LC-MS/MS suggests that hole hopping in cytochrome c peroxidase protects its heme from oxidative modification by excess H$_2$O$_2$†

Meena Kathiresan and Ann M. English*

We recently reported that cytochrome c peroxidase (CcP1) functions as a H$_2$O$_2$ sensor protein when H$_2$O$_2$ levels rise in respiring yeast. The availability of its reducing substrate, ferrocytochrome c (Cyc), determines whether CcP1 acts as a H$_2$O$_2$ sensor or peroxidase. For H$_2$O$_2$ to serve as a signal it must modify its receptor so we employed high-performance LC-MS/MS to investigate in detail the oxidation of CcP1 by 1, 5 and 10 M eq. of H$_2$O$_2$ in the absence of Cyc to prevent peroxidase activity. We observe strictly heme-mediated oxidation, implicating sequential cycles of binding and reduction of H$_2$O$_2$ at CcP1’s heme. This results in the incorporation of ∼20 oxygen atoms predominantly at methionine and tryptophan residues. Extensive intramolecular dityrosine crosslinking involving neighboring residues was uncovered by LC-MS/MS sequencing of the crosslinked peptides. The proximal heme ligand, H175, is converted to oxo-histidine, which labilizes the heme but irreversible heme oxidation is avoided by hole hopping to the polypeptide until oxidation of the catalytic distal H52 in CcP1 treated with 10 M eq. of H$_2$O$_2$ shuts down heterolytic cleavage of H$_2$O$_2$ at the heme. Mapping of the 24 oxidized residues in CcP1 reveals that hole hopping from the heme is directed to three polypeptide zones rich in redox-active residues. This unprecedented analysis unveils the remarkable capacity of a polypeptide to direct hole hopping away from its active site, consistent with heme labilization being a key outcome of CcP1-mediated H$_2$O$_2$ signaling. LC-MS/MS identification of the oxidized residues also exposes the bias of electron paramagnetic resonance (EPR) detection toward transient radicals with low O$_2$ reactivity.

Introduction

There is an expanding list of enzymes with residues that undergo metal-mediated residue oxidation during their normal catalytic cycle. For example, reversible oxidation of tyrosine to a tyrosyl radical (Y$^\cdot$) is well documented in the catalytic cycles of ribonucleotide reductase and prostaglandin H synthase. In the former, Y$^\cdot$ can generate a transient cysteinyl radical (C$^\cdot$) in a substrate over 35 Å away via a hopping mechanism involving the transient formation of new Y$^\cdot$ radicals between the two sites. Similarly, the enzyme MauG oxidizes a substrate separated by 40 Å from its di-heme center by hole hopping through an interfacial tryptophan residue. Recently, Gray and Winkler reported that the chains of tyrosine and tryptophan residues present in many oxidoreductases likely perform a protective function by transporting oxidizing equivalents (or electron holes) away from the active site to the protein’s surface, where they can be scavenged by the reducing power of the cell.

Within the heme peroxidase class of oxidoreductases, there is a dramatic variation in the number of oxidizable residues present in their polypeptides. For example, manganese peroxidase possesses just one tryptophan and no tyrosine, whereas cytochrome c peroxidase (CcP1) is studded with redox-active aromatic residues (Fig. 1). Thus, CcP1 is endowed with a high capacity for hole hopping within its polypeptide, which must be pertinent to its physiological function. In vitro, CcP1 efficiently couples the two-electron reduction of H$_2$O$_2$ to the one-electron oxidation of two ferrocyanochrome c (Cyc$^\cdot$) molecules:

\[
\begin{align*}
\text{CcP1}^{\text{III}} + \text{H}_2\text{O}_2 & \rightarrow \text{CpdI}(\text{Fe}^{\text{IV}}, \text{W191}^{\text{II}+}) + \text{H}_2\text{O} \\
\text{CpdI}(\text{Fe}^{\text{IV}}, \text{W191}^{\text{II}+}) + \text{Cyc}^{\text{II}} & \rightarrow \text{CpdII}(\text{Fe}^{\text{IV}}) + \text{Cyc}^{\text{III}} \\
\text{CpdII}(\text{Fe}^{\text{IV}}) + \text{Cyc}^{\text{II}} + 2\text{H}^+ & \rightarrow \text{CcP1}^{\text{III}} + \text{Cyc}^{\text{III}} + \text{H}_2\text{O}
\end{align*}
\]

CeP$^{\text{III}}$ is the resting ferric enzyme and compound I (CpdI) and compound II (CpdII) are catalytic, high-valent oxyferryl (Fe$^{\text{IV}}$) intermediates. The second of the two oxidizing equivalents (i.e., the two electron holes) in CpdI is localized on W191. This residue forms a stable cationic radical, which was the first tryptophanyl radical identified in a protein. Notably, W191...
lies in the electron-transfer pathway between the Ccp1 and Cyc hemes and mutation of this residue to non-redox active phenylalanine gives the W191F variant, which exhibits negligible Cyp oxidizing ability.11

Ccp1’s catalytic cycle has been examined in exquisite detail in vitro over several decades as a model of heme-peroxidase catalysis.8 Despite the intense focus on Ccp1 as a peroxidase, we have recently reported that it mainly functions as an H2O2 sensor protein in yeast mitochondria.12,13 As yeast cells switch from fermentation to respiration, heme-free apoCcp1 escapes from the mitochondria.12,14 Concomitantly, the activity of the peroxisomal–mitochondrial catalase (Cta1) increases and we have gathered strong evidence that apoCta1 is a recipient of Ccp1’s heme.14 Intracellular H2O2 levels spike as cells begin to respire,12,14 and the proximal heme ligand, H175 (Fig. 1), is extensively oxidized in Ccp1 isolated from respiring yeast.14 Thus, labilization of Ccp1’s heme on H175 oxidation enables its transfer to apoCta1, converting the latter into a powerful catalytic H2O2 scavenger.

