SUPPORTING INFORMATION

Cytochrome *c* as a Peroxidase: Activation of the Pre-Catalytic Native State by H_2O_2-Induced Covalent Modifications

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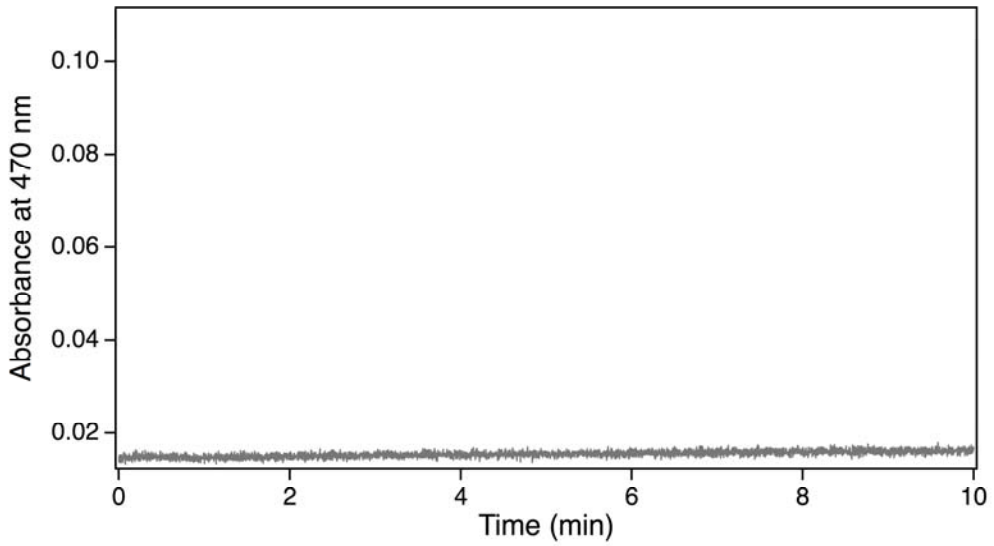
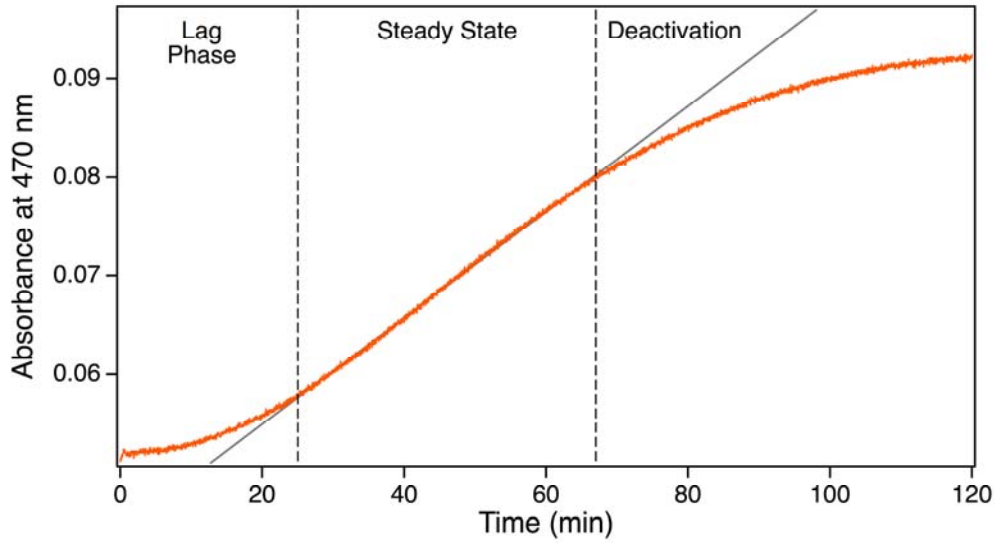
This file contains:

