Outer Coordination Sphere Influences on Cofactor Maturation and Substrate Oxidation by Cytochrome P460

Supporting Information

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Experimental Methods

General Considerations

All buffers and solutions were prepared using Milli-Q deionized water (18.2 MΩ, Millipore). Anaerobic buffers were prepared using three cycles of vacuum followed by bubbling N_2 gas for 10 minutes each. Experiments were carried out in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 8.0 unless otherwise noted. The NO-donor disodium 1-(Hydroxyl-NNO-azoxy)-L-proline (PROLI-NONOate, Cayman Chemicals) was used as an NO source. Stock solutions of PROLI-NONOate were prepared by dissolving 10 mg in 0.01 M NaOH. NO release was quantified by addition of Fe^{II}EDTA to form the Fe(EDTA)–NO complex (ε_{440} = 900 M^{-1} cm^{-1}; EDTA = ethylenediaminetetraacetic acid).[1,2] Disodium diazen-1-ium-1,2,2 triolate ($Na_2N_2O_3$, Angeli's salt, Cayman Chemicals) was used to generate N_2O and was quantified spectrophotometrically (ε_{237} = 6100 M^{-1} cm^{-1}).[3] Hydroxylamine hydrochloride (NH_2OH•HCl, Sigma Aldrich) stock solutions were prepared by dissolving 69 mg in 1 mL of buffer and were quantified by the method of Frear and Burrell.[4] As-isolated Phe41Ala cyt P460 was quantified using a DC protein assay obtained from BIO-RAD. Cross-linked (CL) Phe41Ala cyt P460 was quantified by the extinction coefficient of the Soret peak (ε_{436} = 79 mM^{-1} cm^{-1}). UV/visible (UV/vis) absorption spectra were obtained using a Cary 60 spectrometer (Agilent) equipped with a Peltier cell maintained at 25 °C. Data were fit in Igor Pro version 6.37 (WaveMetrics). For anaerobic experiments, all reagents and protein were allowed to equilibrate in an MBraun N_2-filled glovebox overnight. Stock solutions used for anaerobic reactions were prepared in the glovebox and brought out in septum-sealed GC crimple vials.

Protein Overexpression and Purification

Constructs of *N. europaea* cyt P460 were used as described previously.[2] Mutations were achieved using the primers listed in Table S9 synthesized by Integrated DNA Technologies. Mutated plasmids obtained from PCR were sequenced to ensure they obtained the mutation and not the template DNA. Protein expression and purification were the same as described previously for WT *N. europaea* cyt P460.[2]

Cross-link Forming Reactions

Maturation using lithium peroxide (Li_2O_2) was achieved by addition of 30 μM Li_2O_2 to a cuvette containing 10 μM Phe41Ala cyt P460 that had been equilibrated at 25 °C with stirring for at least 3 minutes. Scans were recorded every 0.2 minutes until no changes in the UV/vis spectra were observed, after which the reaction was quenched with an excess of sodium dithionite ($Na_2S_2O_4$). The cuvette was returned to the glovebox and $Na_2S_2O_4$ was removed by washing with buffer in filtration columns and the protein was re-oxidized using $[Ru(NH_3)_6]Cl_3$. For reactions containing guaiacol, 8 equivalents (64 μM) Li_2O_2 was added to a cuvette containing 8 μM Phe41Ala cyt P460 and 2 mM guaiacol.

Stopped-flow experiments were performed on an Ocean Optics 2000+ high- resolution diode array spectrometer coupled to a KinTek SF-2004 stopped-flow apparatus. O_2 was removed from the mixing chambers by incubation in $Na_2S_2O_4$ solutions for at least one hour. Excess $Na_2S_2O_4$ was removed by washing the lines with 15 mL degassed buffer until the absorbance of $Na_2S_2O_4$ at 315 nm was no longer observed. Solutions of 20 μM Phe41Ala cyt P460 and 80 μM, 120 μM, 160 μM, 200 μM, or 240 μM Li_2O_2 were brought out of the box in syringes and loaded into separate mixing lines. The solutions were mixed 1:1 (v/v) using 60 μL each. Full-wavelength scans were recorded every 0.04 s seconds for 10 seconds. Plots of absorbance at 403 nm versus time were fit to a double exponential.

For all experiments using CL Phe41Ala cyt P460, the cross-link was formed by an optimized reaction. Purified Phe41Ala cyt P460 was diluted to 10 μM in oxygen-saturated buffer with 10 mM NH_2OH and was heated to 30 °C. The reaction was followed by the decay of the 403 nm peak and ingrowth of the 440 nm peak. After there were no spectral changes, the protein was filtered and washed at least twice with buffer in an Amicon filtration column.

Determination of NH_2OH and NO K_d

An anaerobic cuvette containing 10 μM CL Phe41Ala or WT cyt P460 in 2 mL total anaerobic buffer was equilibrated in the UV/vis spectrometer at 25 °C with stirring for five minutes. For the determination of the NH_2OH K_d, the protein solution was titrated with NH_2OH through a Hamilton syringe allowing for five minutes of equilibration before each full wavelength scan was initiated. The absorbance at 414 nm was plotted against the NH_2OH concentration corrected for dilution. The curve was fit using a hyperbolic function (Eq. 1). The same procedure was followed for the determination of the NO K_d using a stock solution of PROLI-NONOate. In this case, the absorbance at 455 nm was used.

$$A_{414} = A_0 + \frac{\Delta A_{414} * [NH_2OH]_0}{K_d + [NH_2OH]_0} \qquad \text{(Eq. 1)}$$

To obtain NO off-rates, the $\{FeNO\}^6$ was generated using 100 μM NO and 10 μM cyt P460. Single-wavelength scans at 455 nm were recorded for two minutes before addition of 200 μM Fe^{II}EDTA. Rates were obtained by fitting the decay at 455 nm to a single exponential.

Electron Paramagnetic Resonance (EPR) Spectroscopy

Continuous-wave EPR spectra were measured at X-band (9.40 GHz) using a Bruker Elexsys-II spectrometer maintained at 10 K by a liquid He cryostat (Oxford). Samples were

prepared in an anaerobic glovebox using 200 μL of 150 μM protein in 50 mM HEPES buffer (pH 8) and without the use of glycerol. Spectra were simulated using SpinCount.[5]

^{57}Fe Enrichment of Cyt P460 and ^{57}Fe Mössbauer Spectroscopy

A 5 mL starter culture was grown overnight in LB supplemented with ampicillin and chloramphenicol. Cells were pelleted at 8000g for 10 minutes, the LB media was decanted, and the cells were resuspended in 5 mL of minimal media by pipetting up and down. The minimal media was based on the recipe established by Liptak et al[6] and was comprised of 38.4 g $Na_2HPO_4 \bullet 7H_2O$, 9.0 g KH_2PO_4, 0.5 g NaCl, 2.0 g NH_4Cl, 20 mL amino acid cocktail, 20 mL 1 M $MgCl_2$, 20 mL vitamin cocktail, 130 mg delta-aminolevulinic acid, 76 mg thiamine HCl, 20 mL 50% glycerol, 5 mL 20% glucose, 20 mL trace metals, 0.0745 g $^{57}FeCl_2$, 200 mg ampicillin, and 200 mg chloramphenicol in 2 L of Milli-Q water. The trace metal solution was composed of 1 M $CaCl_2$, 1 M $MnCl_2 \bullet 4H_2O$, 1 M $ZnSO_4 \bullet 7H_2O$, 0.2 M $CoCl_2 \bullet 6H_2O$, 0.2 M $NiCl_2 \bullet 6H_2O$, 0.1 M $CuCl_2 \bullet 2H_2O$, 0.1 M $Na_2MoO_4 \bullet 5H_2O$, 0.1 M $Na_2SeO_3 \bullet 5H_2O$, and 0.1 M H_3BO_3 in Milli-Q water. The vitamin cocktail was prepared by dissolving 10 mg biotin, 10 mg choline chloride, 10 mg folic acid, 10 mg nicotinamide, 10 mg D-pantothenate, 10 mg pyridoxal, and 1 mg riboflavin in 100 mL Milli-Q water.

