Supplemental Information for:

Quantifying Heme-Protein Maturation from Ratiometric Fluorescence Lifetime Measurements on the Single Fluorophore in Its GFP Fusion

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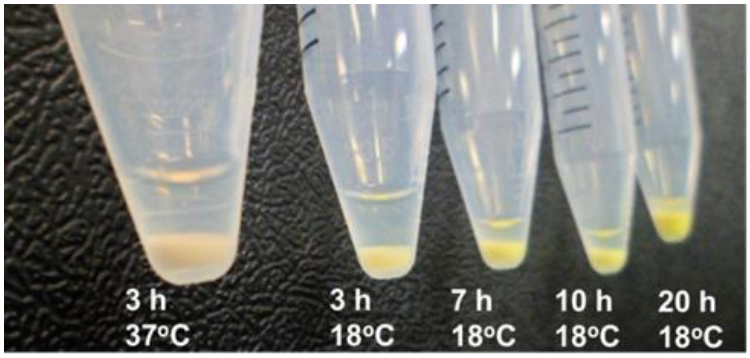

Figure S1. Time and temperature dependence of fluorophore maturation in Ccp1-GFP-His$_6$. Maturation of the GFP fluorophore is complete after 20 h at 18 °C. Note that maturation is not seen in the sample incubated at 37 °C for 3 h. See details of protein expression in the main text.

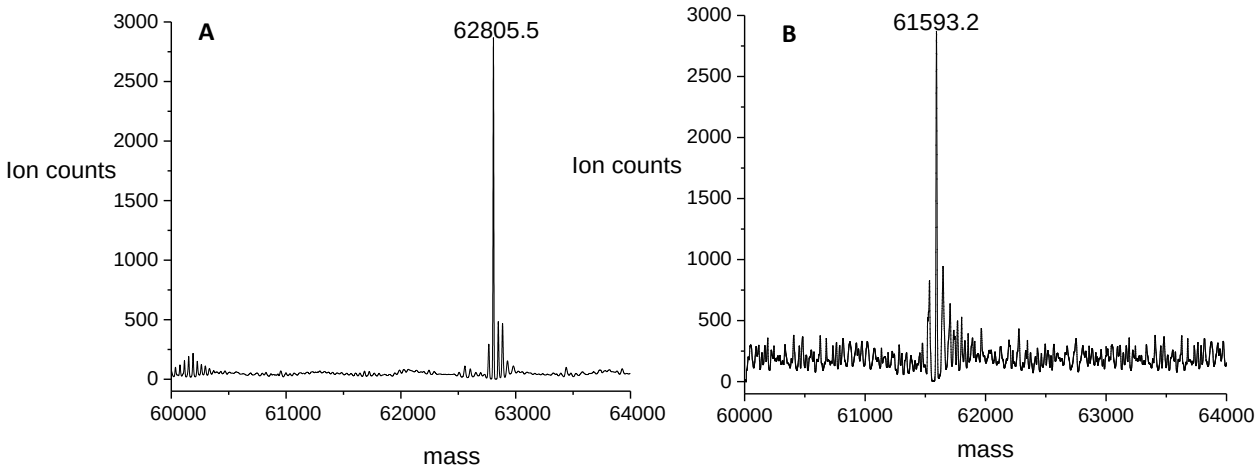

Figure S2. Deconvolved ESI mass spectrum of recombinant apoCcp1-GFP before and after His$_6$ tag removal. Spectra of: (A) Ni-NTA-purified apoCcp1-GFP-His$_6$ and (B) apoCcp1-GFP. To record the mass spectra, a 5-μL aliquot of 2 μM protein in 20 mM KPi plus 100 μM DTPA (pH 7.0) was injected onto a 2.1 x 150-mm C_3 column (Agilent) attached to an Agilent 1100 HPLC. Protein was eluted at 0.2 mL/min into the ESI source of a QToF2 mass spectrometer (Waters) using an acetonitrile gradient in 0.1% aqueous formic acid (0–95% acetonitrile in 15 min then 95% acetonitrile for 5 min). The Q-ToF2 parameters were: capillary +3.5 kV, cone +48 V, source temperature 80 °C, desolvation temperature 300°C, resolution 10,000, m/z range 500–2000. Using apomyoglobin as mass calibrant (Sigma), the mass accuracy was 72 ppm (avg mass: theoretical = 16,951.49 u; observed = 16,952.71 u).