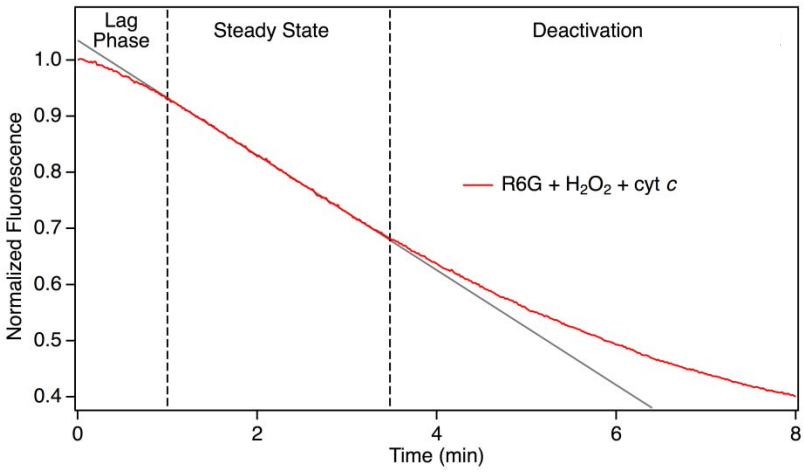
Supporting Figures



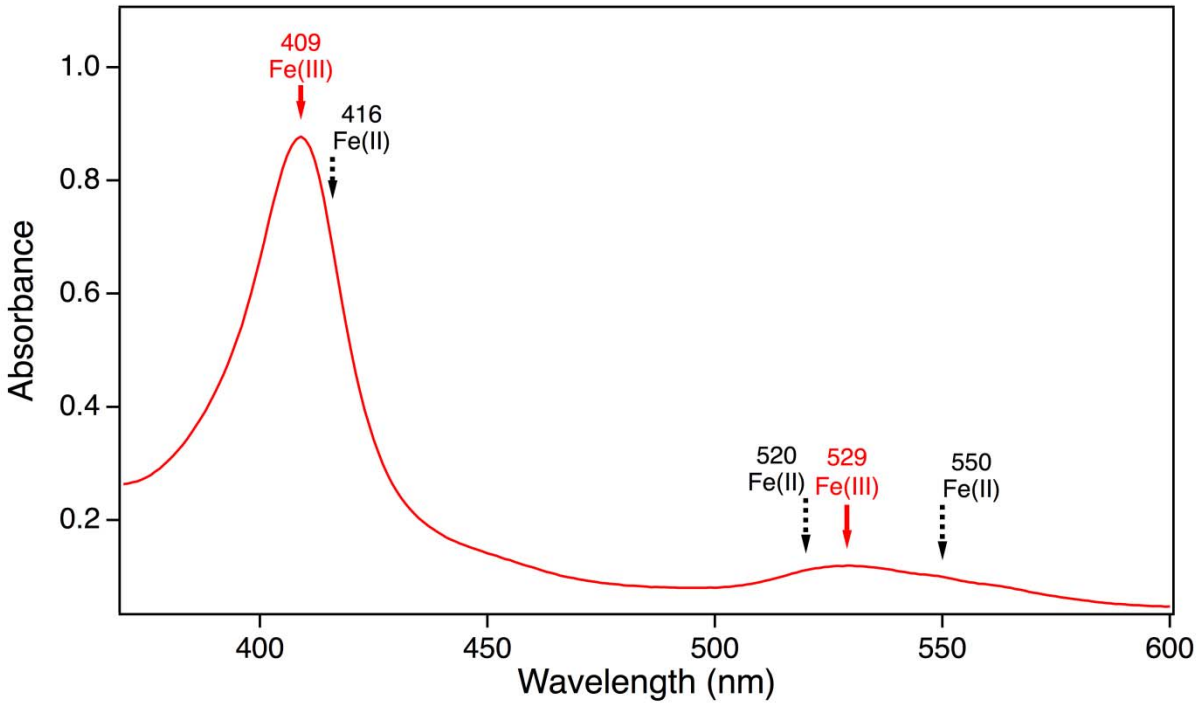
Supporting Figure S1. Peroxidase activity of cyt *c* measured after pre-incubation of the protein with 500 μM H_2O_2 for 15 min, prior to addition of guaiacol. No product formation is detectable under these conditions as a result of oxidative self-degradation.



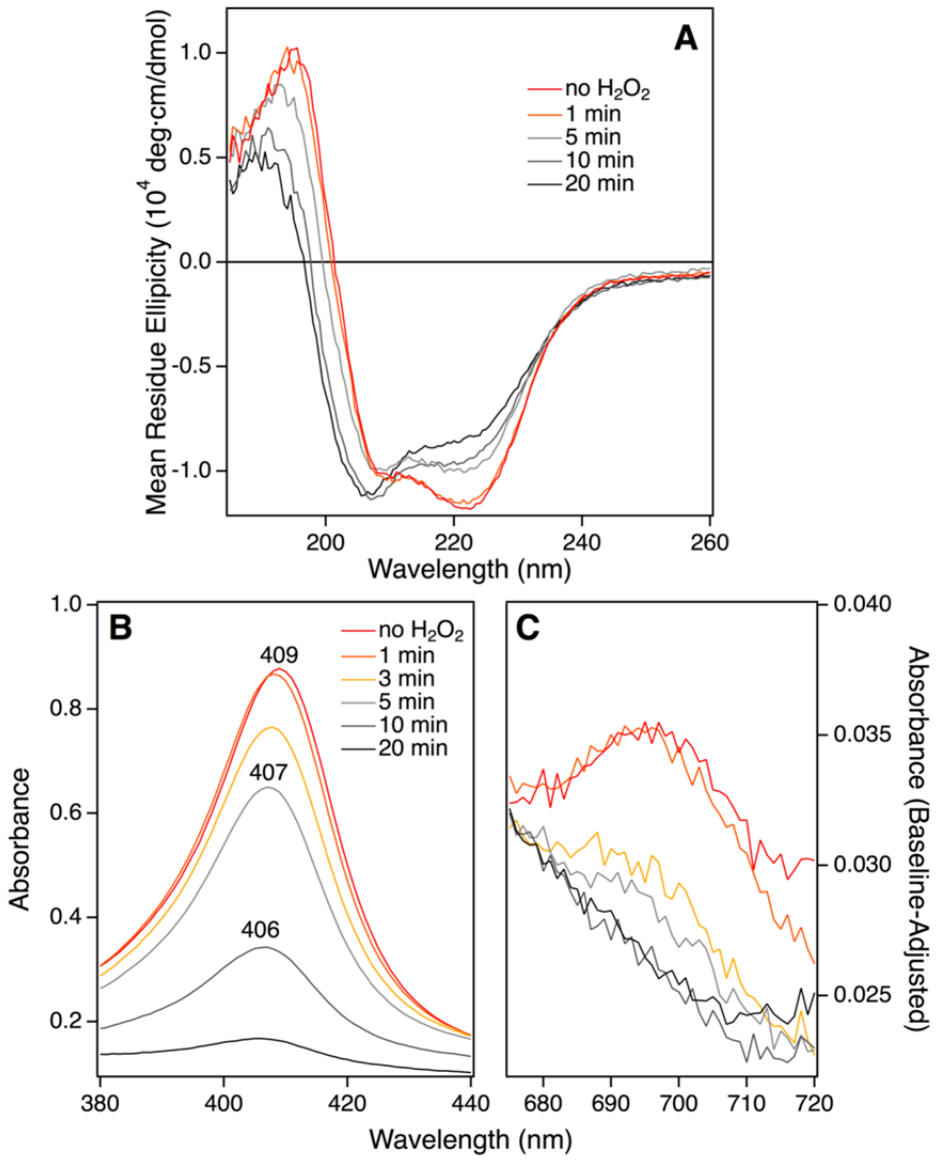
Supporting Figure S2. Peroxidase activity of 1 μM cyt *c* measured by probing the oxidation of guaiacol as in Figure 2 (main text) but in the presence of 50 μM H_2O_2 instead of 500 μM. A fit to the linear region (solid black line) yields a reaction rate of 0.0013 μM s^{-1}.



