Electronic Supporting information for

A single hydrogen bond that tunes flavin redox reactivity and activates it for modification

Debarati Das and Anne-Frances Miller

Emails: dda283@uky.edu, afmill3r2@gmail.com

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1. Experimental Details

Buffers

Lysis Buffer: 50 mM HEPES pH 7.5 + 10 mM imidazole + 1mM (4-(2-aminoethyl) benzenesulfonyl fluoride)hydrochloride protease inhibitor (Chem Impex Intl Cat. Number 21250) + 2 mM benzamidine + 2 mM sodium fluoride (NaF) + 1 μL each of lysozyme (Millipore 71110-1200KU) and DNase (Millipore 71205-25KUN).
Column Wash Buffer: 50 mM HEPES pH 7.5 + 15 mM imidazole.
Elution Buffer: 50 mM HEPES pH 7.5 + 150 mM imidazole.
Default (Working) Buffer: 50mM KPO_4 pH 7.0

2. Supplementary Tables

Table S1 Primers for the H290 variants.[a]

Variant	Forward Primer	Reverse Primer
H290A	TTCTGTACAG**GCG**AAAGCCGGCAT	CCGGAAATACCGCAGGCGAAGTAG
H290E	TTCTGTACAG**GAA**AAAGCCGGCATG	CCGGAAATACCGCAGGCGAAGTAG
H290N	TTCTGTACAG**AAC**AAAGCCGGCATG	CCGGAAATACCGCAGGCGAAGTAG
H290K	TTCTGTACAG**AAA**AAAGCCGGCATGAG	CCGGAAATACCGCAGGCGAAGTAG
H290T	TTCTGTACAG**ACC**AAAGCCGGCATGAG	CCGGAAATACCGCAGGCGAAGTAG
H290F	TTCTGTACAG**TTT**AAAGCCGGCATGAG	CCGGAAATACCGCAGGCGAAGTAG
Y279I	CCCGAAGATC**ATT**TTCGCCTGCGG	GTAACGGTTTTACCGGACTGACCA

[a] Codons comprising the designed mutation are underlined and in bold.

Table S2 Slopes from linear fits of respective phases and the resulting E°s determined at pH 7.5.

	Slope[a], Phase 1	lope, Phase 2	Slope, Phase 3	$E^{\circ}_{Ox/ASQ}$[b] (mV)	$E^{\circ}_{ASQ/AHQ}$ (mV)	$E^{\circ}_{Ox/HQ}$ (mV)
H290F$_{Y279I}$-	0.51	0.56	1.01	-23	-138	-302

[a] Slopes from plots of $\ln\frac{[FAD_{OX}]}{[FAD_{RED}]}$ vs $\ln\frac{[Dye_{OX}]}{[Dye_{RED}]}$ are expected to have values of 0.5 for 1e⁻ events or 1.0 for 2e⁻ events because n_{Dye} = 2 in all cases used.

[b] All values vs. NHE.

Table S3 Quantification of FAD:ETF ratio before and after titration with Ti citrate. [a]

Trial 1	ETF concentration (μM)	FAD concentration (μM)	FAD:ETF ratio (Ideal =2)
H290F$_{Y279I}$ before titration with Ti^{2+} citrate	10.7	21.4	2
H290F$_{Y279I}$ After titration with Ti^{2+} citrate	10.7	10.8	1
Trial 2			
H290F$_{Y279I}$ before titration with Ti^{2+} citrate	45	90.0	2
H290F$_{Y279I}$ After titration with Ti^{2+} citrate	18.3	29.0	1.6

[a] Samples titrated as in Figure 8B were separated from released flavins and the flavin content that remained bound was quantified.