Two 6 L flasks containing 2 L of minimal media were inoculated with 2.5 mL of cells and were grown overnight at 37 °C and 180 rpm. The cells were induced with 200 mg of IPTG after they were grown to saturation. The temperature was reduced to 20-24 °C and the cells were harvested after 24 hours. Cells were harvested by centrifugation at 8000g for 10 minutes, resuspended in lysis buffer containing 20 mM Tris (pH 8.0), 150 mM NaCl, 0.01% Triton X, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and lysed by sonication. The lysate was separated from cell debris by centrifugation at 35,000g for 45 minutes. The supernatant was then

applied to a to HisPur Ni-NTA resin (Thermo Scientific) packed in a gravity column. The bound protein was washed with 10 column volumes of wash buffer comprised of 20 mM MOPS (pH 8.0), 20 mM imidazole, and 150 mM NaCl and was then eluted with using 330 mM imidazole. Fractions containing the purified protein were buffer exchanged using Amicon filtration columns. Ferrous samples were prepared in the box by adding an excess of $Na_2S_2O_4$. Once reduced, the sample was added to a Delrin Mössbauer cup and was quickly frozen in liquid nitrogen.

Data were collected at 4.2 K for ferrous WT *N. europaea* cyt P460 and ferrous Lys70Tyr *N. europaea* cyt P460 while data for Phe41Ala *N. europaea* cyt P460 were collected at 85 K. All data were collected in 0 magnetic field using a SEECo Resonant Gamma-Ray spectrometer (Model W304) equipped with a Janis Research Model SVT-400 Cryostat. The velocity scale was calibrated to iron foil at room temperature. Data were fit using the WMOSS software package[7] with Theoretical Model 3 to fit to quadrupole doublets. Uncertainties were derived from the standard deviation of 200 Monte Carlo simulations for each spectrum.

Resonance Raman Spectroscopy

Resonance Raman spectra were obtained on a home-built spectrometer as previously described.[8] Samples prepared in J. Young NMR tubes were excited using a 405 nm diffraction-limed Lepton IV diode laser (Microlaser Systems) at ca. 30 mW. Data was collected and processed using the Lightfield software package (Princeton Instruments). Spectra for as-isolated Phe41Ala, Phe41Trp, or Phe41Arg cyt P460 were obtained using about 1 mM protein in 50 mM phosphate buffer (pH 8.0) and data were collected by averaging 240 frames at 30 s exposures. For CL Phe41Ala, 900 μM protein was used. Samples were protected from photobleaching by

spinning. Acetaminophen was used to calibrate the Raman shift. Background was removed using the backgrounds macro in Igor Pro version 8.04 (WaveMetrics).

Steady-State Activity Assays

Stock solutions of 800 mM NH_2OH or 100 µM cyt P460 were prepared in the glovebox and removed in crimple vials. Solutions of phenazine methosulfate (PMS) and dichlorophenolindophenol (DCPIP) were prepared in the box and quantified by $\varepsilon_{387} = 26\ M^{-1}cm^{-1}$ and $\varepsilon_{605} = 20.6\ mM^{-1}cm^{-1}$ respectively.[9,10] An anaerobic Spectrosil quartz cuvette (Starna Cells, 10 mm) containing 50 µM DCPIP and 6 µM PMS in 2 mL total was equilibrated in the UV/vis spectrometer at 25 °C with stirring for 5 minutes. A single-wavelength scan at 605 nm was recorded for two minutes before NH_2OH was added using a Hamilton syringe to give final concentrations of 2 mM, 6 mM, 10 mM, and 20 mM. After 90 s, cyt P460 was added using a Hamilton syringe to give a final concentration of 1 µM. Specific activities were determined using a linear regression of the first 10% of oxidant consumption. The average turnover frequencies (TOFs) of at least three trials were plotted as a function of NH_2OH concentration.

N_2O and NO_2^- Quantification

Production of N_2O was determined by gas chromatography-mass spectrometry using a JEOL GCMate equipped with an Rt®-Q-Bond column (30 m, 0.25 mmID, 8 µm df). All reactions were prepared in 2.5 mL septum-sealed headspace vials. For anaerobic reactions, samples were prepared in a glovebox. Turnover conditions were achieved with 5 µM cyt P460, 6 µM PMS, 2 mM hexaaminеruthenium(III) chloride ($[Ru(NH_3)_6]Cl_3$), and 0 µM, 100 µM, 250 µM, 500 µM, or 1000 µM NH_2OH in 500 µL of buffer total. Reactions were initiated by the

addition of NH_2OH, the vial was quickly capped and inverted three times. The headspace was sampled after reactions were allowed to age overnight. N_2O production was quantified by integrating the peak corresponding to N_2O (retention time = 3.75 min).

Nitrite production was quantified using a Griess diazotization assay. Samples were prepared aerobically with 5 μM cyt P460, 6 μM PMS, 2 mM ruthenium hexachloride, and 0, 100 μM, 250 μM, 500 μM, or 1000 μM NH_2OH in 500 μL of buffer total.

Reactions of the {FeNO}^{6} Intermediate

An anaerobic Spectrosil quartz cuvette (Starna Cells, 10 mm) containing 10 μM cyt P460 in 2 mL buffer was allowed to equilibrate for 5 minutes in the UV/vis spectrometer at 25 °C with stirring. The shunted {FeNO}^{6} species was generated by adding 100 μM NO from PROLI-NONOate. Ingrowth of the 455 nm peak was monitored until there was no change in absorbance. After an additional minute, NH_2OH was added through a Hamilton syringe. For reactions with oxidant, DCPIP was added after the generation of the {FeNO}^{6} and spectra were monitored for two minutes before NH_2OH was added. Full-wavelength scans were collected every 0.3 minutes until the reaction was complete. To obtain initial rates, 5 μM WT or Phe41Ala cyt P460 was mixed with 5 μM or 50 μM NO, respectively. Using the same procedure as above, 1 mM, 2 mM, 6 mM, or 12 mM NH_2OH was added to initiate the reaction. Initial velocities were determined using a linear regression of the first 10% of consumption of the {FeNO}^{6}. The initial velocities were then plotted against the concentration of NH_2OH and were fit to the Michaelis-Menten equation. k_{cat} was determined using the initial concentration of the {FeNO}^{6} calculated from the corresponding K_d for WT or Phe41Ala {FeNO}^{6}.

Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectra were collected using a Bruker Vertex V80Vvacuum FTIR system equipped with a nitrogen-cooled MCT detector. All samples were prepared in 50 mM potassium phosphate buffer (pH 7.4). The $\{FeNO\}^6$ was prepared by sparging NO gas with a gas-tight syringe in 1-2.5 mM cyt P460. N-14 NO gas was obtained from Praxair. N-15 NO gas was prepared by treating 10 mg of $Na^{15}NO_2$ with 1 mL anaerobic 0.2 M KI/0.4 M H_2SO_4 in a septum-sealed vial, allowing the reaction to proceed for 3 hours, and drawing gas from the headspace with a gas-tight syringe. The samples were checked by UV-vis to ensure conversion to the $\{FeNO\}^6$ and loaded into an FTIR cell with CaF_2 windows and a 50 μm Teflon spacer. The FTIR sample chamber was purged with nitrogen gas for at least 10 minutes and the buffer background spectra was checked to ensure minimal contribution from water vapor and carbon dioxide. Spectra were collected using a 4 mm aperture, a scanner velocity of 10 kHz, and a 1 cm^{-1} spectral resolution. Samples were averaged over 500 scans.