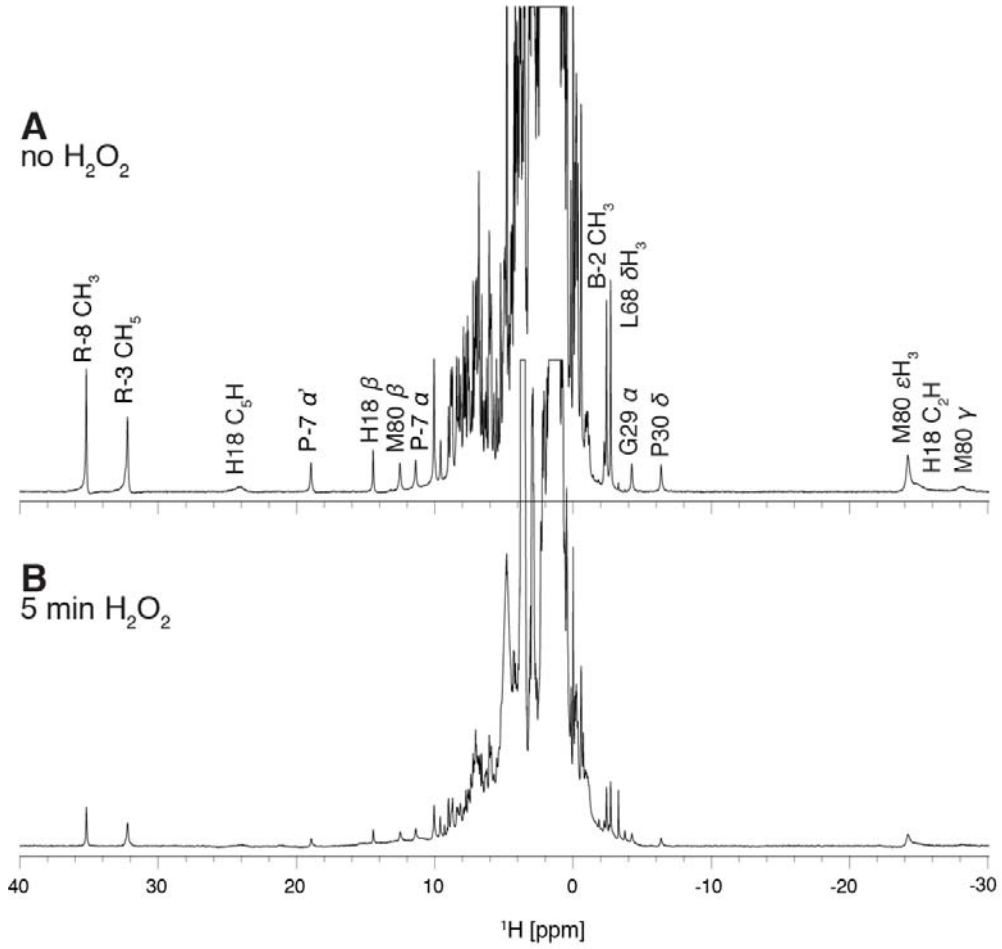
Supporting Figure S3. Peroxidase activity of 10 μM cyt *c* probed by fluorescence bleaching of 10 μM rhodamine 6G (R6G) in the presence of 500 μM H_2O_2. A three-stage kinetic progression similar to that of guaiacol is observed (see Figure 2, main text). Fluorescence experiments were performed on a Fluorolog-3 fluorimeter (Horiba Jobin Yvon; Edison, NJ), using an excitation wavelength of 526 nm, and an emission wavelength of 555 nm.



