LC-MS/MS proteoform profiling exposes cytochrome c peroxidase self-oxidation in mitochondria and functionally important hole hopping from its heme

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SUPPORTING INFORMATION

SI EXPERIMENTAL PROCEDURES

Summary:

Oxidation of recombinant Ccp1 by H_2O_2 in vitro and oxidation of the protein in cells was examined by LC-MS/MS. Recombinant Ccp1 and the protein isolated from cells were digested with trypsin for LC-MS/MS analysis of the tryptic peptides (Figure S1) and identification of the residues oxidized.

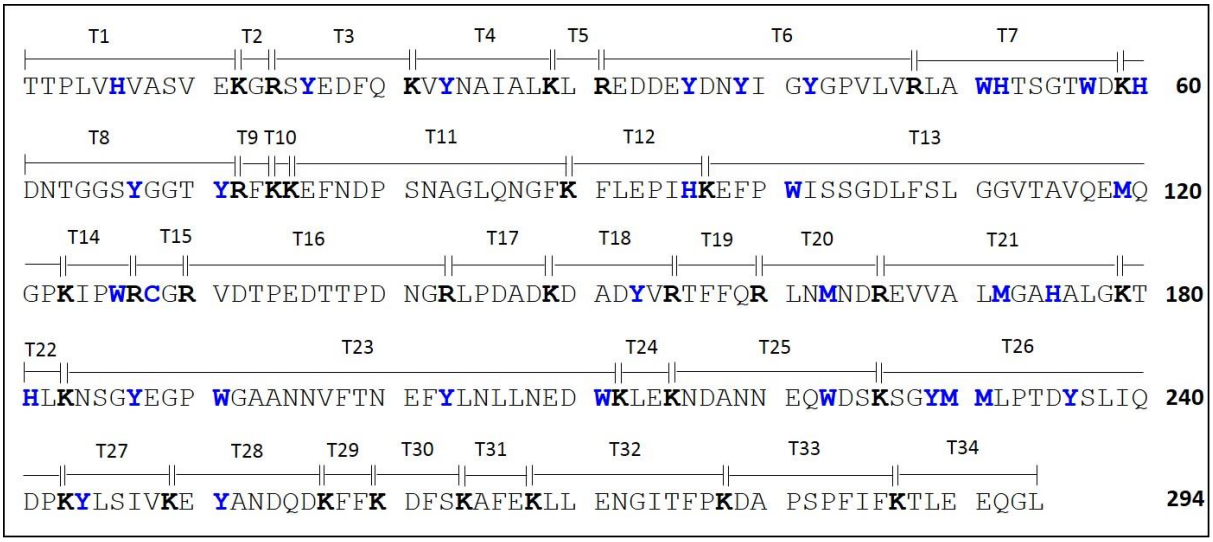

Figure S1. Map of tryptic peptides of Ccp1. The 33 potentially redox-active residues are in **blue font**.

Materials:

Recombinant Ccp1 with MI at positions -2/-1 of the mature protein was prepared as described previously.[1] Chemicals and biochemicals were purchased from Sigma with the following exceptions: sequencing grade modified trypsin (Promega); C18 Zip tips (EMD Millipore), Coomassie (MP Biomedicals); hydrogen peroxide 30%, glucose (ThermoFisher Scientific); yeast extract, peptone, microbiology grade agar, galactose (Bioshop); Zymolase 20T (Amsbio); 7×25 mm HiTrap Q anion-exchange column (GE Healthcare). Immunodot blotting was performed with rabbit anti-Ccp1 serum (kindly provided by Professor David Goodin, University of California, Davis), and HRP-conjugated secondary antibody (goat anti-rabbit, Biorad).

Methods:

Bolus vs. stepwise oxidation of recombinant Ccp1 with H_2O_2 in vitro. As described previously,[2] a 5 µM Ccp1 stock solution was prepared in 20 mM KPi pH 7.5 with 100 µM diethylenetriaminepentaacetic acid (DTPA). H_2O_2 in the same buffer was added to Ccp1 in a single aliquot (bolus) or stepwise in 10 equal aliquots (one every 10 min) to give samples with a final Ccp1 and H_2O_2 concentration of 1 and 10 µM, respectively, at room temperature. Where indicated, 10 mM glutathione (GSH) also was present in the Ccp1 solution before H_2O_2 addition. Ccp1 at 1 µM does not undergo the H_2O_2-induced *inter*molecular dityrosine crosslinking[2] that has been documented at higher

