Supporting Information

Remote Perturbations in Tertiary Contacts Trigger Lysine Ligation to the Heme Iron in Cytochrome *c*

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Table S1. pK_a Values for the Alkaline Transition in Previously Studied Variants of Ferricytochrome *c*[a,1-23]

Species	Variant	pK_a^{alk}	Reference Protein	ΔpK_a[b]	Reference
Horse	WT (1)	9.35	--	--	Ref. 1
	WT (2)	9.22 ± 0.06	(1)	-0.13 ±0 .06	Ref. 2
	H26N/H33N (3)	9.16	(1)	-0.19	Ref. 3
	E4G/H26N/H33N	9.15	(3)	-0.01	Ref. 3
	K8G/H26N/H33N	9.10	(3)	-0.06	Ref. 3
	K22G/H26N/H33N	9.23	(3)	0.07	Ref. 3
	K25G/H26N/H33N	9.27	(3)	0.11	Ref. 3
	H26N/H33N/K39G	9.18	(3)	0.02	Ref. 3
	H26N/H33N/E62G	9.15	(3)	-0.01	Ref. 3
	H26N/H33N/E66G	8.46	(3)	-0.7	Ref. 3
	H26N/H33N/E66A	8.64	(3)	-0.52	Ref. 3
	H26N/H33N/E69G	8.61	(3)	-0.55	Ref. 3
	H26N/H33N/P76G	7.50	(3)	-1.66	Ref. 3
	H26N/H33N/K72G (4)	9.02	(3)	-0.14	Ref. 3
	H26N/H33N/K72G/K73G	9.00	(4)	-0.02	Ref. 3
Horse[c]	Y67F	10.65	(1)	1.3	Ref. 4
	A83P	8.95	(1)	-0.4	Ref. 4
	T78N/A83P	8.25	(1)	-1.1	Ref. 4
	T78N	8.10	(1)	-1.25	Ref. 4
Yeast *iso*-1	WT[d] (5)	8.5	--	--	Ref. 5
	C102T (6)	8.5	(5)	0	Ref. 5
	N52I	9.4	(5)	0.9	Ref. 6
	K72A/C102T (7)	8.5	(6)	0	Ref. 7
	K73A/C102T	8.82 ±0 .02	(6)	0.32 ± 0.02	Ref. 8
	K79A/C102T (8)	8.44 ± 0.01	(6)	-0.06 ± 0.01	Ref. 8
	N52G/K79A/C102S	7.46 ± 0.02	(8)	-0.98 ± 0.02	Ref. 9
	L85A/C102T (9)	7.7 ± 0.1	(6)	-0.8 ± 0.1	Ref. 10
	K72A/L85A/C102S	7.84 ± 0.06	(9)	0.14 ± 0.12	Ref. 10
	F82G/C102T	8.4	(6)	-0.1	Ref. 5

	F82S/C102T	7.7	(6)	-0.8	Ref. 5
	F82L/C102T	7.2	(6)	-1.3	Ref. 5
	F82I/C102T	7.2	(6)	-1.3	Ref. 5
	F82W/C102T	9.95 ± 0.08	(6)	1.45 ± 0.08	Ref. 11
	M80E/C102T	11.55 ± 0.11	(6)	3.05 ± 0.11	Ref. 12
	M80D/C102T	9.25 ± 0.08	(6)	0.75 ± 0.08	Ref. 12
	P76A/C102T	7.83 ± 0.14	(6)	-0.67 ± 0.14	Ref. 13
	G77A/C102T (10)	8.00 ± 0.23	(6)	-0.5 ± 0.23	Ref. 13
	P76A/G77A/C102T	6.88 ± 0.15	(10)	-1.12 ± 0.27	Ref. 13
Yeast *iso*-2	WT[d] (11)	8.45	--	--	Ref. 14
	P76G[d]	6.7	(11)	-1.75	Ref. 14
Human	WT	9.9	--	--	Ref. 15
	WT	9.5	--	--	Ref. 1
	WT	9.3 ± 0.4	--	--	Ref. 16
	WT	9.54 ± 0.03	--	--	Ref. 17
	WT[f] (12)	9.56 ± 0.4	--	--	
	Y46F	8.9	(12)	-0.66 ± 0.4	Ref. 17
	K8R (13)	9.63 ± 0.07	(12)	0.07 ± 0.41	Ref. 17
	K8R/P44S(14)	9.51 ± 0.05	(13)	-0.12 ± 0.09	Ref. 17
	K8R/P44S/Y46F (15)	9.15 ± 0.02	(14)	-0.36 ± 0.05	Ref. 17
	K8R/P44S/Y46F/S47T (16)	9.05 ± 0.01	(15)	-0.1 ± 0.02	Ref. 17
	K8R/P44S/Y46F/S47T/A50E	9.32 ± 0.03	(16)	0.27 ± 0.03	Ref. 17
	Y48E	7.0	(12)	-2.56 ± 0.4	Ref. 18
	Y48I	6.9	(12)	-2.66 ± 0.4	Ref. 18
	Y48F	10.3	(12)	0.74 ± 0.4	Ref. 19
	G41S	7.8 ± 0.3	(12)	-1.76 ± 0.5	Ref. 16
	G41A	8.1 ± 0.5	(12)	-1.46 ± 0.64	Ref. 16
	G41T	6.7 ± 0.2	(12)	-2.86 ± 0.45	Ref. 16
Fruit Fly	WT (17)	9.0	--	--	Ref. 20, 21
	P30A	8.2	(17)	-0.8	Ref. 21
Rat	WT	9.5	--	--	Ref. 22, 23
	WT	9.6	--	--	Ref. 21
	WT (18)[f]	9.55 ± 0.05	--	--	

