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Rewiring the ‘Push-Pull’ Catalytic Machinery of a Heme Enzyme using an Expanded Genetic Code

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ABSTRACT: Nature employs a limited number of genetically encoded axial ligands to control diverse heme enzyme activities. Deciphering the functional significance of these ligands requires a quantitative understanding of how their electron donating capabilities modulate the structures and reactivities of the iconic ferryl intermediates compounds I and II. However, probing these relationships experimentally has proven challenging as ligand substitutions accessible *via* conventional mutagenesis do not allow fine tuning of electron donation and typically abolish catalytic function. Here we exploit engineered translation components to replace the histidine ligand of cytochrome *c* peroxidase (CcP) by a less electron donating *N*_δ-methyl histidine (Me-His) with little effect on enzyme structure. The rate of formation (k_1) and the reactivity (k_2) of compound I are unaffected by ligand substitution. In contrast, proton coupled electron transfer to compound II (k_3) is 10-fold slower in CcP Me-His, providing a direct link between electron donation and compound II reactivity which can be explained by weaker electron donation from the Me-His ligand (‘the push’) affording an electron deficient ferryl-oxygen with reduced proton affinity (‘the pull’). The deleterious effects of the Me-His ligand can be fully compensated by introducing a W51F mutation, designed to increase ‘the pull’ by removing a hydrogen bond to the ferryl-oxygen. Analogous substitutions in ascorbate peroxidase (APX) lead to similar activity trends to those observed in CcP, suggesting a common mechanistic strategy is employed by enzymes using distinct electron transfer pathways. Our study highlights how non-canonical active site substitutions can be used to directly probe and deconstruct highly evolved bioinorganic mechanisms.

Keywords: Heme enzyme, non-canonical ligand, metal-oxo reactivity, proton coupled electron transfer

INTRODUCTION

In nature heme enzymes catalyze a wealth of oxidative transformations. The axial ligand coordinating the heme iron varies across enzyme families and is pivotal to controlling catalytic function.^{1,2} Considerable effort has been devoted to understanding relationships between proximal ligand electron donation, the structures and reactivities of iconic ferryl intermediates compound I and compound II, and overall catalytic function.³⁻⁸ For example, the ability of cytochrome P450s and aromatic perxygenases to functionalize unactivated C–H bonds is dependent upon axial thiolate ligation. Strong electron donation (‘the push’) from the cysteinyl ligand increases electron density on the compound I ferryl-oxygen and thus enhances its reactivity with respect to C–H bond activation (‘the pull’).³⁻⁷

Heme peroxidases utilize a histidine residue as the axial ligand to the heme iron, with a hydrogen bond formed between the non-coordinating *N*_δ atom and a conserved aspartate residue (Figure 1a).^{1,9} This interaction imparts a degree of ‘imidazolate like’ character onto the axial ligand, thus increasing its electron donating capabilities. Despite extensive theoretical and experimental study,^{10,11} the significance of this imidazo-

late ligand in the peroxidase catalytic mechanism remains poorly understood, presenting a missing link in our understanding of heme biochemistry. Directly probing relationships between proximal ligand electron donation and heme enzyme reactivity has proven extremely challenging. Ligand substitutions accessible through standard mutagenesis do not allow fine tuning of electron donating properties and typically eliminate natural catalytic function.¹² Efforts to install a greater range of ligands have often resorted to the creation of ‘cavity mutants,’ involving replacement of large ligands by amino acids with smaller side chains (Gly or Ala) and subsequent diffusion of exogenous ligands into the vacant co-ordination site. These studies have provided valuable insights into the roles of proximal heme ligands. However, the resulting ternary complexes typically fail to deliver catalysts with appreciable activity, demonstrating the importance of covalent attachment of ligands to the protein backbone.^{13,14} For example, coordination of imidazole to the H175G cavity mutant of cytochrome *c* peroxidase (CcP) fully restores its activity with hydrogen peroxide to generate a ferryl intermediate. However, the rate of cytochrome *c* oxidation is unaffected by imidazole com-

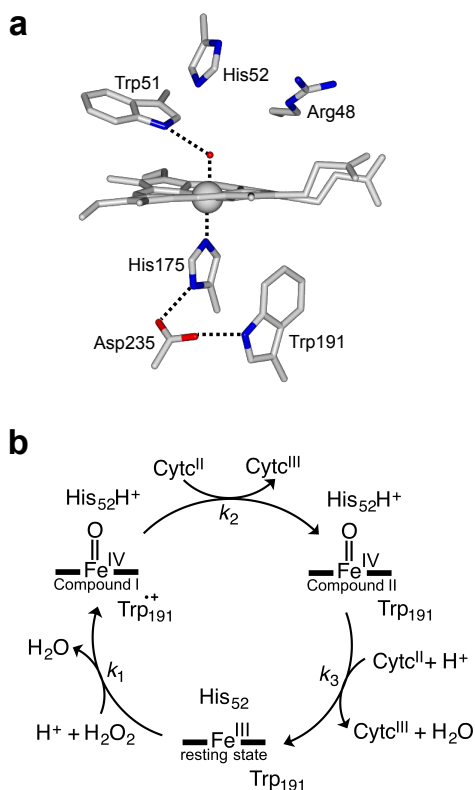


Figure 1. Structure and catalytic mechanism of cytochrome *c* peroxidase (CcP). (a) The active site of CcP (PDB code: 1ZBY).⁹ The hydrogen bond between Asp235 and His175 imparts a degree of imidazolite like character onto the axial ligand, thus increasing its electron donating capabilities. (b) The catalytic cycle of CcP with its biological redox partner ferrous cytochrome *c* (cyt*c*) as an electron donor. His52 is protonated in compound I but not the resting state.¹⁸

plexation, making it challenging to exploit this approach to quantify the impact of ligand substitutions on compound I and compound II reactivity.¹⁴

As a result researchers have turned to synthetic model complexes, which offer greater versatility with respect to local metal co-ordination environment, to validate mechanistic hypotheses.^{2,15} However, the catalytic activities of these small molecule systems are typically orders of magnitude lower than enzymes, which possess highly evolved and sophisticated catalytic mechanisms which are not replicated in synthetic complexes. The ability to selectively install a greater repertoire of metal co-ordinating residues into proteins could therefore offer a powerful and more direct approach to probe complex bioinorganic mechanisms. Here we report the preparation and characterization of a functional cytochrome *c* peroxidase (CcP) with an *N*₅-methyl histidine (Me-His) proximal ligand. In contrast to P450s where strong electron donation is linked to compound I reactivity,³⁻⁶ we show that ‘electron push’ from the ‘imidazolite like’ proximal ligand of CcP has little impact on compound I reactivity, and instead tunes the reactivity of compound II.

RESULTS

CcP recruits electrons from its biological redox partner ferrous cytochrome *c* (cyt*c*) to reduce hydrogen peroxide in mitochondria.¹ The reaction mechanism is comprised of three steps

(Figure 1b); 1) reaction of the resting ferric enzyme with hydrogen peroxide to generate compound I, containing an oxidized ferryl heme and a Trp191 radical cation,¹⁶ 2) single electron reduction of compound I by ferrous cyt*c* to generate compound II and 3) single electron reduction of compound II by a second equivalent of ferrous cyt*c*. Compound II reduction is coupled with proton transfer to the ferryl oxygen.¹⁷ The distal pocket His52, which is protonated in compound I but not in the resting state,¹⁸ is a likely source of the required proton.

