SUPPORTING INFORMATION

Surveying the scope of aromatic decarboxylations catalyzed by prenylated-flavin dependent enzymes

Anushree Mondal[1], Pronay Roy[1], Jaclyn Carrannatto[1], Prathamesh M. Datar[1], Daniel J. DiRocco[1], Katherine Hunter[1] and E. Neil G. Marsh[1,2]

[1]Department of Chemistry, University of Michigan, Ann Arbor, Michigan, USA

[2]Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan, USA

Abbreviations

FMN, Flavin mononucleotide; prFMN, prenylated FMN; FDC, Ferulic acid decarboxylase; SSN, Sequence similarity network; DMAP, Dimethylally phosphate; DMAPP, Dimethylally pyrophosphate; IPTG, isopropyl-β-D-1-thiogalactopyranoside; SDS-PAGE, Sodium dodecyl-sulfate polyacrylamide gel electrophoresis; NMR, Nuclear magnetic resonance; MS, Mass spectrometry; HPLC, High performance liquid chromatography; tPAD, truncated phenylacrylic acid decarboxylase; ESI, Electrospray ionization; APCI, Atmospheric pressure chemical ionization; DMSO, Dimethyl sulfoxide; singlet (s); doublet (d); triplet (t); quartet (q); doublet of doublets (dd); triplet of doublets (td); doublet of triplets (dt); multiplet (m).

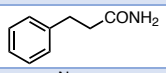
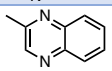
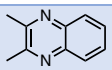
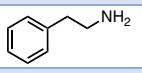
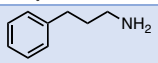
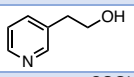
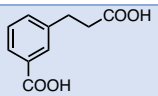
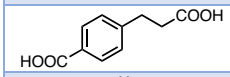
%M_D with respect to M			
Substrate	Full Reaction	No Cofactor	No Enzyme
	0.53 (±0.03)	0.1	0.1
	0.03 (±0.01)	n.d	n.d
	0.7 (±0.02)	n.d	n.d
	0.25 (±0.02)	0.04	0.04
	0.06 (±0.01)	n.d	n.d
	0.17 (±0.01)	0.02	0.03
	0.05 (±0.01)	n.d	n.d
	0.08 (±0.01)	n.d	n.d
	0.11 (±0.01)	0.02	0.01
	0.13 (±0.02)	0.02	0.02
	1.04 (±0.02)	n.d	n.d
	0.05 (±0.01)	0.01	n.d
	1.50 (±0.03)	0.34	0.20
	0.05 (±0.02)	0.02	0.01
	0.10 (±0.01)	n.d	n.d

Table S1. Table showing %M_D incorporation in full reactions and controls of different substrate when the reactions were done with purified protein. (n.d implies no deuterium incorporation was detected).

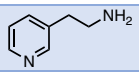
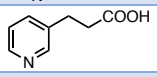
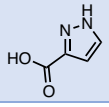
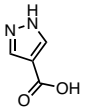
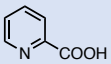
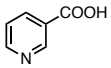
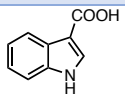
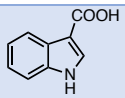
%M_D with respect to M			
Substrate	Full Reaction	No co factor	No Enzyme
	0.31 (±0.11)	0.03	0.03
	13.28 (±0.36)	n.d	0.03
	4.74 (±0.52)	0.03	n.d
	0.31 (±0.13)	n.d	n.d
	2.04 (±0.16)	0.23	0.3
	27.32 (±1.35)	0.37	0.81
	17.78 (±1.4)	0.30	0.14
	2.77 (±0.12)	0.25	0.20
	2.63 (±0.18)	0.16	0.4
	49.58 (±1.29)	1.3	1.5
	28.05 (±0.53)	1.2	1.1
	79.87 (±1.47)	1.1	1.6
	37.75 (±0.66)	0.67	0.74
	5.72 (±0.51)	0.12	0.17

Table S2. Table showing %MD incorporation in full reactions and controls of different substrate when the reactions were done with crude protein powders. (n.d implies no deuterium incorporation was detected).