P30A	8.5	(18)	-1.05 ± 0.05	Ref. 21
P30V	5.9	(18)	-3.65 ± 0.05	Ref. 21
Y67F	10.7	(18)	1.15 ± 0.05	Ref. 22, 23
N52I	9.5	(18)	-0.05 ± 0.05	Ref. 6

[a]The list is not comprehensive, only variants related to reference proteins by a single point mutation are listed. [b]ΔpK_a = pK_a^{alk} (variant) – pK_a (reference protein). [c]Semisynthetic protein prepared by reaction of the synthetic peptide with the cyt *c* fragment after CNBr-treatment. [d]With Cys at position 102. [e]Cys102 modified by treatment with methyl methanethiosulfonate. [f]Average value of multiple values reported for WT (listed above).

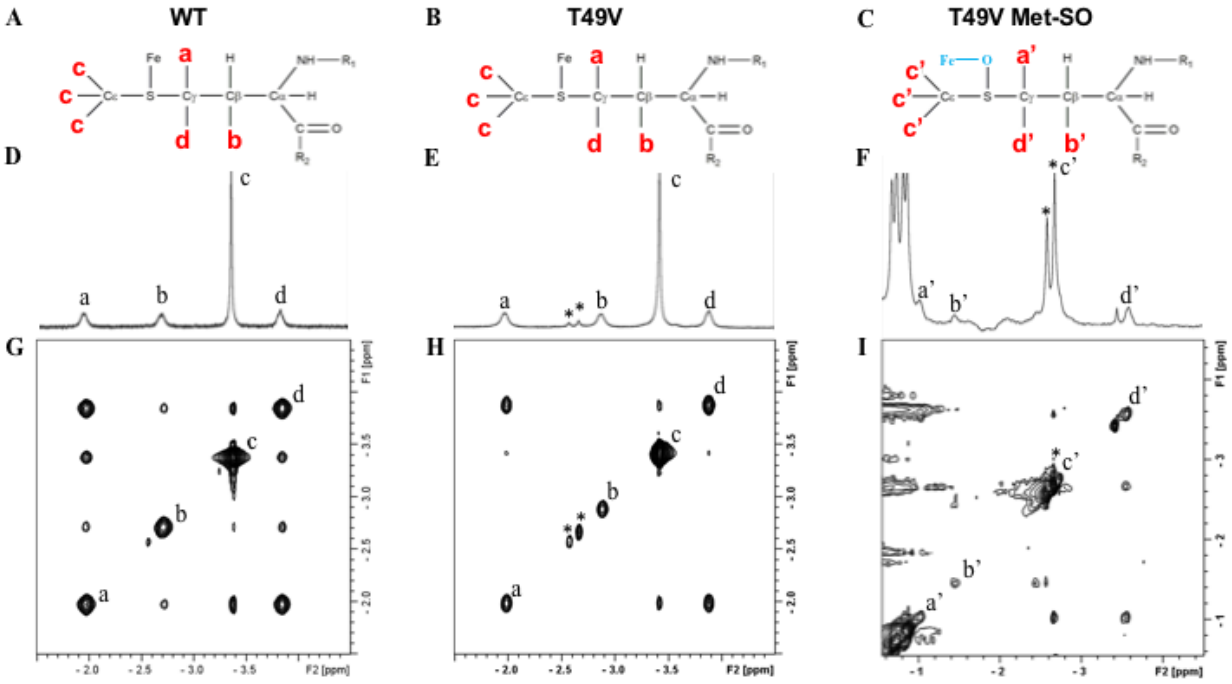

Figure S1. ^{1}H NMR spectra of ferrous WT, T49V, and T49V Met-SO in a 50 mM sodium phosphate buffer at pH 7.4 and 25 °C. (A)(B)(C) schematic representation of the Met (or Met-SO) ligand (lower case letters representing protons corresponding to labeled peaks in the spectra); (D)(E)(F) ^{1}H and (G)(H)(I) 2D ^{1}H NOESY NMR spectra for WT, T49V, and T49V Met-SO.