Characterization of the Phe41Trp mutant

Expression and purification of the Phe41Trp mutant yielded a primarily CLD protein whose Soret band was centered at 403 nm but was significantly broader than other CLD mutants (**Figure S13**). There was a small component at 440 nm indicating that this mutant is capable of being cross-linked. However, cross-link formation could not be driven by reaction with Li_2O_2 and only resulted in heme degradation (**Figure S14**). The gentler reaction with O_2-saturated buffer and NH_2OH resulted in a slow conversion to CL protein, but complete maturation could not be achieved. It is possible that the Trp side-chain is too bulky and impedes cross-link formation. Resonance Raman spectroscopy using excitation at 405 nm showed that the spin state marker band (ν_3) had shifted to 1495 cm^{-1} compared to the 1485 cm^{-1} band for the Phe41Ala mutant (**Figure S15**). The ν_3 energy for Phe41Trp cyt P460 is in the range of 5-coordinate high-spin hemes while the 1485 cm^{-1} ν_3 band for Phe41Ala cyt P460 is in the range of 6-coordinate high-spin hemes. A change in coordination is consistent with the more open active site of the Phe41Ala mutant being accessible to solvent while access to the active site of Phe41Trp cyt P460 is blocked by the Trp side-chain.

Supplementary Figures

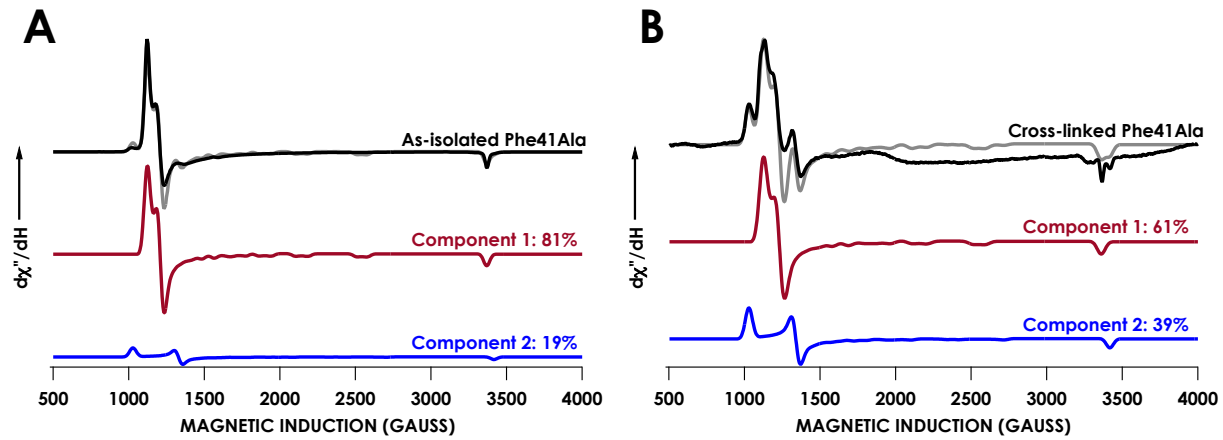

Figure S1. Continuous-wave X-band (9.4 GHz) EPR spectra obtained at 10 K for A) as-isolated Phe41Ala cyt P460 and B) cross-linked Phe41Ala cyt P460 formed by reaction with O_2-saturated buffer and NH_2OH at 30 °C. Experimental spectra are shown in black and simulated spectra are shown in gray. Individual components of the simulations are shown as red and blue traces.

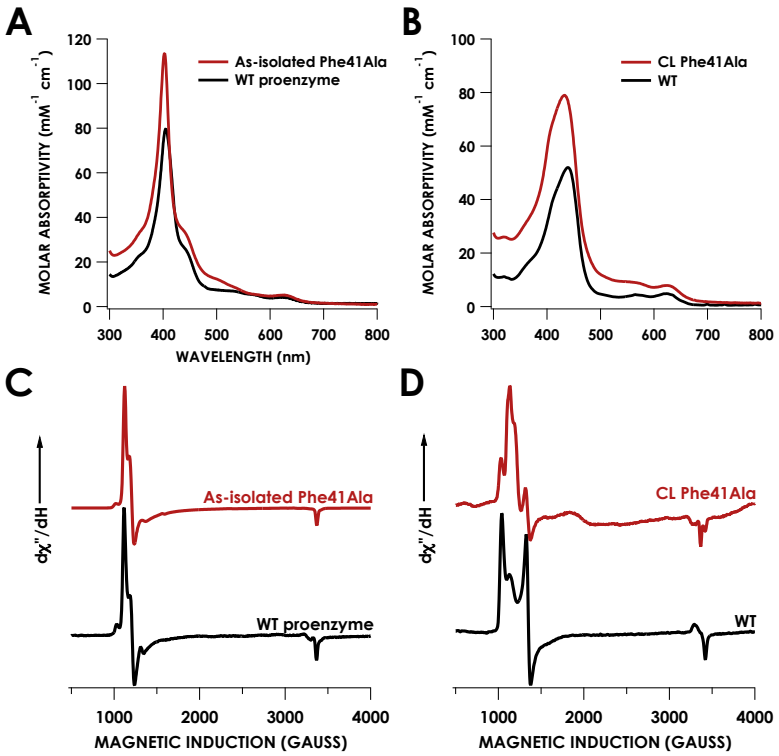

Figure S2. Comparison of the Phe41Ala mutant and WT cyt P460. A) UV/vis spectra of as-isolated Phe41Ala cyt P460 and the WT proenzyme. B) UV/vis spectra of cross-linked Phe41Ala cyt P460 and WT cyt P460. C) X-band EPR spectra obtained at 10 K for as-isolated Phe41Ala cyt P460 and the WT proenzyme. D) X-band EPR spectra obtained at 10 K for cross-linked Phe41Ala cyt P460 and WT cyt P460.

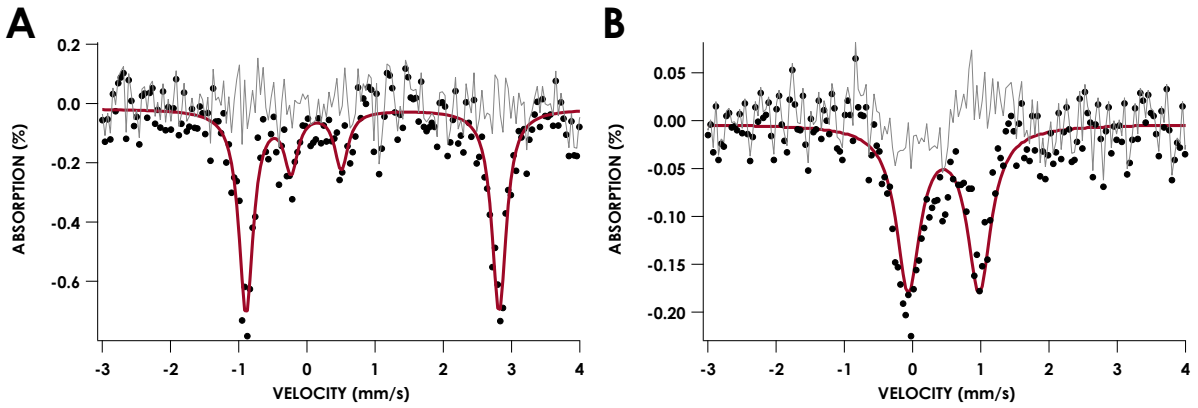

Figure S3. Mössbauer spectra obtained at 4.2K and 0 field for A) WT *N. europaea* cyt P460 and B) Lys70Tyr *N. europaea* cyt P460. Experimental spectra are shown as black dots, simulated spectra are shown in red, and the residual is shown in gray.