Supporting Figures

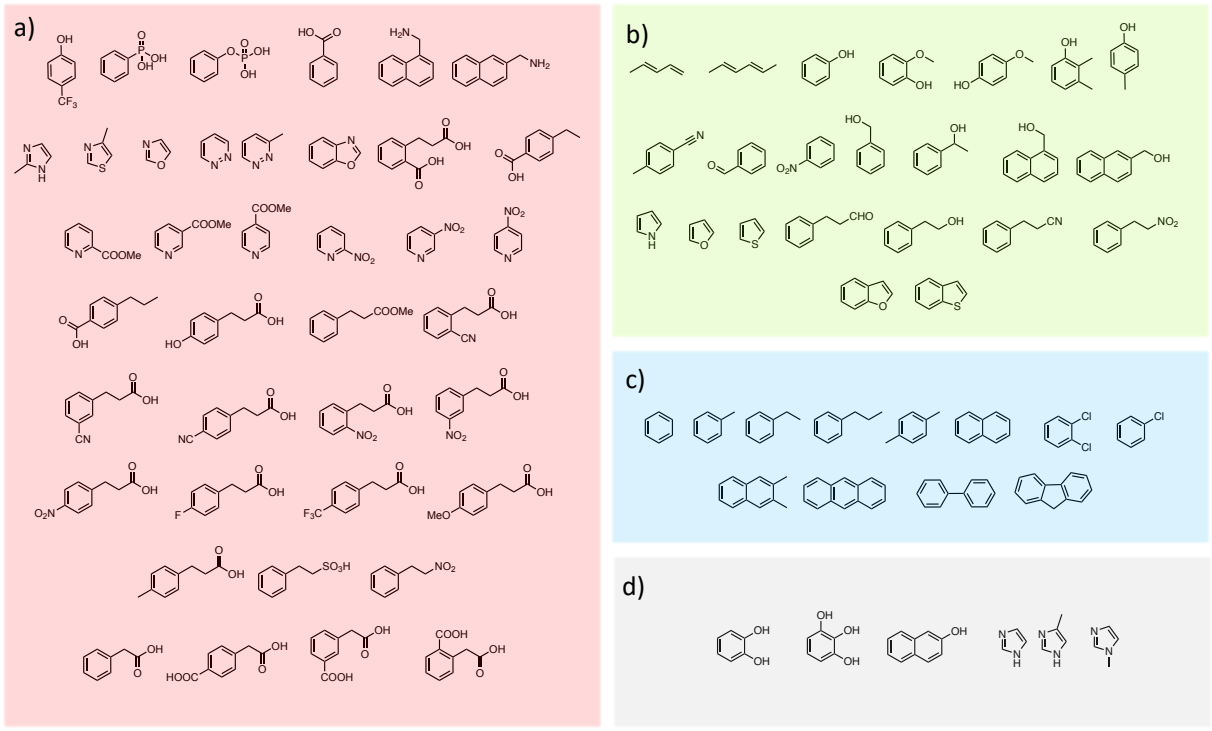


Fig. S1. Substrate sets for H/D exchange reaction screening that showed no deuterium incorporation or inconclusive results. **a)** Substrates that showed no deuterium incorporation. Analyzed by HESI ionization method on a Thermo Scientific Orbitrap Fusion Lumos Tribrid Mass spectrometer **b)** Substrates that were not detected on the mass spectrometer due to low flying efficiency or ionization issues. Analyzed by either HESI ionization method on a Thermo Scientific Orbitrap Fusion Lumos Tribrid Mass spectrometer or with an ESI/APCI ionization method on an Agilent Q-ToF HPLC MS or by electron impact ionization method on a Micromass autospec Ultima Magnetic Sector Mass Spectrometer. **c)** Substrates for which results were inconclusive because of poor ionization, and/or the mode of MS analysis was not able to distinguish between M_{13C} and M_D peaks. Analyzed by electron impact ionization method on a Micromass autospec Ultima Magnetic Sector Mass Spectrometer **d)** Substrates showing high backgrounds for deuterium incorporation in control reactions. Analyzed HESI ionization method on a Thermo Scientific Orbitrap Fusion Lumos Tribrid Mass spectrometer.