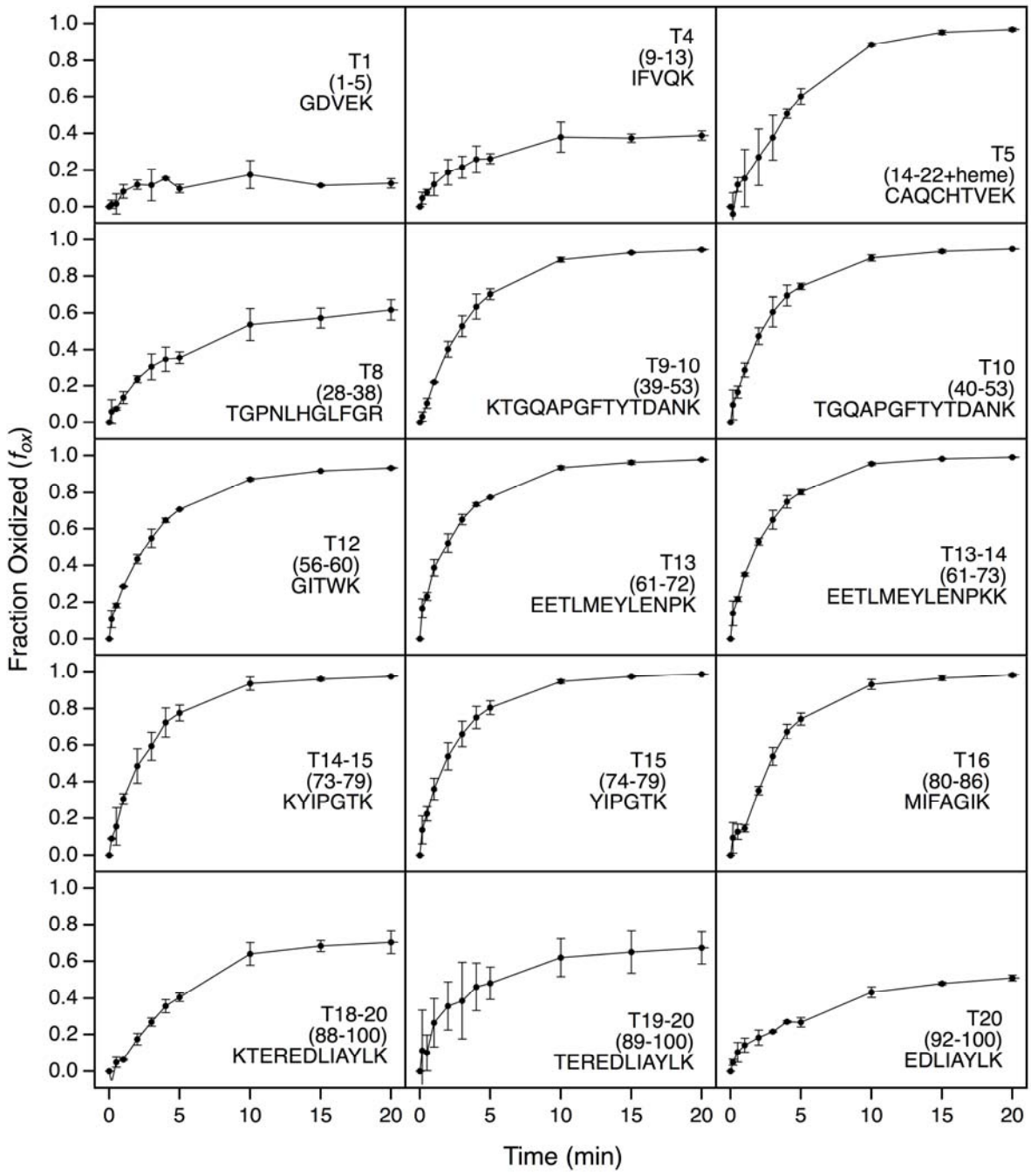
Supporting Figure S4. UV-Vis absorption spectrum of cyt *c* used in this work, prior to H_2O_2 exposure. These data confirm the oxidation state of the heme iron as Fe(III). The measured spectral maxima (red) are consistent with literature values for the Fe(III) form, whereas there is no match with the peak positions expected for Fe(II) cyt *c* (black).[1,2]



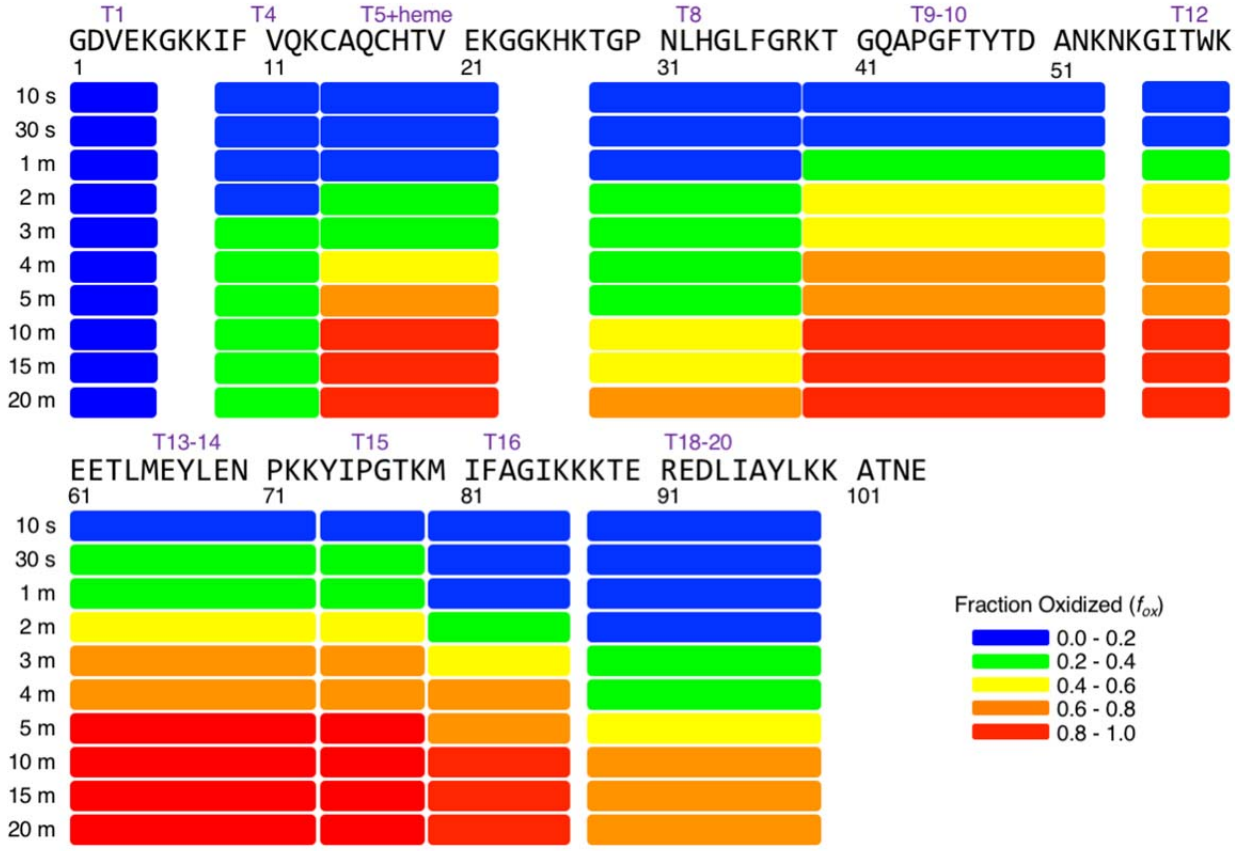
Supporting Figure S5. Spectroscopic evidence for cyt *c* time-dependent structural changes in 500 μM H_2O_2. (A) Far-UV CD spectra. (B) UV-Vis absorption spectra of the heme Soret band. (C) Absorption spectra of the A695 band, which reports on the Met80-Fe interaction. For better visualization spectra in panel C were base-line shifted such that they all had the same absorbance at 670 nm.