To better understand this unprecedented mechanism of H2O2-regulated heme transfer, detailed characterization of Ccp1 modification by excess H2O2 in the absence of substrate was undertaken. Heme-mediated reduction of H2O2 by endogenous donors in Ccp1 in the absence of Cyc21 is well-documented in vitro25 but the oxidized forms of Ccp1 have been poorly characterized. Repeated two-electron reduction of H2O2 by Ccp1 requires repeated intramolecular radical transfer or hole hopping from the oxidized heme. This will generate new transient radicals in the polypeptide, which will hop to new sites before being trapped as stable oxidation products (eqn (5)) or the nascent radicals may be trapped (eqn (6)):15

$$\text{Ccp}^{\text{III}} + \text{H}_2\text{O}_2 \rightarrow \text{CpdI} + \text{H}_2\text{O} \quad (4)$$

$$\text{CpdI} \rightarrow \text{Ccp}^{\text{III}}(\text{A'}, \text{B'}) \rightarrow \text{Ccp}^{\text{III}}(\text{C}_{\text{ox}}, \text{D}_{\text{ox}}) \quad (5)$$

$$\text{Ccp}^{\text{III}}(\text{C}_{\text{ox}}, \text{D}_{\text{ox}}) + \text{H}_2\text{O}_2 \rightarrow \text{Ccp}^{\text{III}}(\text{C}_{\text{ox}}, \text{D}_{\text{ox}}, \text{E}_{\text{ox}}, \text{F}_{\text{ox}}) + \text{H}_2\text{O} \quad (6)$$

Ccp1’s ability to endogenously reduce up to 10 M eq. of H2O2 stems from its abundance of oxidizable residues (Fig. 1).7 Polypeptide oxidation has been confirmed by the detection of transient radicals in CpdI and overoxidized Ccp1 (defined here as Ccp1 oxidized by >1 M eq. of H2O2) by spectroscopic studies on the wild-type protein and its variants.16–21 A broad EPR signal at 4 K was unequivocally assigned to W191C and a narrow EPR signal to Y71 and Y236.16–21 Trapping of radicals provides additional evidence for oxidation of Ccp1 at multiple residues. Spin adducts can be characterized by mass spectrometry (MS),22–26 and Y radicals trapped in Ccp1 by 2-methyl-2-nitrosopropane (MNP) give MNP mass adducts that were localized to tyrosine-containing tryptic peptides T6 (Y36, Y39, Y42) and T26 (Y229, Y236),23 as well as to specific residues, Y39, Y236 and Y153.27 Efficient radical quenching by TEMPO’ (2,2,6,6-tetramethylpiperidinyl-1-oxyl) also generates mass adducts amenable to MS analysis.28 We isolated several TEMPO-labeled peptides from digests of overoxidized Ccp1, including T6, T14 + T15 (W126), T18 + T19 (Y153), T23 (Y187, W191, Y203, W211), T27 + 28 (Y244, Y251) and T28 (Y251), where the oxidizable residues are in brackets, but the actual residue(s) labeled in each peptide was (were) not identified.26

EPR investigations detect the more stable radicals in proteins, notably those with low O2-reactivity, as the results of this study suggest. Spin trapping and scavenging can identify less stable radicals but those that react with spin traps and scavengers tend to be exposed on a protein’s surface because of steric hindrance. We rationalized that high-performance MS, the current method of choice for the qualitative and semi-quantitative characterization of oxidative protein modifications,27–30 would allow us to identify all residues in Ccp1 that serve as endogenous donors, including those that undergo only small mass changes on oxidation. Indeed, the LC-MS/MS results described here provide a comprehensive map of the residues modified on heme-mediated oxidation of Ccp1 by 1 M eq. of H2O2, which generates CpdI (eqn (4)),25 and by 5 and 10 M eq. of H2O2. Multiple cycling of the heme back to its ferric form by hole transfer to the polypeptide (eqn (5) and (6)) enables repeated H2O2 activation and reduction at the heme iron, leading to a highly overoxidized protein.15,22,23,31–35

The chemical nature of the stable oxidation products and their location within Ccp1’s polypeptide are identified by LC-MS/MS. The results are interpreted by considering both the intrinsic reactivity of the amino acid radicals formed on hole hopping from the heme as well as their proximity to conserved internal waters and to regions of O2 density found by molecular dynamics (MD) simulations. Overall, our results reveal that

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**Fig. 1** Oxidizable residues in Ccp1. PyMOL-generated cartoon of Ccp1 (PDB 1ZBY) showing the protein’s 14 tyrosines (Y, green), 7 tryptophans (W, blue), 6 histidines (H, orange), 5 methionines (M, grey) and the single cysteine (C, magenta). Solvent-exposed residues are underlined.
extensive H2O2-initiated hole hopping can be accommodated in a seemingly controlled manner within a relatively small protein matrix. We also elucidate how heme-mediated oxidation of Ccp1 supports its remarkable role in yeast as a H2O2 sensor and signaling molecule that partakes in H2O2-regulated heme transfer. Until recently, H2O2 was viewed as a toxic by-product of aerobic metabolism and associated with many pathologies and biological aging. However, H2O2 signaling is now known to mediate many physiological processes via thiol- and metal-catalyzed protein oxidation. Furthermore, our study suggests that extensive protein oxidation may be physiological and not just pathophysiological.