3. Supplementary Figures

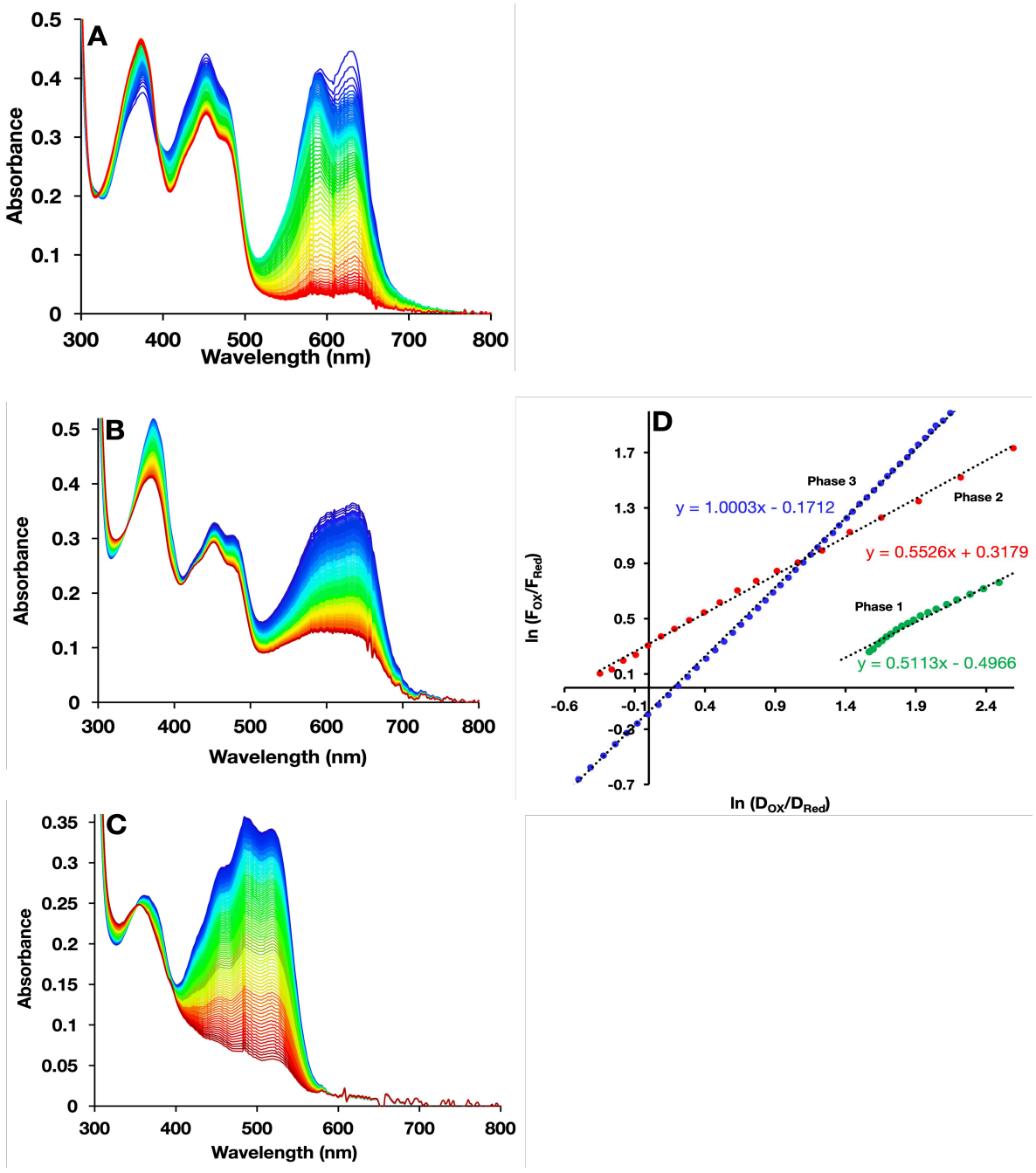

Fig. S1 Co-reduction of H290FY279I ETF in 50mM KPO_4 at pH 7.0 using Xanthine/Xanthine oxidase in equilibrium with A, New Methylene blue for phase 1; B, Nile blue for phase 2; C, Safranin O using Xanthine/Xanthine oxidase for phase 3 and D; linear fits of plots $\ln(D_{ox}/D_{Red})$ vs $\ln(F_{ox}/F_{Red})$ for individual phases to calculate the mid-point potential.[1,2,3]