Ccp1 concentrations. DTPA was present in all samples to inhibit catalysis of H_2O_2 or O_2 oxidation of Ccp1's residues and of GSH by trace metal impurities in the buffers. Catalase (0.1 nM) was routinely added although H_2O_2 was not detected in the samples by the HRP/ABTS assay following incubation for 100 min.[2]

Yeast growth conditions. The *S. cerevisiae* BY4741 strain (Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; EUROSCARF) was grown in YPD liquid medium (1% yeast extract, 2% peptone and 2% glucose) at a medium-to-flask ratio of 1:5. The cultures at an initial OD_{600} of 0.01 were incubated at 30°C with shaking at 225 rpm. After 72 h, the spent YPD medium was replaced with 0.85% NaCl solution (w/v) to switch cells to stationary phase.[3]

Subcellular fractionation of the yeast lysates. Mitochondria-enriched (P10) and mitochondria-depleted (S10) subcellular fractions were isolated from yeast as described previously.[4] Briefly, cells were grown in 500 mL of YPD medium for up to 7 days at 30 °C as described above, harvested at 2,000 x g and washed twice with aqueous 0.85% NaCl. The cell pellets were resuspended in 50 mL of pre-warmed 100 mM Tris-H_2SO_4 (pH 9.4) with 10 mM DTT, incubated for 10 min at 30 °C and 80 rpm, harvested at 2,000 x g, washed twice with 20 mL of 10 mM KPi (pH 7.4) containing 1.2 M sorbitol, and treated with 12 mg of Zymolyase per g of wet cells at 30 °C. Spheroplast formation was monitored by light microscopy and was complete after 1-2 h incubation at 30 °C with gentle agitation. The resulting spheroplasts were washed twice with 20 mL of the same buffer and resuspended in 20 mL of 10 mM Tris (pH 7.4) containing 0.6 M sorbitol, 1 mM EDTA, 1 mM PMSF and protease inhibitor cocktail. Spheroplasts at 4 °C were disrupted by 15 strokes of a glass-Teflon homogenizer, the homogenates were spun at 2,000 x g and the supernatants, which correspond to the denucleated lysates (S2 fractions), were collected. The S2 fractions were further centrifuged at 10,000 x g for 15 min at 4°C, and the mitochondria-depleted supernatants (S10 fractions) were separated from the mitochondria-enriched pellets (P10 fractions).

Enrichment by anion-exchange of Ccp1 from the P10 and S10 extracts and immunodot blotting. Extracts from 500 mL cultures of 16-h, 30-h and 7-day cells were dialyzed against 20 mM KPi/100 µM DTPA (pH 6.1) overnight and centrifuged at 14,000 x g for 10 min. The supernatants (0.5–1 mg total protein) were applied to the 7×25 mm HiTrap Q anion-exchange column equilibrated with the same buffer and attached to an ÄKTApurifier 10 (GE Healthcare). Proteins were eluted from the column using a linear 0–1 M NaCl gradient over 25 min at a flow rate of 2.0 mL/min, 1-mL fractions were collected and 15 µL of each was dot blotted onto a methanol-soaked PVDF membrane (BioRad) and allowed to dry for 10 min at room temperature. The membrane was blocked for 1 h in TBST (50 mM Tris, 150 mM NaCl and 0.05% Tween 20 v/v, pH 7.6) containing 5% skimmed milk (w/v), and incubated with the rabbit anti-Ccp1 antibody (dilution 1:10,000) in 1% milk/TBST overnight at 4° C. After washing with TBST, the membrane was incubated for 1 h with the goat anti-rabbit secondary antibody (dilution 1:10,000) in 1% milk/TBST. Ccp1-containing fractions were detected by chemiluminescence using an ECL kit (ThermoFisher Scientific) and the membrane was scanned on the AlphaImager (ProteinSimple) with an exposure time of 30 s.

Immunodot blotting with anti-Ccp1 of the P10 extracts from 16-h cells shows that Ccp1 eluted from the anion-exchange column mainly in fractions F8 and F9 (Figure S2A,B). No Ccp1 was detected in the S10 extracts (Figure S2B), which supports the mitochondrial location of Ccp1 in exponentially growing cells.[1] Immunodot blotting of the extracts from 30-h and 7-day cells (Figure S3) reveals that most of Ccp1 also is eluted from the anion-exchange column in two fractions.

