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Geometric and Electronic Structure Contributions to O–O Cleavage and the Resultant Intermediate Generated in Heme-Copper Oxidases

Andrew W. Schaefer[†], Antonio C. Roveda Jr.[†], Anex Jose[†], and Edward I. Solomon[†]

[†] Department of Chemistry, Stanford University, Stanford, California 94305, United States

Abstract

This study investigates the mechanism of O–O bond cleavage in heme-copper oxidase (HCO) enzymes, combining experimental and computational insights from enzyme intermediates and synthetic models. It is determined that HCOs undergo a proton-initiated O–O cleavage mechanism where a single water molecule in the active site enables proton transfer (PT) from the cross-linked tyrosine to a peroxo ligand bridging the heme Fe^{III} and Cu^{II}, and multiple H-bonding interactions lower the tyrosine p*K*_a. Due to sterics within the active site, the proton must either transfer initially to the O(Fe) (a high-energy intermediate), or from another residue over a ~10 Å distance to reach the O(Cu) atom directly. While the distance between the H⁺ donor (Tyr) and acceptor (O(Cu)) results in a barrier to PT, this separation is critical for the low barrier to O–O cleavage as it enhances backbonding from Fe into the O–O σ* orbital. Thus, PT from Tyr precedes O–O elongation and is rate-limiting, consistent with available kinetic data. The electron transfers from tyrosinate after the barrier via a superexchange pathway provided by the cross-link, generating intermediate P_M. P_M is evaluated using available experimental data. The geometric structure contains an Fe^{IV}=O that is H-bonded to the Cu^{II}-OH. The electronic structure is a singlet, where the Fe^{IV} and Cu^{II} are antiferromagnetically coupled through the H-bond between the oxo(Fe) and hydroxo(Cu) ligands, while the Cu^{II} and Tyr[•] are ferromagnetically coupled due their delocalization into orthogonal magnetic orbitals on the cross-linked His residue. These findings provide critical insights into the mechanism of efficient O₂ reduction in HCOs, and the nature of the P_M intermediate that couples this reaction to proton pumping.

Graphical abstract

Corresponding Authors edward.solomon@stanford.edu.

ASSOCIATED CONTENT

Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org> Structural details of the computational model Calculations for kinetics, isotope effects, and magnetic couplings Proton transfer via two water molecules The authors declare no competing financial interests.

1. INTRODUCTION

Heme-copper oxidases (HCOs) comprise a superfamily of metalloenzymes, most notably cytochrome c oxidase (CcO) and ubiquinol oxidase (Ubo), which catalyze the 4 e^- reduction of O_2 to H_2O throughout biology. As this reaction and its reverse are critically relevant for a variety of energy conversion processes, with applications including fuel cells, electrolyzers, solar energy, and biofuels, considerable effort has been committed to understanding the mechanism of O_2 reduction in these enzymes.^{1,2} Employing cytochrome c or ubiquinol as the biological electron source, this reaction is highly exergonic, yielding a net free energy gain of ~ 46 kcal/mol, which is efficiently used to drive a proton pumping process that is critical in ATP synthesis.³ The active site for O_2 reduction, termed the binuclear center (“BNC”, Figure 1), consists of a His-ligated heme (heme a_3) and a closely situated Cu ion (Cu_B) coordinated by three His residues, one of which is covalently linked to a nearby Tyr (Tyr244 using bovine CcO numbering). The Tyr residue is H-bonded to the hydroxyl group of the heme a_3 farnesyl chain, approximately 5.5 Å from the Fe_{a_3} and Cu_B .⁴ Additional metal centers in HCOs include a low-spin heme (heme a) and a binuclear Cu (Cu_A , although absent in ubiquinol oxidase), which are responsible for electron transport to the BNC.

