Supplemental Material for

The second coordination sphere of FIH controls hydroxylation

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1. Thermal stability of FIH variants

The thermal stability of FIH variants was measured by differential scanning calorimetry, as described in the text.

Table S1. DSC melting temperature analysis of FIH and its mutants.

	$T_{M(app)}$ (°C)
WT FIH	54.5
N205A	58.6
N294A	58.9
R238M	56.7
Q239N	56.2

FIH (50 μM) in 50 mM HEPES pH 7.50, temperature range: 25-75 °C, scan rate: 60 °C/hour.

2. Co^{II} binding thermodynamics to FIH variants.

Metal titration data fitting:

The binding affinity of (Co^{2+}+αKG)FIH for Co^{2+} was obtained by competitive titration in 50 mM HEPES, pH 7.50 at 23ºC. A 200 μL solution of apo FIH (20 μM), citrate (1.00 mM), and αKG (100 μM) was temperature equilibrated in a fluorescence cuvette, while a separate solution of $CoCl_2$ (1.00 mM) with citrate (1.00 mM) in buffer was loaded into a titrating syringe. As FIH-1 utilizes αKG as a co-substrate as well as a bidentate ligand for metal, αKG was included to complete the relevant ligand set of (Co^{2+}+αKG)FIH. This experiment used citrate to buffer the concentration of free Co^{2+}, or $Co(H_2O)_6^{2+}$, as the log β values for Co(II) binding to citrate are well defined.[1] Upon addition of small volumes of $CoCl_2$, apo FIH bound the available αKG and Co^{2+}.

The fluorescence intensity of FIH-1 at 340 nm (F_{340}) was plotted against $log[Co^{2+}]_{free}$, exhibiting the sigmoidal shape characteristic of a binding equilibrium. The binding curve was fitted to a simple 1:1 binding equilibrium in which the Co^{2+} dissociation constant of (Co^{2+}+αKG)FIH (K_D) is with respect to $Co(H_2O)_6^{2+}$ (Eq. 1). The fluorescence intensity (F_{340}) was fitted to Eq. S2[2] which yielded log K_D (x) relative to the $log[Co^{2+}]_{free}$ (c), using a cooperativity parameter (B) which accounted for both chemical cooperativity involved with dimeric FIH-1 as well as the spectroscopic cooperativity involved with fluorescence signal from the eight Trp residues of FIH. The results are in Table S2.

$$(Co^{2+}+\alpha KG)FIH\text{-}1 \rightleftharpoons Co(H_2O)_6^{2+} + \text{apo FIH-1} \qquad \text{(S1)}$$

$$F_{340} = F_{min} + (F_{max} - F_{min}) / (1 + 10^{(c-x)B}) \qquad \text{(S2)}$$

Table S2. Co^{II} binding affinity of FIH-1 mutants in the presence of αKG

	K_D (M)	B
WT FIH	$1.38(6) \times 10^{-7}$	2.2 ± 0.2
N205A	$1.63(3) \times 10^{-7}$	1.6 ± 0.1
N294A	$1.01(4) \times 10^{-7}$	2.3 ± 0.2
Q239N	$1.45(8) \times 10^{-7}$	1.9 ± 0.2
R238M	$1.9(2) \times 10^{-7}$	1.3 ± 0.1

$CoCl_2$ (1 mM) / citrate (1 mM) was titrated into FIH (20 μM), αKG (100 μM) in 50 mM HEPES pH 7.50.

References

[1] Martell, A. E., and Smith, R. M. (eds). (1974) *Critical stability constants*, Plenum Press, New York

[2] Mills, S. A., and Marletta, M. A. (2005) *Biochemistry* **44**, 13553-13559