The theoretical mass of Ccp1-GFP-His$_6$ is 62,801.4 u based on its sequence (https://web.expasy.org/compute_pi/) and a 20-u mass loss[1,2] on reaction of S65, Y66 and G67 to form the GFP fluorophore.[3] The observed mass of apoCcp1-GFP-His$_6$ was 62,805.5 ± 1.0 (65 ppm error), confirming that GFP was in its mature form. Removal of the His$_6$ tag (GSLEH$_6$) decreases the mass by 1,209.2 u to give a theoretical of 61,592.2 u vs an observed mass of 61,593.2 ± 0.7 u for apoCcp1-GFP (18 ppm error).

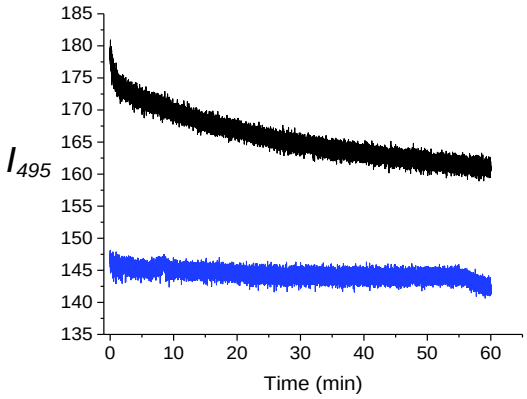

Figure S3. Room-temperature steady-state fluorescence at 495-nm of Ccp1-GFP over 60 min ± NaCl. Emission from 0.4 µM Ccp1-GFP in 20 mM MES pH 6.5, 100 µM DTPA without (black line) and with 100 mM NaCl (blue) as monitored following excitation at 488 nm using the Kinetics Module of the Cary Eclipse spectrofluorometer with a 15-W Xenon flash lamp with 1–1.5 µS flash duration. The emission intensity was captured every 0.1 s with 5-nm slits and the PMT at 600 V. Note that Cl^- binds near the GFP fluorophore and quenches fluorescence by electrostatically inhibiting its deprotonation, which lowers the concentration of the deprotonated, fluorescent form.[1] This property of FPs is exploited in halide-ion sensing.[2]