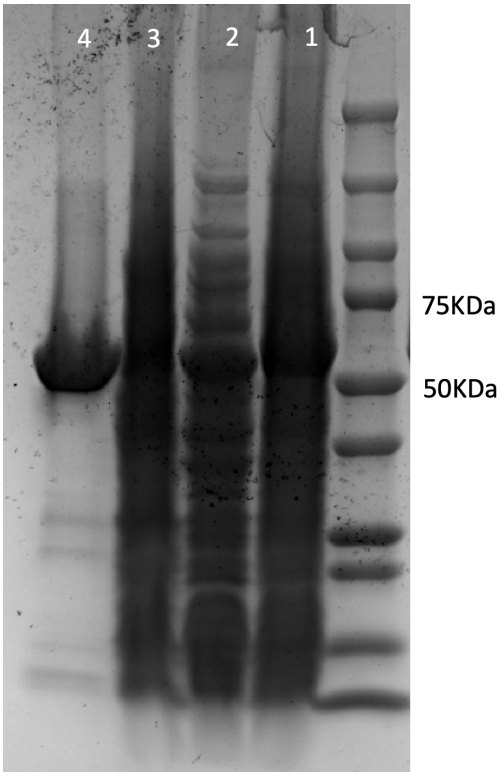


Figure S2. SDS PAGE of crude protein powders. 1- FDC, 2- FDC I330S and 3 - ΔUbix FDC, 4 – FDC purified protein as a standard. Molecular weight of FDC is 57.9 KDa.

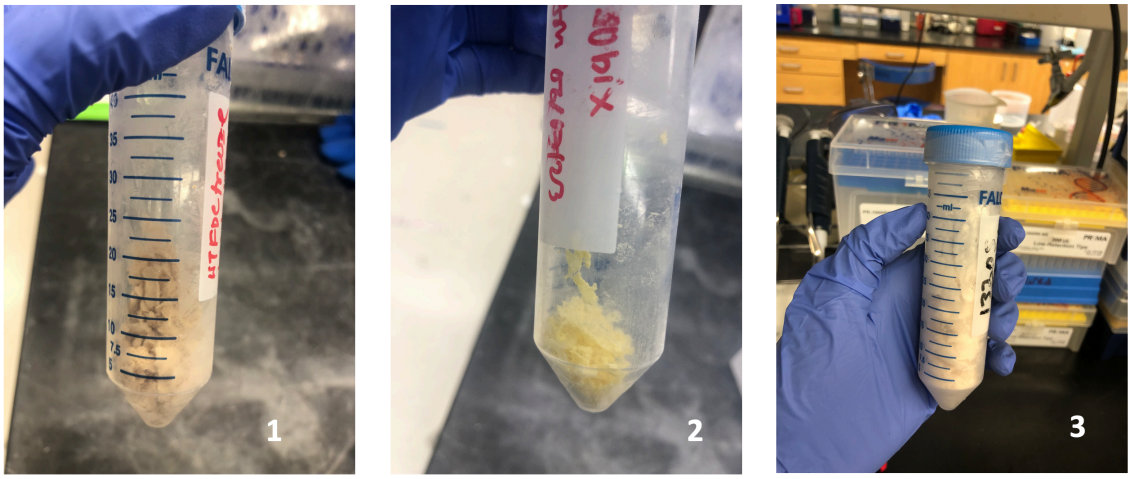


Figure S3. Crude protein powders of FDC (1), ΔUbix FDC (2) and FDC I330S mutant (3).