Decades of experimental data have contributed to the current consensus mechanism, in which several intermediates have been observed spectroscopically.^{2,5} As illustrated in Figure 2, O_2 binds to the fully reduced active site (**R**), forming a heme-superoxo complex (**A**), which rapidly undergoes O–O cleavage to yield intermediate **P_M**. When O_2 binds to the “mixed-valent” form of the enzyme (where only the active site is reduced), O–O cleavage occurs rapidly (yielding **P_M**, where the M subscript denotes deriving from the mixed-valent reduced state), indicating that all 4 e^- s required for O–O cleavage must be near the BNC.^{6,7} As only 3 e^- s are supplied by heme a_3 (Fe^{II} to $Fe^{IV}=O$) and Cu_B (Cu^I to $Cu^{II}-OH$), it is believed that the fourth e^- derives from Tyr244, thus P_M is formulated as $Fe^{IV}=O/Cu^{II}-OH/Tyr^*$.⁸ Following re-reduction by cytochrome c or ubiquinol, H^+/e^- delivery to the active site generates the **F** intermediate, and an additional H^+/e^- returns the enzyme to the oxidized state (**O_H**).

Based on kinetic and computational studies, it is generally viewed that the rate-limiting step in O₂ reduction involves breaking the O–O bond.⁹ This has attracted great interest in how HCOs perform this step with a low barrier. Although an intermediate between **A** and **P_M** has not been observed experimentally, extensive investigations with synthetic and computational models have suggested the involvement of a highly reactive peroxo-level species (**I_P** in Figure 2).^{9–11} It has been proposed that this peroxo species serves as the active H⁺ acceptor (rather than the superoxo ligand believed to be present in **A**), highlighting the importance of the H⁺ in O–O cleavage.^{12–14} In order to evaluate the feasibility of a peroxo intermediate and elucidate factors that may contribute to its reactivity, numerous model studies have been devoted to understanding the behavior of such a species.¹⁵ Despite the wealth of literature employing computational and synthetic HCO models, and in some cases a combination of the two, many details of this process remain unresolved with regards to the H⁺ and e[−] during O–O cleavage, including their source, timing, and means of delivery to the bound O₂ molecule.

While it is widely viewed that the source of the H⁺ is Tyr244 (supported by FTIR data and thermodynamics calculations),^{10,16–19} and that the means of H⁺ transfer likely involves a shuttle through water molecules, its predicted timing during O–O cleavage has varied considerably throughout the literature. For instance, Sharma et al.²⁰ and Blomberg et al.⁹ have recently proposed mechanisms where proton transfer from Tyr occurs *prior* to formation of **I_P**, such that the peroxo intermediate is protonated and the barrier involves cleavage of an FeO–O(H)Cu species. In an earlier study, the same authors presented a similar mechanism except where the proton on the peroxo in **I_P** derived from an external donor (delivering an “extra” proton to the active site), and the Tyr remained protonated until after the barrier for O–O(H) cleavage.¹² In contrast, Noodleman et al. have suggested that proton transfer to **I_P** (an unprotonated Fe–O–O–Cu) comprises the rate-limiting step, *early* in O–O cleavage.²¹ Presenting a fourth possibility, our recent investigation into reactivity in a synthetic model (employing a Fe–O–O–Cu complex and an exogenous phenol) showed experimentally that PT can occur after the barrier and *late* in O–O cleavage.¹⁰

Although direct evidence has remained elusive, the fourth e[−] required for O₂ reduction is generally considered to come from Tyr244 (although some reports^{22,23} have suggested other protein residues). Since oxidation of a protonated Tyr would be thermodynamically uphill, the timing of ET can be loosely defined as after the Tyr is deprotonated. However, the mechanism of ET from Tyr[−] to the cleaving peroxo and its possible contribution in the barrier have scarcely been addressed in the literature.

We have therefore carried out DFT calculations that build on our past experimentally validated study on a hemeperoxo–Cu model complex that undergoes O–O bond cleavage upon addition of phenolic substrates.¹⁰ The present study specifically probes these unresolved questions, drawing on experimental data and comparing results with past computational studies. In the first of three sections, we investigate the mechanism of O–O bond cleavage using a computational model of the active site in HCOs. We evaluate key features of the active site in terms of their functional role in O–O cleavage, dissecting the reaction into three primary components: proton transfer, electron transfer, and O–O elongation. We evaluate possible mechanisms that could enable rapid O–O cleavage and

their thermodynamic/kinetic properties to correlate with experimental data. Our findings offer new insight into the nature of the rate limiting step for O–O cleavage in HCOs, and how the cross-linked Tyr functions as an ideal biological proton donor.