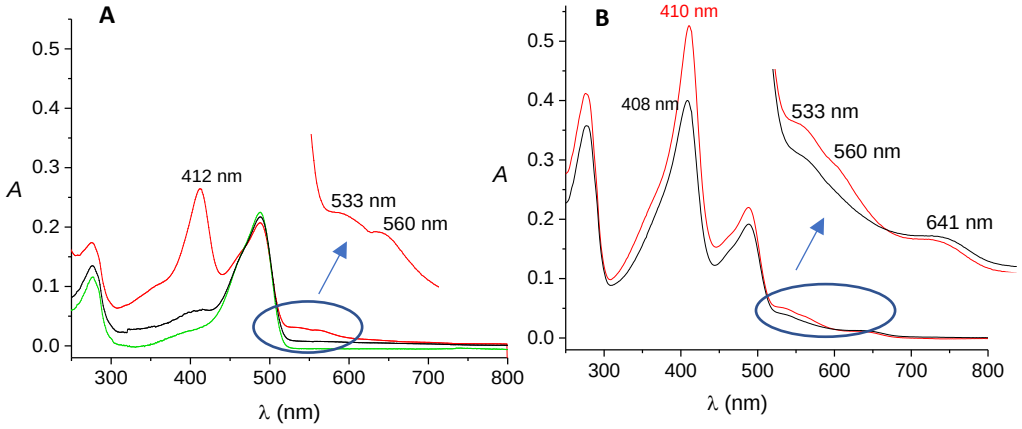

Figure S4. Effects of the His_6 tag on heme absorption of GFP and holoCcp1-GFP. (A) UV-vis spectra of: (A) 3 µM GFP-His_6 (green trace), GFP-His_6 + heme (red trace) and GFP + heme (black trace). (B) 5 µM holoCcp1-GFP-His_6 (red line) and holoCcp1-GFP (black line). The inserts show a 20X expansion of the visible regions. Spectra were recorded in 20 mM MES with 100 mM NaCl and 100 µM DTPA (pH 6.5) after removal of excess heme on a DEAE column as outlined under *Materials and Methods* of the main text.

Panel A: Adding heme to GFP-His_6 gives bands at 533 and 560 nm indicative of 6-coordinate heme. **Panel B:** holoCcp1-GFP-His_6 also exhibits bands at 533 and 560 nm as well as a band at 641 nm, which is assigned to the 5-coordinate heme of Ccp1. After any excess heme is removed by DEAE, holoCcp1-GFP and holoCcp1-GFP-His_6 exhibit Soret maximum at 408 and 410 nm plus a 408/280 ratio of 1.1 and 1.3, respectively, indicative of heme binding to the His_6 tag.

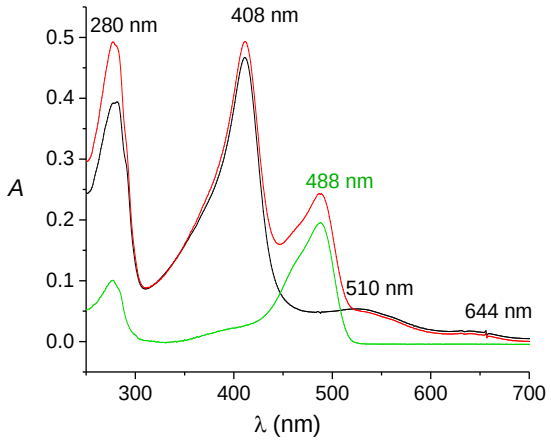

Figure S5. Sum of the absorption spectra of GFP and holoCcp1. The room-temperature UV-vis spectrum of 5 µM GFP (green trace) was added to that of 5 µM holoCcp1 (black trace) to predict the spectrum of holoCcp1-GFP (red trace). Proteins were dissolved in 10 mM KPi, 10 mM MES with 100 mM NaCl and 100 µM DTPA (pH 7.0) and spectra were recorded in a 1-cm pathlength.