To probe the influence of proximal ligand electron donation on the CcP catalytic mechanism, the axial His175 ligand of *S. cerevisiae* CcP was replaced with a non-canonical Me-His residue using an engineered pyrrolysyl-tRNA synthetase/pyrrolysyl-tRNA pair (PylRS^{Me-His}/tRNA^{Pyl}), which selectively incorporates Me-His in response to the amber UAG stop codon (Figure S1).¹⁹ The CcP Me-His X-ray crystal structure, refined to a resolution of 1.90 Å (Figure S2, Table S1), superimposes well with a previously reported CcP His structure⁹ (Figure 2, PDB code: 1ZBY; secondary structure superpose, RMS deviation of 0.11 Å). Comparison of the CcP His and CcP Me-His structures demonstrates that His → Me-His constitutes a highly conservative mutation, with the geometry and environment of the heme cofactor and key active site residues well preserved in the modified enzyme. Arg48 within the distal pocket adopts two conformations as observed previously in ferric CcP (Figure 2).⁹ Proximal ligand substitution disrupts the conserved Asp-His proximal pocket hydrogen bond responsible for increasing the electron donating properties of His ligands in peroxidases.^{1,10-11} Asp235 undergoes a minor conformational adjustment to accommodate the additional methyl substituent on the proximal histidine ligand. The proximal Me-His adopts a similar conformation to the histidine residue in previously reported structures, thus maintaining π -stacking interactions with Trp191. Significantly, the environment sur-

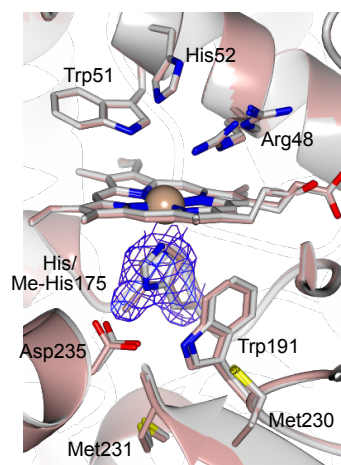


Figure 2. Structural characterization of CcP His and CcP Me-His. Overlay of CcP (PDB code: 1ZBY)⁹ and CcP Me-His (PDB code: 6H08) active sites (using secondary structure superpose, RMS deviation of 0.107 Å). The CcP His heme cofactor and key residues are shown as atom colored sticks with grey carbons. The protein backbone is represented in cartoon format (colored in grey). The CcP Me-His heme cofactor and key residues are shown as atom colored sticks with pink carbons. The protein backbone is represented in cartoon format (colored in pink). The $2F_o - F_c$ electron density map corresponding to the Me-His residue is contoured at 1σ (blue mesh).

rounding the redox active Trp191 residue is fully conserved in the CcP Me-His structure, including the hydrogen bonding interaction with Asp235 and interactions with nearby Met230 and Met231 residues that are important for the stability of the compound I Trp191 radical cation.¹ This contrasts with previous attempts to modulate proximal ligand electron donation in CcP through mutation of Asp235, which resulted in dramatic reorientation of the Trp191 residue.²⁰

Introduction of a proximal Me-His ligand resulted in a 22-fold reduction in k_{cat} for the oxidation of ferrous cytc from horse heart (pH 6.0, 25°C) ($k_{\text{cat}} = 805 \pm 25 \text{ s}^{-1}$ for CcP His, $k_{\text{cat}} = 38 \pm 5 \text{ s}^{-1}$ for CcP Me-His) with only modest changes in K_M (Figure 3a and Table 1). Despite the reduction in k_{cat} , CcP Me-His is able to oxidize >10,000 equivalents of ferrous cytc with no evidence of enzyme deactivation. Mutation of the proximal pocket Trp191 residue to phenylalanine in CcP Me-His and CcP His eliminates catalytic activity, demonstrating that this redox active residue retains an essential role in the catalytic function of the modified enzyme. In contrast to the substantially reduced k_{cat} observed with the biological redox partner ferrous cytc, proximal ligand substitution has minimal effects on the kinetics of guaiacol (*ortho*-methoxyphenol) oxidation (Figure S3b), suggesting that the non-canonical active site modification specifically perturbs long-range electron transfer from cytc mediated through Trp191.

Replacement of the proximal His ligand by Me-His increases

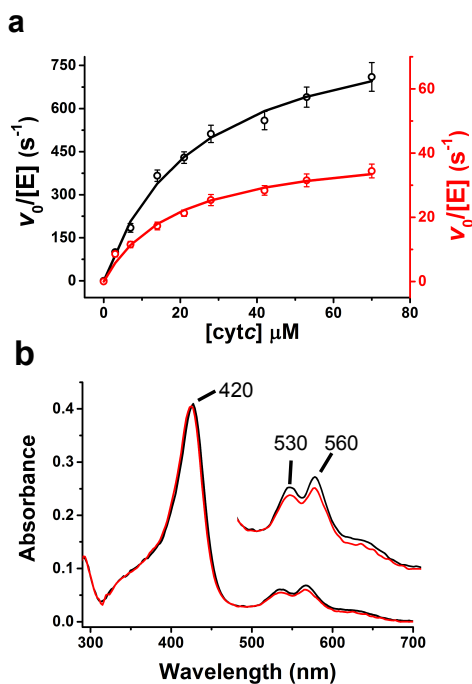


Figure 3. Kinetic and spectroscopic characterization of CcP His and CcP Me-His. (a) Michaelis-Menten plots of cytc oxidation by CcP His (black, $k_{\text{cat}} = 805 \pm 25 \text{ s}^{-1}$, $K_M^{\text{cytc}} = 18 \pm 3 \mu\text{M}$) and CcP Me-His (red, $k_{\text{cat}} = 38 \pm 5 \text{ s}^{-1}$, $K_M^{\text{cytc}} = 17 \pm 2 \mu\text{M}$). Measurements at pH 6, 25°C, error bars are SEM $n=3$. (b) Overlay of the UV-vis spectra of the compound I states of CcP (black) and CcP Me-His (red).

Table 1. Cytc oxidation kinetic parameters for CcP His, CcP Me-His and their W51F variants^a

Variant	$k_{\text{cat}} \text{ (s}^{-1}\text{)}$	$K_M \text{ (}\mu\text{M)}$
CcP	$805 \pm 25 \text{ (}590 \pm 21\text{)}^b$	$18 \pm 3 \text{ (}16 \pm 2\text{)}$
CcP Me-His	$38 \pm 5 \text{ (}25 \pm 4\text{)}$	$17 \pm 2 \text{ (}18 \pm 2\text{)}$
CcP W191F	ND ^c	-
CcP W191F Me-His	ND	-
CcP W51F	$2,350 \pm 100$	14 ± 2
CcP W51F Me-His	$1,170 \pm 30$	18 ± 2

^aAll measurements were carried out at 25°C in potassium phosphate (50 mM) at pH 6.0. ^bValues in parentheses correspond to measurements in D₂O. ^cND: no activity detected above background.

the midpoint reduction potential for the heme Fe^{III}/Fe^{II} couple by $+35 \pm 1 \text{ mV}$ (Figure S4), consistent with reduced electron donation from the proximal ligand. This increased potential can be attributed to loss of a hydrogen bond between the proximal ligand and Asp235. Despite this reduced electron donation, the UV-Vis spectra of CcP Me-His in the ferric, com-