In the second section, we investigate the cross-linked Tyr in terms of its role as the electron donor, with a particular focus on how and when the electron from Tyr[–] transfers to complete the 4-electron reduction of O₂. Our calculations further highlight the importance of the cross-link in HCO function, and reveal a mechanistic dependence on spin state.

In the third section we follow the O–O cleavage reaction coordinate to completion and evaluate the final product, intermediate **P_M**. We generate a series of possible **P_M** structures and evaluate their geometric and electronic structures based on available experimental data. The DFT calculations offer valuable insights into the H-bonding interactions and magnetic couplings in **P_M**. Combining spectroscopic data on **P_M** and crystallographic data on the 1-electron reduced analog (**P_R**) from recent literature,^{24–27} together with the mechanistic insights from our synthetic and computational modelling of O–O cleavage, we converge on a likely geometric and electronic structure of **P_M**.

The findings presented below reveal several structure-function relationships that are critical for O–O cleavage in HCOs, and highlight how nature has optimized the heme/Cu active site to perform efficient O₂ reduction. Recent experimental data are applied to the possible **P_M** structures from DFT, contributing further insight into the reaction mechanism in the enzyme. As the intermediates following **P_M** (**F**, **O_H**, **E**, **R**) are not only spectroscopically observable, but also active in proton pumping, unraveling the geometric and electronic structure of **P_M** and how its structure reflects O–O cleavage marks a critical step in understanding how HCOs couple O₂ reduction to proton pumping.

2. METHODS

DFT Calculations.

Density functional theory (DFT) calculations were performed with the electronic structure package Gaussian16, D.01. These calculations were performed using the B3LYP functional including Grimme dispersion corrections (GD3BJ) within the spin-unrestricted formalism. Basis sets employed for geometry optimizations were as follows: 6–311g* on Fe/Cu, 6–311+g* on O, 6–31+g* on all metal-bound N-atoms and O-bound H atoms, and 6–31g on all remaining atoms. This method has been experimentally validated by O–O cleavage performed by an analogous synthetic model.¹⁰ SCF used tight convergence criteria on an ultrafine integration grid, and solvation was included using a polarized continuum model with a dielectric of 4.0. Unless otherwise stated, energetics given in the text are calculated on the singlet surface; triplet energetics differed by less than 1 kcal/mol and are given in the SI. Thermodynamics were calculated by removing contributions from frozen atoms, which would otherwise introduce artifacts from additional imaginary frequencies. All transition state structures contained one imaginary frequency, and all reactants, intermediates, and products contained only real frequencies. Free energies were calculated at 298 K. For calculation of coupling constants, energies for broken-symmetry states were corrected for

spin contamination using the Yamaguchi approximate spin projection method.²⁸ Visualization of orbitals was performed with Lumo.²⁹ See SI for additional details.

3. RESULTS AND ANALYSIS

3.1 Mechanism of O–O Cleavage in HCOs

Computational evaluation of O–O bond cleavage in CcO was carried out using a model of the active site constructed from a recent crystal structure of bovine CcO (PDB 3WG7),⁴ shown with a bound peroxo ligand in Figure 3A, ($\{0\}$ - I_p), the $\{0\}$ indicates that no water molecules are included in the model). To effectively mimic structural constraints imposed by the protein secondary structure, each amino acid residue was truncated at the α -carbon by replacing the backbone with H atoms, which were then frozen in the calculations. While it is generally absent in HCO computational model studies in the literature, the conserved Val243 residue (sequentially adjacent to Tyr244) was included in the calculations herein, as it lies in close proximity to Cu_B and can sterically inhibit access to the O_2 molecule bound in the BNC. Building upon insights from synthetic and computational model studies,^{10,12,21,30} the starting structure for O–O cleavage comprises an $\text{Fe}^{\text{III}}\text{-O}_2^2\text{-Cu}^{\text{II}}$ core and a protonated Tyr244, denoted as I_p (Figure 3A). In accordance with the consensus mechanism, the product structure following O–O cleavage (intermediate P_M) is described as a $\text{Fe}^{\text{IV}}=\text{O}$ ($S=1$)/ $\text{Cu}^{\text{II}}\text{-OH/Y}^*$ species, as depicted in Figure 3B. Importantly, while additional H^+ and e^- donors are available near the active site, it has been recently demonstrated that the tyrosine must serve as the H^+ and e^- donor in the net reaction, and that the tyrosine must be deprotonated prior to transferring the electron.¹⁰