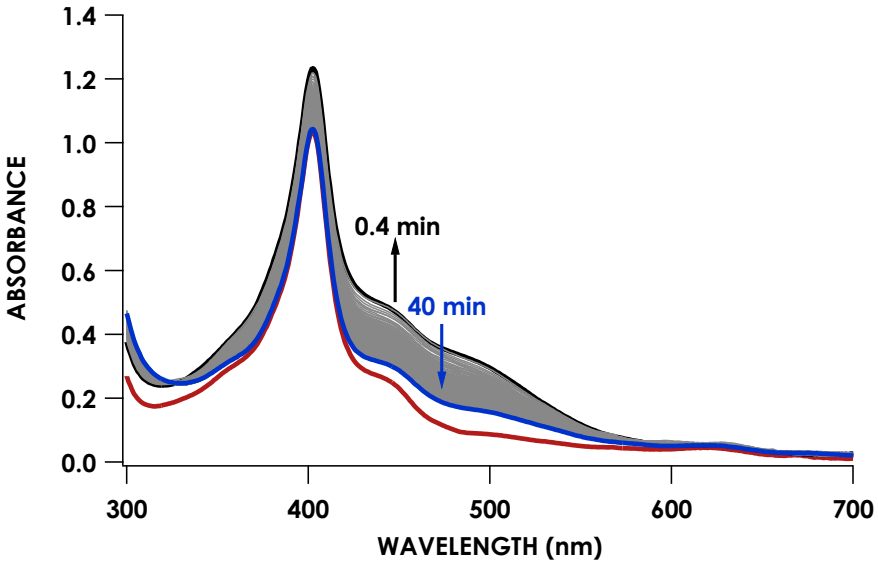

Figure S4. Full-wavelength UV/vis scans for the reaction of 8 μM as-isolated Phe41Ala cyt P460 with 2 mM guaiacol and 64 μM Li_2O_2. The red trace is before addition of Li_2O_2, the black trace is 0.4 minutes after addition of Li_2O_2, and the blue trace is the final scan after 40 minutes. Grey traces represent scans every 0.2 minutes.

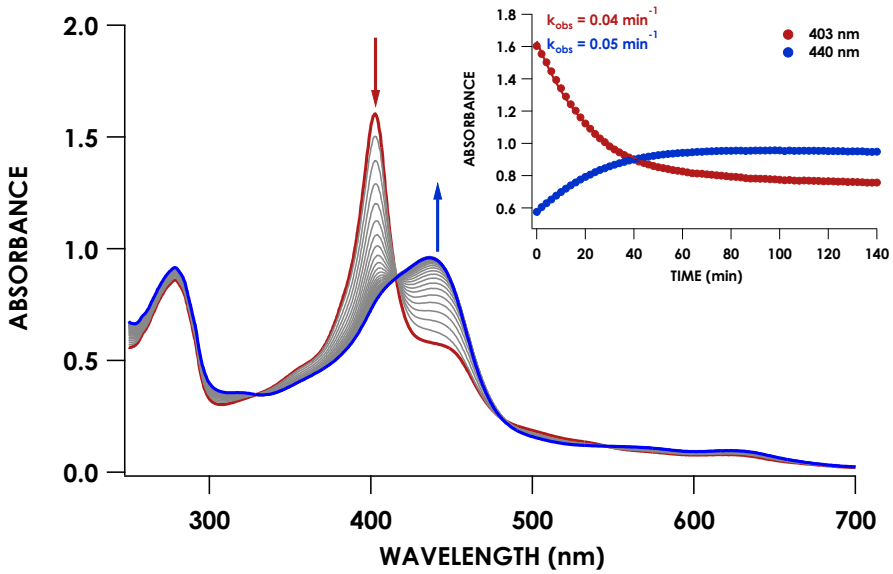

Figure S5. Cross-link forming reaction achieved using 10 µM CLD Phe41Ala cyt P460 and 10 mM NH_2OH in O_2-satured 50 mM HEPES (pH 8.0) at 30 °C. The red trace is as-isolated Phe41Ala cyt P460 immediately after NH_2OH was added and the blue trace is the final spectrum. The inset shows the time-course of the reaction at 403 nm (red) and 440 nm (blue) fit to a single exponential.

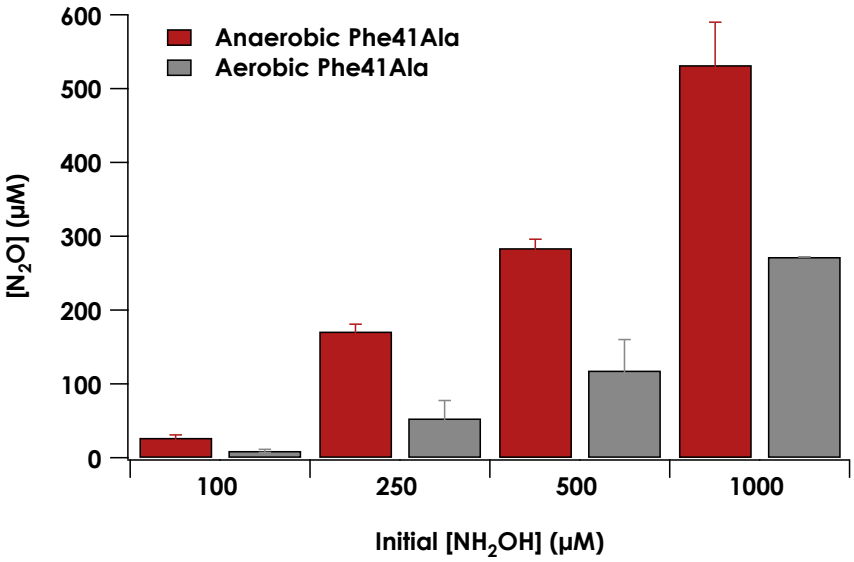

Figure S6. N_2O production measured by GC-MS after the reaction of 5 μM CL Phe41Ala cyt P460, 6 μM PMS, and 2 mM $[Ru(NH_3)_6]Cl_3$ with varying concentrations of NH_2OH under anaerobic (red) and aerobic (grey) conditions. Error bars represent standard deviations of three trials.

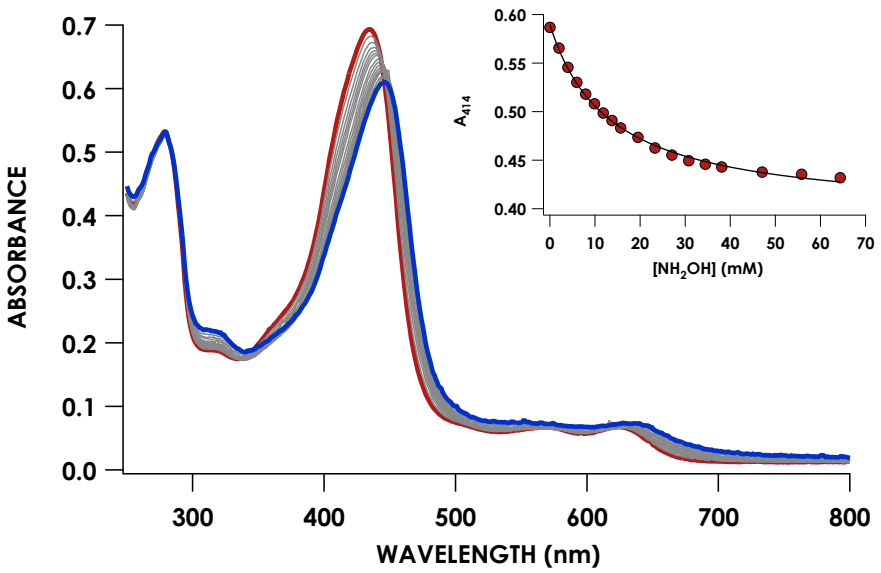

Figure S7. Titration of CL Phe41Ala cyt P460 with NH_2OH. The red trace is before NH_2OH was added, grey traces represent each addition of NH_2OH, and blue is the final NH_2OH-adduct. The inset shows the absorbance at 414 nm vs. [NH_2OH] fit to a hyperbolic function.