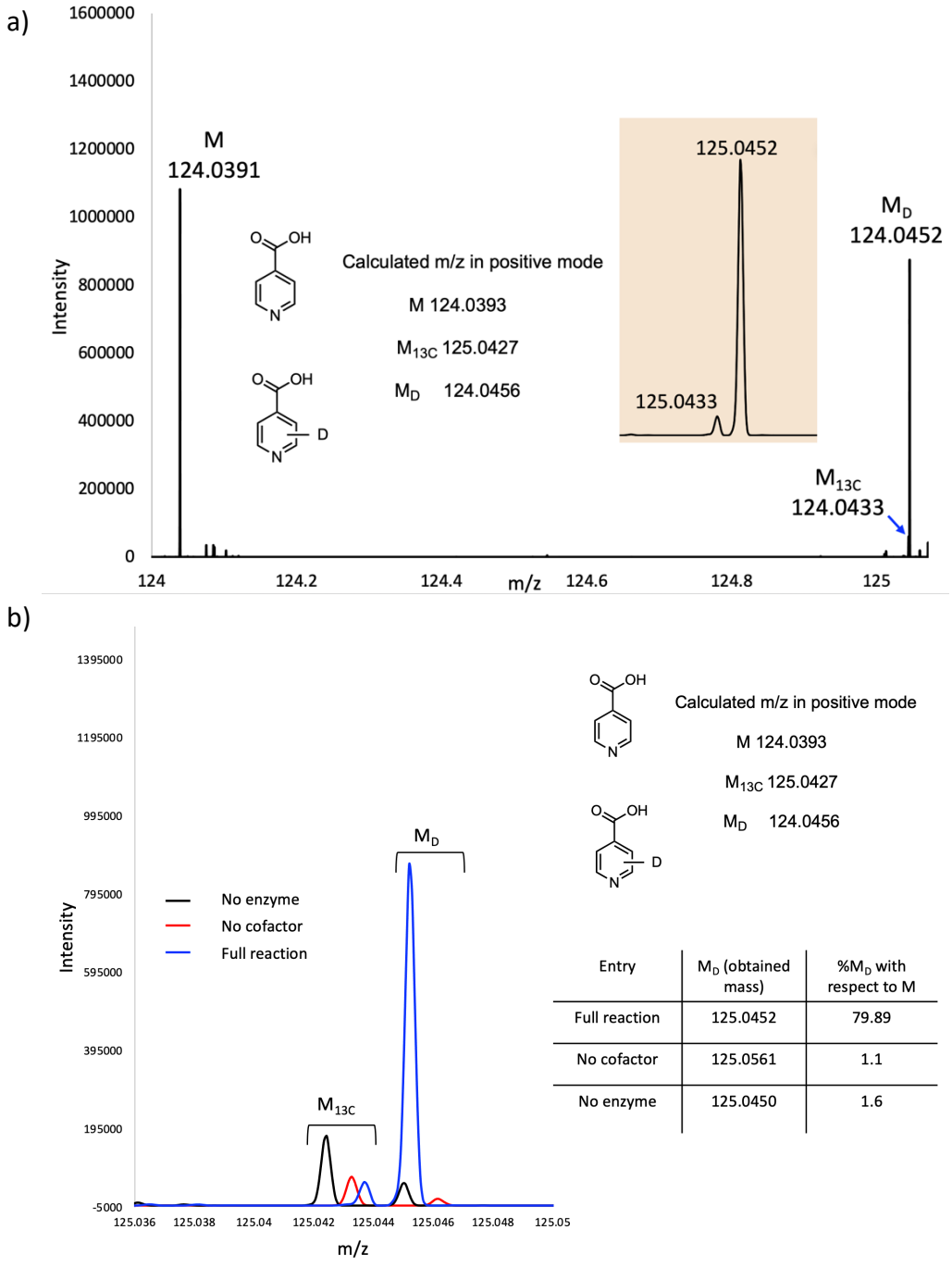


Fig. S4 Additional data for H/D exchange with crude protein powders. Mass spectrum showing the deuterium incorporation in pyridine 4-carboxylic acid substrate. **a)** Calculated and obtained M, M_{13C} and M_D peaks for pyridine 4-carboxylic acid substrate in full reaction. **b)** Mass spectra of full reaction, no enzyme, and no cofactor reaction overlayed together for comparison. The $\%M_D$ obtained in controls reactions are also shown in an inset table.

Reaction conditions for H/D exchange of pyridine-4-carboxylic acid with crude protein powder for NMR analysis

H/D exchange reactions for NMR analysis of pyridine 4-carboxylic acid with FDC crude protein powder were performed in 20 mM potassium phosphate D2O buffer (pD 6.5) containing FDC crude powder (~60-70 μM) and substrate (5 mM, stock made in DMSO) for 16-18 hour at 30°C with shaking at 1000 rpm.[1, 2] A small fraction of the reaction was quenched by addition of acetonitrile followed by centrifugation to remove the protein powder. The supernatant was analyzed by mass spectrometry to detect deuterium incorporation on the substrate. The other major fraction of the reaction was diluted with addition of the same D_2O buffer (to a final substrate concentration of 1mM), followed by centrifugation and the supernatant solution was analyzed by NMR to detect deuterium incorporation into the substrate. ^{1}H NMR (800 MHz, D2O): δ 8.63 (d, 2H, J= 4.8 Hz), 7.77 (d, 2H, J=4.8 Hz).