As this net reaction bears close resemblance to that of our recent experimentally-correlated DFT investigation of exogenous phenol-induced O–O bond cleavage in a heme-peroxo-Cu model complex ($\{[(\text{DCHIm})(\text{Fs})\text{Fe}^{\text{III}}]\text{-(O}_2^{2-})\text{-[Cu(II)(AN)]}\}^+$, “LS-AN”),¹⁰ key insights from the synthetic system can be applied to the CcO model. In particular, the study with LS-AN showed that a low barrier to O–O cleavage could be accomplished via two different mechanisms, distinguished fundamentally by whether PT (from phenol interacting directly with the peroxide bridge) occurs before or after the barrier. It was experimentally demonstrated (via a kinetic isotope effect) that in the reaction of the LS-AN model complex, PT occurred primarily after the barrier, indicating that an H-bonding interaction was sufficient to yield a low barrier. This presents a particularly interesting issue with respect to HCOs, since the computational literature has generally invoked a mechanism wherein PT to the bound O_2 molecule precedes O–O cleavage.^{9,12,21}

Taking advantage of our past results on the well-defined model system, $\{\text{LS-AN} + \text{PhOH}\}$, the process of O–O cleavage was evaluated in CcO. Calculation of the overall thermodynamics for I_p to P_M in CcO (Figure 3A to B) yields a ΔG of -11.7 kcal/mol (calculated at 298 K), nearly identical to the value recently reported for $\{\text{LS-AN} + \text{PhOH}\}$ ($\Delta G = -10.7$ kcal/mol),¹⁰ signifying strong similarity between the enzyme and the synthetic model. It is worth recognizing, however, that the experimental O–O cleavage barrier ($\Delta G^\ddagger$) in CcO is < 12.4 kcal/mol,³¹ while the experimental value for $\{\text{LS-AN} + \text{PhOH}\}$ is ~ 15 kcal/mol. This indicates that the CcO active site provides a lower intrinsic barrier (i.e. the difference in $\Delta G^\ddagger$ is not due to thermodynamics). To evaluate this reaction computationally,

the B3LYP functional was employed with Grimme dispersion corrections, a method found to provide good agreement with experimental data (KIE and $G^\ddagger$) for {LS-AN + PhOH}.

3.1.1 H-Bond Assisted Mechanism—To correlate the kinetic and mechanistic insights from {LS-AN + PhOH} to HCOs, the H-bond Assisted mechanism for O–O cleavage was first evaluated for the HCO active site ({0}-Ip, Figure 3A). A key distinction between the synthetic model and HCOs, however, is that the phenol in {LS-AN + PhOH} can interact directly with the peroxo core (Figure 4A), which lowers the barrier to O–O homolysis through an H-bonding interaction with the peroxo. In contrast, the donor in HCOs (widely considered Tyr244) is covalently constrained ~5 Å away from the BNC, and O–O homolysis without an H-bond to the peroxo yields a barrier too high relative to experimental data on CcO ($G^\ddagger_{\text{calc}} = 21.9$ kcal/mol, $G^\ddagger_{\text{exp}} < 12.4$ kcal/mol). However, water molecules around the active site (crystallographically observed, and generated during turnover)^{19,24} have often been proposed to participate in H⁺ delivery to O₂, and can approach to H-bond directly to the bridging peroxo. Moving a water molecule from bulk into the active site in {0}-Ip (as in {1C}-Ip in Figure 4B; {1C} indicates the inclusion of one water molecule near the Ocu atom) is calculated to be a nearly thermoneutral process ($H = -8$ kcal/mol, $G = +2$ kcal/mol).