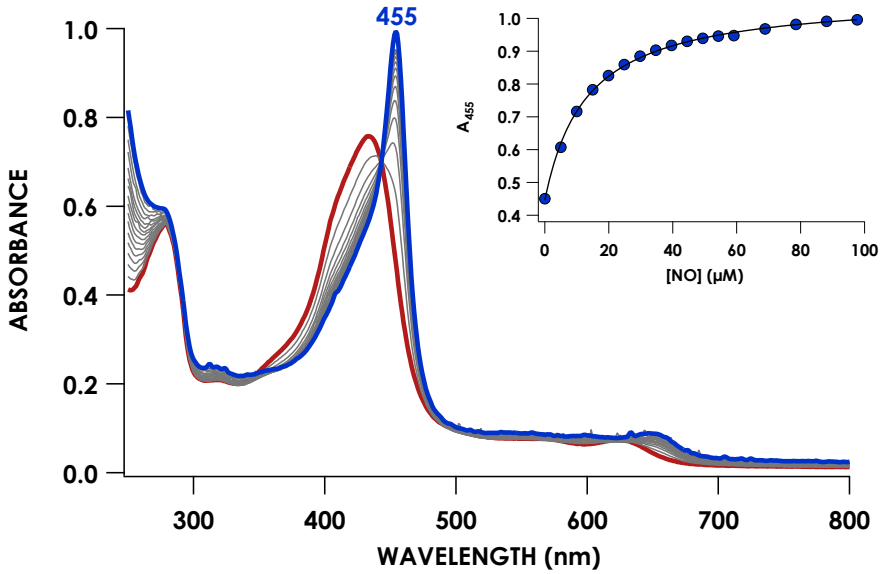

Figure S8. Titration of CL Phe41Ala cyt P460 with NO. The red trace is before NH_2OH was added, grey traces represent each addition of NO, and blue is the final NO-adduct. The inset shows the absorbance at 455 nm vs. [NO] fit to a hyperbolic function.

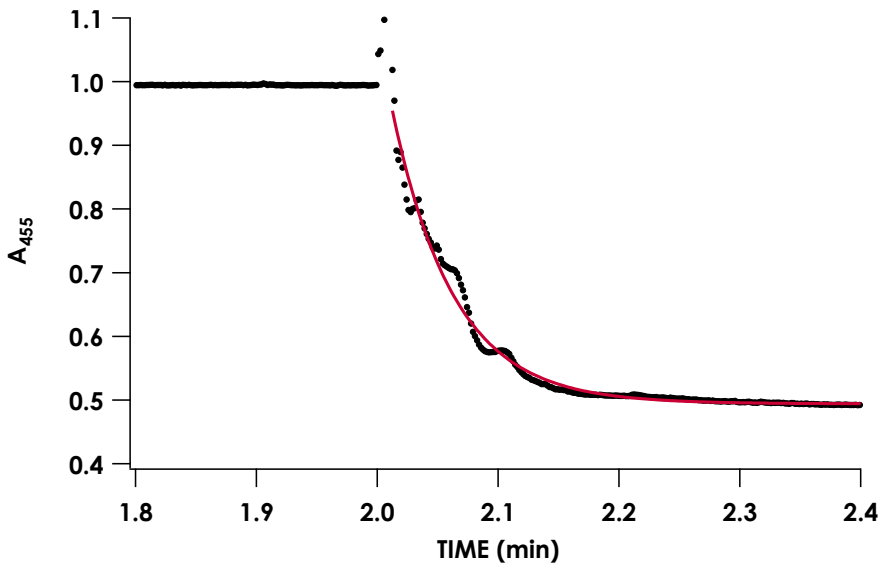

Figure S9. Time-course of the absorbance at 455 nm of a shunted {FeNO}^{6} generated with 10 µM WT cyt P460 and 100 µM NO from a NO-saturated solution reacted with 200 µM Fe^{II}EDTA. The black points represent the absorbance recorded every 0.1 seconds and the red curve is an exponential fit.

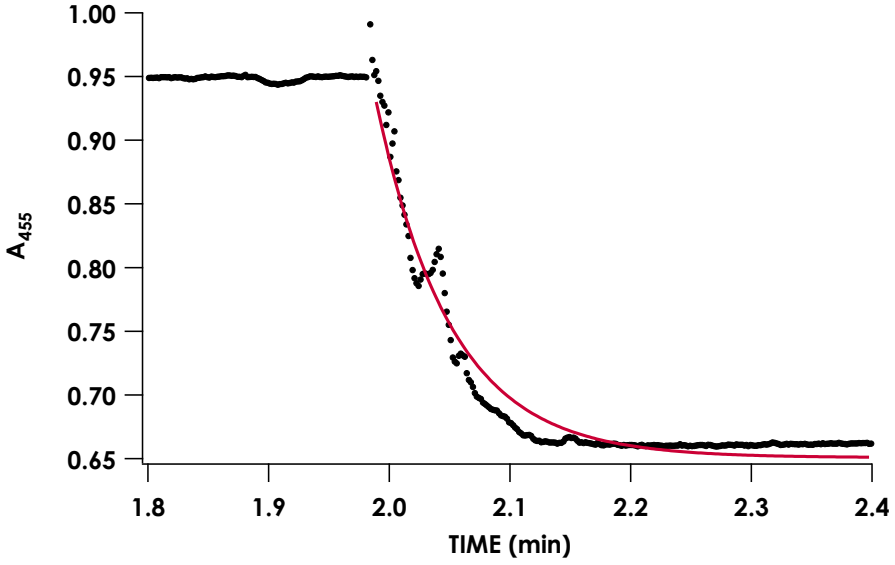

Figure S10. Time-course of the absorbance at 455 nm of a shunted {FeNO}^{6} generated with 10 µM CL Phe41Ala cyt P460 and 100 µM NO from a NO-saturated solution reacted with 200 µM Fe^{II}EDTA. The black points represent the absorbance recorded every 0.1 seconds and the red curve is an exponential fit.

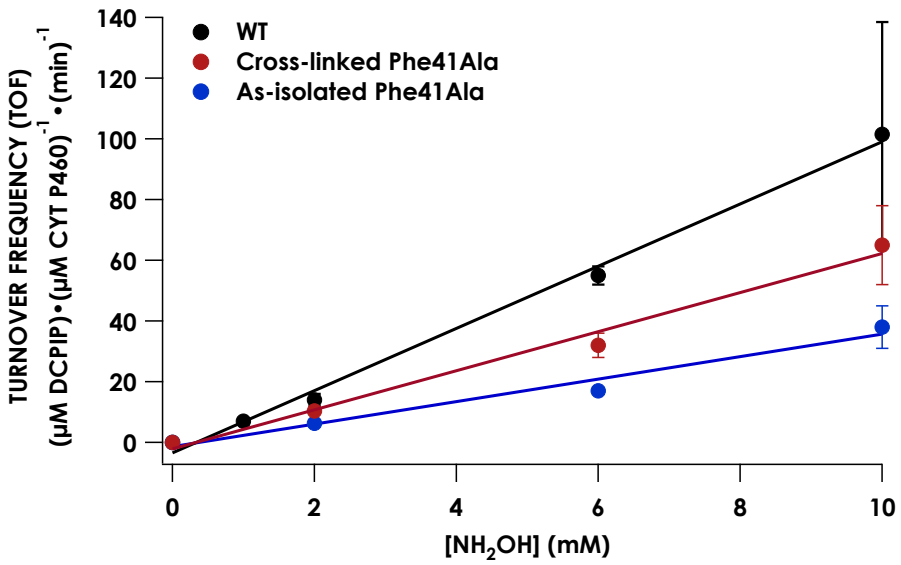

Figure S11. Steady-state activity plot of CL Phe41Ala (red), as-isolated Phe41Ala (blue), and WT (black) *N. europaea* cyt P460. Turnover frequencies were obtained by fitting the first 10% of DCPIP consumption to a line. All data points are averages of three trials.