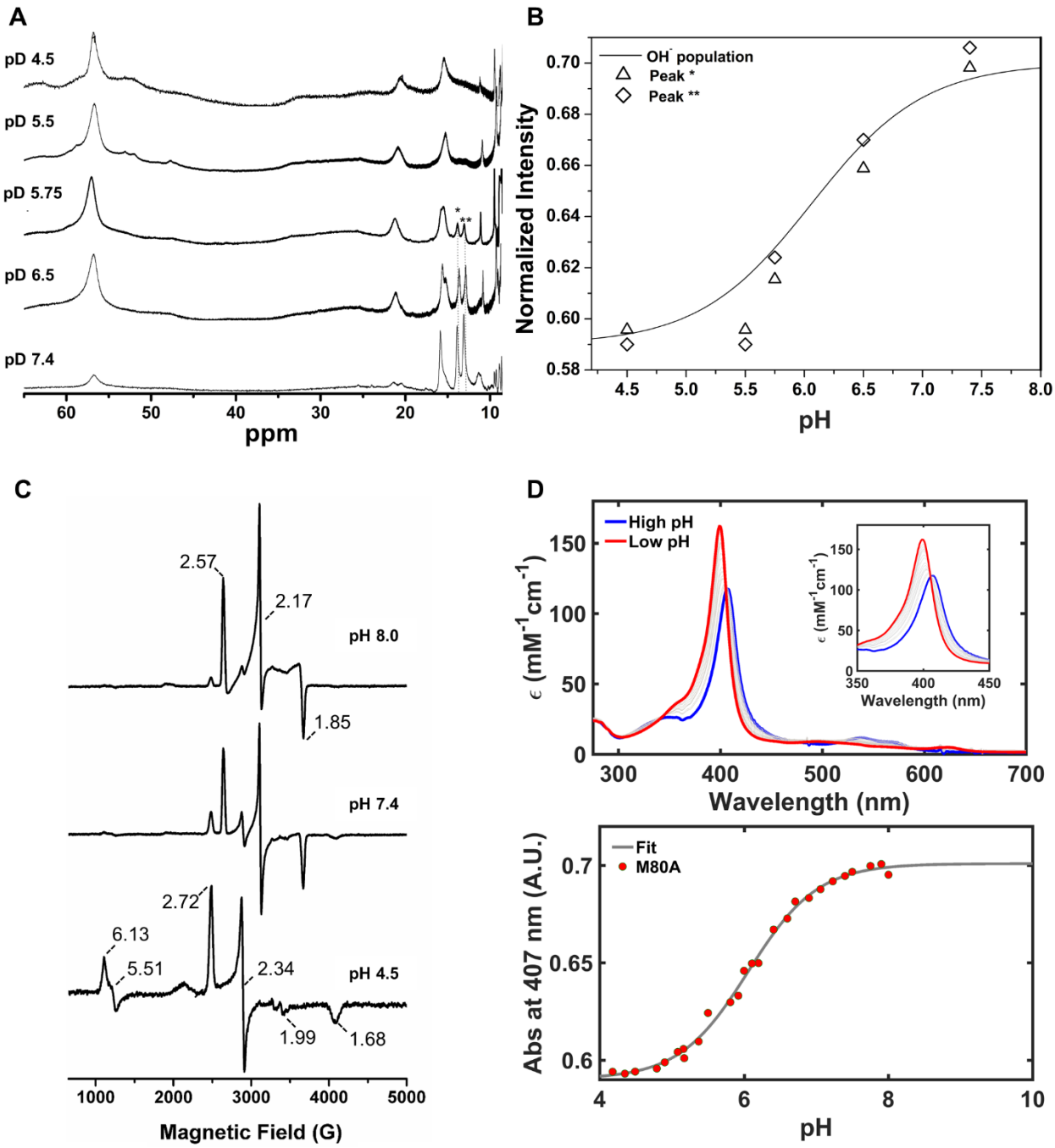

Figure S2. Spectra of ferric M80A at different pH values: (A)^{1}H NMR spectra at 25 °C; (B) normalized intensities of the selected NMR peaks, plotted along the fraction of the hydroxide-ligated form of M80A as a function of pH from UV-visible experiments; (C) EPR spectra at 10 K; (D) UV-visible spectra at 22 ± 2 °C (*top*) and fit of the spectral changes (*bottom*).