The H-bond Assisted mechanism can therefore be mimicked in the enzyme by elongating the O–O bond of the structure shown in Figure 4B. However, the calculated barrier including this H-bond (“TS_{hb}” in Figure 5) is still too large ($G^\ddagger = 21.2$ kcal/mol). While the H-Bond Assisted mechanism gave a lower barrier in {LS-AN + PhOH} ($G^\ddagger = 16.5$ kcal/mol), the higher $G^\ddagger$ for H-Bond Assisted cleavage in CcO can be attributed to weaker H-bonding from H₂O relative to phenol (using H₂O with LS-AN raises its $G^\ddagger$ to 19.2 kcal/mol). Further, the calculations show that deprotonating the active site Tyr in the CcO model (thereby providing a low-potential electron source) does not result in a lower barrier for O–O homolysis, as the electron does not transfer from Tyr[–] before the barrier (vide infra). It should be noted that the above energetics were calculated on the broken-symmetry singlet surface; calculation on the triplet surface yields higher barriers to O–O homolysis because the Cu-oxyl species that is forming in the transition state is a singlet on the overall triplet surface, which is less stable than the triplet Cu-oxyl formed on the overall singlet surface. In all, these results indicate that an H-bond from H₂O would not lower the barrier to O–O cleavage enough to agree with the observed kinetics in HCOs.

3.1.2 Proton-Initiated Mechanism—The second mechanism evaluated for the O–O cleavage reaction of {LS-AN + PhOH}, “Proton-Initiated” (PI), involves PT from phenol to the peroxo early in O–O cleavage. In the LS-AN model this was found to produce a higher O–O cleavage barrier and incorrect KIE relative to experiment. For CcO, the PI mechanism can be accomplished by transferring a H⁺ from the Tyr to the peroxo bridge via a Grotthuss mechanism (a concerted movement of protons through water molecules). It has been reported that no H⁺ uptake is observed in CcO before O–O cleavage, meaning the H⁺ must derive from an internal donor.³² Most computational HCO models in the literature have invoked a bridge from Tyr244 to the o_{cu} atom consisting of one or two water molecules, shuttling the tyrosyl proton to O_{cu} (which has a higher H⁺ affinity than O_{Fe} by ~9 kcal/mol).

¹² However, our calculations reveal that a valine residue (Val243) conserved in the active sites of HCOs sterically prohibits a direct bridge between Tyr244 and O_{Cu} (region marked by a gray X in Figure 6), yet this residue has not been included in the past computational HCO models. Nevertheless, a water molecule may form a bridge between Tyr244 to the O_{Fe} atom of the peroxo (marked by the red circle in Figure 6, corresponding to structure {1F}-I_p ({1F} includes one water molecule able to interact with the O_{Fe} and/or Tyr) Section 3.2.1 below), where both O_{Tyr} ... O_{water} and O_{water} ... O_{Fe} distances are < 2.7 Å. It is thermodynamically favorable to bind the water molecule in {1F}-I_p from bulk, with a calculated $G_{\text{binding}} = -0.4$ kcal/mol ($H_{\text{binding}} = -9.7$ kcal/mol).

3.1.2.1 Proton Transfer from Tyr: To evaluate proton transfer from Tyr to the peroxo O_{Fe} via a water molecule ({1F}-I_p → {1F}-H_{Fe1}, Figure 7), a 2-dimensional potential energy surface (PES) was calculated involving motion of both protons active in transfer (O_{Tyr}...H...O_{water} and O_{water}...H...O_{Fe}). This 2D PES led to the convergence of a true transition state for PT through the bridging water molecule, yielding a barrier of $G^\ddagger = 13.7$ kcal/mol ($H^\ddagger = 10.4$ kcal/mol). The thermodynamic cost of transferring the H⁺ to O_{Fe} (forming {1F}-H_{Fe1}, Figure 7) is $G = +12.7$ kcal/mol ($H = +9.4$ kcal/mol).