**Experimental**

**Materials**

Proteins were obtained from the following suppliers: bovine catalase, horse heart cytochrome c (Cyc) type III (Sigma), sequencing grade modified trypsin (Promega) and thrombin (EMD Millipore). Recombinant Ccp1 with MI at positions −2 and −1 of the mature protein was overexpressed as the apo-protein in BL21(DE3) cells, purified and reconstituted with hemin as described previously. Suppliers of (bio)chemicals were as follows: Coomassie (MP Biomedicals); hemin chloride, HPLC grade acetonitrile, diethylenetriamine-pentaacetic acid (DTPA) (Sigma Aldrich); and 30% hydrogen peroxide (Fisher Scientific).

**Ccp1 oxidation.** A 5 μM Ccp1 stock solution was prepared in 20 mM KPi pH 7.5 with 100 μM DTPA (KPi/DTPA) and mixed with a stock H2O2 solution in the same buffer to give 1 μM Ccp1 with the desired H2O2 concentration. DTPA was added to all buffers to inhibit catalysis of H2O2 or O2 oxidation of Ccp1’s residues by trace metal impurities in the buffers. Catalase (0.1 nM) also was routinely added to remove residual H2O2 although none was detected in the samples after 1 h by the HRP/ABTS assay, which agrees with our previous report that Ccp1 rapidly consumes H2O2 using endogenous donors (eqn (4), (5) and (6)). The CCP activity of oxidized Ccp1 was determined by monitoring the oxidation of horse heart CycII by H2O2 as reported.

**MS analyses.** LC-MS analysis of intact apo- and holoCcp1 ± H2O2 and of Ccp1-derived heme is described in the ESI. Details of the LC-MS/MS analysis of tryptic digests (Fig. S1†) of oxidized Ccp1 also are provided in the ESI.† Sequest filters, XCorr (>2) and false discovery rate (<0.01), and the mass filters in Table S1,† were implemented for confident peptide identification, and donor residues were identified by sequencing the oxidized tryptic peptides.

**Semi-quantification of residue oxidation.** Label-free semi-quantification was performed at the MS1-level. Peptide ion intensity is expressed as the integrated peak area (PA) extracted within a 10 ppm window from the digest mass chromatogram. Four peptides consistently found unmodified (Table S2†) were used as internal standards to correct for changes in PA due to variation in instrument response. The normalized yield of an oxidized form of a residue (Xox) identified by MS/MS is given by:

\[
\text{Normalized} \% \ X_{\text{ox}} = \frac{100 \text{ PA}_{\text{ox}}}{\text{SUM} \text{PA}_{\text{ox}} + \text{SUM} \text{PA}}
\]

The numerator sums the normalized PAs of all peptides containing Xox (PAox) and the denominator sums the normalized PAs of all peptides containing any form of X. The relative standard deviation of the reference peptide PAs is ∼4% (Table S2†), which reflects the precision in the percent oxidation reported here.

**Purification and dityrosine fluorescence of monomeric oxidized Ccp1.** Intramolecular dityrosine crosslinking in oxidized Ccp1 was investigated by dityrosine fluorescence. Following its separation from higher molecular weight species by gel filtration chromatography, the steady-state fluorescence of monomeric oxidized Ccp1 was monitored at 410 nm, the maximum emission of dityrosine, as outlined in the caption to Fig. S2.† Oxidized Ccp1 also forms intermolecular dityrosine crosslinks but, given the high sensitivity of MS, we could work at 1 μM Ccp1 and suppress H2O2-induced intermolecular crosslinking (Fig. S2A†). Since dilution effectively attenuates the rapid bimolecular reaction between Y+ radicals (Table 1) on different protein molecules, we assume that other bimolecular reactions are likewise repressed.

**Molecular dynamics simulation of O2 diffusion in Ccp1.** Accessibility of O2 to internal regions of Ccp1 was examined using MD simulations as outlined in the ESL †

**Results**

**Oxidation of Ccp1 by H2O2 is heme mediated**

Following oxidation, the mass spectrum of intact holoCcp1 exhibits new peaks with incremental mass shifts of +16 u (Fig. 2). We assign these peaks to oxidized forms of the protein