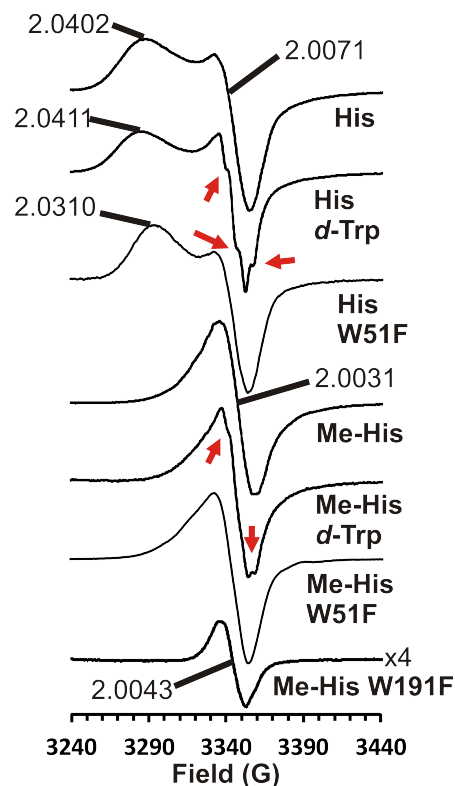


Figure 4. X-band continuous wave EPR spectra of the compound I state of CcP His and CcP Me-His with and without the additional W51F mutation. EPR spectra recorded at 6 K showing the effect of tryptophan-(indole-*d*₅) on the spectra and a comparison with the CcP Me-His W191F variant; *g* values are marked and red arrows indicate partially resolved hyperfine splitting.

compound I and compound II states are highly similar to the corresponding spectra of the wild-type enzyme (Figure 3b, Figure S5 and Figure S6). The compound I and compound II states of CcP are not distinguishable by UV-vis spectroscopy, with spectral features consistent with a neutral ferryl heme (Soret maxima at 420 nm and associated Q bands at 530 and 560 nm).

In CcP His, the second oxidising equivalent of compound I is stored as a stable Trp191 radical cation, which is an essential intermediate along the electron transfer pathway from ferrous cytc.¹⁶ To confirm the identity of the protein based radical cation in CcP Me-His, compound I was characterized by X-band cw EPR spectroscopy. CcP His compound I has previously been shown to produce a distinctive EPR signal at 10 K or below that arises from a distribution of scalar magnetic or exchange coupling (J) values between the $S = 1$ ferryl (FeIV=O) heme and the $S = 1/2$ tryptophan cation radical spin systems.^{16,21} That EPR signal was reproduced in our experiments (Figure 4), having a shoulder at $g = 2.04$ and crossing point at $g = 2.00$ at 6 K. Global replacement of tryptophan residues in CcP His with L-tryptophan-(indole- d_5) gave rise to

changes in the EPR signal arising from hyperfine coupling to the C_{β} -protons that are now resolved in the absence of the hyperfine contributions from the indole ring protons. Additionally, deuteration leads to a slight narrowing and shift of the shoulder at $g = 2.04$ due to a reduction in unresolved proton hyperfine coupling. These effects confirm the contribution made by the tryptophan cation radical to the compound I signal. This signal is present at 94-97% of heme concentration in the CcP His preparations as judged by double integration at non-saturating powers against Cu(II)-EDTA standards. The 6 K compound I EPR signal associated with CcP Me-His (present at 83-86% of heme concentration) lacks the shoulder at $g = 2.04$, however, L-tryptophan-(indole- d_5) substitution gives rise to similar effects to those observed in CcP His, though naturally less dramatic on the narrower CcP Me-His signal. Spectra of CcP Me-His compound I bearing the W191F mutation are consistent with the formation of relatively low yielding (~15% of heme concentration) adventitious tyrosine radicals remote from the heme, as previously observed in the W191F and W191G variants of CcP His.^{22,23} Therefore, despite exhibiting an EPR signal line shape different from that

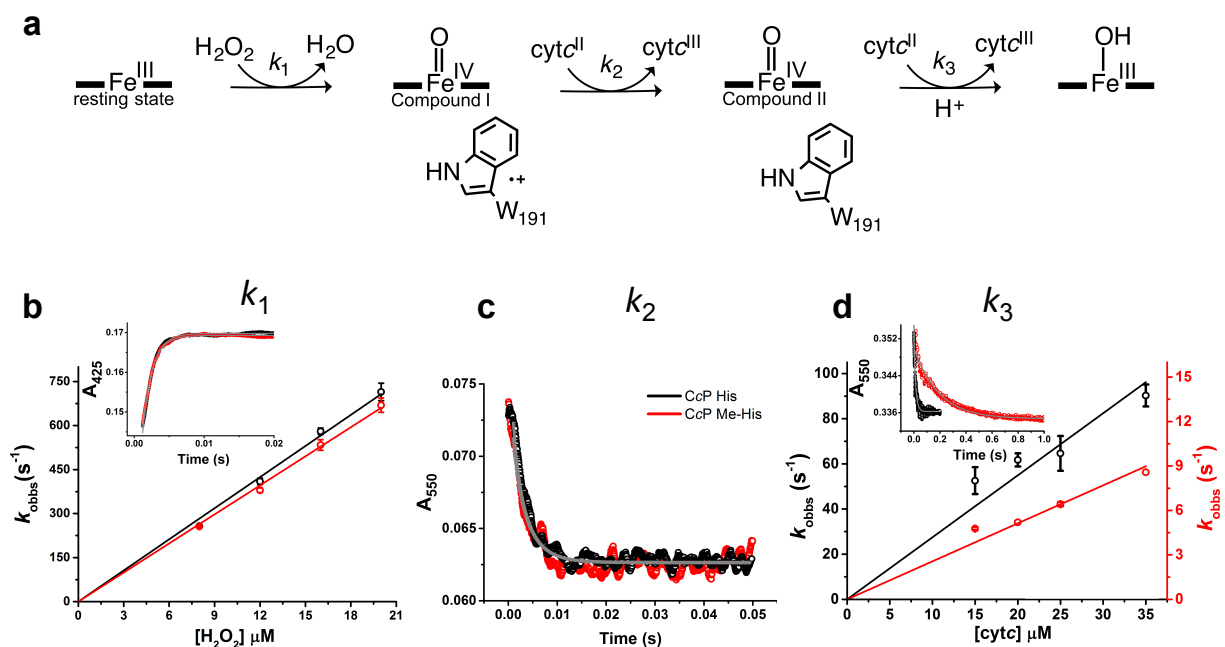


Figure 5. Pre-steady state kinetic characterization of CcP His and CcP Me-His. (a) The catalytic mechanism of CcP. (b) Observed rate constants for compound I formation at varying H_2O_2 concentrations. Representative kinetic traces at $20 \mu M H_2O_2$ are shown (inset). A linear fit of k_{obs} vs $[H_2O_2]$ was used to derive a bimolecular rate constants of $k_1 = (3.5 \pm 0.7) \times 10^7 M^{-1} s^{-1}$ for CcP His (black) and $k_1 = (3.3 \pm 0.4) \times 10^7 M^{-1} s^{-1}$ for CcP Me-His (red). Error bars represent SD $n=3$. (c) Averaged kinetic traces ($n=3$) for compound I reduction for both CcP His (black) and CcP Me-His (red). Conditions: CcP His/CcP Me-His ($4 \mu M$), H_2O_2 ($8 \mu M$), delay time of 1 s before reaction with cytc ($1.5 \mu M$) (post-mixing concentrations). Reactions were monitored by reduction in absorbance at 550 nm due to oxidation of ferrous cytc. These data are fitted to an integrated second order rate equation (grey lines) to derive an apparent intrinsic rate constants of $k_2 = (1.16 \pm 0.12) \times 10^8 M^{-1} s^{-1}$ in CcP His and $k_2 = (1.14 \pm 0.25) \times 10^8 M^{-1} s^{-1}$ in CcP Me-His. The instrument dead time for the determination of k_2 is <1.5 ms. (d) Observed rate constants for compound II reduction at varying cytc concentrations. Representative kinetic traces at $35 \mu M$ are shown (inset) and all data were fitted to: $A_{550} = A_0 + \Delta A(e^{-k_2[cytc]_0 t} + e^{-k_{obs} t})$ with k_2 fixed to the value determined above. A linear fit of k_{obs} vs. $[cytc]$ was used to derive a bimolecular rate constants for CcP His (black, $k_3 = (2.7 \pm 0.2) \times 10^6 M^{-1} s^{-1}$) and CcP Me-His (red, $k_3 = (2.6 \pm 0.1) \times 10^5 M^{-1} s^{-1}$). Error bars are SD ($n=2$ for CcP Me-His, $n=3-5$ for CcP His). All measurements are at pH 6, $4^\circ C$.

of CcP His, CcP Me-His forms a compound I characterized by a ferryl heme exchange-coupled to a Trp191 cation radical, with the differences in line shape being attributable to a change in that ferromagnetic coupling. Houseman *et al*²¹ state in their original determination of the value(s) of J for CcP His that ‘given the tiny energies associated with the heme-radical spin coupling, it is hardly surprising that the EPR spectrum of compound I should be exquisitely sensitive to small perturbations in the protein’s surroundings.’