SDS-PAGE of the anion-exchange fractions. 1D SDS-PAGE under reducing conditions on 6% stacking and 12% resolving (8 cm x 5.8 cm x 1 mm) gels served to further decomplexify the anion-exchange fractions containing Ccp1. After 1-h electrophoresis at 150 V, the gels were Coomassie stained and a representative gel of the F4–F9 fractions from the P10 extract from 16-h cells is shown in Figure S2C. The entire F8 and F9 lanes were cut into 2x2 mm gel slices since post-translational modifications (PTMs) may alter the migration of Ccp1 in the gel.

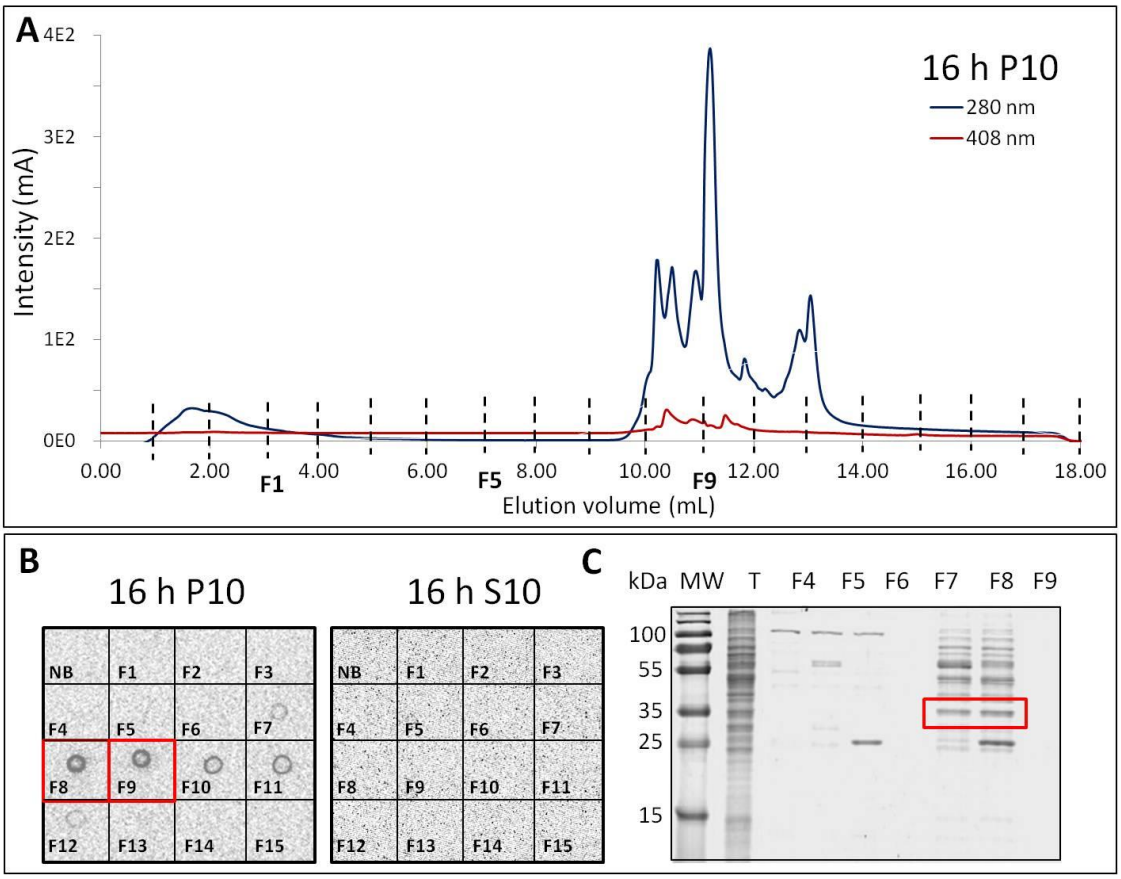

Figure S2. Purification of Ccp1 from 16-h yeast cells. (A) Chromatogram of a P10 extract from 16-h mitochondria. Extract (1 mL) in 20 mM KPi/100 µM DTPA (pH 6.1) containing 0.5 mg of total protein was loaded onto the HighTrap Q anion-exchange column, and eluted with a linear 0-1 M NaCl gradient at a flow rate of 1 mL/min and detected at 280 and 408 nm. (B) Representative anti-Ccp1 immunodot blots of the anion-exchange fractions of P10 mitochondrial and S10 extramitochondrial extracts from 16-h cells. Most of the Ccp1 present in P10 was eluted in fractions F8 and F9 (red boxes) and no Ccp1 was detected in S10. (C) Reducing SDS-PAGE analysis of 25-µL aliquots of fractions F4–F9 from the P10 extract in panel B. The gel shown is representative of those obtained for the P10 extracts from three independent cultures.