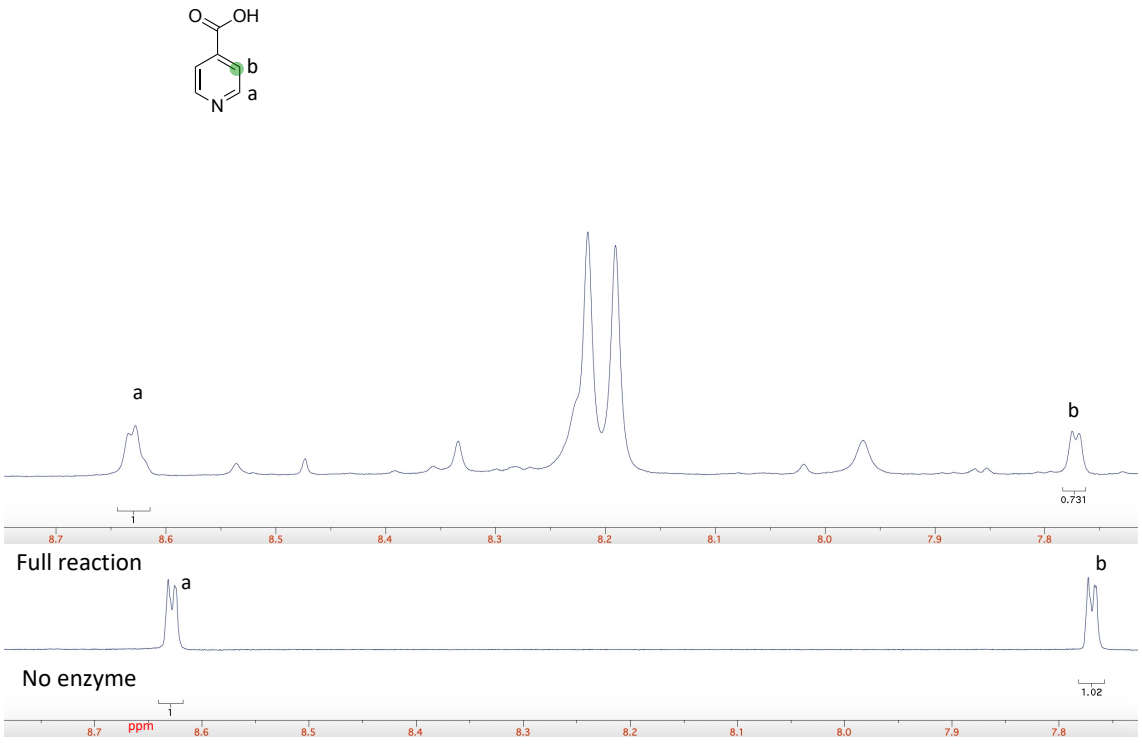


Fig. S5. NMR spectra of pyridine 4-carboxylic acid full reaction and no enzyme showing the ratio of Ha and Hb protons changed from 1: 1.02 in no enzyme to 1:0.731 in full reaction (calculated H_a to H_b ratio is 1:0.826, based on 53.5% deuterium incorporation in full reaction obtained from MS analysis of the same sample). This indicated H_b-proton is partially exchanged with deuterium in the full reaction.

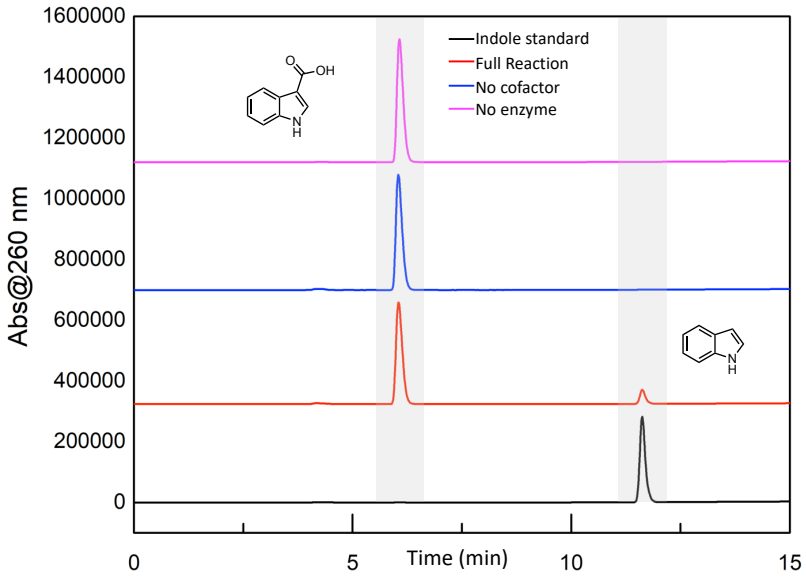


Fig. S6 HPLC chromatograms at 260 nm showing decarboxylation of Indole 3-carboxylic acid with FDC I330S mutant (16% conversion was observed as determined by peak area). Other substrates that showed decarboxylase activity were Pyrrole 2-carboxylic acid (86% conversion, with FDC), indole, benzofuran and benzothiophene 2-carboxylic acids (15%, 90% and 90% conversion respectively with FDC I330S mutant) and 2-naphthoic acid (5.8% conversion with FDC I330S mutant).