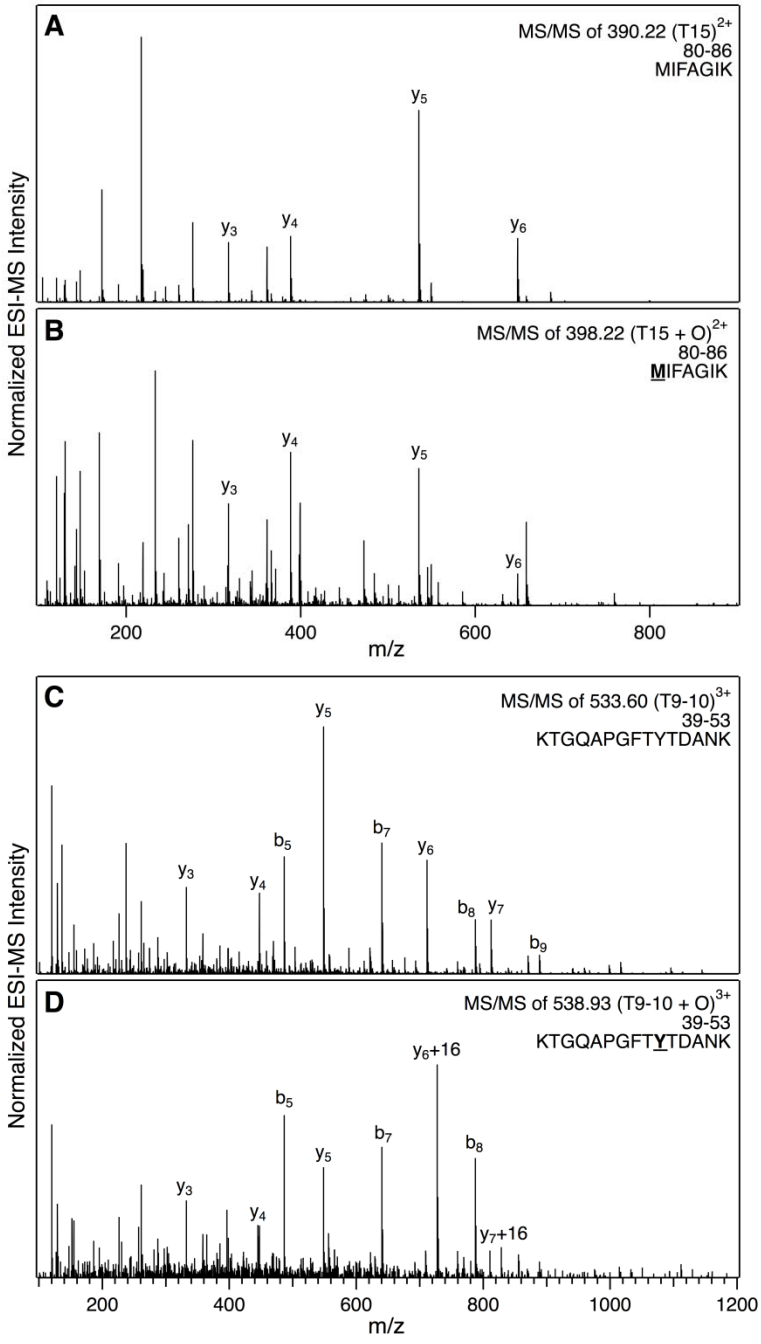
Supporting Figure S6. 1H-NMR spectra of cyt *c*. (A) No H_2O_2 added. (B) After 5 min of incubation in 500 μM H_2O_2 and subsequent catalase quenching. Peaks were assigned and denoted according to Feng et al.[3] A global loss in signal intensity after H_2O_2 incubation is consistent with partial heme degradation, as also confirmed by optical data of Figure 3B in the main text.