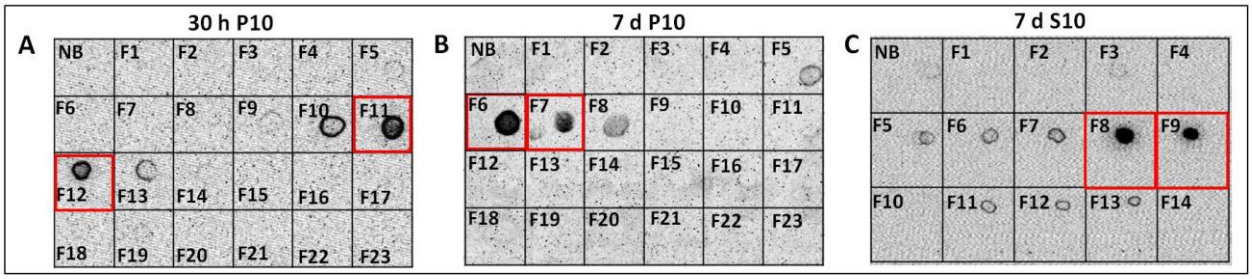

Figure S3. Purification of Ccp1 from 30-h and 7-day yeast cells. Representative anti-Ccp1 immunodot blots of the anion-exchange fractions of (*A*) the P10 extract from 30-h cells, and of the (*B*) mitochondria-enriched P10 and (*C*) extramitochondrial S10 extracts from 7-d cells. Most of Ccp1 was eluted in the fractions boxed in red. Note that no Ccp1 was detected in the 30-h S10 extract so the immunodot blot of this extract is omitted. See the *SI Experimental Procedures* for details.

In-gel Ccp1 digestion and LC-MS/MS analysis of the tryptic peptides. Coomassie stained SDS-PAGE gels were excised down their entire length, destained in a solution of 50% acetonitrile in 25 mM aqueous ammonium bicarbonate (pH 8.0), reduced with 10 mM DTT for 30 min at 50 °C and alkylated with 55 mM iodoacetamide for 30 min in the dark at room temperature. For example, the entire F8 and F9 lanes of the representative gel in Figure S2C were cut into 2x2 mm gel slices in case PTMs alter Ccp1 migration. The gel slices were incubated overnight for in-gel digestion with 12.5 ng/µL trypsin at 37°C and the peptides were extracted with 5% formic acid in 50% aqueous acetonitrile.

The tryptic peptides (Figure S1) were desalted on C18 Zip Tips and injected (5 µL/injection) onto a homemade reversed-phase C18 capillary column (100 µm x 6.5 cm) equilibrated with 2% aqueous acetonitrile/0.1% formic acid and attached to a NanoLC (Easy-nLC II, Thermo Scientific). Peptides were eluted at a flow rate of 200 nL/min into the nanoESI source of an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific) using a 2–94% acetonitrile gradient and analyzed in full-scan mode (m/z 350–2000) in the Orbitrap high-resolution mass analyzer (R = 60,000 at m/z 400). Other instrumental parameters were: electrospray voltage 3 kV, CID collision energy 35 V and heated capillary temperature 200 °C. Precursor peptide ions were selected in MS1 using a mass exclusion threshold of 10 ppm and fragmented in the LTQ at a collision energy of 35 V. MS2 fragments with an intensity count of ≥ 20 were analyzed with a mass tolerance of 0.8 u using Proteome Discoverer 1.3.0 software (Thermo Scientific) and the Sequest search engine with mass filters for the modifications listed in Table S1 plus cysteine alkylation by iodoacetamide (+ 57 u). Dynamic exclusion was enabled with a repeat count of 1, a repeat duration of 30 s and an excluded list size of 500. Sequest correlates the MS2 spectra with peptide sequences in the Ccp1 Fasta file downloaded from the NCBI website (ftp://ftp.ncbi.nlm.nih.gov/). Also, Sequest's XCorr (> 2) and False Discovery Rate (< 0.01) filters were implemented for confident peptide identification.[2]