Enzymatic activity of lyophilized protein powders

Crude protein powders were tested for standard cinnamic acid decarboxylation activity before using them for general -H/-D exchange reaction screening. Decarboxylation with crude protein powder was performed in 20 mM potassium phosphate buffer (pH 6.5) containing FDC or FDC I330S mutant crude powder (~5 µM) and substrate (5 mM, stock made in DMSO) for 5 minutes at room temperature.[3] The reaction was quenched by addition of acetonitrile followed by centrifugation to remove the protein powder. The supernatant was analyzed by HPLC (method 1) to detect cinnamic acid consumption and styrene production. A no enzyme reaction was run in absence of any crude protein powder and a no cofactor control was run with ΔUbix FDC protein powder. Chromatograms are shown in Fig. S7.

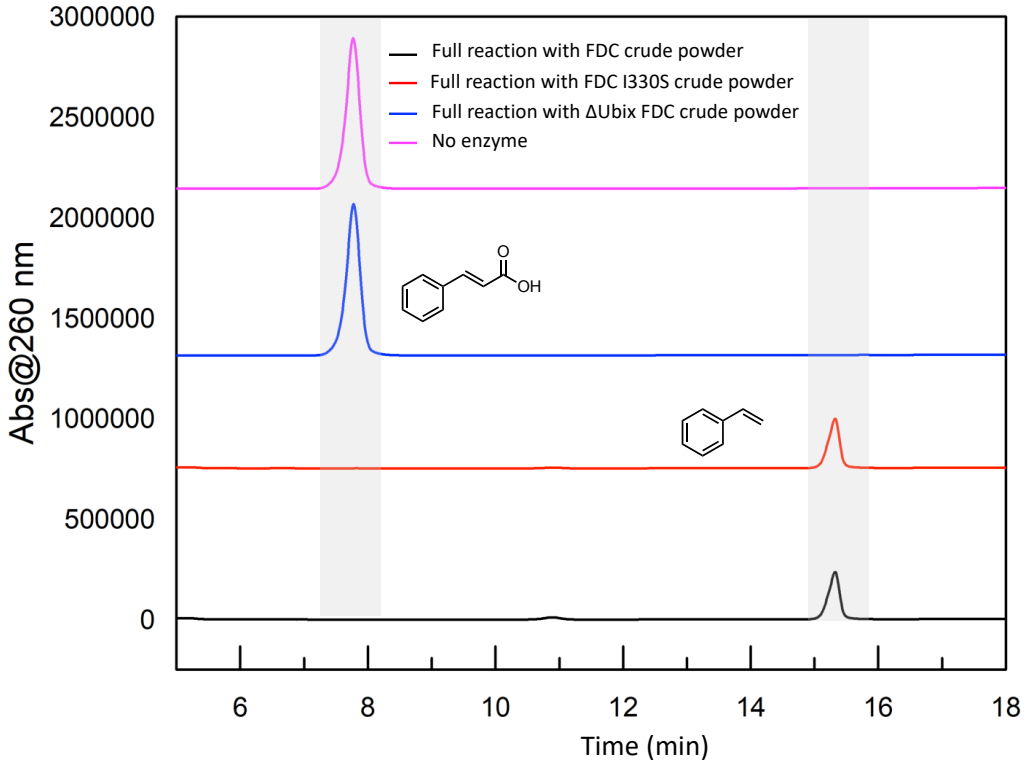


Fig. S7 HPLC chromatogram at 260 nm showing the decarboxylation of cinnamic acid to styrene using FDC and FDC I330S mutant crude powders.

References

(1) Wang, L.; Lou, Y.; Xu, W.; Chen, Z.; Xu, J.; Wu, Q. Biocatalytic Site-Selective Hydrogen Isotope Exchange of Unsaturated Fragments with D2O. *ACS Catalysis* **2021**, *12*, 783-788.

(2) Ferguson, K. L.; Arunrattanamook, N.; Marsh, E. N. G. Mechanism of the novel prenylated flavin-containing enzyme ferulic acid decarboxylase probed by isotope effects and linear free-energy relationships. *Biochemistry* **2016**, *55*, 2857-2863.

(3) Lin, F.; Ferguson, K. L.; Boyer, D. R.; Lin, X. N.; Marsh, E. N. G. Isofunctional enzymes PAD1 and UbiX catalyze formation of a novel cofactor required by ferulic acid decarboxylase and 4-hydroxy-3-polyprenylbenzoic acid decarboxylase. *ACS Chemical Biology* **2015**, *10*, 1137-1144.