Supporting Information for:

Using an artificial tryptophan “wire” in cytochrome c peroxidase for oxidation of organic substrates

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1. Protein Expression and Purification

Proteins were expressed in BL21(DE3) *E. coli* cells in Luria broth supplemented with 30 µg/mL kanamycin. Expression cultures (1L) were inoculated with 5 mL of an overnight starter culture and grown to OD<sub>600</sub> = 0.7 at 37°C, followed by overnight induction with 1 mM IPTG at 31°C. Cells were harvested via centrifugation, resuspended in lysis buffer (50mM Tris•HCl, 100mM NaCl, 5mM imidazole, 1mM PMSF, pH 8) and lysed using ultrasonication. Following centrifugation to remove cellular debris, the supernatant was adjusted to pH 8 and loaded onto a Ni-NTA column. The column was washed with 3 column volumes of equilibration buffer (20mM Tris-HCl, 0.5M NaCl, 5mM imidazole, pH 8). The protein was eluted by increasing the imidazole concentration to 250mM. The buffer was exchanged to 100 mM sodium phosphate pH 7.5 and 1 equivalent of hemin chloride was added. Following a 3 hour incubation at 4°C, proteins were dialyzed overnight against 5 mM sodium phosphate pH 6. After ultrafiltration concentration, proteins were purified using ion exchange chromatography (DEAE was used for all proteins, except for A193W/Y229W, where a Q ion exchange column was used). Proteins were eluted using a NaCl gradient. Fractions were assayed by UV-Vis and MALDI-TOF. Pure fractions were pooled and concentrated using ultrafiltration.
Optical spectroscopy

The optical spectra for WT CcP and mutants were collected in 20 mM sodium phosphate buffer, pH 6. The extinction coefficients of the Soret bands were determined using pyridine hemochrome assays.\textsuperscript{1} Cpd I was generated by addition of approximately one equivalent of hydrogen peroxide, and spectra were collected immediately. To generate CN\textsuperscript{−} bound CcP, approximately one equivalent NaCN was added to ferric CcP solutions and spectra were collected immediately.

2. Optical spectra – CcP resting state

\textit{Figure S1.} Optical spectra of resting state WT Ccp and Trp-modified mutants.

\textsuperscript{1} E. A. Berry, B. L. Trumpower. \textit{Anal. Biochem.} 1987, \textbf{161}, 1-15.
3. Optical spectra – CcP compound I

![Optical spectra of Compound I WT Ccp and Trp-modified mutants.](image)

*Figure S2.* Optical spectra of Compound I WT Ccp and Trp-modified mutants.

4. Optical spectra – CcP ferric-CN\(^{-}\) complexes

![Optical spectra of WT Ccp and mutants coordinated to CN\(^{-}\).](image)

*Figure S3.* Optical spectra of WT Ccp and mutants coordinated to CN\(^{-}\).
5. Circular dichroism spectroscopy

Figure S4. Far UV CD spectra of WT CcP and each Trp-modified mutants. For all proteins, a total of five CD scans were taken and averaged with a step size of 1 nm and 0.5 seconds per point. Raw data were converted using the following formula: $\theta = \theta M/(n C l)$ where $M$, $n$, $C$, and $l$ represent molecular weight, number of residues, concentration in mg/ml and the cuvette path length, respectively. Values are reported as (deg$^2$ cm mol$^{-1}$) against wavelength (nm). All data were collected in 20 mM sodium phosphate buffer, pH 6.
6. Tryptophan Fluorescence

![Tryptophan Fluorescence Spectra](image)

**Figure S5.** Tryptophan fluorescence spectra of WT CcP and each Trp-modified mutants. All spectra were collected at 10 µM protein concentration in 20 mM sodium phosphate buffer, pH 6. Proteins were excited at 280 nm.
Mass spectrometry

Electrospray ionization mass spectrometry was initially used to confirm protein identity and purity. For rapid identification in routine protein expression experiments, matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry was used. Sinapinic acid was used as a MALDI matrix and mixed in equal amounts with protein solution using the dried droplet method. For reference, the WT Ccp construct expressed here has a predicted mass of 34416 Da. Observed (ESI-MS): 34418 Da. Observed (MALDI): 34425 Da.