Rate constants for the three elementary steps (k_1 , k_2 and k_3 , Figure 5a) in the catalytic cycle of CcP His and CcP Me-His were determined at 4°C using established stopped-flow techniques.²⁴ Introduction of the less electron donating Me-His ligand has negligible impact on the rate constant for compound I formation ($k_1 = (3.5 \pm 0.7) \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ for CcP and $k_1 = (3.3 \pm 0.4) \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ for CcP Me-His, Figure 5b, Figure S7a-d and S7i). Likewise, the rate constant for compound I reduction is unaffected by proximal ligand substitution ($k_2 = (1.16 \pm 0.12) \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ in CcP His and $k_2 = (1.14 \pm 0.25) \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ in CcP Me-His, Figure 5c and Figure S7l), demonstrating that the natural electron transfer pathway from ferrous cytc to Trp191 is maintained in the modified enzyme and that the redox potential of Trp191 has not been significantly altered by axial ligand substitution. In contrast, compound II reduction is 10-fold slower in CcP Me-His ($k_3 = (2.6 \pm 0.1) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ vs $(2.7 \pm 0.2) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ for CcP His, Figure 5d, Figure S7e-h and S7j). This 10-fold reduction in k_3 also correlates with a comparable reduction in k_{cat} in steady-state assays

at 4°C ($k_{\text{cat}} = 235 \pm 35 \text{ s}^{-1}$ for CcP His, $k_{\text{cat}} = 22 \pm 5 \text{ s}^{-1}$ for CcP Me-His, Figure S3c and S3d).

The relationship between axial ligand substitution and compound II reactivity cannot be rationalized through consideration of the driving force for electron transfer, which is greater in CcP Me-His due to the increase in heme redox potential. However, differences in electronic coupling between Trp191 and the heme center upon ligand substitution could potentially give rise to reduced electron transfer rates due to modulation of the electron transfer pathway. Alternatively, compound II reduction in heme peroxidases is thought to be coupled to proton transfer to the ferryl oxygen, with significant solvent kinetic isotope effects (KSIEs) reported previously for ascorbate peroxidase and *Leishmania* peroxidase (LmP).^{25,17} The less electron-donating Me-His ligand could give rise to a less basic ferryl-oxygen and consequently perturb proton coupled electron transfer to the ferryl heme (Figure 6c and Figure 6d). The $\text{p}K_{\text{a}}$ of metal-oxo bonds is known to be exquisitely sensitive to the electron donating capabilities of ancillary ligands.²⁶

To distinguish between these two mechanistic hypotheses, we performed activity assays in buffered H₂O and D₂O. Kinetic characterization of CcP His and CcP Me-His (pH/pD 6.0) revealed a KSIE of 1.4 and 1.5, respectively, with no effect on K_{M} in either variant (Figure 6a). We also investigated the influence of pH/pD on CcP His and CcP Me-His activity. For both variants, initial reaction velocities increase with decreasing pH/pD between 6 and 5 (Figure S8a and S8b). The increased reaction rates also correlate with increasing KSIEs

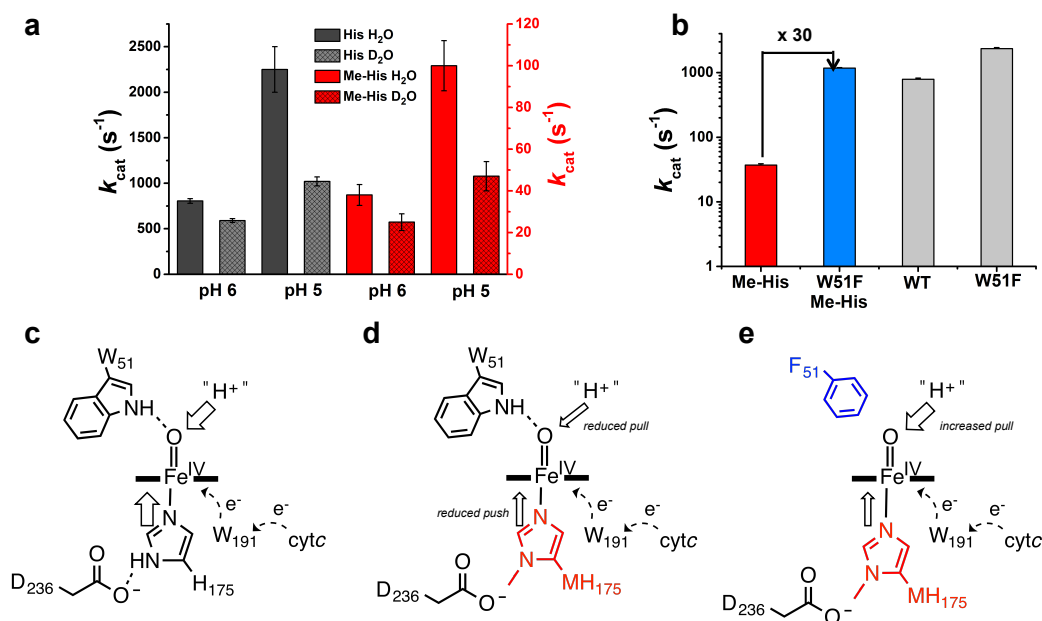


Figure 6. Solvent kinetic isotope effects and the role of Trp51 in controlling compound II proton affinity and reactivity. (a) Bar chart showing the solvent kinetic isotope effect on k_{cat} for cytc oxidation by CcP His, CcP Me-His at pH/pD = 6 and at pH/pD = 5. CcP His: KSIE = 1.4 (pH/pD = 6) and KSIE = 2.2 (pH/pD = 5). CcP Me-His: KSIE = 1.5 (pH/pD = 6) and KSIE = 2.1 (pH/pD = 5) (b) Bar chart showing the kinetics (k_{cat}) of cytc oxidation by CcP His, CcP Me-His and their W51F variants. (c-e) Schemes for proton coupled compound II reduction in (c) CcP His, (d) CcP Me-His and (e) CcP Me-His W51F, highlighting the importance of axial ligand electron donation (the ‘push’) and hydrogen bonding interactions with Trp51 in controlling the proton affinity of the ferryl oxygen (the ‘pull’). The proton (“H”) is likely transferred from His52 via an ordered water molecule.¹⁷

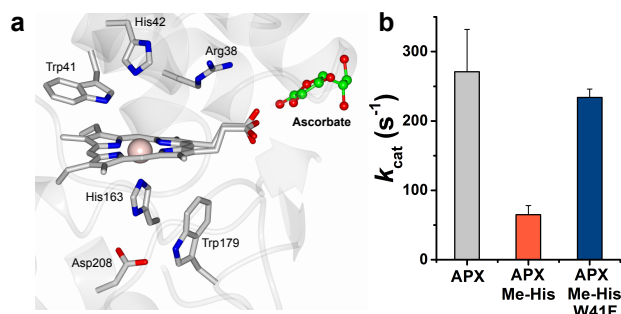


Figure 7. Kinetic characterization of ascorbate peroxidase (APX) and key active site mutants. (a) The active site of APX showing the substrate ascorbate bound at the γ -heme edge (PDB code: 1OAF).³¹ (b) Bar chart showing the kinetics (k_{cat}) of ascorbate oxidation by APX His, APX Me-His and APX Me-His W51F. Measurements are recorded at 25°C in phosphate buffer (50 mM, pH 6).

across the series, with values of 2.2 and 2.1 determined for CcP His and CcP Me-His, respectively, at pH/pD = 5 (Figure 6a). These data demonstrate that proton transfer is involved in rate limiting compound II reduction in CcP His and CcP Me-His, and suggest that the observed electron donation-compound II reactivity trend can be explained by considering the proton affinity (‘the pull’) of the ferryl-oxygen (Figure 6c and 6d).