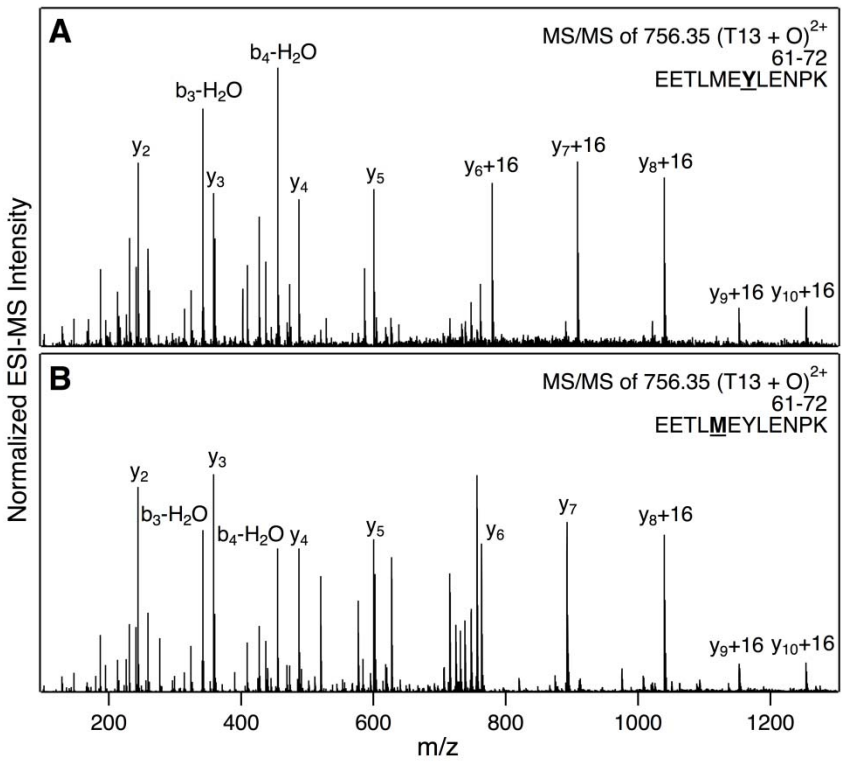
Supporting Figure S7A. Fraction oxidized (f_{OX}) plots of tryptic cyt *c* peptides following incubation in 500 μM H_2O_2 and catalase quenching. Standard deviations of two replicates are depicted as error bars.



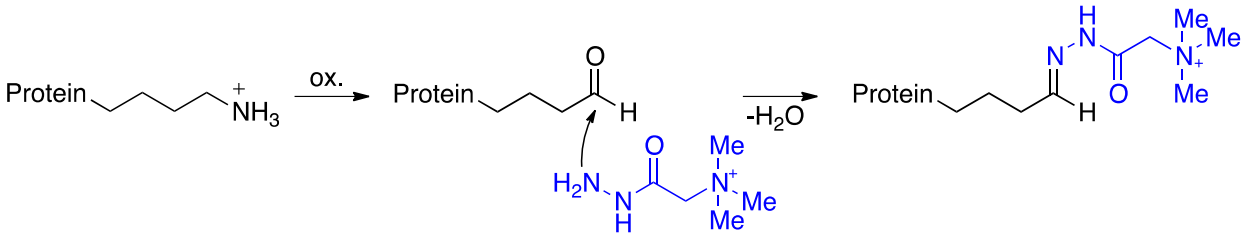
Supporting Figure S7B. Graphic representation of f_{OX} data as a function of H_2O_2 exposure time (from panel A), using the same color-coded layout as in Figure 4B (main text).



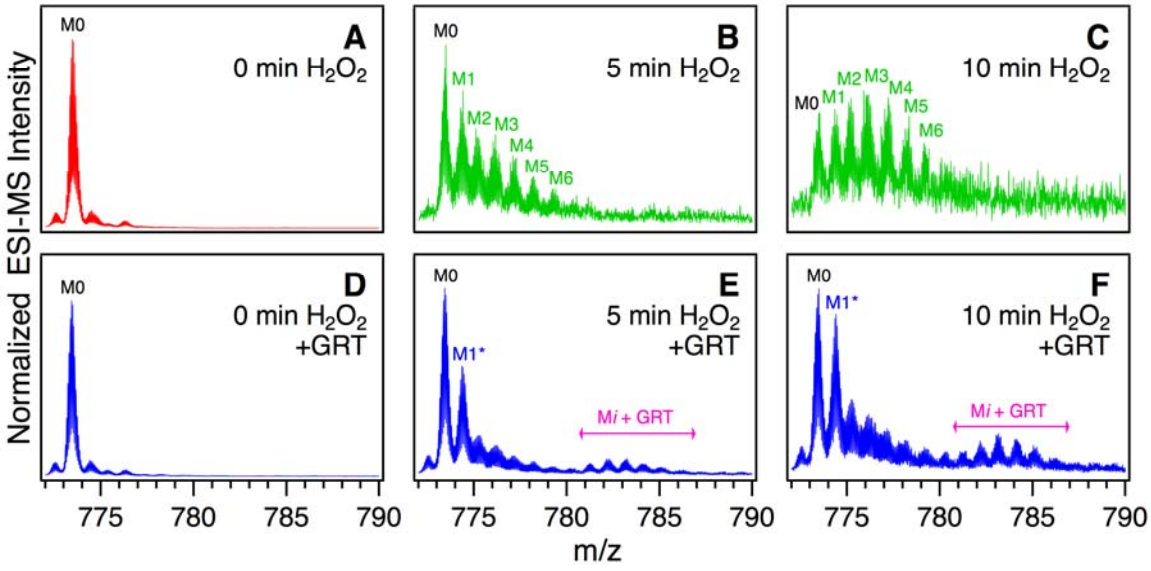
Supporting Figure S8. Representative tandem mass spectra of tryptic peptides, illustrating the identification of oxidation sites. MS/MS data of oxidized peptides were obtained after 30 min of cyt *c* incubation in 500 μM H_2O_2, followed by tryptic digestion. (A) Unmodified T15. (B) T15 oxidized at Met80 (MetO formation, +16 Da, bold/underlined). (C) Unmodified T9-10. (D) T9-10 oxidized at Tyr48 (+16 Da, bold/underlined).