Table S2 summarizes the $(M+H)^+$ ions of all the tryptic peptides detected in the MS1 spectra pf the Ccp1 proteoforms. Ccp1 was identified with high confidence from the spectra of the tryptic peptides isolated from a single slice that bracketed Ccp1's expected molecular weight of ~34 kDa (e.g., Figure S2C). This confirms the absence of any high-mass modification such as covalent dimerization of the protein. Notably, 16–40 unique

peptides were found and sequence coverage from independent samples was routinely 65-85% (Table S3), with the lower values being typically for Ccp1 from 16-h fermenting cells and higher values for 30-h and 7-day respiring cells, which express more Ccp1.

Table S1. Modifications considered in database searching [a]

Modification: candidate amino acids [b]	Δm (u) [c]
Monoxidation (ox): K, R, C, M, Y, H, P, W, F, D, N	15.9949
Dioxidation (diox): K, R, C, M, Y, H, P, W, F	31.9898
Trioxidation (triox): C	47.9847
Carbonylation: R, E, Q, I, L, K, V, W	13.9793
Carbamidomethylation: C [d]	57.02
Hydroxykynurenine: W	19.9898
Kynurenine (kyn): W	3.9949
Pyrrolidinone: P	30.0105
Pyroglutamic acid: P	13.9792
Aspargine: H	23.0159
Aspartic acid: H	22.0319
Aspartylurea: H	10.0320
Formylaspargine: H	4.9790
Aspartate semialdehyde: M	32.0085
Homocysteic acid: M	33.9691
Dehydro (deH): K, R, C, M, Y, H, P, W, F, D, N [e]	1.00783
Phosphorylation: S, T, Y	79.9799
Nitration: Y, W, C, M	44.98

[a] Table modified from Ref 2.

[b] Products of single amino acid oxidation, phosphorylation or nitration.

[c] Difference in monoisotopic mass of the oxidized and native form of the indicated amino acid.

[d] All cysteines were modified with 2-iodoacetamide as outlined in the *SI Experimental Procedures.*

[e] Crosslinked residues undergo loss of a hydrogen atom.

Table S2. $(M+H)^+$ ions of the tryptic peptides of the Ccp1 proteoforms

Tryptic peptide [a]	Residues [a]	Residue(s) oxidized [b]	Obs $(M+H)^+$ (*m/z*) [c]	Calc $(M+H)^+$ (*m/z*) [c]	ppm error [c]
T2+T3	13-21	None	1129.5283	1129.5273	0.88
		Y16 (ox)	1145.5232	1145.5222	0.73
T3	15-21	None	916.4047	916.4047	0.00
		Y16 (ox)	932.4005	932.3996	0.96
T4	22-29	None	891.5290	891.5298	-0.90
		Y23(ox)	907.5249	907.5247	0.22
T5 + T6	30-48	None	2286.1085	2286.1037	2.01
		Y42 (ox)	2302.1020	2302.0986	1.47
		Y36-Y42 (deH)	2284.0933	2284.0881	2.27
		Y36-Y39 (deH); Y42 (ox)	2300.0878	2300.0830	2.08
T6	32-48	None	2016.9213	2016.9185	1.39
		Y36-Y39 (deH)	2014.9103	2014.9029	3.67
		Y36 (ox); Y39 (ox)	2048.9111	2048.9083	-1.36
T7	49-59	None	1301.6250	1301.6273	-1.76
		W51 (ox)	1317.6189	1317.6222	-2.50
		H52 (ox)	1317.6233	1317.6222	0.83
		W57 (ox)	1317.6189	1317.6222	-2.50
T8	60-72	None	1384.5866	1384.5876	-0.72
		Y71 (ox)	1400.5875	1400.5825	3.57
		Y67 (ox)	1400.5815	1400.5825	-0.71
		Y67-Y71 (deH)	1382.5746	1382.572	1.88
T12	91-97	None	883.5023	883.5036	-1.47
		H96 (ox)	899.5007	899.4985	2.45
T13	98-123	None	2780.3692	2780.3600	3.30
		M119 (ox)	2796.3678	2796.3549	4.61
		W101 (diox)	2812.3389	2812.3498	-3.87
		W101 (ox); M119 (ox)	2812.3516	2812.3498	0.64
		W101 (diox); M119 (ox)	2828.3536	2828.3447	3.15
T14+T15	124-130	W126 (ox)	903.4606	903.4618	-1.32
		W126 (ox); C128 (triox)	951.4405	951.4465	-4.20
T15+T16	128-143	None	1732.7501	1732.7555	-3.11
		C128 (triox)	1765.7404	1765.7453	-2.77
T16+T17+T18	131-155	None	2775.2678	2775.2704	-0.93
		Y153 (P)	2855.2296	2855.2367	-2.48
T17+T18	144-155	None	1377.6631	1377.6645	-1.01
		Y153 (ox)	1393.6577	1393.6594	-1.22
		Y153 (P)	1457.6317	1457.6308	0.62
T20	161-166	None	762.3561	762.3563	-0.26
		M163 (ox)	778.3512	778.3512	0.00
T21	167-179	None	1295.7134	1295.7140	-0.46
		M172 (ox)	1311.7090	1311.7089	0.08
		H175 (ox)	1311.7049	1311.7089	-3.04
		M172 (ox); H175 (ox)	1327.6989	1327.7038	-3.70