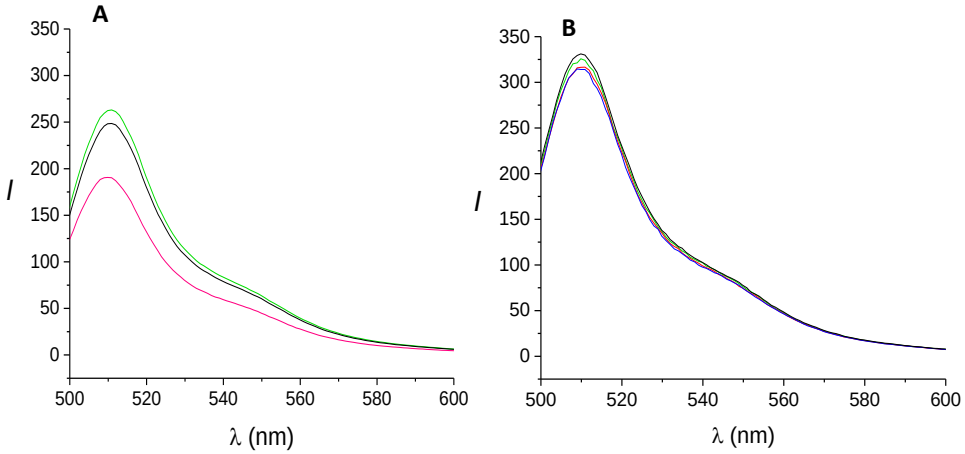

Figure S6. Effects of heme and heme proteins on the steady-state fluorescence of GFP and GFP-His$_6$. Emission spectra of: (A) GFP (green line), GFP-His$_6$ (green line; note the emission of both samples is identical), GFP plus heme (black line) and GFP-His$_6$ plus heme (red line) in 10 mM KPi, 10 mM MES (pH 7.0); (B) GFP-His$_6$ (green trace), GFP-His$_6$ plus holoCcp1 (black trace), GFP-His$_6$ plus holomyoglobin (red trace), and GFP-His$_6$ plus bovine serum albumin (blue trace) in 20 mM KPi (pH 7.0). All proteins were 0.4 µM, and 100 mM NaCl plus 100 µM DTPA also were present in all samples. BSA serves as a non-heme-protein control. Heme was added (as hemin protoporphyrin IX chloride) in 1.1 molar excess to the 1-cm cuvettes where indicated. Spectra were recorded at room temperature with 488-nm excitation and 5-nm slits (see caption to Figure 2 of the main text).