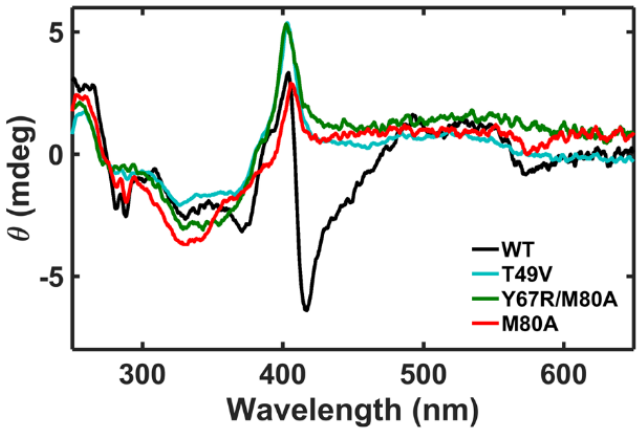

Figure S3. Circular dichroism spectra in the Soret region of WT (*black*), T49V (*cyan*), Y67R/M80A (*green*), and M80A (*red*) at pH 7.4 and 22 ± 2 °C. Protein concentrations were 50 μM and the path length *l* was 2 mm in these experiments.

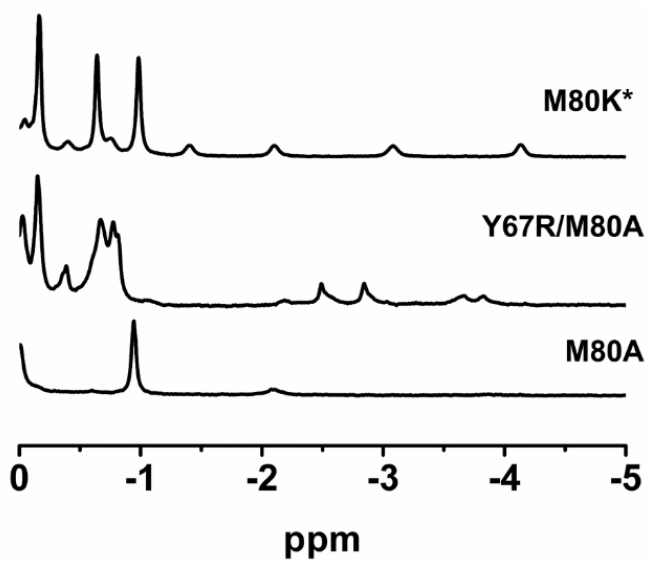

Figure S4. Upfield region of the ^{1}H NMR spectra of ferrous yeast iso-1-cyt *c* K73A/K79G/M80K, horse heart cyt *c* variants Y67R/M80A and M80A at 25 ºC in a 50 mM sodium phosphate buffer at pD 7.4 containing 10% D_2O v/v.

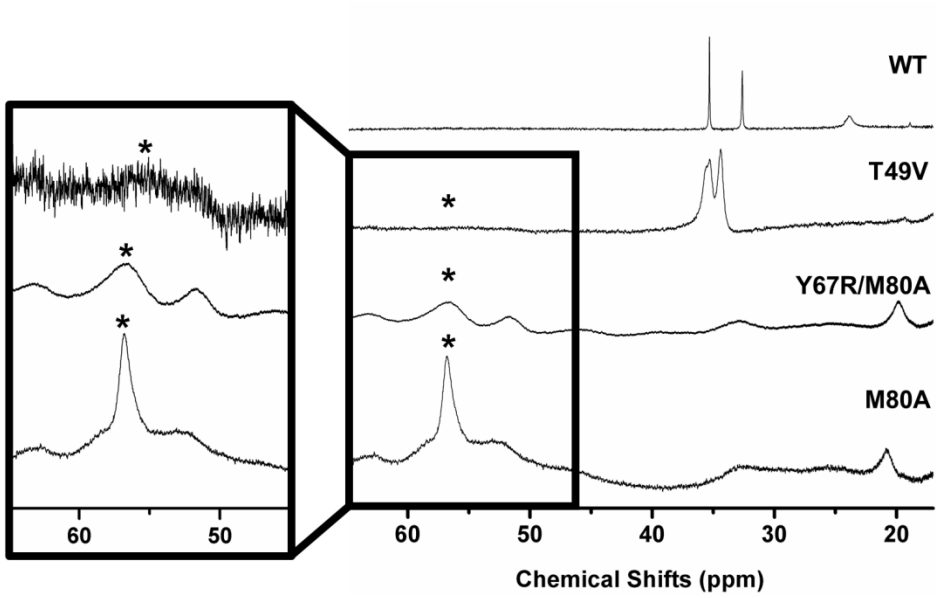

Figure S5. ^{1}H NMR spectra of ferric WT, T49V, Y67R/M80A and M80A variants in a 50 mM sodium acetate (d_6) at pD 4.5 and 25 ºC. The signal associated with the high-spin heme iron species is labeled by *.