We therefore considered alternative factors that are likely to govern the $\text{p}K_{\text{a}}$ of compound II. The N-H group of Trp51 forms a hydrogen bond to the ferryl oxygen,¹⁸ an interaction that can be expected to withdraw electron density from the Fe(IV)=O unit. We reasoned that deletion of this hydrogen bond using a W51F mutation in CcP His and Me-His would provide a means of increasing the $\text{p}K_{\text{a}}$ of the ferryl oxygen without causing significant disruption of the electron transfer pathway from Trp191 to the heme iron. Indeed, EPR spectra at 6 K of compound I generated in W51F CcP and W51F CcP Me-His demonstrate formation of a ferryl heme:Trp191 exchange coupled species (Figure 4). Consistent with previous studies,²⁷ a W51F mutation in CcP His leads to a 3-fold increase in cytc oxidation activity ($k_{\text{cat}} = 2,350 \pm 100 \text{ s}^{-1}$). This activity has been previously shown to be strictly dependent on the presence of Trp191, demonstrating that the natural electron transfer pathway is maintained in the W51F mutant.²⁸ The W51F substitution in CcP Me-His leads to a more dramatic 32-fold increase in k_{cat} ($1,170 \pm 30 \text{ s}^{-1}$, ferrous cytc oxidation) (Figure 6b, 6e and Figure S3f). Thus the deleterious effects of reduced electron donation from the Me-His axial ligand can be fully compensated by removing a single hydrogen bonding interaction to the ferryl oxygen (Figure 6d and 6e) to afford a variant (W51F CcP Me-His) with catalytic features reminiscent of the wild-type enzyme, further underscoring the importance of Fe(IV)=O $\text{p}K_{\text{a}}$ in governing compound II reactivity.

A strongly electron donating ‘imidazolate-like’ axial ligand is a conserved feature across the heme peroxide family. To investigate whether this ligand plays an important role in tuning Fe(IV)=O $\text{p}K_{\text{a}}$ and catalytic activity in family members other than CcP, we elected to re-engineer the heme coordination environment of ascorbate peroxidase (APX). We have previously shown that introducing a Me-His ligand into an evolved ascorbate peroxidase (APX2) has negligible effects on

the efficiency of guaiacol oxidation, but increases the robustness of the enzyme towards irreversible deactivation during catalysis.²⁹ The active site structure and overall catalytic mechanism of APX is highly similar to that of CcP. Compound II reduction is rate-limiting,³⁰ and electron transfer from ascorbate is thought to be coupled with proton transfer to the ferryl-oxygen.²⁵ However, unlike CcP, the proximal pocket Trp179 of APX does not participate in redox chemistry and electron transfer to the heme occurs directly from the small molecule substrate ascorbate bound at the γ -heme edge (Figure 7).³¹ Consequently, we reasoned that axial ligand substitution in APX would provide a means of tuning Fe(IV)=O $\text{p}K_{\text{a}}$ without causing significant perturbations to the pathway of electron transfer from substrate to the heme center. His \rightarrow Me-His ligand substitution in APX leads to a substantial reduction in k_{cat} for ascorbate oxidation (pH 6.0, 25°C) ($k_{\text{cat}} = 270 \pm 71 \text{ s}^{-1}$ for APX His, $k_{\text{cat}} = 66 \pm 13 \text{ s}^{-1}$ for APX Me-His, respectively) with negligible changes in K_{M} (Figure 7, Fig S9). We have previously shown that this substitution causes only minor structural changes similar to those observed in CcP.²⁹ Significantly, the activity of APX Me-His can be restored to wild-type levels by introducing a W41F substitution designed to increase Fe(IV)=O $\text{p}K_{\text{a}}$ ($k_{\text{cat}} = 234 \pm 12 \text{ s}^{-1}$ for APX Me-His W41F). These activity trends mirror the trends observed in CcP (Figure 6b), suggesting a common mechanistic strategy is employed by enzymes using distinct electron transfer pathways. Combined, these data provide further evidence of the relationships between Fe(IV)=O basicity and reactivity.

DISCUSSION

The ability to expand upon nature’s genetic code to selectively install non-canonical ligands into proteins provides exciting new opportunities to directly probe metalloenzyme mechanisms.³² For example, selenocysteine has proven to be a valuable surrogate for axial heme thiolate ligands.³³⁻³⁵ We have recently demonstrated that introduction of a Me-His proximal ligand into an evolved ascorbate peroxidase (APX2)²⁹ and into the oxygen binding protein myoglobin³⁶ improves catalytic performance towards non-biological oxidations of model phenolic substrates.

An expanded genetic code has now allowed us to elucidate the functional significance of the ‘imidazolate like’ axial ligand of a heme peroxidase. Replacement of the proximal histidine ligand of CcP by Me-His provides a means of disrupting the hydrogen bond between Asp235 and the axial histidine while preserving catalytic function and crucially, the environment surrounding the key redox active Trp191 residue. This contrasts with previous attempts to modulate proximal ligand electron donation though mutation of Asp235, which resulted in dramatic reorientation of the Trp191 residue and complete loss of activity.²⁰ Surprisingly, replacement of the His ligand by Gln has previously been shown to have little effect on the rate of cytc oxidation.³⁷ However, whilst a W191F mutation in CcP His or CcP Me-His abolishes activity, the CcP H175Q W191F double mutant maintains high levels of activity. The H175Q mutation thus appears to open up alternative electron pathways from cytc to the ferryl heme of CcP that are not utilized in the natural catalytic cycle.

The availability of a functional CcP with a modified axial ligand allowed us to directly probe and quantify the impact of altered ligand electron donation on the reactivity of the iconic ferryl intermediates compound I and compound II. Replacement of the proximal histidine ligand of CcP by a less electron

donating Me-His has little effect on the rate of formation of compound I. This contrasts with the substantial increase in rate constant for the formation of ferryl intermediates observed upon His → Me-His ligand substitution in myoglobin³⁶ an oxygen binding protein that has served as a valuable model system to probe heme enzyme structure-function relationships. Introduction of the Me-His ligand into CcP also has negligible effects on the rate constant for compound I reduction, demonstrating that the natural electron transfer pathway from ferrous cytc to Trp191 is maintained in the modified enzyme. In contrast, compound II reduction is 10-fold slower in CcP Me-His, providing a direct link between axial ligand electron donation and compound II reactivity. Our combined data present a compelling body of evidence in support of Fe(IV)=O pK_a being a key determinant of compound II reactivity in heme peroxidases, with reduced electron donation from the non-canonical Me-His ligand ('the push') affording an electron-deficient ferryl-oxygen with decreased proton affinity ('the pull') compared with the wild-type enzyme. The deleterious effects of the Me-His ligand can be fully compensated by introducing a W51F mutation, designed to increase 'the pull' by removing a hydrogen bond to the ferryl-oxygen. This ability to rationally rewire the catalytic machinery of CcP NMH provides strong evidence to support our mechanistic interpretations. The W51F mutation in CcP His has previously been shown to increase the reactivity of the ferryl heme center. However, the molecular origins of this increased reactivity are not well defined. For example, it has been suggested that introduction of the smaller Phe residue provides more space and flexibility in the heme pocket that could give rise to the observed activity changes.³⁸ Elsewhere, the increased rate of compound II reduction has been ascribed to an increase in activation entropy, which the authors suggest may arise due to a more facile release of the ferryl oxygen atom as water.³⁹ We instead propose that the W51F substitution serves to facilitate proton transfer to the ferryl oxygen, which is required prior to the dissociation of water. This interpretation is consistent with the large increase in cyt *c* oxidation activity due to W51F mutation in CcP Me-His, and the substantial kinetic solvent isotope effects observed for CcP His and CcP Me-His.