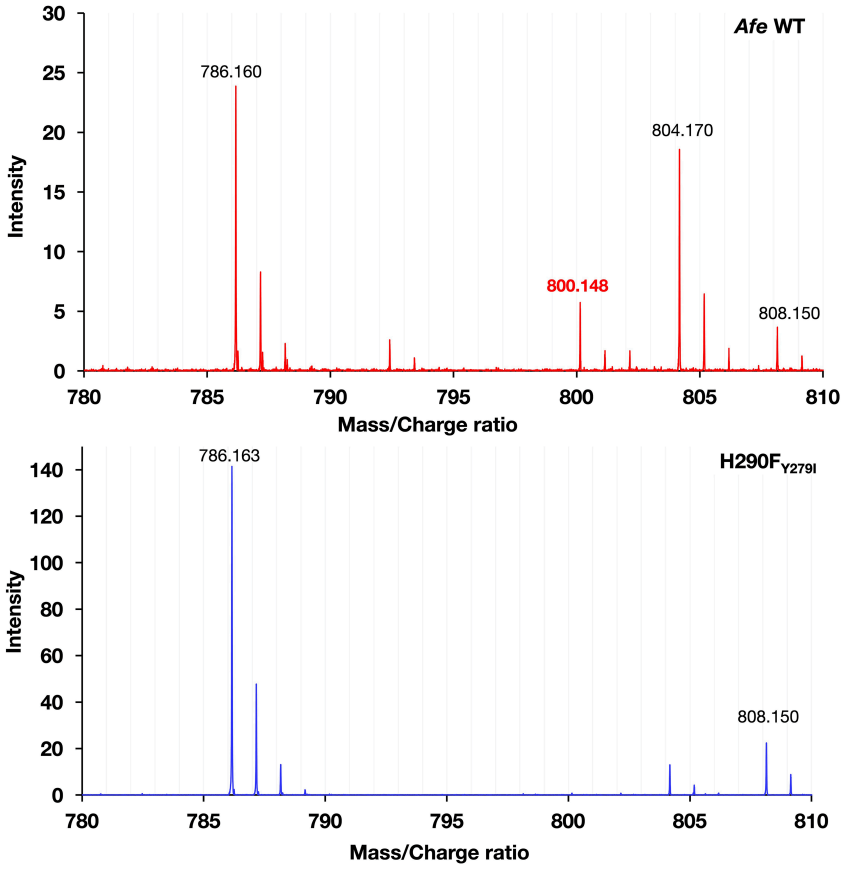

Fig. S2 Identification of 8-formyl flavin in the flavin released from WT *Afe*ETF but not the H290FY79I variant. WT- or H290FY79I -ETF were allowed to incubate anaerobically in darkness at 4 °C for two weeks (Figure 9), and then were denatured to release their non-covalently bound flavins. These were analysed by mass spectrometry (MS), and the data shown above reveal the presence of 8-formyl flavin (8fF, 800.148 Da) in the WT-ETF but not in H290FY79I -ETF.

Released flavins were first resolved by XID chromatography and the peak eluting from 4.3-4.7 minutes was seen to include both natural FAD and 8fF, for ET-ETF. This fraction was therefore analysed by MS, which reveals an additional m/z of 800.148 in the WT in addition to the m/z of 786.160 expected for FAD. FAD is seen in flavins released from both samples but the m/z of 800.148 is unique to WT. This m/z value of 800.148 has been assigned to 8fF.[4,5]

4. References for ESI

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2. N. Mohamed-Raseek and A.-F. Miller, Contrasting roles for two conserved arginines: Stabilizing flavin semiquinone or quaternary structure, in bifurcating electron transfer flavoproteins, Journal of Biological Chemistry, 2022, 298, 101733.
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5. N. Mohamed-Raseek, C. van Galen, R. Stanley and A. F. Miller, Unusual reactivity of a flavin in a bifurcating electron-transferring flavoprotein leads to flavin modification and a charge-transfer complex, J. Biol. Chem., 2022, 298, 102606.