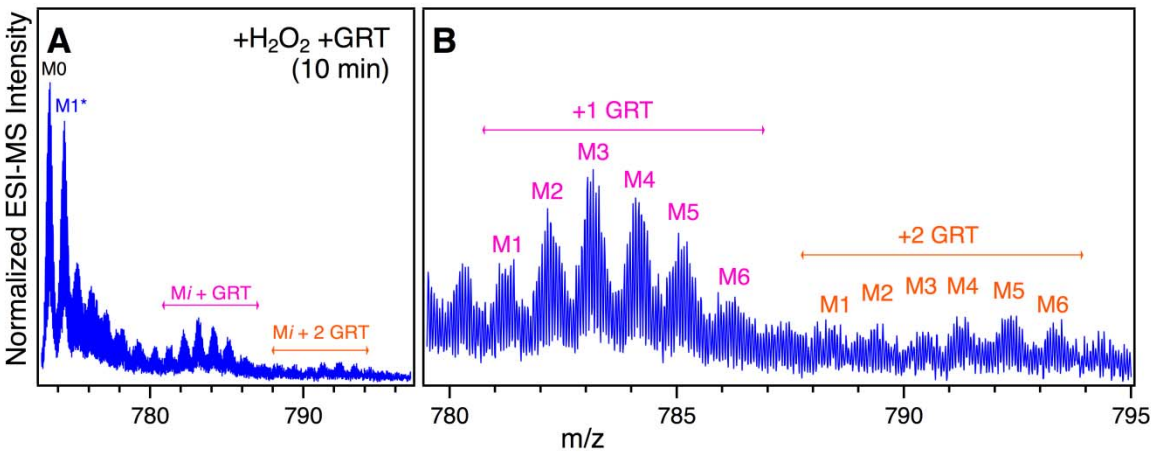
Supporting Figure S9. Representative tandem mass spectra of tryptic cyt *c* peptides obtained in control experiments. (A): Y67 oxidation (+16 Da) generated after incubation of pre-digested tryptic peptides in H_2O_2 (30 min). (B) M65 oxidation (+16 Da) generated after cyt *c* oxidation using chloramine-T.[4] Chloramine-T treatment was performed by adding 2.5 mM of the oxidant to buffered protein solution, followed by room temperature incubation for 1 hour. Reactions were stopped by exchange into phosphate buffer using 10 kDa MWCO centrifuge filters that were spun 3 times, each cycle being 15 minutes at 10 kG.



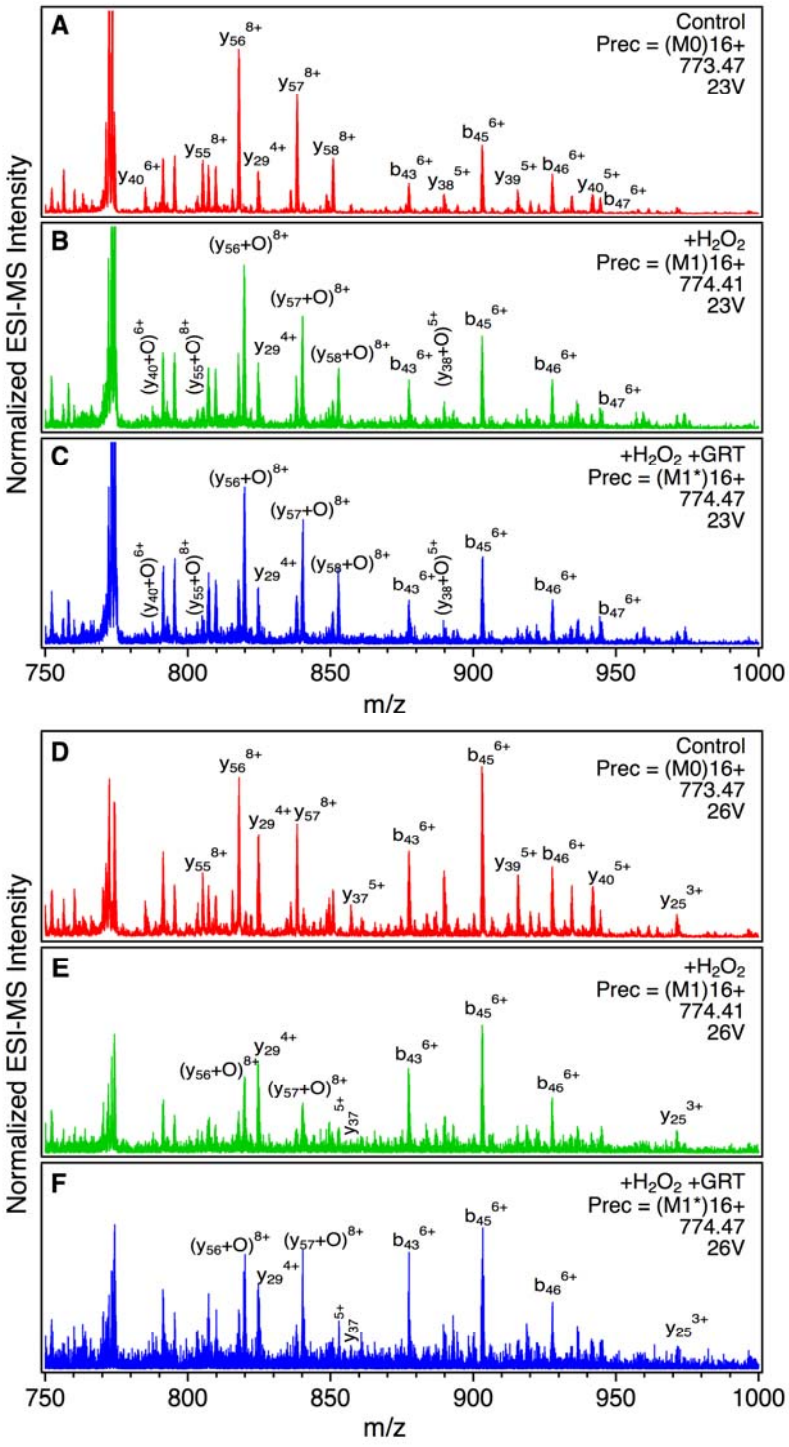
Supporting Figure S10. Assay for Lys carbonylation, i.e., oxidation to aminoadipic semialdehyde, Δm = -1.032 Da. Covalent attachment of the hydrazide-containing label, Girard's Reagent T (GRT) takes place via Schiff base formation. This results in $\Delta m/z$ = +112.063, when going from a Lys^+-containing reactant to the GRT-labeled product. The quaternary ammonium group adds a permanent cationic moiety to facilitate MS detection. Also, it possesses a higher solubility in aqueous solution compared to the previously used Girard's reagent P.[5]