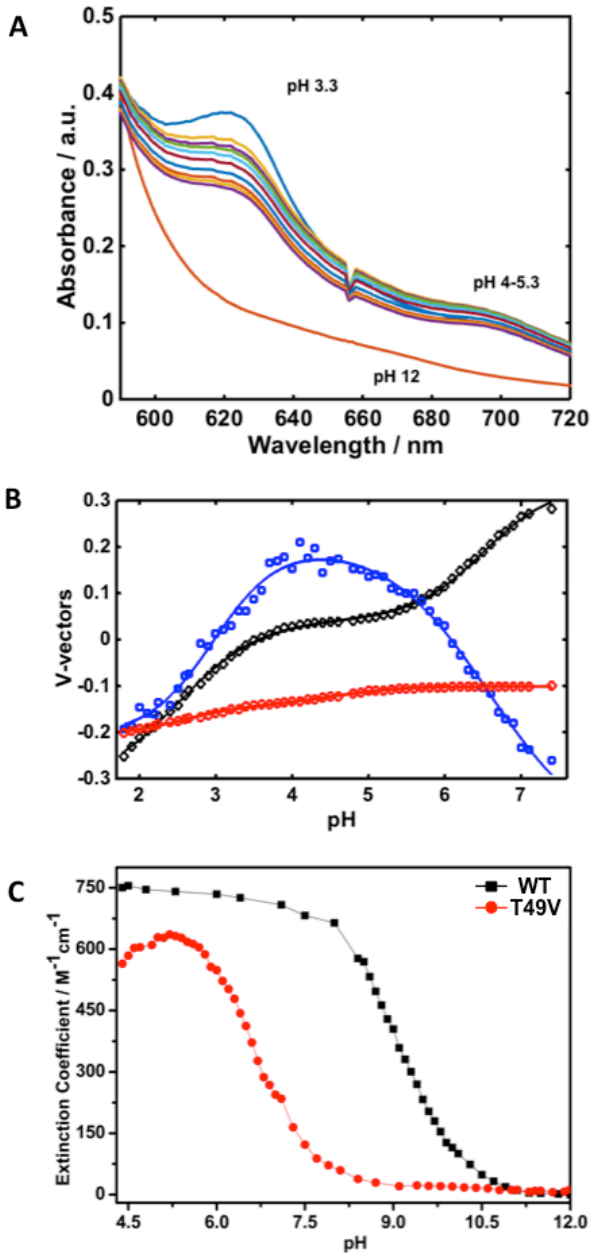

Figure S6. (A) pH dependence of the charge-transfer region of the absorption spectra of ferric T49V at 22 ± 2 °C (pH 3.3 to 12.0), (B) plots of the V-vectors (V1 (*blue*), V2 (*black*), and V3 (*red*)) from SVD analysis of these spectra , and (C) pH dependence of the extinction coefficient at 695 nm of ferric WT and T49V.

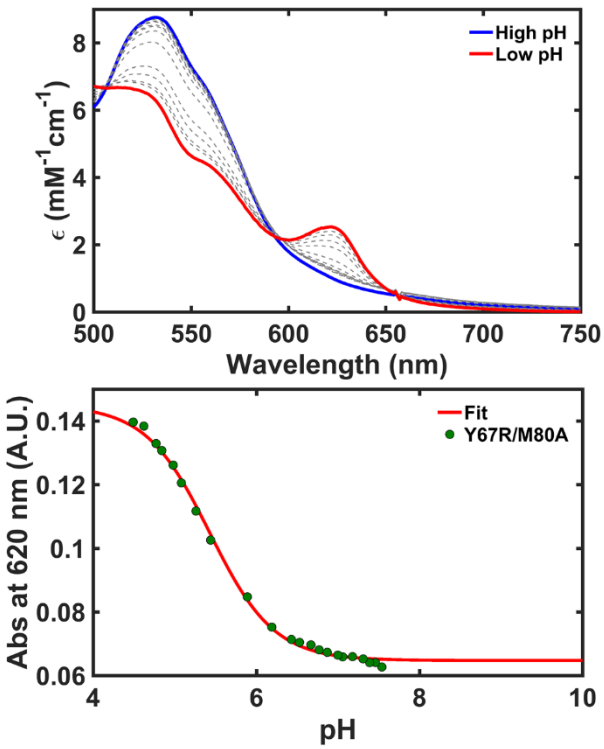

Figure S7. (*Top*) pH dependence of the charge transfer region of Y67R/M80A absorption spectra at 22 ± 2 °C: spectra at high (pH=8.05, *blue*), low (pH=3.94, *red*) pH, and several intermediate (*gray*) pH values are shown. (*Bottom*) Absorbance values at 620 nm versus pH (*green*) and corresponding fit. Points below pH 4.5 and above 7.5 were omitted from the analysis due to large changes in the spectral baselines suggesting protein denaturation at increasingly acidic and basic conditions.