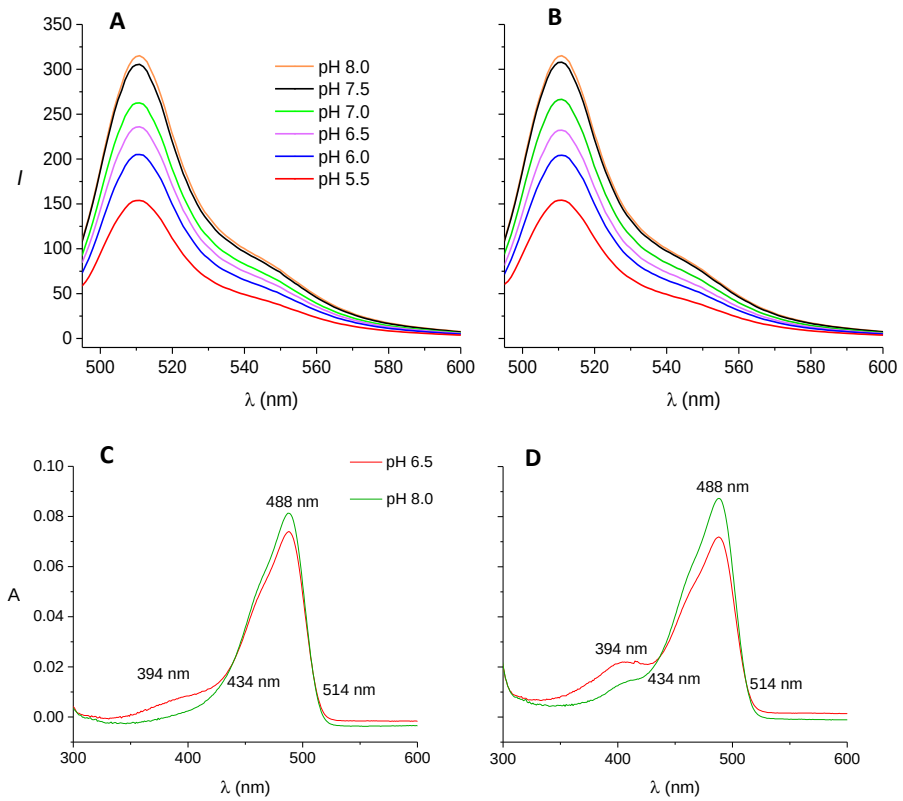

Figure S7. Changes in the steady-state fluorescence and absorption of GFP and apoCcp1-GFP due to the pH-sensitive equilibrium between the nonfluorescent A and fluorescent B ground states of the fluorophore. The emission spectrum following 488-nm excitation of 0.4 µM: (A) GFP-His_6 and (B) apoCcp1-GFP-His_6 in 10 mM KPi, 10 mM MES with 100 mM NaCl and 100 µM DTPA. The absorption spectrum at pH 6.5 (red trace) and 8.0 (green trace) of: (C) GFP-His_6 and (D) apoCcp1-GFP-His_6 in a 1-cm cuvette at room temperature. To adjust the pH, HCl or NaOH was added to 2 µM protein in 20 mM KPi, 100 µM DTPA (no NaCl here). The 394-nm band of the protonated form A of GFP, which is nonfluorescent,[1] grows in at the expense of the 488-nm band of the deprotonated, fluorescent state B as the pH is decreased. Also, weak heme absorption can be seen at ~ 400 nm in panel D since apoCcp1-GFP-His_6 is isolated with trace heme. Fluorescence spectra were recorded as outlined in the caption to Figure 2C of the main text.

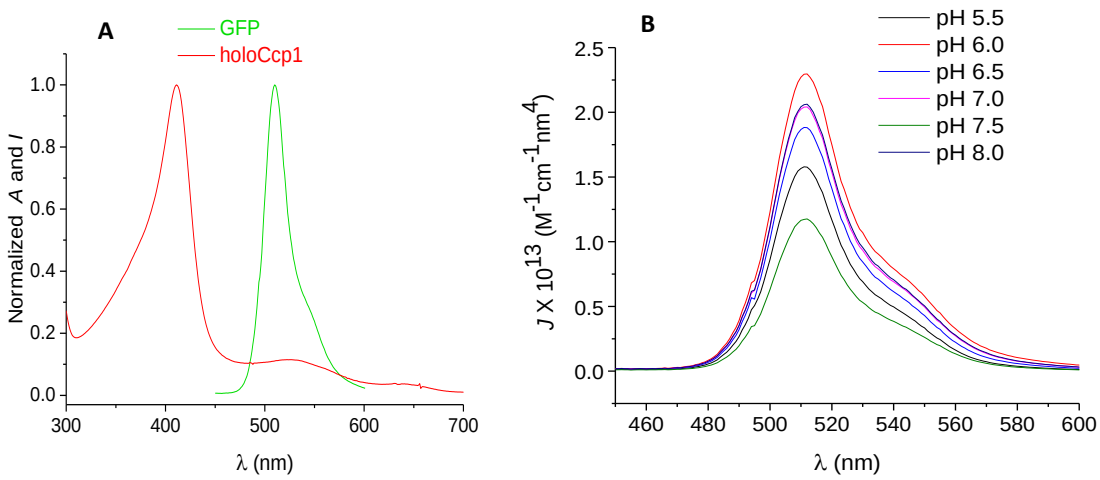

Figure S8. Overlap of GFP emission with holoCcp1 absorption and plot of spectral overlap integrals. (A) Emission spectrum of 0.4 μM GFP and absorption spectrum of 3 μM holoCcp1 at pH 6.5 in a 1-cm cuvette at room temperature. (B) Spectral overlap integral *J* ($M^{-1}cm^{-1}nm^4$) vs λ between GFP emission and holoCcp1 absorption at pH 5.5–8.0. All samples were prepared in 10 mM KPi, 10 mM MES with 100 mM NaCl and 100 μM DTPA. The pH was adjusted with NaOH or HCl.