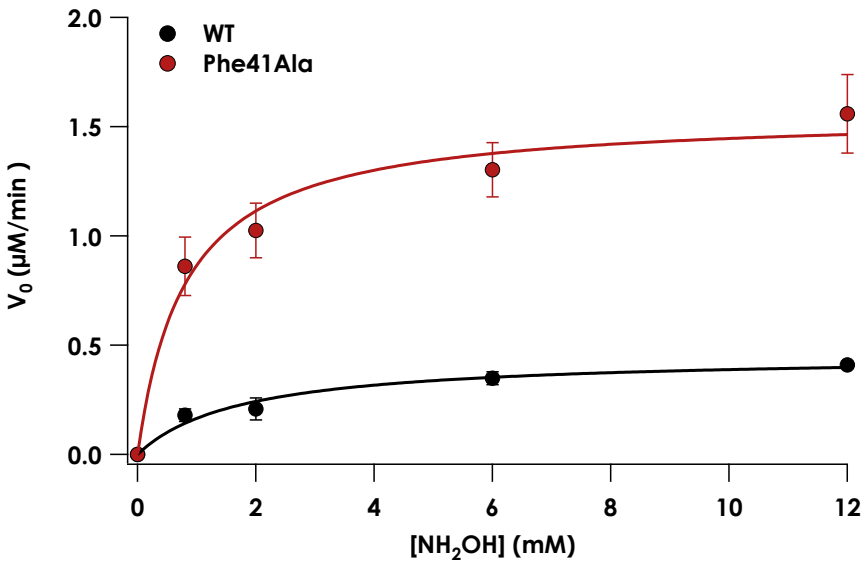

Figure S12. Initial velocities for the reaction of the shunted {FeNO}^{6}, formed by mixing 5 μM WT or Phe41Ala cyt P460 with 5 μM or 50 μM NO respectively, with several concentrations of NH_2OH. The solid lines represent fits to the Michaelis-Menten equation. Error bars represent the standard deviations of at least three trials.

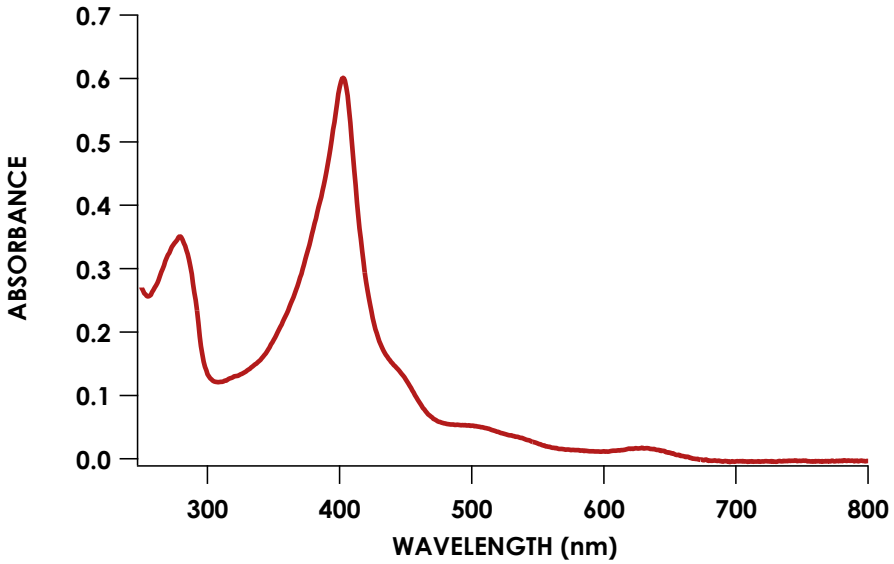

Figure S13. UV/vis spectrum of as-isolated Phe41Trp cyt P460.

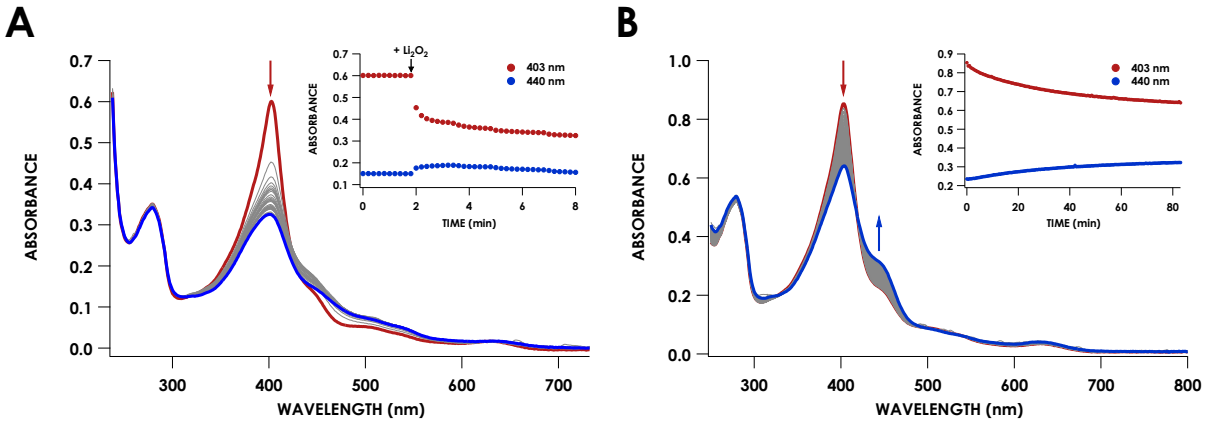

Figure S14. Full-wavelength UV/vis scans for the reaction of A) Phe41Trp cyt P460 with 3 equivalents of Li_2O_2 and B) Phe41Trp cyt P460 with O_2-saturated buffer and 10 mM NH_2OH. The red trace is the first scan and the blue trace is the final scan. Insets show the time-course at 403 nm (red) and 440 nm (blue).

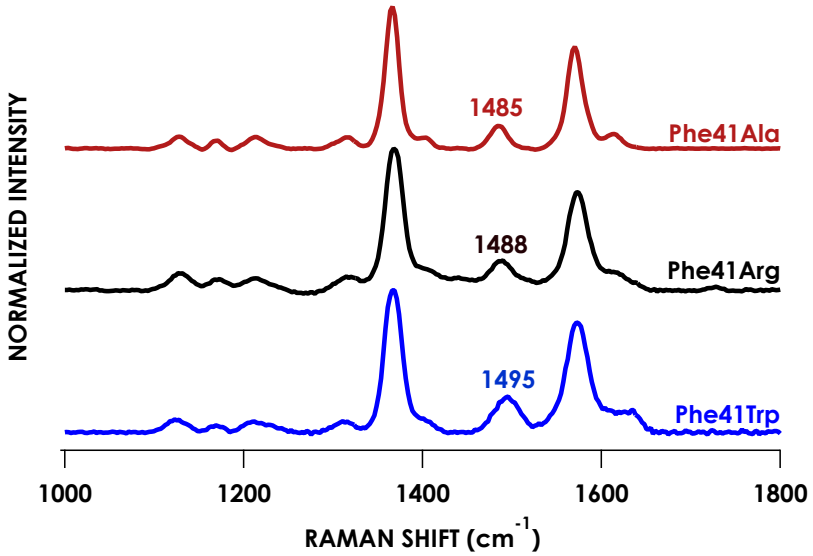

Figure S15. Resonance Raman spectra obtained via excitation at 405 nm for as-isolated Phe41Ala cyt P460 (red), Phe41Arg cyt P460 (black), and Phe41Trp cyt P460 (blue).

