Investigating Substrate Promiscuity of 6-Hydroxynicotinate 3-monooxygenase: NicC Activity with 5-Chloro-Coumalate

Aqlesia Aregawi and Mark J. Snider; Program of BCMB, The College of Wooster, Ohio

Background and Significance

- *N*-heterocyclic aromatic compound (NHACs) are one of the most pervasive contaminants that can exist either naturally or stem from synthetic NHACs, as found in industrial productions.
- FMOs such as 6-Hydroxynicotinate 3-monooxygenase (NicC), catalyze the electrophilic aromatic substitution in NHAC such as pyridine, quinoline, lutidine, *etc.* as they are degraded by bacteria.
- The efficiency with which FMOs reduce the flavin with NAD(P)H and release NAD(P)$^+$ is enhanced when a substrate is activated (such as ionized) upon binding.

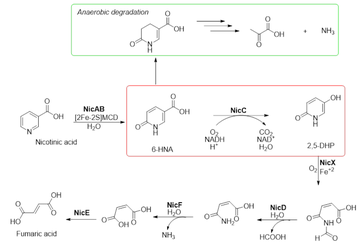


Figure 1: Nicotinic acid (NA) degradation in aerobic and anaerobic bacteria[1].

NicC Activity with Alternate Substrates

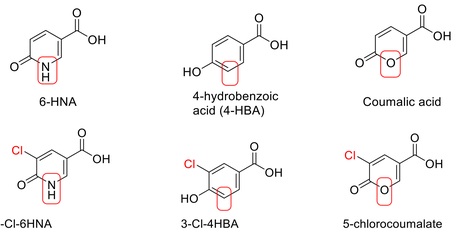


Figure 2: Structures of alternate substrates for NicC showing the chlorinated version along the bottom row.

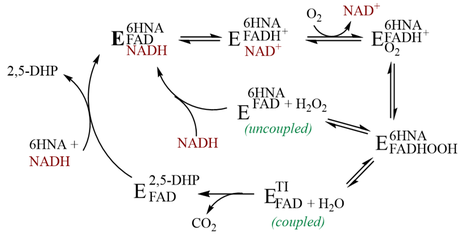


Figure 3: Simplified kinetic scheme of the reaction mechanism of NicC with 6-HNA[1].

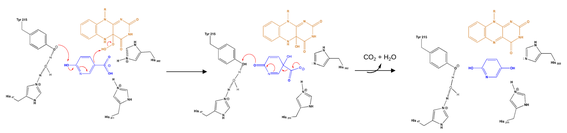


Figure 4: NicC mechanism for the decarboxylative hydroxylation of 6-HNA to 2,5 DHP[1].

Hypothesis & Research Objectives

- **Can the chlorination of a substrate enhance the catalytic activity of NicC in the decarboxylative-hydroxylation of 6-HNA analogues? Would this apply to a poor substrate?**

Aim 1: **Determine if there is product formation when NicC acts on 5-Cl-coumalate. If so, what is the coupling extent and catalytic efficiency?**

Aim 2: **Use transient state and steady state kinetic analysis of NicC's reaction with 5-Cl-coumalate to determine effects of the chlorination.**

Hypothesis: Based on previous data with alternative substrates, chlorination of the substrate would result in higher catalytic efficiency.

Can NicC catalyze the decarboxylative hydroxylation of 5-Cl-coumalate?

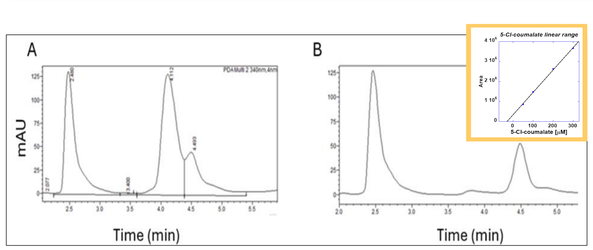


Figure 5: HPLC chromatogram of reaction containing 5-chloro coumalate, NicC, and NADH observed at 340nm. (A) Chromatogram of the reaction at time ~ 0 min. (B) Chromatogram at reaction time = 90 min.