The visible heme bands at 510 and 644 nm of holoCcp1 (Figure S5) overlap GFP emission at 510 nm (Figure S8A). Table S1 lists the value of *J*, the overlap integral, summed over all wavelengths between the GFP donor and Ccp1-heme acceptor for each pH. The average value over the pH range examined is 7.32 x 101^4 M^{-1} cm^{-1} nm^4. This yields an average R_o of 43.80 Å (Table S2) using Hink's method,[4] which assumes values of 0.67 for *k* (random orientation of the donor and acceptor transition dipoles) and 1.4 for the refractive index (*n*) of the medium between the donor and acceptor. R_0 was calculated by filling in the following information into the template[4] in Hink's method: the emission spectrum of the donor (GFP), the absorption spectrum of the acceptor (heme of holoCcp1), the extinction coefficient of heme at its lambda max and the quantum yield of GFP (0.64)[5]. From the FRET efficiency and τ_1 and τ_2 of holoCcp1-GFP, the heme-fluorophore separation ***r*** can be calculated from the following equations:

$$E = 100\left(1 - \frac{\tau_n}{\tau_0}\right)$$

$$r = \sqrt[6]{\frac{R_0{}^6 - ER_0{}^6}{E}}$$

Table S2 summarizes the values of *E* and *r* calculated at each pH for both τ_1 and τ_2. For E_1 = 0.66 ± 0.03 and E_2 = 0.12 ± 0.01 the average values of *r* are 39 ± 2 and 61 ± 2 Å, respectively. These distances correspond closely to the separation expected between the heme and GFP fluorophore with the GRRIPGLIN linker in helical and extended conformations, respectively (Figure 7 of the main text).

Table S1. Spectral overlap integral *J* between GFP emission and holoCcp1 absorption at pH 5.5–8.0

pH	Spectral overlap integrand (J) $M^{-1}cm^{-1}nm^4$	R_0 Å
5.5	5.79×10^{14}	42.19
6.0	8.93×10^{14}	45.36
6.5	7.06×10^{14}	43.61
7.0	7.82×10^{14}	44.36
7.5	6.41×10^{14}	42.91
8.0	7.94×10^{14}	44.48
Average	$7.32 \times 10^{14} \pm 1.04$	43.80 ± 1.05

Table S2. Average separation (*r*) between GFP fluorophore and Ccp1 heme in holoCcp1-GFP at pH 5.5–8.0 [a]

pH	8.0	7.5	7.0	6.5	6.0	5.5	Average
τ_2 (ns)	2.57	2.54	2.51	2.49	2.49	2.48	2.51 ± 0.04
τ_1 (ns)	1.09	1.04	1.00	0.97	0.93	0.86	0.98 ± 0.07
a_2	66	64	63	62	61	58	62 ± 2.5
a_1	34	36	37	38	39	42	38 ± 2.5
E_2	0.11	0.12	0.12	0.13	0.13	0.14	0.12 ± 0.01
E_1	0.62	0.64	0.65	0.66	0.68	0.70	0.66 ± 0.03
r_2 (Å)	64	60	61	60	62	57	61 ± 1.5
r_1 (Å)	41	39	40	39	40	37	39 ± 1.5

[a] Average values of τ and fractional amplitude *a* for three independent samples of holoCcp1-GFP at each pH. Lifetimes were measured by TCSPC (see Figure 3 of the main text). *E* and *r* values were calculated from the equations given above.