T23	184-212	None	3362.5557	3362.5389	4.99
		Y187 (ox)			
		W203 (ox)			
		W211 (ox)	3378.5353	3378.5338	0.44
		W191 (ox)	3378.5353	3378.5338	1.14
		W211 (diox)	3410.5275	3410.5236	-0.29
T24+T25	213-226	Native	1690.7662	1690.7667	2.11
		W223 (ox)	1706.7652	1706.7616	2.43
		W223 (diox)	1722.7607	1722.7565	0.07
T25	216-226	Native	1320.5452	1320.5451	0.08
T25+T26	216-243	Native	3260.461	3260.4511	3.03
		W223 (ox)	3276.460	3276.4460	4.27
T26	227-243	Native	1958.9241	1958.9230	0.56
		Y229 (ox)	1974.9265	1974.9187	3.95
		M231 (ox)	1974.9280	1974.9187	4.71
		M230 (ox); M231 (ox)	1990.9131	1990.9136	-0.25
		Y229 (ox); M230 (ox)	1990.9129	1990.9136	-0.35
		Y229(ox); M230 (ox); M231 (ox)	2006.9088	2006.9077	0.55
		Y229 (P); M231 (ox)	2054.8985	2054.9077	-4.47
		Y229 (deH); M230 (ox); Y236 (deH)	1972.9034	1972.9023	0.56
T27	244-249	None	722.4442	722.4447	-0.69
		Y244 (ox)	738.4380	738.4396	-2.17
		Y244 (diox)	754.4352	754.4345	0.92
T28	250-257	None	982.4139	982.4112	2.74
T28+T29	250-260	None	1404.6418	1404.6430	-0.85
		Y251 (ox)	1420.6401	1420.6379	1.55
T28+T29+T30	250-264	None	1881.8640	1881.8654	-0.74
		Y251 (ox)	1897.8589	1897.8603	-0.73

[a] Figure S1 shows the tryptic map of Ccp1 and the sequence of tryptic peptides T1-T34.

[b] Observed residue modifications are assigned based on the data in Table S1.

[c] The observed monoisotopic *m/z* value is listed for each ion and the error in ppm is:
(Obs *m/z* – Calc *m/z*)/Calc *m/z*) x 10^6

Table S3. Summary of figures of merit for the LC-MS/MS analyses of Ccp1 peptides from the yeast extracts

Extract [a]	16-h P10	30-h P10	7-d P10	7-d S10
Score [b]	71.15	91.53	122.55	168.88
% sequence coverage [c]	68.9 ± 8.3	83.0 ± 8.1	75.14 ± 8.2	83.38 ± 8.3
Unique peptides [d]	16	20	38	40
Tryptic peptides not observed [e]	**T13**, **T14**, T19, **T22**, **T23**, T24, T30,T31, T34	**T22**, T24, T31	**T22**, T29, T30, T1, T32, T34	**T14**, **T15**, **T22**, T30, T31

[a] Ccp1 proteoforms from mitochondrial (P10) and extramitochondrial (S10) fractions were extracted from three independent cultures at each time point, fractionated by anion-exchange chromatography (Figure S2A,B and Figure S3) and SDS-PAGE (Figure S2C), in-gel digested with trypsin and analyzed by LC-MS/MS. This table provides representative figures of merit from three cultures.