In summary, this study sheds light on long standing questions in heme biochemistry and advances our fundamental understanding of biological electron transfer events. Proton coupled electron transfers and related H atom transfers to high valent metal-oxo intermediates are thought to be ubiquitous in biological systems, and have been implicated as key steps in the catalytic mechanisms of several histidine ligated metalloenzymes.^{40,41} Our methodology is thus expected to provide a versatile strategy to deconstruct these complex and sophisticated bioinorganic mechanisms and can be extended to probe and tune the reactivities of isoelectronic heme carbene and nitrene intermediates.⁴² Such insights into the subtle interplay between active site structure and catalytic activity will be critical for future efforts to rationally create functional metalloenzymes.

EXPERIMENTAL PROCEDURES

Materials. All materials were obtained from Sigma-Aldrich unless otherwise stated. Oligonucleotides were synthesized by MWG Eurofins (Ebersberg, Germany). Ferric cytc from horse heart was obtained from Sigma-Aldrich and used throughout the study.

Construction of pET-11a_CcP, pET-11a_CcP_Me-His and their variants. The gene encoding cytochrome *c* peroxidase (Ccp1p from the *S. cerevisiae* YJM1444 genome) was PCR amplified from plasmid pLeics03CCP39 (a modified version of an original CcP clone)⁴³ using Phusion High-Fidelity DNA polymerase (New England BioLabs, UK). Primers contained a N-terminal His tag and complimentary sequences to allow insertion of the PCR product into pET-11a plasmid using In-Fusion® Advantage PCR Cloning via *Nde*I and *Bam*HI restriction sites, yielding pET-11a_CcP. The His175Me-His mutation was introduced into the pET-11a_CcP by replacing the His175 codon with a TAG stop codon. Site-directed mutagenesis using the Q5® Site-Directed Mutagenesis Kit (New England BioLabs, UK) of pET-11a_CcP yielded pET-11a_CcP_Me-His. W191F and W51F mutations were introduced into pET-11a_CcP and pET-11a_CcP_Me-His using the Q5® Site-Directed Mutagenesis Kit. Correct DNA sequences were confirmed by DNA sequencing (MWG Eurofins).

Construction of pET-29b_APX, pET-29b_APX_Me-His. An engineered monomeric soybean ascorbate peroxidase (APX) containing K14D, E112K and A134P mutations was used throughout this study.⁴⁴ We have previously created the p29b_APX2 and p29b_APX2_Me-His constructs required for the production of APX W41F and APX Me-His W41F, respectively.²⁹ To create pET-29b_APX and pET-29b_APX Me-His, a F41W mutation was introduced into p29b_APX2 and p29b_APX2_Me-His using assembly PCR followed by restriction cloning using *Nde*I and *Xho*I sites.

Protein production and purification. For expression of CcP and its variants, BL21(DE3) *E. coli* were transformed with pET-11a_CcP, pET-11a_CcP_W51F and pET-11a_CcP_W191F and the cells were plated onto LB agar (Formedium, Norfolk, UK) plates containing 50 µg/ml ampicillin. For expression of APX, BL21(DE3) *E. coli* was transformed with pET-29b_APX and the cells were plated onto LB agar (Formedium, Norfolk, UK) plates containing 50 µg/ml kanamycin. A single colony of freshly transformed cells was cultured for 18 h in 10 ml of LB medium containing 50 µg/ml ampicillin or kanamycin (for CcP and APX, respectively). 4 ml of the culture was used to inoculate 400 ml of 2xYT medium (Formedium, Norfolk, UK) supplemented with 1 mM δ-aminolevulinic acid and 50 µg/ml of the appropriate antibiotic. The culture was incubated for ~2 h at 37°C with shaking at 180 rpm. When the OD₆₀₀ of the culture reached ~0.5, IPTG was added to a final concentration of 100 µM. The induced cultures were incubated for ~20 h at 25°C, and the cells were subsequently harvested by centrifugation at 7000 *g* for 10 min.

For expression of CcP Me-His and its variants, BL21(DE3) *E. coli* were co-transformed with pET-11a_CcP_Me-His/pET-11a_CcP_Me-His_W191F/pET-11a_CcP_Me-His_W51F and pEVOL_PylRSM-His²⁹ and the cells were plated onto LB agar plates containing 50 µg/ml ampicillin and 34 µg/ml chloramphenicol. For expression of APX Me-His and APX Me-His W41F, BL21(DE3) *E. coli* were transformed with pET-29b_APX Me-His and p29b_APX2_Me-His and the cells were plated onto LB agar (Formedium, Norfolk, UK) plates containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol. A single colony of freshly transformed cells was cultured for 18 h in 10 ml of LB medium containing 50 µg/ml ampicillin or kanamycin (for CcP and APX, respectively) and 34 µg/ml chloramphenicol. 4 ml of the culture was used to inoculate 400 ml of 2xYT medium supplemented with 1 mM δ-aminolevulinic acid, 10 mM H-His(3-Me)-OH (Me-His)

(Bachem, Saint Helens, UK), 50 $\mu\text{g}/\text{ml}$ of the appropriate antibiotic and 34 $\mu\text{g}/\text{ml}$ chloramphenicol. The culture was incubated for ~ 2.5 h at 37°C with shaking at 180 rpm. When the OD_{600} of the culture reached ~ 0.5 , IPTG and arabinose were added to a final concentration of 100 μM and 0.05%, respectively. The induced cultures were incubated for ~ 20 h at 25°C , and the cells were subsequently harvested by centrifugation at 7000 g for 10 min.

The pelleted bacterial cells were suspended in phosphate buffer (50 mM KPi, 300 mM NaCl, 10 mM imidazole pH 7.5) supplemented with lysozyme (1 mg/ml), DNase (0.1 U/ml) and a Complete EDTA free protease inhibitor cocktail tablet (Roche) and subjected to sonication (13 mm probe, 15 mins, 20 s on, 40 s off, 40% amplitude). Cell lysates were centrifuged at 27,000 g for 30 min and the supernatants subjected to affinity chromatography using Ni-NTA Agarose (Qiagen, West Sussex, UK). His-tagged CcP and APX variants were eluted by 50 mM KPi, 300 mM NaCl, pH 7.5 buffer containing 300 mM imidazole. The purified protein was desalted using a 10DG desalting column (Bio-Rad, Hertfordshire, UK) into 50 mM KPi pH 6 buffer. To maximize heme occupancy, variants were reconstituted with hemin chloride. Cell suspensions were mixed with 0.5 mM of hemin chloride (50 mM stock solution in 10 mM NaOH) for 30 mins at RT and filtered through a 0.45 micron membrane. The CcP and CcP Me-His variants were then subjected to anion exchange chromatography with 50 mM KPi pH 6 buffer using a ResourceTM Q column on an AKTA Fast Protein Liquid Chromatography (FPLC) system (both GE Healthcare, Buckinghamshire, UK). Proteins were eluted by a linear gradient of NaCl in the concentration range 0-500 mM and the eluate was collected in 2 ml fractions. The purest fractions were selected after spectral analysis, pooled and concentrated using Vivaspin 20 centrifugal concentrators (Generon, Berkshire, UK) with a 10 kDa molecular weight cut off membrane. The protein was aliquoted, flash frozen in liquid nitrogen and stored at -80°C . The concentrations of CcP and APX proteins were determined assuming an extinction coefficient of 101 $\text{mM}^{-1}\text{cm}^{-1}$ at 408 nm and 128 $\text{mM}^{-1}\text{cm}^{-1}$ at 405 nm, respectively.^{2,29} The heme occupancies of purified proteins and their variants are comparable, with similar Rz (A410/A280) values of ~ 1.5 .