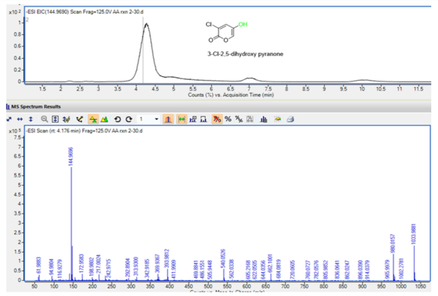
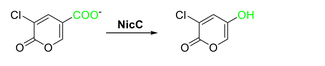


Figure 6: Analysis by mass spectrometry of a reaction of 5-Cl-coumalte and NADH with NicC to confirm production of 3-Cl-2,5-dihydroxy pyran-one.



What is the coupling extent when NicC acts on 5-Cl-coumalate?

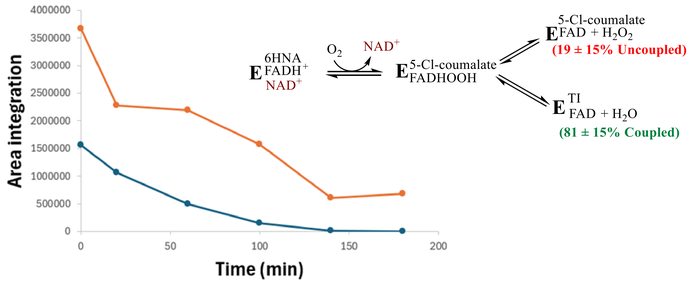


Figure 7: Plot of the integration of NADH and 5-Cl-coumalate elution peaks showing the difference in utilization by NicC. The orange line follows disappearance in 5-Cl-coumalate, the blue line follows NADH disappearance. Coupling extent was approximated from fractional disappearance of NADH and 5-Cl-coumalate in a 1:1 reaction.

Steady state Kinetics of NicC with 5-Cl-coumalate

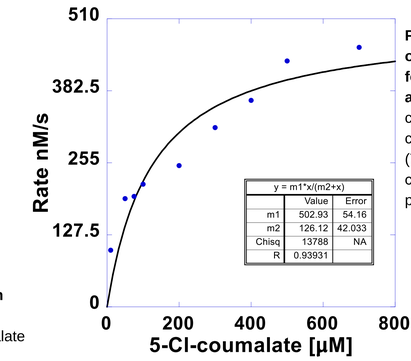


Figure 8: Initial rate of NADH oxidation measured by following linear change of rate at 380 nm. 5-Cl-coumalate concentrations (10 –700 µM), a constant concentration of NADH (700 µM), and constant NicC concentration of 100 nM at 25 ˚C pH 7.5.

Kinetic constants for alternate substrates

Table 1: Steady state kinetic comparison of substrate analogues[1,2,3].

Substrate	K_M (µM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)	K_d (µM)	Product coupling ratio
6-HNA	118	5.1	4.2×10^4	90	0.9
5-Cl-6-HNA	3.9	2.2	5.6×10^5	7	1
4-HBA	600	0.0074	1.2×10^2	355	0.52
3-Cl-4-HBA	41	6.2	1.5×10^5	49	1
Coumalate	180	<1.2	6.4×10^3	5	0.2
5-Cl-coumalate	126	5 ± 0.5	$(3.9 \pm 1.3) \times 10^4$	ND	≥ 0.70

Table 2: Comparison of the rate of reduction of NicC by NADH in the presence of a substrate to the rate of reduction of NicC by NADH in the absence of any substrate[1,2,3].

