for NADH.

- **Figure 8** depicts the regression analysis follows a non-linear fit to the Michaelis-Menten equation, allowing for the determination of kinetic parameters.
- Notably, the K_m for 6-HNA was 5 ± 3 mM, which is significantly higher than the wild-type value of 97 ± 12 μM, suggesting a substantial reduction in substrate affinity in the R108K variant. Overall k_{cat} = 0.05 ± 0.01 s^{-1} derived from **Table 2**.
- **Figure 9** is a summary of K_m values obtained by ITC kinetic analysis. Data reported in **Table 2** Overall Km = 50 ± 14 mM.

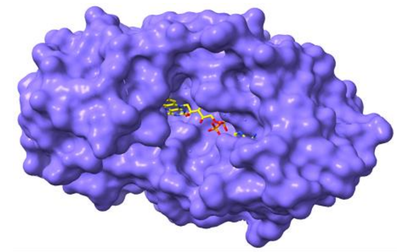
Arg 108 Conclusions

Arg 108 is important to the binding of NADH by nicC at the FAD binding site.

- The **replacement of Arg 108 with Lys** alters **electrostatic interactions** and **hydrogen bonding networks** in the active site.
- NicC relies on **NADH as an electron donor to reduce FAD**, which then facilitates the hydroxylation reaction.
- The **R108K mutation likely changes NADH positioning** relative to FAD, leading to **inefficient electron transfer**.

Future Research

- **Stopped-Flow Transient State Kinetics:** Provide insight into the binding affinity of NADH by NicC (how tightly bound).
- Isolate reductive half-reaction to see how this variant affects hydride transfer without molecular oxygen.



- Investigate other residues within the FAD binding pocket.
- Accumulate research on multiple variants in this binding pocket to elucidate the binding of NADH by nicC.

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References

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