Production of CcP and CcP Me-His containing L-tryptophan-(indole- d_5). For expression of L-tryptophan-(indole- d_5) CcP (*d*-Trp CcP) and L-tryptophan-(indole- d_5) CcP Me-His (*d*-Trp CcP Me-His); BL21(DE3) *E. coli* were transformed with pET-11a_CcP or pET-11a_CcP_Me-His and pEVOL_PyIRSMe-His, respectively, and single colonies were cultured for 18 h in 10 ml of LB medium containing the appropriate antibiotics. 2 ml of the culture was used to inoculate 200 ml of M9 minimal medium; containing 33.7 mM Na_2HPO_4 , 22.0 mM KH_2PO_4 , 8.55 mM NaCl, 9.35 mM NH_4Cl , 0.4% glucose, 1 mM MgSO_4 , 0.3 mM CaCl_2 , 1 $\mu\text{g}/\text{L}$ biotin and 1 $\mu\text{g}/\text{L}$ thiamin, 1 x trace elements solution (100 x trace element solution - 13.4 mM EDTA, 3.1 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.62 mM ZnCl_2 , 76 μM $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 42 μM $\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$, 162 μM H_3BO_3 , 8.1 μM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$), 0.08% -Trp DROP OUT Complete Supplement Mixture (ForMedium) and 10 mM L-tryptophan-(indole- d_5). Cultures were induced and pelleted as above and the proteins were purified as described above. Complete replacement of all tryptophans with L-tryptophan-(indole- d_5) was confirmed by MS (Figure S1).

MS analysis. Purified protein samples were buffer-exchanged into 0.1% acetic acid using a 10K MWCO Vivaspin (Sartori-

us) and diluted to a final concentration of 0.5 mg ml^{-1} . MS was performed using a 1200 series Agilent LC, 5 μl injection into 5% acetonitrile (with 0.1% formic acid) and desalted inline for 1 min. Protein was eluted over 1 min using 95% acetonitrile with 5% water. The resulting multiply charged spectrum was analyzed using Agilent QTOF 6510 and deconvoluted using Agilent MassHunter Software. All data is presented in Figure S1.

Crystallization, refinement and model building. CcP Me-His was crystallized at 50 mg ml^{-1} in 50 mM KPi, pH 6 buffer. Initial crystallization conditions were identified using the JCSG+ matrix screen (Molecular dimensions). Crystals suitable for diffraction experiments were obtained by sitting drop vapour diffusion at 4°C in 400 nl drops containing equal volumes of protein and a solution containing 5 mM cobalt(II) chloride hexahydrate, 5 mM cadmium chloride hemi(pentahydrate), 5 mM magnesium chloride hexahydrate, 5 mM nickel(II) chloride hexahydrate, 0.1 M HEPES pH 7.5 and 12% w/v PEG 3350. The crystals were cryoprotected by the addition of 10% PEG 200 to the mother liquor and flash-cooled in liquid nitrogen. Data were collected on beamline IO4-1 (wavelength 0.9159 \AA) at the Diamond Light Source Facility and reduced and scaled with the X-ray Detector Software suite (XDS37). The CcP Me-His crystal structure was determined by molecular replacement using the PHASER program in the CCP4 suite⁴⁵ using WT CcP structure as the starting model (PDB code: 2CYP). The CcP Me-His model was completed by iterative cycles of manual model building and real space refinement using the program Coot and crystallographic refinement using Refmac. The processing and final refinement statistics are presented in Table S1. Coordinates and structure factors have been deposited in the Protein Data Bank under accession number 6H08.

Spectroscopic characterization of CcP and CcP Me-His variants. UV-vis absorption analysis was carried out on a Cary 50Bio UV-vis spectrophotometer (Varian, CA, USA), using a 1 cm path length quartz cuvette, recording spectra between 250 and 700 nm and typically with CcP at 4 μM in 50 mM KPi pH 6 buffer (See Figure S5).

Steady state enzyme kinetic assays. Steady state enzyme assays were carried out on a Cary 50Bio UV-vis spectrophotometer (Varian, CA, USA) in 50 mM KPi pH 6 using a 1 cm path length, 1 ml quartz cuvette. For cytc oxidation assays CcP, CcP W51F, CcP Me-His, and CcP W51F Me-His variants were diluted to 1.0 μM on the basis of the heme absorption, respectively. CytcII was prepared by reduction with dithionite and the excess reductant was removed with a 10DG desalting column. The concentration of cytcII was calculated using the extinction coefficient; $\epsilon_{550} = 27.7 \text{ mM}^{-1}\text{cm}^{-1}$.⁴⁴ At each concentration of cytc (0–100 μM), CcP, CcP W51F, CcP Me-His, and CcP W51F Me-His variants were diluted to 1, 1, 10, and 1 nM, respectively. H_2O_2 (100 μM) was added to initiate the reaction and the rate of change in absorbance at 550 nm was monitored over 1 min at 25°C . The difference in absorptivity of cytcII and cytcIII was calculated using $\Delta\epsilon_{550} = 19.5 \text{ mM}^{-1}\text{cm}^{-1}$.⁴⁶ Reactions were performed in triplicate (at RT and 4°C) to produce a mean rate calculated as moles of cytcII oxidized per mol CcP per second. These data were plotted against the relevant cytcII concentration and fitted using the Michaelis–Menten hyperbolic function within Origin software. Reported values are corrected for background cytc oxidation in the absence of enzyme. For CcP His W191F and CcP W191F Me-His at 50 nM no activity was observed above background.

For ascorbate oxidation assays APX, APX Me-His and APX Me-His W41F variants were diluted to 1 μM on the basis of the heme absorption, respectively. At each concentration of ascorbate (0–1100 mM), APX, APX Me-His and APX Me-His W41F variants were diluted to 1 nM and 100 μM H_2O_2 was added to initiate the reaction. Product formation was monitored at 290 nm over 5 min at 25°C and calculated using $\epsilon_{290} = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$.⁴⁷ All reactions were performed in triplicate to produce a mean rate calculated as moles of substrate oxidized per mol APX per second. These data were plotted against the relevant substrate concentration and fitted using the Michaelis–Menten hyperbolic or Hill functions within Origin software (see Figure S9).