7. Mass Spectrometry – CcP Y229W

![MALDI-TOF mass spectrum of Y229W. Predicted: 34439 Da. ESI-MS: 34442 Da. MALDI: 34431 Da.](image)

**Figure S6.** MALDI-TOF mass spectrum of Y229W. Predicted: 34439 Da. ESI-MS: 34442 Da. MALDI: 34431 Da.
8. Mass Spectrometry – CcP A193W

*Figure S7.* MALDI-TOF mass spectrum of A193W. Predicted: 34531 Da. ESI-MS: 34539 Da. MALDI: 34593 Da.
9. Mass Spectrometry – CcP Y229W/A193W

*Figure S8.* MALDI-TOF mass spectrum of CcP A193W/Y229W. Predicted: 34554 Da. ESI-MS: 34542 Da. MALDI: 34542 Da.
10. Compound I stability – pH 6

To assess stability of Cpd I, the absorbance at 424 nm was monitored over the course of 20 minutes. The reaction mixture contained 6.5 μM ferric CcP and 2 equivalents of H₂O₂. Data collection was initiated immediately upon addition of hydrogen peroxide. Protein samples were in 20 mM sodium phosphate buffer at pH values of 6 or 8.

![Absorbance over time graph](image)

*Figure S9.* Decay of absorbance at 424 nm upon addition of 2 equivalents of H₂O₂ to WT CcP and Trp-modified mutants at pH 6.
11. Compound I stability – pH 8

*Figure S10.* Decay of absorbance at 424 nm upon addition of 2 equivalents of H$_2$O$_2$ to WT CcP and Trp-modified mutants at pH 8.
12. EPR Spectra of Compound I for WT and A193W/Y229W CcP

EPR spectra were collected with samples held at 100 K. Cpd 1 was generated by the addition of 2 equivalents of H$_2$O$_2$ to 700 µM proteins samples in 20 mM sodium phosphate buffer, pH 6. Samples were flash frozen in liquid nitrogen immediately (≥1 min) after mixing. Microwave frequency = 9.382964 GHz

**Figure S11.** EPR spectra of WT CcP and A193W/Y229W Ccp at pH 6. Spectra are offset for clarity.

**Figure S12.** EPR spectra of WT CcP and A193W/Y229W Ccp at pH 8. Spectra are offset for clarity.
13. Veratryl alcohol oxidation assay – pH 4

Figure S13. Oxidation assay of veratryl alcohol to veratryl aldehyde. Traces are absorbance changes at 310 nm due to production of veratryl aldehyde. Assays contain: 10 µM protein, 2 mM veratryl alcohol and 400 µM H$_2$O$_2$ in 20mM sodium phosphate buffer, pH 4. The thin black line shows the same reaction without CcP.

Figure S14. Oxidation assay of veratryl alcohol to veratryl aldehyde. Traces are absorbance changes at 310 nm due to production of veratryl aldehyde. Assays contain: 10 µM protein, 2 mM veratryl alcohol and 400 µM H₂O₂ in 20mM sodium phosphate buffer, pH 6.
15. Expanded kinetics traces for pH 8 veratryl alcohol oxidation

**Figure S15.** Log(time) plot for oxidation assay of veratryl alcohol to veratryl aldehyde. Traces are absorbance changes at 310 nm due to production of veratryl aldehyde. Assays contain: 10 μM protein, 2 mM veratryl alcohol and 400 μM H$_2$O$_2$ in 20mM sodium phosphate buffer, pH 8.

**Figure S16.** Log(time) plot of the initial 60 seconds for oxidation assay of veratryl alcohol to veratryl aldehyde. Traces are absorbance changes at 310 nm due to production of veratryl aldehyde. Assays contain: 10 μM protein, 2 mM veratryl alcohol and 400 μM H$_2$O$_2$ in 20mM sodium phosphate buffer, pH 8.
16. Dye Decolorization Assays

**Figure S16.** Decolorization of ~64 µM Reactive Black 5. Traces are absorbance changes at 610 nm. Assays contain: 10 µM protein and 400 µM H₂O₂ in 20 mM sodium phosphate buffer, pH 8.

**Figure S17.** Decolorization of ~16 µM Coomassie Brilliant Blue. Traces are absorbance changes at 620 nm. Assays contain: 10 µM protein and 400 µM H₂O₂ in 20 mM sodium phosphate buffer, pH 8.
Figure S18. Decolorization of 42 µM azo violet (p-nitrophenylazoresorcinol). Traces are absorbance changes at 466 nm. Assays contain: 10 µM protein and 400 µM H$_2$O$_2$ in 20mM sodium phosphate buffer, pH 8.
Figura S19. Mapa de salto para A193W/Y229W CcP. Los parámetros ET utilizados para construir el mapa se proporcionan en el pie de la figura.