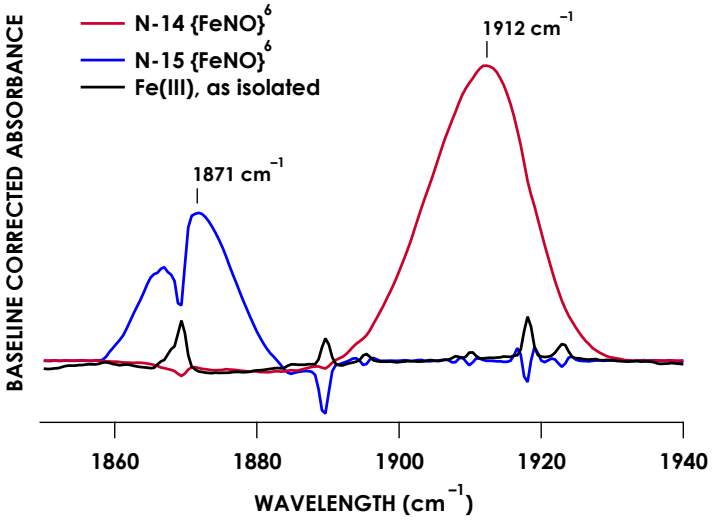

Figure S16. FTIR spectra obtained of as-isolated ferric cyt P460 (black) and its N-14 (red) and N-15 (blue) $\{FeNO\}^6$ derivatives.

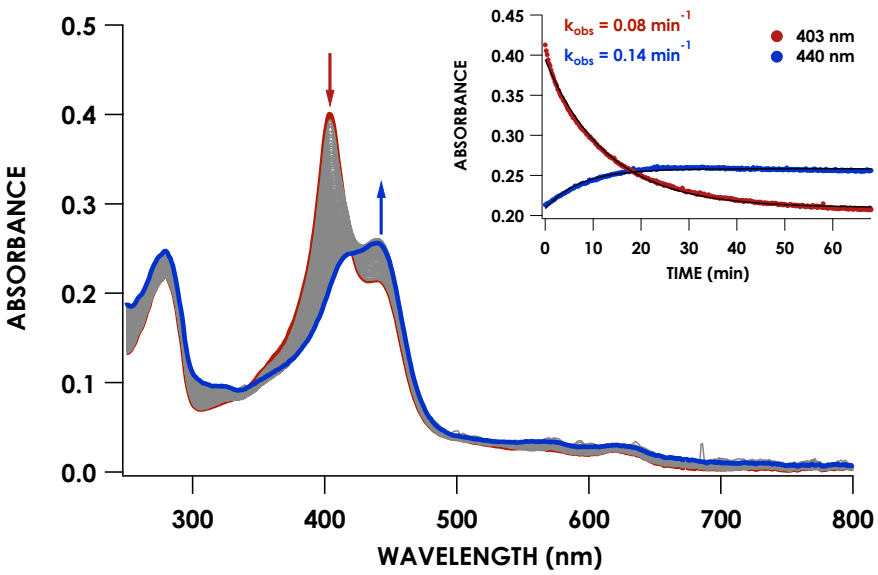

Figure S17. Full-wavelength UV/vis scans for the reaction of Phe41Arg with O_2-saturated buffer and 10 mM NH_2OH. The red trace is the first scan and the blue trace is the final scan. The inset shows the time-course at 403 nm (red) and 440 nm (blue).

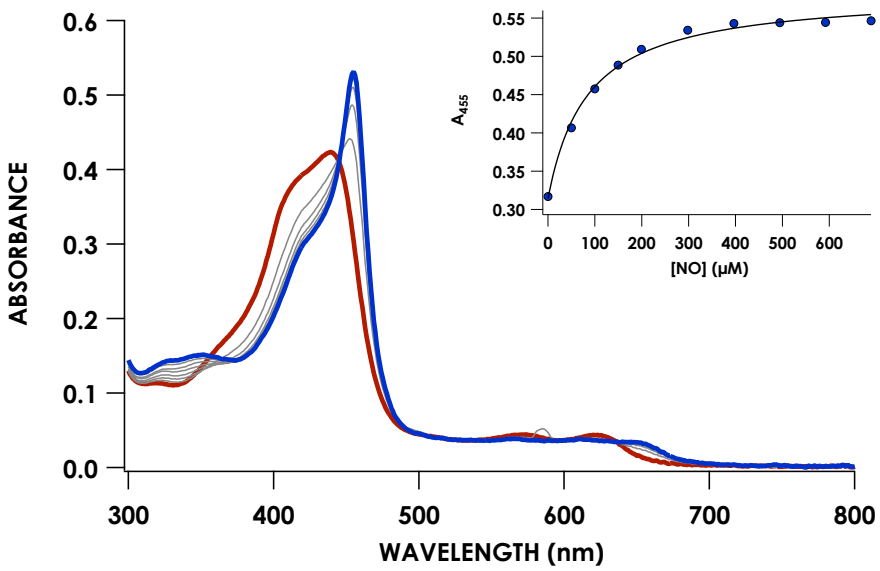

Figure S18. Titration of CL Phe41Arg cyt P460 with NO. The red trace is before NH_2OH was added, grey traces represent each addition of NO, and blue is the final NO-adduct. The inset shows the absorbance at 455 nm vs. [NO] fit to a hyperbolic function.

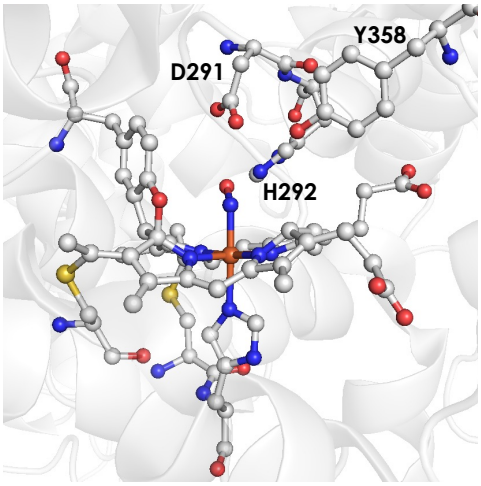

Figure S19. Active site of *N. europaea* HAO with NH_2OH bound (PDB 4N4O), highlighting residues predicted to play a role in NH_2OH oxidation.

Supplementary Tables

Table S1. UV/vis absorption spectral data.

Cyt P460 Variant	λ_{max} (nm)	Q-bands (nm)
WT	440, 414 (sh) (ε_{440} = 52 mM^{-1} cm^{-1})	570, 627
WT, Fe^{III}-NH_2OH	445	561, 633 (broad)
WT, $\{FeNO\}^6$	455	554, 603, 652
Phe41Ala, CLD	403	501, 533, 627
Phe41Ala, CL	436, 408 (sh) (ε_{440} = 79 mM^{-1} cm^{-1})	570, 628
Phe41Ala, CL Fe^{III}-NH_2OH	446	570, 639 (broad)
Phe41Ala, CL $\{FeNO\}^6$	455	555, 600, 650
Phe41Arg, CLD	402	500, 534, 572, 627
Phe41Arg, CL	440, 413 (sh)	572, 623
Phe41Trp, CLD	403 (broad)	503, 535, 631

Table S2. Spin Hamiltonian parameters obtained from fitting X-band EPR spectra of cyt P460 variants. AL212 = Nitrosomonas sp. AL212.