### Table 1 Properties of amino acid radicals

| R° | pK_{a} of R° | Reduction potential E° of R° at pH 7 (V) | Peroxy radical reported | k (M⁻¹ s⁻¹) for R° + O₂ reaction |
|----|-------------|----------------------------------------|-------------------------|-------------------------------|
| Y° | −2 [ref. 49] | 0.93 [ref. 50 and 51] | Yes (ref. 52) | <10⁻⁵ [ref. 53] |
| W° | 4 [ref. 50] | 1.01 [ref. 50, 51 and 54] | Yes (ref. 55) | <10⁻⁶ [ref. 56] |
| C° | NR | 0.92 [ref. 57] | Yes (ref. 58 and 59) | 6.1 × 10⁻⁴ [ref. 60] |
| H° | 5–7 | 1.17 [ref. 61] | Yes (ref. 62) | NR |
| M° | −6 [ref. 63] | 1.5 [ref. 63] | NR | NR |

a R° is the neutral amino acid radical of tyrosine (Y), tryptophan (W), cysteine (C), histidine (H) and methionine (M). No k = 5 × 10⁶ M⁻¹ s⁻¹ for dimerization of Y° to dityrosine. NR, not reported.
that have incorporated an oxygen atom at an increasing number of residues. For example, Ccp1 treated with 10 M eq. of H₂O₂ forms up to 20 covalent adducts (Fig. 2C), signifying extensive overoxidation of its polypeptide by H₂O₂ in the absence of CycII (eqn (5) and (6)) as we and others reported previously.\(^{15,22,23,31–35}\)

In sharp contrast, no mass adducts are detected for heme-free, apoCcp1 incubated with 10 M eq. of H₂O₂ (Fig. 2D), demonstrating that oxidation of the holoprotein by H₂O₂ is strictly mediated by its heme.

**Localization of the oxygen adducts in oxidized Ccp1**

Using the mass filters in Table S1,\(^\dagger\) the peptides identified in tryptic digests of oxidized Ccp1 based on their monoisotopic m/z values are listed in Table S3.\(^\dagger\) Importantly, the <5 ppm error in m/z ensures high confidence in peptide identification. Four (M119, M163, M172, M231, Fig. 1) of the five methionine residues in Ccp1 are oxidized to the sulfoxide (MetO; +16 u) above the 5% level in peptides from untreated Ccp1 (Fig. 3A). With the exception of M163, MetO levels increase to \(~\text{40–100%}\) in oxidized Ccp1 (Fig. 3A), suggesting that these residues are major donors to the heme. It has been reported that 60 M eq. of H₂O₂ extensively oxidizes M119, M230 and M231 in apoCcp1 at pH 4 and that the reconstituted holoenzyme exhibits negligible reaction with H₂O₂ (eqn (4)).\(^{46}\) However, the mass spectrum of apoCcp1 treated with 10 M eq. of H₂O₂ in KPi/DTPA is essentially identical to that of the untreated protein (Fig. 2A vs. D), which does not support H₂O₂-induced MetO formation in the apoprotein under the experimental conditions examined here.

A single buried cysteine residue (C128) is located >20 Å from the heme in the distal domain. In CpdI \(~\text{3%}\) of C128 is oxidized to CysSO₂H/CysSO₃H, and the oxidized forms sum to \(\text{60%}\) and \(\text{100%}\) on treatment with 5 and 10 M eq. of H₂O₂, respectively (Fig. 3B), revealing that C128 also acts as a donor to the heme.

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**Fig. 2** Deconvolved mass spectra showing that Ccp1 oxidation by H₂O₂ is mediated by its heme. Oxidized Ccp1 (1 μM) was diluted 5-fold into the MS solvent and 5 μL aliquots were analyzed by LC-MS on a Waters QToF3 mass spectrometer. Mass spectra of (A–C) holoCcp1 oxidized with 0, 1 and 10 M eq. of H₂O₂ and (D) apoCcp1 oxidized with 10 M eq. of H₂O₂. The observed mass of the unoxidized polypeptide is 33 730.50 ± 1.35 u (calc. 33 730.33 u) and overoxidation of holoCcp1 gives incremental mass shifts of +16 u, which are not observed for apoCcp1 (panels B and C vs. D). Experimental details are provided in the ESI.\(^\dagger\)

**Fig. 3** Methionine and cysteine oxidation. Ccp1 (1 μM) in KPi/DTPA was treated with the indicated molar ratio of H₂O₂ for 1 h at room temperature, digested with trypsin and the peptides were analyzed by LC-MS/MS as described in the ESI.\(^\dagger\) Percent (A) methionine oxidation to MetO (+16 u); (B) C128 oxidation to CysSO₂H (+32 u) and CysSO₃H (+48 u). Yields are based on peptide PAs (eqn (7)) from three independent experiments (\(n = 3\)) and presented as averages ± SD. Solvent-exposed methionines are underlined in panel A.

**Fig. 4** Tryptophan residues undergo extensive mono- and dihydroxylation. Percent tryptophan oxidation to (A) TrpOH, (B) Trp(OH)₂ and kynurenine (inset). Experimental details are given in the caption to Fig. 3. Solvent-exposed tryptophans are underlined.
Ccp1’s seven tryptophans undergo extensive H$_2$O$_2$-induced hydroxylation and up to 15% of W223 is additionally converted to kynurenine (Fig. 4). Notably, W191, W211, W223 proximal to the heme are 24–40% oxidized by 1 M eq. of H$_2$O$_2$, whereas the distal W57 and W126 are extensively oxidized by 5 M eq. of H$_2$O$_2$ but W51 at 3.1 Å from the heme is modified only in protein exposed to 10 M eq. of H$_2$O$_2$ (Fig. 4). Furthermore, oxidized W51 and W57 are detected solely as Trp(OH)$_2$ (dihydroxytryptophan), from which we infer that their TrpOH form is readily oxidized as hole hopping to the distal domain increases in overoxidized Ccp1. W101, located on Ccp1’s distal surface at >25 Å from the heme, undergoes little oxidation (Fig. 4) probably because hole hopping to this residue is blocked by O$_2$ scavenging of radicals on W126 or C128 or other residues closer to the heme (Fig. 1).