Redox potentiometry. Optically transparent thin layer electrochemistry (OTTLE) techniques were used to determine the CcP heme redox potentials.^{48,49} Briefly, the proteins were exchanged into 100 mM KCl, 50 mM potassium phosphate, 10% glycerol, pH 6, and the following redox mediators were added to ensure efficient equilibration in the electrochemical cell: 20 μM anthraquinone-2-sulfonate, 20 μM phenazine, 25 μM 2-hydroxy-1,4-naphthoquinone and 6 μM indigotrisulfonate. The solution containing protein and mediators was added to a home-built OTTLE cell, constructed from a flat quartz EPR cell (WilmaD, USA) calibrated against cytc, a platinum gauze working electrode, platinum counter electrode and an Ag/AgCl reference electrode (BASi, USA). Potentials were applied for 30 minutes each across the cell using a Biologic SP-150 potentiostat, and titrations were carried out in both reductive and oxidative directions to ensure good equilibration. UV/visible spectra were acquired at each potential using an Agilent Cary 60 UV/visible spectrometer. Heme redox potential was determined by plotting the absorbance at 439 nm (corresponding to the ferrous heme Soret band) against applied potential and fitting to a standard 1-electron Nernst function: $f(x) = (A + B \times 10^{((E_m - x)/59)}) / (1 + 10^{((E_m - x)/59})$.

Where A and B are the y-axis values representing 0 and 100% reduced CcP respectively. E_m corresponds to the heme reduction potential. Once it was established that the E_m for both reductive and oxidative titrations was the same, the data was combined and plotted as proportion reduced vs applied potential (vs NHE) and then fitted to the equation above.

Electron paramagnetic resonance (EPR) analysis. Continuous wave EPR spectra were recorded at X-band (~ 9.4 GHz) using a Bruker ELEXSYS E500/E580 EPR spectrometer (Bruker GmbH, Rheinstetten, Germany). Temperature was maintained using an Oxford Instruments ESR900 helium flow cryostat coupled to an ITC 503 controller from the same manufacturer. EPR sample tubes were 4 mm Suprasil quartz supplied by WilmaD (Vineland, NJ). Compound I was formed using 200 μM protein with 180 μM H_2O_2 in 50 mM KPi pH 6 buffer. EPR experiments employed 10 μW microwave power (non-saturating), 100 KHz modulation frequency and 1 G (0.1 mT) modulation amplitude to avoid diminution of any partially resolved hyperfine coupling. The experimental temperatures were as given in the text and figure captions.

Stopped-flow kinetics. Stopped-flow absorbance experiments were performed on an Applied Photophysics SX18 stopped-flow spectrophotometer (Applied Photophysics Ltd., Leatherhead, UK) equipped with a xenon arc lamp and a 1 cm path length in 50 mM KPi pH 6 buffer. To follow the spectra evolution of CcP and CcP Me-His ferric or ferrous to compound I and compound II intermediates, respectively, the drive syringes were loaded with separate solutions of 16 μM ferric or fer-

rous protein (reduced with minimum dithionite) and stoichiometric or 64 μM H_2O_2 .⁵⁰ Multiple wavelength data were collected at RT using a (PDA) detector and XSCAN software (see Figure S6).

Second-order rate constants for formation of CcP and CcP Me-His compound I were derived as follows. The drive syringes of the stopped-flow were loaded with separate solutions of 4 μM ferric protein and 16, 24, 32 and 40 μM H_2O_2 . Compound I formation was monitored by an increase in absorbance at 425 nm at 4°C. Values are an average of three individual measurements (fitted values are given in the Figure S7i) and a linear fit of k_{obs} vs $[\text{H}_2\text{O}_2]$ was used to derive a bimolecular rate constant (k_1).

Second-order rate constants for CcP and CcP Me-His compound I reduction were determined in double mixing stopped flow experiments, using an excess of compound I (over cytc) to avoid subsequent reduction of compound II. CcP/CcP Me-His (16 μM) was mixed 1:1 with H_2O_2 (32 μM) and aged for 1 s to allow complete compound I formation. CytcII (3 μM) was mixed 1:1 with compound I. Oxidation of ferrous cytc to the ferric state was monitored by a reduction in absorbance at 550 nm. Consistent with previous studies, determination of k_2 under pseudo first order conditions was not possible due to the rapid nature of this electron transfer process.^{24,51} The compound I reduction reaction was kinetically modelled by the equation within Figure S7k. Fitting was performed using Mathematica 11 (Wolfram Research Inc.) using the NDSolve and Findfit functions. The time evolution of [cytcII] was fitted by: $A(t) = A_0 + \Delta\epsilon_{\text{app}} \times [\text{cytc}](t)$. There are 3 fitting parameters: k , the apparent intrinsic rate constant, described by the ODEs; $\Delta\epsilon_{\text{app}}$ the apparent extinction coefficient difference between reactant and products; and A_0 the background sample absorbance. Experiments were measured under a single set of conditions with $[\text{CcP}] = 4 \mu\text{M}$, $[\text{H}_2\text{O}_2] = 8 \mu\text{M}$ and $[\text{cytcII}] = 1.5 \mu\text{M}$. The model assumes [compound I] = 4 μM at t_0 i.e. all CcP is converted to compound I prior to the second stopped-flow push at t_0 (the conversion to compound I was shown to be complete within 0.2 s in single mixing experiments under comparable conditions). Each experiment was performed in triplicate and fitted values are given in the Figure S7l. Second-order rate constants for CcP and CcP Me-His compound II reduction were determined in double mixing stopped flow experiments. CcP His/CcP Me-His (6.8 μM) was mixed 1:1 with 6 μM H_2O_2 and aged for 1 s to allow complete compound I formation. Compound I (3 μM) was subsequently mixed 1:1 with 30, 40, 50, 70 μM cytcII. Oxidation of ferrous cytc to the ferric state was monitored by a reduction in absorbance at 550 nm (Figure S7e). Values reported are an average of two–five individual measurements and all data were fitted to: $A_{550} = A_0 + \Delta A(e^{-k_2[\text{cytc}]_0 t} + e^{-k_{\text{obs}} t})$ with k_2 fixed to the value determined above (fitted values are given in the Figure S7j). A linear fit of k_{obs} vs. [cytc] was used to derive a bimolecular rate constant (k_3).

Solvent kinetic isotope effects and pH profiling. KSIE and pH profile experiments were performed in a three component buffer system (37.5 mM acetic acid, 37.5 mM MES, 75 mM Tris)^{52,53} with virtually constant ionic strength over a large pH range (4.00–7.50). Deuterated buffers were prepared in 99.9% D_2O , and pD (pH in D_2O) was adjusted according to the following relationship: $\text{pD} = \text{pH}_{\text{obs}} + 0.38$. Buffers were prepared at the following pH/pD values 4.50, 4.75, 5.00, 5.25, 5.50, 5.75, 6.00, 6.25, 6.50, 6.75. Concentrated stock solutions of cytc and H_2O_2 were prepared in H_2O and D_2O at pH/pD 6.0

and were used for all assays. Concentrated stock solutions of CcP His and CcP Me-His solutions were prepared in a 50:50 mixture of H₂O (pH 6.0) and D₂O (pD 6.0) and was used for all assays. For pH profiling, assays were performed at a fixed concentration of cytc (70 μM). Initial velocities at each pH/pD were determined as described above (steady state enzyme kinetic assays), using the following assay components: 940 μl of appropriate buffer, 10 μl enzyme (1 nM for CcP His and 10 nM for CcP Me-His), 25 μl cytc (70 μM) and 25 μM H₂O₂ (100 μM). The pH was recorded after each measurement. Reported values are corrected for background cytc oxidation in the absence of enzyme at the appropriate pH.

For KSIE at pH/pD 5.0 (37.5 mM acetic acid, 37.5 mM MES, 75 mM Tris) and pH/pD 6.0 (50 mM KPi) steady state reactions were performed in triplicate to produce a mean rate calculated as moles of cytcII oxidized per mol CcP per second. These data were plotted against the relevant cytcII concentration and fitted using the Michaelis–Menten hyperbolic function within Origin software (see Figure S9).

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Notes

The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. MS data, UV–vis spectral data, redox potentiometry, kinetic analysis, and structure statistics are described. The crystal structure of CcP Me-His was deposited in the RCSB Protein Data Bank (PDB) under accession number 6H08.

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