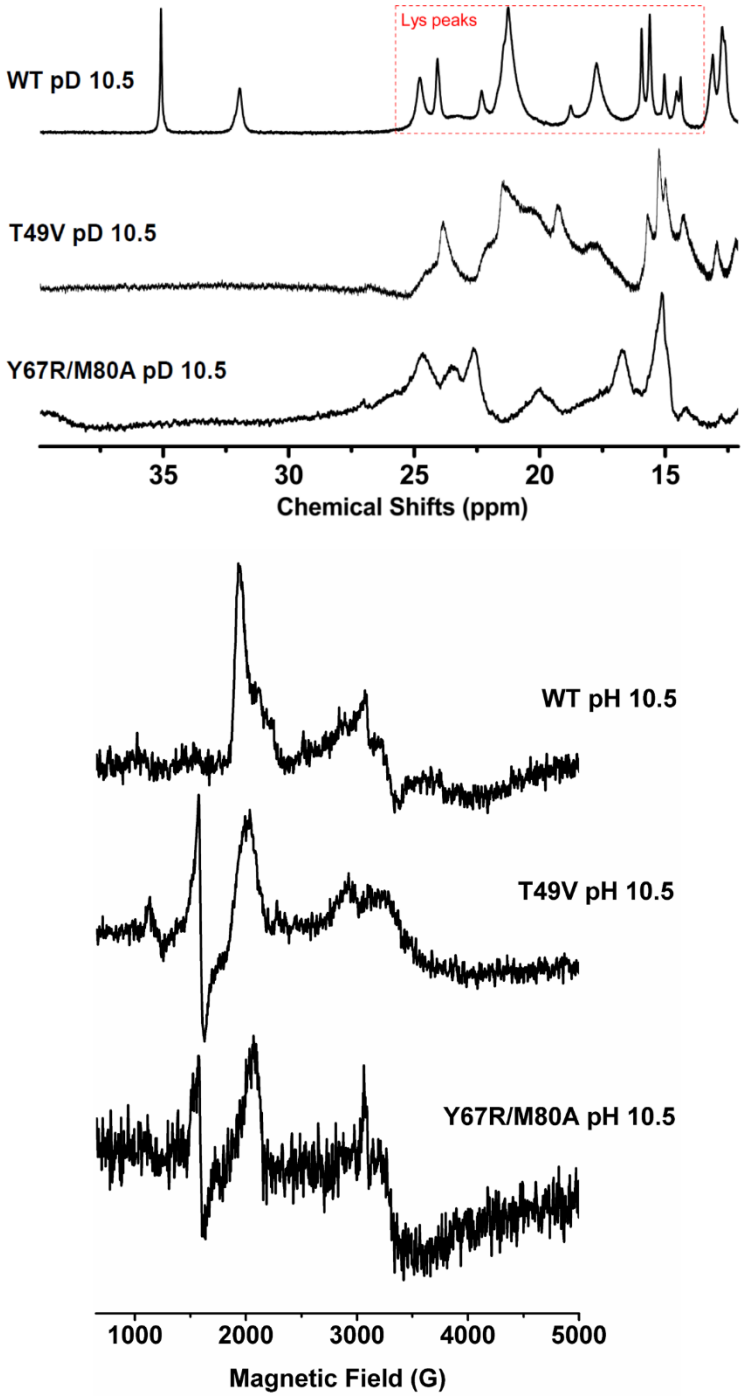

Figure S8. ^{1}H NMR spectra at 25 °C (*top*) and EPR spectra at 10 K (*bottom*) of ferric WT, T49V, and Y67R/M80A in 50 mM sodium borate buffer at pD (or pH) 10.5. EPR samples contained 20% glycerol v/v.