**Tyrosine is oxidized mainly to dityrosine**

Oxygen uptake by tyrosine contributes minimally to the +16 peaks in Fig. 2C since only Y229 proximal to the heme forms TyrOH (hydroxytyrosine) in high yield. Y16 and Y251 are 10–30% converted to TyrOH (Fig. 5A) but none of the remaining 11 tyrosines appear to undergo hydroxylation. Peptides T4 (Y23), T8 (Y153) and T27 (Y244) exhibit similar MS1 PAs in untreated and oxidized Ccp1 (data not shown), revealing that these tyrosines escape oxidation. In contrast, the PAs of T6 (Y36, Y39, Y42) and T8 (Y67, Y71) decrease 100-fold when Ccp1 is overoxidized with 10 M eq. of H$_2$O$_2$ (Fig. S2D) so we assumed that Y$_c$ is quenched by intramolecular dityrosine crosslinking, which is supported by fluorescence measurements. Dityrosine emits strongly at 410 nm above pH 7, and oxidized monomeric Ccp1 (Fig. S2B) exhibits increasing 410 nm emission up to a H$_2$O$_2$:Ccp1 ratio of 10 (Fig. S2C), consistent with H$_2$O$_2$-induced dityrosine formation within its polypeptide.

**Fig. 5** Tyrosine oxidation products include TyrOH and dityrosine. Percent tyrosine oxidation to (A) TyrOH (+16 u) and (B) dityrosine (−2 u) in T6 (Y36, Y39, Y42), T8 (Y67, Y71) and T26 (Y229, Y236). Experimental details are given in the caption to Fig. 3. Solvent-exposed tyrosines are underlined in panel A.

**Fig. 6** LC-MS/MS analysis of dityrosine formation in tryptic peptide T6. MS2 spectrum of the (M + 3H)$_3^+$ ion of: (A) native T6 at m/z 672.9784 and (B) oxidized T6 at m/z 672.3047. The T6 precursor ions (green) were fragmented by CID (30 V) to give $b_n$ (red) and $y_n$ (blue) sequence ions. The $y_{10}^{2+}$ and $y_{13}^{2+}$ ions encircled in panel B have masses consistent with loss of an H atom (−1 u) from both Y36 and Y39. The peptide sequence in each panel shows Y36, Y39 and Y42 in red font and the observed fragmentations are mapped onto the sequence. Note the absence of fragmentation between crosslinked Y36 and Y39 in panel B. For clarity, low abundance ions are not mass labeled in the spectra but a complete list of the identified sequence ions is provided in Tables S6 and S7.
The doubly and triply charged ions of peptides T6 and T8 from untreated Ccp1 show high intensity MS1 signals (data not shown). Overoxidized Ccp1 has peptide ions at two mass units lower (−2 u) than the untreated protein which, based on the MS2 spectra (Fig. 6 and S3A and B†), are assigned to peptide T6 and T8 that have lost an H atom (−1 u) from each of two tyrosines. Notably, no b or y sequence ions arising from peptide bond fragmentation between the oxidized tyrosines appear in the MS2 spectra (Fig. 6B and S3B†). In fact, the stability of the cyclic peptide region identifies Y36–Y39 and Y36–Y42 as cross-links in T6 (Table S3†). The yield of crosslinked T6 and T8 is >70% in overoxidized Ccp1 (Fig. 5B) and, in addition to M230/M231 oxidation (Fig. 3A and Table S3†), ~10% of Y229–Y236 undergoes crosslinking in T26 (Fig. 5B and S3D†) in competition with Y229 hydroxylation (Fig. 5A). Intramolecular dityrosine crosslinking has not been reported for overoxidized Ccp1 previously but intermolecular crosslinking involving the T6 tyrosines (Y36, Y39, Y42)23,34,35 and Y23636 is documented. Presumably, the Ccp1 dimers and trimers detected here (Fig. S2A†) contain such intermolecular crosslinks.

Oxidation of the proximal iron ligand H175 and of the catalytic distal H52

Solvent-exposed H6, H60, H96 and H181 are <2% oxidized (+16 u) in overoxidized Ccp1 (data not shown). In contrast, the proximal H175, which coordinates the heme iron, is up to 40% oxidized (Fig. 7A). The absorption spectrum of untreated Ccp1 in KPi/DTPA at pH 8.1 shows a Soret maximum at 410 nm and visible bands at 505 and 645 nm (Fig. 7B), which is indicative of pentacoordinate high-spin heme.47 Immediately upon addition of 1 M eq. of H2O2, the spectrum converts to that of CpdI with a Soret at 419 nm and 530/560 nm visible bands (Fig. 7B), which is stable over 1 h. The spectrum of the protein overoxidized with 10 M eq. of H2O2 initially resembles that of CpdI but with time the Soret drops in intensity and blue shifts to 414 nm, which we tentatively associate with the partial oxidation of H175 to HisO.47 The heme released in the MS solvent at low pH from overoxidized Ccp1 has the same exact mass (616.1742 ± 0.00025 u) and peak intensity as that from untreated Ccp1 (Fig. S4†). Importantly, these MS results reveal that (i) essentially all of the heme escapes irreversible oxidation by 10 M eq. of H2O2 and (ii) any time-dependent changes in the absorption spectrum of overoxidized Ccp1 are not due to heme modification.