[b] The score calculated by the Proteome Discoverer software is the probability that the identified protein is a correct match based on a comparison of its experimental and theoretical MS/MS spectra. Scores are 69–169 for the three independent experiments.

[c] The % sequence coverage is the number of different residues in all the Ccp1 peptides detected by MS divided by 293 residues in yeast Ccp1. Sequence coverage is 68–94% for the three independent experiments.

[d] Unique peptides are those present in Ccp1 only and absent from all other proteins in the NCBI yeast database (ftp://ftp.ncbi.nlm.nih.gov/).

[e] Ccp1 tryptic peptides not found in the MS1 spectra. A map of the tryptic peptides of Ccp1 is found in Figure S1. Peptides containing oxidizable residues (Figure 1 of the main text) are in **blue font**.

***Semiquantitation of residue oxidation*.** Label-free semiquantitation was performed at the MS1-level as described previously.[2] Briefly, peptide ion intensity is expressed as the integrated peak area (*PA*) extracted within a 10 ppm window from the mass chromatogram of the tryptic digest. The yield of an oxidized form of a residue (X_{ox}) identified by MS/MS is given by:

$$\% X_{ox} = \frac{100 \sum PA_{ox}}{\sum PA_{ox} + \sum PA} \quad \text{Eq. S1}$$

The numerator sums the *PA*s of all peptides containing X_{ox} (PA_{ox}) and the denominator sums the *PA*s of all peptides containing any form of residue *X*.