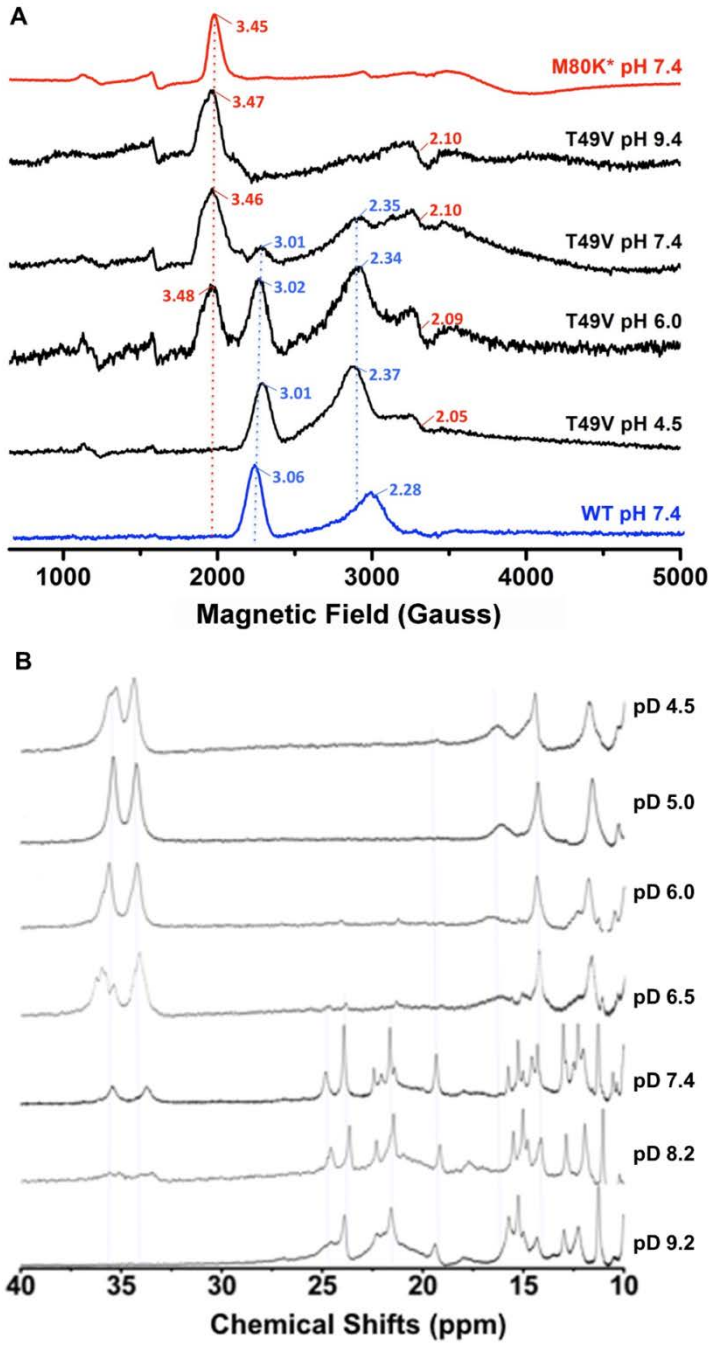

Figure S9. (A) EPR spectra of yeast iso-1- K73A/K79G/M80K cyt *c* at pH 7.4; horse heart T49V cyt *c* at pH 9.4, pH 7.4, pH 6.0, and pH 4.5; and horse heart WT cyt *c* at pH 7.4. Each sample contained around 300 μM protein in 50 mM borate buffer, pH 9.4; sodium phosphate buffer, pH 7.4; MES buffer, pH 6.0; or sodium acetate buffer, pH 4.5, with 30% v/v glycerol (except for K73A/K79G/M80K). Spectra were obtained at 10 K. Signals attributed to Lys-bound and Met-bound species are labeled in *red* and *blue*, respectively. (B) pH dependence of the downfield region of the ^{1}H NMR spectra of ferric T49V at 25 °C. Buffers were 50 mM sodium acetate at pD 4.5-6.0; 50 mM sodium phosphate at pD 6.5-7.4; 50 mM Tris buffer at pD 8.2; and 50 mM sodium borate at pD 9.2.