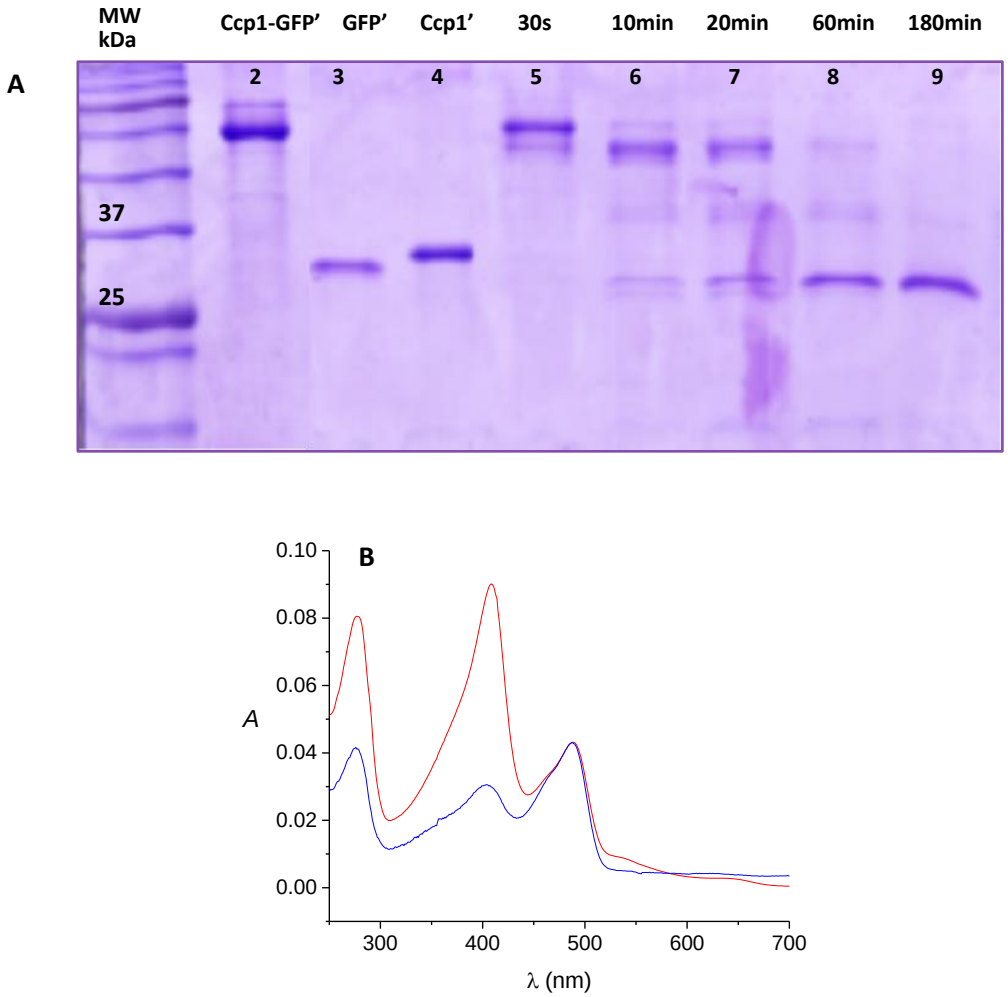

Figure S9. Tryptic digestion of holoCcp1-GFP-His$_6$ releases intact GFP. (A) A 25-μL aliquot of 1 μM holoCcp1-GFP-His$_6$ (lane 2, labeled Ccp1-GFP'), GFP-His$_6$ (lane 3, labeled GFP'), holoCcp1-His$_6$ (lane 4, labeled Ccp1'), and a 30-s, 10–180-min tryptic digests of 1 μM holoCcp1-GFP-His$_6$ (lanes 5–9) were loaded on a 12% SDS-page gel. Tryptic digestion was performed with 1:50 tryspin : protein at 37 °C in 20 mM KPi, pH 7.4, 100 μM DTPA. (B) UV-vis spectrum of the 30-s (red trace) and 180-min (blue trace) tryptic digests of holoCcp1-GFP-His$_6$. The 180-min digest was added to a 10 x 2-cm P10 column to remove molecules < 5 kDa by gel filtration before its spectrum was recorded. Also, 500 μL of Ni-NTA resin was added to 100 μL of the 30-s digest but protein remained in the supernatant, indicating that the His$_6$ tag was rapidly cut by trypsin.

Note that the main species in the 180-min digest (lane 9) appears just slightly lower in the gel than GFP-His$_6$ (lane 3), indicating that it is tag-free GFP, which is resistant to trypsin, as confirmed by no loss of GFP absorption at 488-nm in panel B (blue vs red trace). In contrast, no holoCcp1 remains in the 180-min digest (lane 9) but the remaining Soret absorption (panel B, blue trace) suggests that some of the digestion products of Ccp1 bind heme.

Supplemental references

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