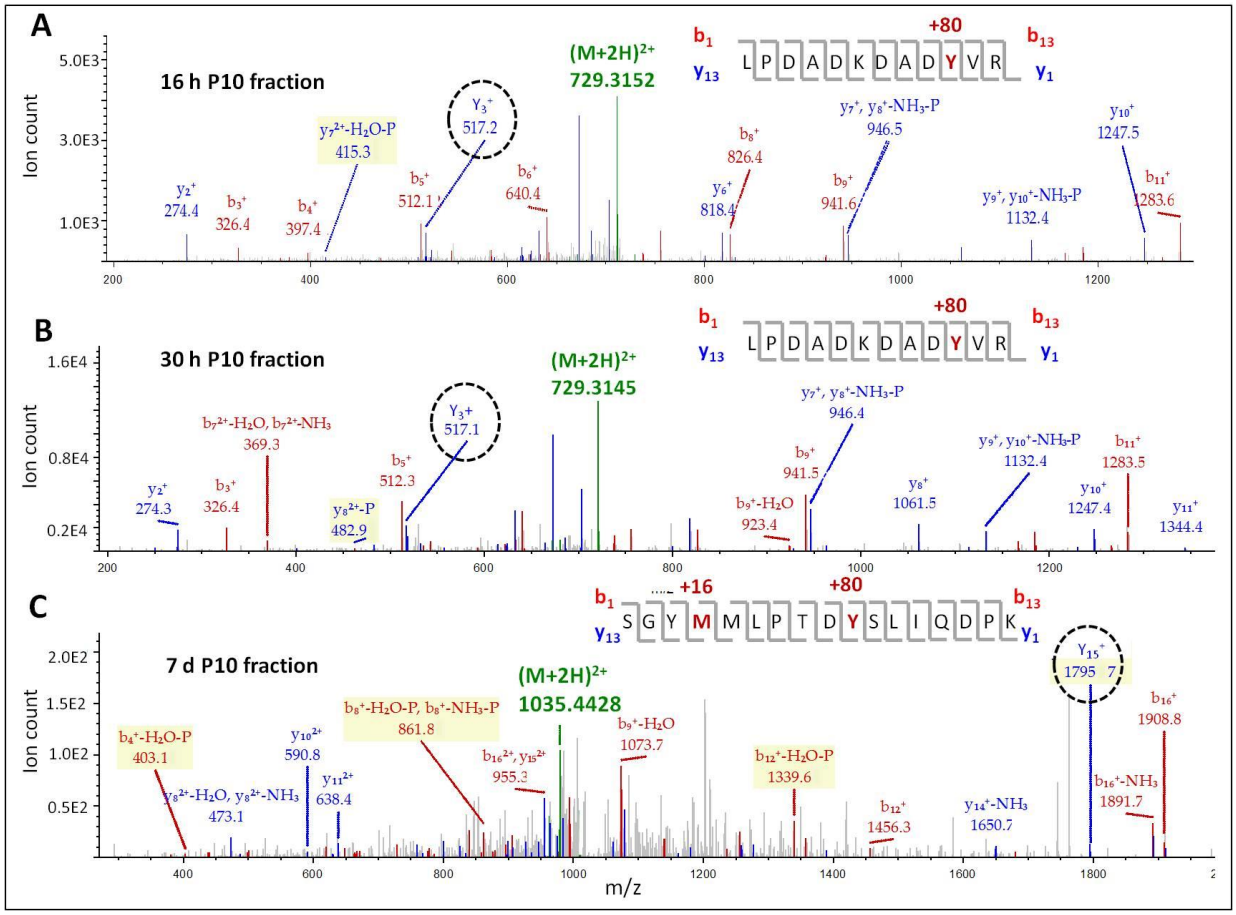

Figure S4. LC-MS/MS analysis of Y153 and Y229 phosphorylation. MS2 spectrum of the $(M+2H)^{2+}$ ion of phosphorylated tryptic peptide T17+T18 at *m/z* 729.3152 of mitochondrial Ccp1 from (A) 16-h and (B) 30-h cells; (C) phosphorylated tryptic T26 at *m/z* 1035.4428 of mitochondrial Ccp1 from 7-day cells. The P10 extracts were treated as described in the *SI Experimental Procedures* and the peptide precursor ions (green) were fragmented by CID (30 V) to give b_n (red) and y_n (blue) sequence ions. The encircled ions have masses indicative of phosphorylation (+80 u) at Y153 in panels A, B and at Y229 in panel C. Spectra are representative of those recorded in 3 independent experiments.

Residue oxidation in recombinant Ccp1 in vitro on bolus vs. stepwise addition of H_2O_2. Tables S4 and S5 summarize residue oxidation in Ccp1 in vitro when H_2O_2 is added in a single dose (bolus) or stepwise as described in the SI *Experimental Procedures*.

Table S4. Percent dityrosine and TyrOH formation on bolus vs. stepwise addition of H_2O_2 to recombinant Ccp1 in vitro[a]

Residue[b]	*Y16*	*Y23*	*Y36*[e]	*Y39*[e]	*Y42*[e]	*Y67*[e]	*Y71*[e]	*Y153*	Y187	Y203	Y229[e]	Y236[e]	Y244	Y251
bolus 10X H_2O_2[c]	*25*	*0*	***72*** *2*	***72*** *2*	***72*** *3*	***83*** *0*	***83*** *0*	*0*	0	0	**9** 86	**9** 86	0	12
Stepwise 1x10 H_2O_2[d]	*5*	*14*	***14*** *3*	***14*** *11*	***14*** *34*	***10*** *47*	***10*** *5*	*7*	0	0	**1** 82	**1** 23	0	11

[a]Average percent residue oxidation based on peptide abundance (SI Eq. S1) from three independent experiments (n = 3).

[b]See location of residues in Figure 1 of the main text. Residues distal to the heme are in italics. Buried residues are in red font.

H_2O_2 (10 μM) was added [c]in a single step (bolus addition) or [d]stepwise in 1-μM increments at 10-min intervals to 1 μM recombinant Ccp1 (final concentration) in 20 mM KPi pH 7.5/100 μM DTPA.

[e]The first and second number in these columns is the percent oxidation of Tyr to **dityrosine** (**bold font**) and to TyrOH, respectively. The remaining Tyr were oxidized to TyrOH only.

Table S5. Percent oxidation of Trp, Met, His and Cys on bolus vs. stepwise addition of H_2O_2 to recombinant Ccp1 in vitro[a]

Residue[b]	*W51*	*W57*	*W101*	*W126*	W191	W211	W223	M119	*M163*	M172	M230	M231	*H52*	H175	*C128*
Bolus 10X H_2O_2[c]	**100*	**100*	*18*	*100*	66	100	64	100	*27*	48	66	65	*57*	42	**100*
Stepwise 1x10 H_2O_2 [d]	*80*	*74*	*4*	*100*	52	83	100	68	*32*	48	34	67	*35*	55	**100*

[a-d]See corresponding footnotes to Table S4.

*Asterisk indicates extensive oxidation of Trp to $Trp(OH)_2$ as well as Cys to $CysO_2H$ plus $CysO_3H$ (3).

SI References

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