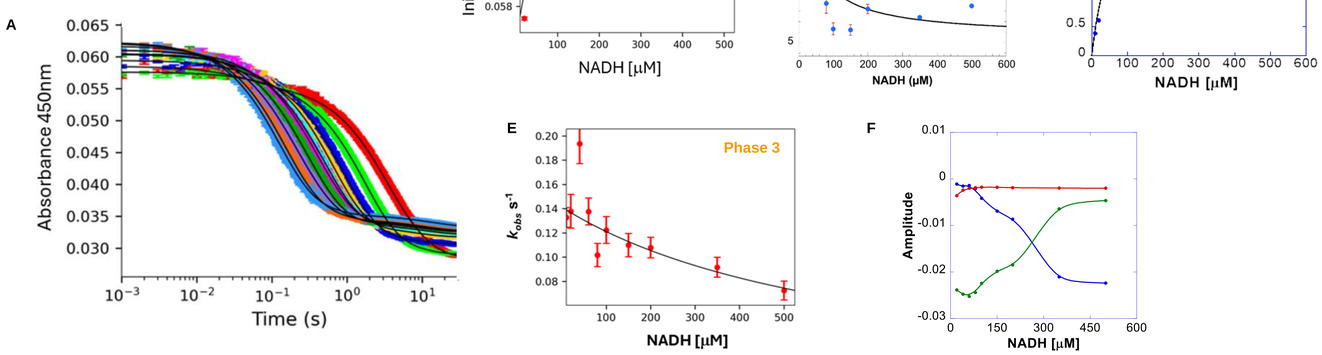
Substrate	K_d^S) µM) of E_S•NADH	NADH binding enhancement BE = $(\frac{K_d}{K_d^S})$	Hydride transfer k_{red}^S)s^{-1})	Reduction rate enhancement RE = $(\frac{k_{red}^S}{k_{red}})$	Overall enhancement (BE × RE)
6-HNA	3.5	220	42	28	6200
5-Cl-6HNA	50	15	44	29	435
4-HBA	303	2.5	12	8	20
3-Cl-4-HBA	15	50.6	15	10	506
Coumalate	670	1.13	37	25	28
5-Cl-coumalate	39	19	3.5	2.3	45

$K_{d,NADH}$ in absence of substrate = 770 µM; k_{red} in absence of substrate = 1.2 s^{-1}

Anaerobic reduction of NicC by stopped-flow spectroscopy in the presence of 5-Cl-coumalate

Figure 9: (A) Time course for reduction half reaction in anoxic solution with NADH by NicC·5Cl-coumalate at 450 nm absorbance. (B) Initial change in amplitude, (C) first phase, (D) second phase, and (E) third phase of reductive half reaction fit with hyperbolic equation. (F) has the change in amplitudes for phase 1 (red), phase 2 (green), and phase 3 (blue) fitted.

- Reaction mechanism was predicted based on the change in amplitude observed in the three phases.



deadtime of measurement (<1ms)

(maybe CTC)

$$\mathbf{E}_{ox}\cdot\mathbf{S} + NADH \underset{K_d=54mM}{\overset{rapid}{\rightleftharpoons}} \mathbf{E}_{ox}\cdot\mathbf{S}.NADH \underset{k_{-1}=19s^{-1}}{\overset{k_1=9s^{-1}}{\rightleftharpoons}} \mathbf{E}_{ox}\cdot\mathbf{S}\cdot NADH \underset{k_{-2}\sim 0}{\overset{k_2=3.5\ s^{-1}}{\rightleftharpoons}} \mathbf{E}_{red}\cdot\mathbf{S}\cdot NAD^+ \underset{k_{-3}=0.14s^{-1}}{\overset{k_3=0.11s^{-1}}{\rightleftharpoons}} \mathbf{E}_{red}\cdot\mathbf{S} + NAD^+$$

Phase 1 | Phase 2 | Phase 3

Future Research

- Investigate the binding activity of NicC and the formation of charge transfer complex.
- Investigate the oxidative half reaction to further elucidate the effects of chlorination on NicC.
- Determine NicC activity on substrates that have the chlorine substituent at different positions to understand how conformation is affecting catalytic activity.

Acknowledgements

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References

(1) Nakamoto, K. D.; Perkins, S. W.; Campbell, R. G.; Bauerle, M. R.; Gerwig, T. J.; Gerislioglu, S.; Wesdemiotis, C.; Anderson, M. A.; Hicks, K. A.; Snider, M. J. Mechanism of 6-Hydroxynicotinate 3-Monooxygenase, a Flavin-Dependent Decarboxylative Hydroxylase Involved in Bacterial Nicotinic Acid Degradation. *Biochemistry* **2019**, *58* (13), 1751–1763.

(2) Hlaing, M. Z. Investigating Substrate Specificity for 6-Hydroxynicotinate 3-Monooxygenase (NicC) with Alternative Substrates: Consequences of Catalysis of Replacing or Removing the Ring Nitrogen. Senior Thesis, 2022.

(3) Redick, J. C. Characterization of Substrate Promiscuity for 6-Hydroxynicotinate 3-Monooxygenase: A Transient Kinetic Investigation of the Decarboxylative-Hydroxylation of 5-Chloro-6-Hydroxynicotinic Acid. Senior Thesis, 2021.