Close to 60% of the distal H52 is present as HisO in Ccp1 oxidized with 10 M eq. of H2O2 (Fig. 7A). The low CCP activity (11%) of this sample (Table S4†) reflects the critical function of H52 as an acid–base catalyst in holoCcp1 and, as evidenced by the 105-fold lower H2O2 reactivity of the HisO variant.48 The extensive heme loss in Ccp1 exposed to 100 M eq. of H2O2 (Fig. S4E†) may result from attack by the OH− produced on homolytic H2O2 cleavage catalysed by the heme or heme-deriven iron following H52 oxidation.

Discussion

This study provides a comprehensive profile of Ccp1 oxidation by 1, 5 and 10 M eq. of H2O2. Using high-performance LC-MS/MS, we identify and semiquantitate stable oxidative modifications on 24 of Ccp1’s 294 residues (Fig. 3–5 and 7). Key findings include the oxidation of tyrosine to dityrosine, tryptophan to TrpOH, Trp(OH)2 and kynurenine, histidine to HisO, methionine to MetO and cysteine to CysSO2H and CysSO3H. Formation of these products is likely triggered by a superoxide, allowing the hypovalent cations to trap water and deprotonate to give the +16 u mass products detected by MS (Schemes S1–S3†). Of key interest is how intrinsic radical reactivity is modulated by the local protein environment as this dictates the preferred donor residues in Ccp1. This question is explored in the following sections before we discuss how overoxidation by H2O2 may enable Ccp1 to perform its remarkable H2O2 sensing and signaling function in the cell.

Intrinsic radical reactivity

Table 1 summarizes the properties of the radicals derived from the one-electron oxidation of the free amino acids that constitute the redox-active residues in proteins. These properties, combined with the protein microenvironment, determine the oxidized forms of the donor residues found by LC-MS/MS in oxidized Ccp1.

Free methionine and many methionine residues are oxidized to MetO with H2O2 as a typical oxidant.49 Nonetheless, MS analysis provides no convincing evidence for more MetO formation in H2O2-treated apoCcp1 than in the untreated apo-protein (Fig. 2D vs. A). This we attribute in part to inhibition of trace-metal activation of H2O2 by the 100 µM DTPA present in the buffer. Tryptic digestion was also performed in the presence of DTPA but 2–20% MetO is detected in peptides from untreated holoCcp1 (Fig. 3A), which may signal methionine oxidation catalysed by the heme released during proteolysis. With the
advantageously, the presence of several unmodified tyrosines in overoxidized Ccp1 aids in mapping hole-hopping pathways from the heme and in delimiting donor zones within the polypeptide (Fig. 8).

Since the pK_a of free H^+ is ~5–7 and H^+ has an E_c of 1.17 V (Table 1), oxidation of histidine residues also will be sensitive to the local protein environment. Hydrogen bonding to D235 imbibes H175 with imidazolate character, which promotes donation of electron density to the hypervalent heme. However, we detect <5% H175 oxidation in Ccp1 treated with 1 M eq. of H_2O_2 (Fig. 7A) but this increases to ~50% in overoxidized Ccp1 as discussed in the next section. The stable HisO product is likely formed via a peroxyl radical (Scheme S3^†) as proposed for the other oxidizable residues.

**Hole-hopping pathways and electron-donor zones in Ccp1**

The primary donor to the ultra-short-lived porphyrin π-cation formed on the initial two-electron oxidation of Ccp1’s heme is W191 (eqn (4)). This catalytic residue is surrounded by oxidizable residues, most notably M230 and M231 with their sulfur atoms at ~4 Å from the indole ring. Methionine-aromatic

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823 x 1158 | 152-308 | 1158 | 1152-1162
interactions (~1–3 kcal mol\(^{-1}\)), present in 33% of proteins in the PDB, stabilize protein structure\(^{23}\) and also may regulate the redox properties of aromatic residues as suggested over 25 years ago for W191.\(^{19,24,25}\) Our MD simulations located no O\(_2\) docking site within 5 Å of W191 (Fig. S6 and Table S8\(^\dagger\)) which, combined with its positive charge, serve to protect this key residue from scavenging by O\(_2\). Hence, hole hopping can continue from the transient W191\(^{•+}\) radical to M231 and to residues at the surface of the proximal domain, including M230, W223, W211 and Y229. These solvent-exposed residues form stable oxidation products detectible at 1 : 1 H\(_2\)O\(_2\) : Ccp1 (Fig. 3, 4 and 5A) and they are clustered in a region of the polypeptide that we label zone 1 (Fig. 8).

Oxidation of M230 and/or M231 converts W191 into a poorer electron donor as seen on mutation of these residues.\(^{17,18}\) Hence, hole hopping from the heme to the distal region (zones 2a and 2b, Fig. 8) opens up. Both W126 and C128 act as major distal donors in overoxidized Ccp1 (Table S5\(^\dagger\)) and these neighboring residues are in a favorable environment for hole formation and subsequent termination via O\(_2\) scavenging (Schemes S1 and S4\(^\dagger\)), being close to 2–3 internal waters and O\(_2\) docking sites (Fig. S6 and Table S8\(^\dagger\)). W57 and Y67/Y71 are additional termination sites in zone 2a whereas zone 2b contains solvent-exposed Y36, Y39 and Y42 that undergo efficient hole termination by crosslinking once oxidized to Y\(^•\) (Fig. 5B and Scheme S2\(^\dagger\)). The sequence of hole hopping to zones 2a and 2b is not resolved in our study but the clustering of donors into these subzones suggests two distinct routes from the heme via transient hole formation on W51 and on M119, respectively (Fig. 8).