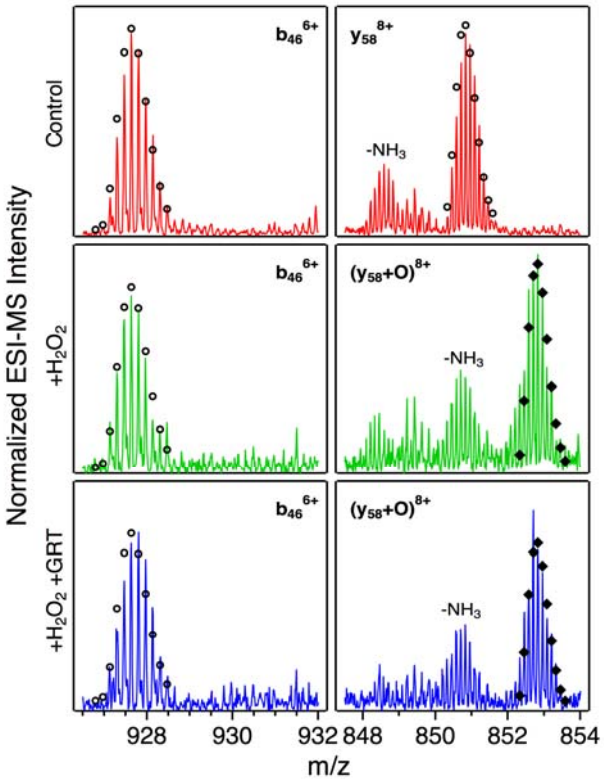
Supporting Figure S11. Intact protein mass spectra of cyt c^{16+} following H_2O_2 incubation and subsequent labeling with GRT. (A) unmodified cyt *c*. (B, C) After increasing lengths of H_2O_2 incubation. (D) – (F) show spectra of the corresponding samples after GRT labeling. Note that GRT labeling is detectable only after H_2O_2 exposure, reflecting the formation of reactive carbonyl groups.



Supporting Figure S12. Mass spectral data for cyt *c* following 10 min incubation in 500 μM H_2O_2 and subsequent GRT labeling. The populations of singly and doubly-adducted GRT are highlighted in magenta and orange, respectively. (A) Overview of the 16+ range. (B) Expanded view of the m/z range corresponding to GRT addition.



Supporting Figure S13. Complete top-down tandem mass spectra produced by CID of cyt c^{16+}. The data of Figure 8 (main text) are a subset of the spectra shown here. (A) Spectrum obtained after fragmentation of (A) M0, (B) M1, and (C) M1* at a collision voltage of 23 V. Panels (D) – (F) show spectra obtained for the same precursor ions, but at a slightly elevated collision voltage of 26 V.



Supporting Figure S14. Representative top-down MS/MS fragment ions of cyt *c* preceding and following H_2O_2 incubation and/or GRT labeling. The complementary fragments b_{46} and y_{58} completely cover the protein sequence, confirming the minor signal in y_{58} to arise from neutral (ammonia) loss, not oxidation site heterogeneity.

SI References

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(2) Gu, J.; Shin, D.-W.; Pletneva, E. V. *Biochemistry* **2017**, *56*, 2950−2966.
(3) Feng, Y. Q.; Roder, H.; Englander, S. W. *Biophys. J.* **1990**, *57*, 15-22.
(4) Capdevila, D. A.; Marmisolle, W. A.; Tomasina, F.; Demicheli, V.; Portela, M.; Radi, R.; Murgida, D. H. *Chem. Sci.* **2015**, *6*, 705-713.
(5) Yang, Y.; Stella, C.; Wang, W. R.; Schoneich, C.; Gennaro, L. *Anal. Chem.* **2014**, *86*, 4799-4806.