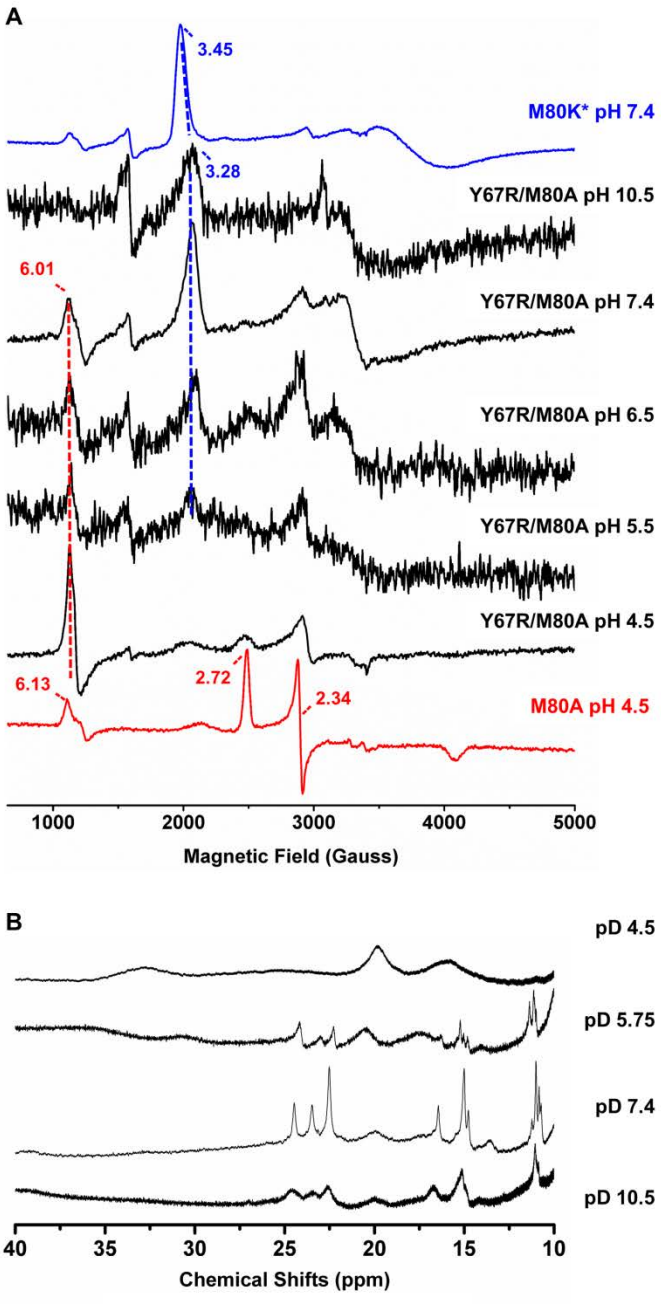

Figure S10. (A) EPR spectra of yeast iso-1 K73A/K79G/M80K cyt *c* pH 7.4; horse heart Y67R/M80A cyt *c* at pH 10.5, pH 7.4, pH 6.5, pH 5.5, and pH 4.5; horse heart M80A cyt *c* pH 4.5. Each sample contained 200-300 μM protein in 50 mM borate buffer, pH 10.5; sodium phosphate buffer, pH 7.4-6.5; MES buffer, pH 5.5; sodium acetate buffer, pH 4.5, with 20% v/v glycerol (except for K73A/K79G/M80K). Spectra were obtained at 10 K. Signals attributed to Lys-bound and high-spin H_2O species are labeled in *blue* and *red*, respectively. (B) pH dependence of the downfield region of the ^1H NMR spectra of ferric Y67R/M80A at 25 °C. Buffers were 50 mM sodium acetate, pD 4.5-5.75; 50 mM sodium phosphate, pD 7.4; 50 mM sodium borate, pD 10.5.

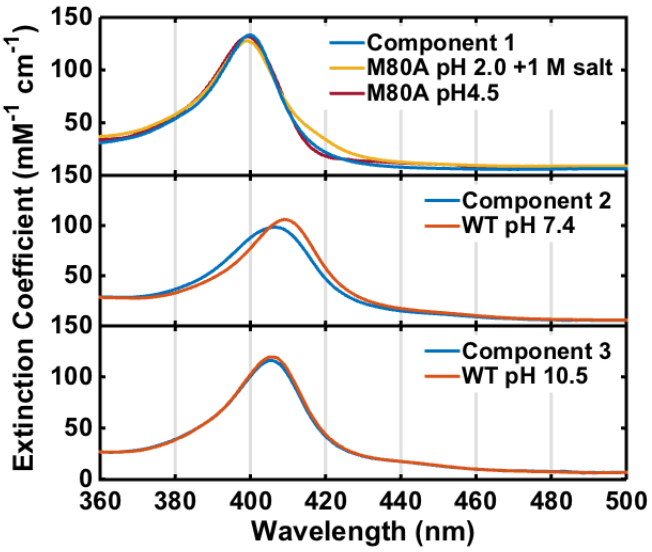

Figure S11. Spectra of H_2O-ligated M80A at pH 2.0 with 1 M salt and at pH 4.5, Met-ligated native WT at pH 7.4 and Lys-ligated WT at pH 10.5 at 22 ± 2 °C are compared with the three components obtained by input-independent SVD analysis of the T49V spectra.

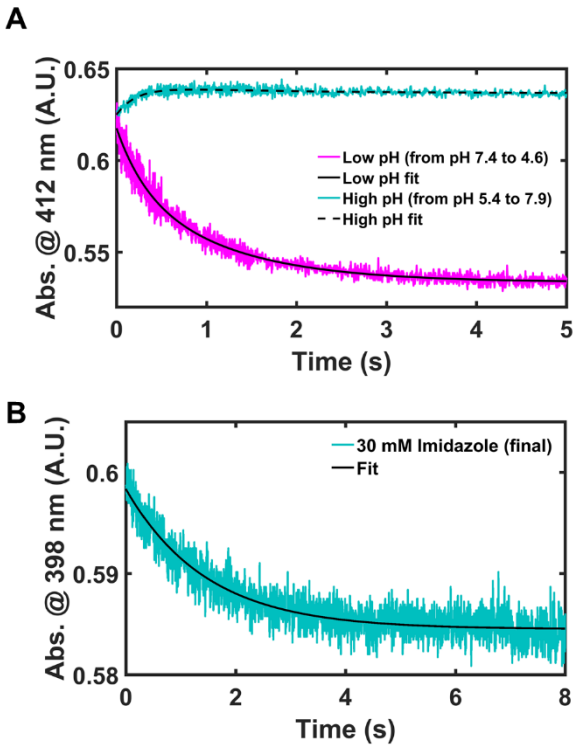

Figure S12. Representative kinetic traces (shown are results for Y67R/M80A) from (A) pH jumps from pH 7.4 to 4.6 and from pH 5.4 to 7.9 and (B) measurements of imidazole binding kinetics at pH 7.4. Mixing experiments were performed at 22 ± 2 °C.

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