W51, W191 (Fig. 4) and H52 (Fig. 7) kick in as major endogenous donors in Ccp1 treated with 10 M eq. of H\(_2\)O\(_2\). Hole-hopping from the heme presumably terminates at these active-site residues following oxidation of residues further from the heme. We group W51, W191, H52 and M119 into zone 3 (Fig. 8) together with M172 and H175, which are maximally oxidized (~50%) in Ccp1 exposed to 5 M eq. of H\(_2\)O\(_2\) (Fig. 3A and 7A). Significantly, M172 and H175 are, for the most part, oxidized in different Ccp1 molecules (Table S5\(^\dagger\)). Also, the relative orientation of H175 and W191 is fixed by hydrogen bonds to D235, which additionally modulates coupling of W191\(^{•+}\) to the heme.\(^{72}\) Thus, the susceptibility to oxidation of these three proximal residues must be interdependent. Given its probable critical importance in labilizing the heme,\(^{14}\) computational investigations of H175 oxidation, including the roles of M230 and M231 that stabilize W191\(^{•+}\),\(^{9,17,26}\) are underway. Oxidation of the distal H52 seen in the 10 : 1 H\(_2\)O\(_2\) : Ccp1 sample (Fig. 7A) also is a vital step since this histidine is essential for H\(_2\)O\(_2\) heterolytic cleavage by Ccp1.\(^{48}\) Shutting-down this rapid reaction by H52 oxidation is necessary to defend the heme as the endogenous donors become exhausted.

So far we have only considered hole hopping from the heme as a mechanism of residue oxidation in Ccp1. For the sake of completeness, we note that once H52 is oxidized (Fig. 7), slow homolytic H\(_2\)O\(_2\) cleavage may generate some ‘OH at the heme. A likely target would be W51 just above the heme (Fig. 8) as highly reactive ‘OH radicals do not diffuse far from their site of generation. It is noteworthy that W51 is 100% dihydroxylated in the 10 : 1 H\(_2\)O\(_2\) : Ccp1 sample (Fig. 4B), which may reflect some oxidation of this residue by ‘OH. However, we anticipate very little ‘OH formation in the H\(_2\)O\(_2\)–Ccp1 incubations examined here since H\(_2\)O\(_2\) is consumed rapidly.\(^{22}\) In CuZn-superoxide dismutase, on the other hand, homolytic cleavage of H\(_2\)O\(_2\) at the catalytic copper results in oxidation of histidines ligated to the catalytic center.\(^{62,74,75}\) In fact, histidines are oxidized in the dismutase during cell aging,\(^{76,77}\) and HisO is viewed as a biological marker for evaluating protein modification from oxidative stress.\(^{78}\)

Comparison of MS and EPR studies on Ccp1 oxidation

Radicals are detected by EPR, which directly confirms their transient presence in proteins during catalysis. Catalytic Y\(^•\) radicals that are well-characterized by EPR include those in ribonucleotide reductase, prostaglandin H synthase, photosystem II and dopamine b-monoxygenase.\(^{27,29}\) A recent elegant EPR study on H\(_2\)O\(_2\) oxidation of Ccp1 variants with multiple mutations identified Y71\(^{•}\) as a catalytic radical and Y236\(^{•}\) as a non-catalytic radical in peroxidase turnover with guaiacol as a reducing substrate.\(^{20}\) Our LC-MS/MS product analysis identifies Y71 as a major donor in zone 2a but establishes Y229 and not Y236 as a major donor in zone 1 (Fig. 5B and 8). Efficient scavenging of Y229\(^•\) by O\(_2\) (Fig. 5A) would compete with its detection by EPR as would hole hopping from Y236 to neighboring W223 and W211, which are extensively oxidized (Fig. 4).

We also locate tyrosine donors in zone 2b (Fig. 8) where a second molecule of guaiacol is known to bind near residue I40.\(^{40}\) Rapid quenching of these Y\(^•\) radicals by dimerization would likely preclude their detection by EPR but, as we demonstrate here, dityrosine-crosslinked peptides as well as the specific tyrosines involved in the crosslinking can be readily identified by high-performance LC-MS/MS (Fig. 6 and S3\(^\dagger\)).

The assignment of W\(^•\) radicals by EPR can be challenging due to peak broadening.\(^{40}\) Moreover, their efficient scavenging by O\(_2\) as seen in oxidized Ccp1 (Fig. 4) will compete with their detection by EPR. In fact, W191\(^{•+}\) with low O\(_2\) reactivity is the only indolyl radical reported in EPR studies of CpdI and overoxidized Ccp1.\(^{20,21}\) Furthermore, MnP succeeded in trapping Y153, Y39\(^•\) and Y236\(^•\) but no W\(^•\) radicals in Ccp1,\(^{22,23}\) revealing that spin trapping also is biased toward Y\(^•\) with low O\(_2\) reactivity. Thus, the current results underscore the complementarity of MS and EPR for studies of protein-based radicals. EPR can directly detect relatively long-lived radicals and provide information on their stability and, in some instances, the specific residues oxidized can be selected from EPR spectra.\(^{42}\) MS, on the other hand, can identify all oxidized residues and additionally characterize their stable end products. This sheds light on radical-quenching mechanisms and also on possible hole-hopping pathways from heme or other redox-active metal centers in proteins.