Variant	Resting Fe^{III} (component 1)		Resting Fe^{III} (component 2)		Reference
	g_{eff}	E/D	g_{eff}	E/D	
WT *N. europaea*	6.57, 5.09, 1.97	0.03	N/A	N/A	[2]
WT *N. europaea* proenzyme	6.52, 5.06, 1.97	0.03	6.02, 5.54, 1.99	0.01	[11]
WT AL212	6.39, 5.13, 1.97	0.03	6.00, 5.52, 1.99	0.012	[12]
AL212 A131E	6.40, 5.14, 1.97	0.03	6.00, 5.51, 1.99	0.012	[12]
AL212 A131N	6.51, 5.12, 1.97	0.03	6.03, 5.53, 1.99	0.012	[12]
As-isolated *N. europaea* Phe41Ala	6.54, 5.05, 1.97	0.03	5.99, 5.55, 1.99	0.01	This work
CL *N. europaea* Phe41Ala	6.54, 5.01, 1.97	0.03	5.98, 5.45, 2.00	0.01	This work

Table S3. Resonance Raman shifts of porphyrin marker bands from *N. europaea* cyt P460 variants obtained using laser excitation at 405 nm.

Cyt P460 Variant	ν_3 (cm^{-1})	ν_4 (cm^{-1})	ν_{10} (cm^{-1})	Reference
Lys70Tyr	1501	1372	NR	[8]
WT	1504	1359	1614	[11]
WT proenzyme	1488	1369	1616	[11]
Arg44Ala	1487	1367	1618	[11]
As-isolated Phe41Ala	1485	1367	1614	This work
CL Phe41Ala	1501	1367	1614	This work
Phe41Trp	1495	1367	1611, 1633	This work
Phe41Arg	1488	1368	1612	This work

Table S4. Mössbauer parameters for Fe^{II} *N. europaea* cyt P460 variants obtained from fitting. Errors represent the standard deviation of 200 Monte Carlo simulations.

Cyt P460 Variant	Temp (K)	Reduced χ^2	Component	δ (mm/s)	ΔE_Q (mm/s)	Γ (mm/s)	Relative area
WT	4	1.00(0.01)	major	0.96(0.01)	3.71(0.01)	0.25(0.02)	78%
			minor	0.13(0.03)	0.75(0.06)	0.25(0.06)	22%
40% CL Phe41Ala*	85	0.92(0.06)	CLD	0.920(0.01)	2.27(0.01)	0.43(0.02)	64%
			CL	0.96(0.02)	3.65(0.03)	0.61(0.06)	36%
60% CL Phe41Ala*	85	0.97(0.07)	CLD	0.97(0.02)	2.23(0.04)	0.66(0.06)	44%
			CL	0.96(0.01)	3.90(0.01)	0.39(0.02)	56%
Lys70Tyr	4	0.99(0.07)	n/a	0.46(0.01)	1.04(0.02)	0.42(0.03)	100%

*Quantification of CL percentage determined by UV/vis spectroscopy

Table S5. Observed rates obtained by fitting the decay of the 403 nm Soret band to a double exponential following the stopped-flow reaction of 10 μM cyt P460 with Li_2O_2.

[Li_2O_2] (μM)	**k_{obs1} (s^{-1})**	**k_{obs1} average (s^{-1})**	**k_{obs2} (s^{-1})**	**k_{obs2} average (s^{-1})**
40	6.8, 6.6, 7.0	6.8 ± 0.2	0.44, 0.46, 0.46	0.45 ± 0.01
60	8.5, 11.3, 10.8	10.2 ± 1.5	0.51, 0.54, 0.54	0.53 ± 0.02
80	15.3, 11.7, 13.3	13.4 ± 1.8	0.62, 0.60, 0.59	0.60 ± 0.02
100	14.2, 14.7, 16.7	15.2 ± 1.3	0.63, 0.66, 0.64	0.64 ± 0.02
120	17.3, 25.0, 16.3	19.5 ± 4.8	0.70, 0.71, 0.67	0.69 ± 0.02

Table S6. Concentrations of NO_2^- measured by a Griess Diazotization assay for reactions under aerobic turnover conditions using 5 μM WT or Phe41Ala cyt P460, 2 mM $[Ru(NH_3)_6]Cl_3$, 6 μM phenazine methosulfate (PMS), and varying amounts of NH_2OH.

	$[NO_2^-]$ (μM)			
$[NH_2OH]$ (μM)	**WT**	**WT, average**	**Phe41Ala**	**Phe41Ala, average**
0	0.8, 0.7, 0.8	0.8 ± 0.1	40, 41, 39	40 ± 1
100	53, 51, 57	53 ± 3	100, 104, 103	103 ± 2
250	150, 157, 148	152 ± 5	180, 175, 186	180 ± 5
500	301, 321, 306	309 ± 8	283, 284, 286	284 ± 2
1000	537, 522, 494	518 ± 15	420, 414, 421	421 ± 8

Table S7. Concentrations of N_2O measured by GC-MS for reactions under aerobic turnover conditions using 5 μM WT or Phe41Ala cyt P460, 2 mM $[Ru(NH_3)_6]Cl_3$, 6 μM phenazine methosulfate (PMS), and varying amounts of NH_2OH.

	$[N_2O]$ (μM)			
$[NH_2OH]$ (μM)	**WT**	**WT, average**	**Phe41Ala**	**Phe41Ala, average**
100	ND	ND	8, 12, 8	9 ± 2
250	5, 7, 8	6 ± 1	54, 55, 50	53 ± 3
500	39, 21, 25	28 ± 10	108, 146, 101	118 ± 24
1000	94, 118, 107	107 ± 12	320, 246, 249	272 ± 42

Table S8. Concentrations of N_2O measured by GC-MS for reactions under anaerobic turnover conditions using 5 μM WT or Phe41Ala cyt P460, 2 mM $[Ru(NH_3)_6]Cl_3$, 6 μM phenazine methosulfate (PMS), and varying amounts of NH_2OH.

	$[N_2O]$ (μM)			
$[NH_2OH]$ (μM)	**WT**	**WT, average**	**Phe41Ala**	**Phe41Ala, average**
100	ND	ND	35, 29, 27	30 ± 4
250	94, 123, 63	93 ± 30	180, 175, 160	171 ± 10
500	235, 276, 271	261 ± 22	301, 306, 284	297 ± 12
1000	393, 377, 394	388 ± 9	418, 457, 532	469 ± 58

Table S9. Primer sequences for generation of the *N. europaea* cyt P460 Phe41Ala, Phe41Trp, and Phe41Arg mutants. Fwd = forward; Rev = reverse.

Primer	Sequence
Phe41Ala Fwd	5′-GGT AAA GCT CCG GCT ACC GAA ATT CGC-3′
Phe41Ala Rev	5′-AGC CGG AGC TTT ACC-3′
Phe41Trp Fwd	5′-TAA AGC TCC GTG GAC CGA AAT TC-3′
Phe41Trp Rev	5′-CCA TCA TTC AGT TCG TTC GGC-3′
Phe41Arg Fwd	5′-TAA AGC TCC GCG TAC CGA AAT TCG-3′
Phe41Arg Rev	5′-CCA TCA TTC AGT TCG TTC GGC-3′

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