In addition to confirming W191\(^{•+}\) as the main radical species in CpdI, a recent QM/MM computational analysis\(^{85}\) proposed Y203/Y251 and Y236 as secondary radical sites in two possible pathways from W191\(^{•+}\). Y203/Y251 together with Y153 and Y244 are clustered in proximal zone 4 (Fig. 8) and undergo little or no oxidation detectable by MS (Fig. 5). However, we did detect
Characterization of overoxidized Ccp1 provides insights into its possible physiological functions

The safeguarding of heme integrity in Ccp1 is evocative of the antioxidant role proposed for the chains of tyrosine and tryptophan residues present in many oxidoreductases. Nonetheless, heme hopping to residues remote from the heme is not protective of CCP activity (Table S4†). Instead, the physiological importance of Ccp1’s multiple hole-hopping pathways is related to its function in H2O2-regulated heme transfer. We report that in respiring yeast mitochondria, Ccp1 donates its heme directly or indirectly to catalase A (CatA). H2O2 levels spike ~10-fold when yeast begin to respire23,44 causing Ccp1 to become overoxidized45 since synthesis of its reducing substrate, Cyc146 (eqn (2) and (3)), is under O2/heme control,41 unlike Ccp1 itself. Oxidation by excess H2O2 of Ccp1’s proximal ligand, H175, labilizes its heme as seen in H175 variants with weakened axial ligation.46 The current study shows that diverting the oxidizing equivalents derived from H2O2 away from the heme (Fig. 8) spares it from irreversible oxidative modification (Fig. S4†). This allows Ccp114 to function as a H2O2-regulated donor of unmodified heme, a role unique to Ccp1 until now.

Intriguingly, ascorbate peroxidase (APX) possesses a very similar active-site structure to Ccp1 with W41 and W179 occupying the same distal and proximal locations as W51 and W191, respectively, in Ccp1.47–48 However, heme crosslinking to W41 occurs in oxidized APX,49 which is associated with stabilization of a transient π-cation radical on the porphyrin rather than on W179.47 Ccp1W191F also forms a transient porphyrin π-cation radical and heme crosslinking to W51 has been reported in this variant.49 Thus, ultra-rapid radical transfer from the porphyrin appears to be key in preventing heme crosslinking to the distal tryptophan in Ccp1. A role other than that of catalytic H2O2 scavenger has not been proposed for APX whereas our recently published targeted proteomics study provides additional support for Ccp1’s participation in heme transfer.49

Intramolecular dityrosine crosslinking is prevalent in Ccp1 overoxidized with 5 and 10 M eq. of H2O2 (Fig. 5B). Crosslinked Ccp1 may signal oxidative stress in yeast given that dityrosine is becoming increasingly identified as a marker of oxidative stress and is linked to a number of pathologies including amyloid fibril formation.93

Conclusions

Building on previous studies16–23,26,32–35 that pinpointed a limited number of donors to the heme in the endogenous oxidation of Ccp1 by H2O2, here we identify an unprecedented number of donor residues (24) by characterizing their stable end products by in-depth LC-MS/MS analysis. This large number of proximal and distal donors delineates numerous hole-hopping pathways emanating from Ccp1’s heme that protect it from irreversible modification. All routes may not be operative or lead to polypeptide oxidation in cells because of the efficient repair of protein radicals by glutathione and ascorbate92 and/or the reversible phosphorylation of tyrosine residues. Importantly, we have already demonstrated that Ccp1’s heme is labilized in respiring mitochondria by H175 oxidation.14 Here we report that the heme is spared irreversible oxidative modification by H2O2 and illuminate the structural mechanism of how this is achieved during the reduction of up to 10-fold excess H2O2 by endogenous donors in Ccp1.

Interestingly, Fe-catalyzed histidine oxidation is implicated in H2O2 sensing by the peroxide resistance protein (PerR) from B. subtilis. H2O2 binding to the non-heme Fe2+ center of PerR results in oxidation of the Fe ligands (H37 and H91), promotes Fe release and apoPerR dissociation from DNA to turn on genes such as that encoding the catalase, KatA.93,94 Thus, both PerR and Ccp1 can be viewed as metalloenzyme proteins that enable their ligand, H2O2, to regulate its own consumption by a catalase: H2O2 regulates heme transfer from Ccp1 to apoCatA1 and Fe loss from PerR to initiate KatA translation. Although these mechanisms are fascinatingly diverse, they share a common essential step: oxidation by H2O2 of Fe-ligated histidine to trigger heme or Fe release from the metalloenzyme.

Finally, on a methodological note, we reiterate that our detailed analysis of oxidized Ccp1 using a high-performance universal detection/characterization method such as MS exposes a bias in EPR detection toward protein-based radicals like Y′ with low O2 reactivity. This should be borne in mind when mapping hole-hopping pathways in proteins based on EPR monitoring of transient radicals.

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