Elongating the O-O bond with the proton on O_{Fe} yields a high barrier to O-O cleavage, indicating that the proton must rearrange to bind the O_{Cu} atom of the peroxo (as in {1F}-H, far right in Figure 7) before O-O cleavage. Note that the water in {1F}-I_p in Figure 7 cannot access the O_{Cu} atom directly without a large distortion in the Cu ligand field (which would give a $G^\ddagger > 20$ kcal/mol), supporting that the proton from Tyr should transfer initially to the O_{Fe} atom. After PT from Tyr to yield {1F}-H_{Fe1}, the H⁺ on O_{Fe} rotates away from the water molecule (for which $G^\ddagger = 13.5$ kcal/mol, {1F}-H_{Fe1} → {1F}-H_{Fe2} in Figure 7). Finally, a barrierless proton shift from O_{Fe} to O_{Cu} yields {1F}-H in Figure 7.

While increasing the number of bridging water molecules has been shown to lower the barrier for Grotthuss mechanisms,^{33,34} our calculations show that incorporating a second water molecule in the bridge between Tyr and O_{Fe} (yielding the model denoted {2F}, see SI Section 2 for additional details) would not effectively lower the PT barrier in CcO. This is due to the H₂O...HO_{Fe} H-bond in {1F}-H_{Fe1} being broken in the H-O_{Fe} rotation step (Figure 7, {1F}-H_{Fe1} → {1F}-H_{Fe2}), which causes this step to have a similar barrier and transition state structure for one or two bridging water molecules. Nevertheless, the barrier to PT calculated above for one bridging water molecule (13.7 kcal/mol) is sufficiently close to the 12.4 kcal/mol experimental limit to be a good candidate for the active mechanism.

3.1.2.2 O-O Cleavage of Fe^{III}-O-O(H)-Cu^{II}: Evaluating the barrier to O-O bond cleavage of {1F}-H (Figure 8), where the H⁺ from Tyr is on O_{Cu} and a water molecule is H-bonded to the Tyr, a transition state was found at an O-O distance of 1.84 Å with an energy of $G^\ddagger$ of 11.5 kcal/mol (on both the singlet and triplet spin surfaces). This result has two crucial implications. First, O-O cleavage of {1F}-H involves a sufficiently low barrier to be consistent with kinetic data on CcO. Second, it presents a lower barrier to O-O cleavage than the H-Bond Assisted mechanism (vide supra), indicating that a Proton-Initiated mechanism is favored in HCOs, in contrast to the behavior observed in {LS-AN + PhOH}. This change in mechanism for the enzyme relative to the model can be understood based on two key

factors responsible for favoring one mechanism over the other that were identified from evaluation of {LS-AN + PhOH}. Most notably, the Proton-Initiated mechanism is favored by: 1) employing a more acidic proton donor, and 2) by enhancing electron donation into the peroxo, which increases its basicity. Applying these insights to HCOs, we evaluate below how the active site in {1F}-H enables O-O cleavage with a barrier that is half that of the Proton-Initiated O-O cleavage of LS-AN (11.5 vs. 25 kcal/mol, **TS_{PI}** in Figure 8), highlighting key structure-function relationships in HCOs.

The change in behavior calculated for O-O cleavage of {1F}-H compared to {LS-AN + PhOH} derives from a combination of structural and electronic differences in their Proton-Initiated potential energy surface. While the overall thermodynamics for product formation (**I_P** → **P_M**, Figure 8) are very similar, approximately $G^\circ = -11$ kcal/mol, the energetic cost of PT from Tyr to O_{cu} (i.e. **I_P** → **H**) is much lower in {1F} (4 kcal/mol) than in {LS-AN + PhOH} (13 kcal/mol). This difference is attributable to the lower pK_a of the Tyr in CcO compared to the phenolate in LS-AN, as the Tyr in {1F} has two H-bonds to stabilize the deprotonated anion (one from the water molecule and one from the farnesyl hydroxyl group, see {1F}-H in Figure 8), whereas the phenol in LS-AN is stabilized by only one H-bond (from the O_{cu}). Removing the water molecule in {1F} raises the energetic cost of transferring the Tyr H⁺ by ~7 kcal/mol. This result also highlights a likely function of the hydroxy group on the farnesyl side chain of the active site heme, as it is favorably positioned to H-bond to the tyrosinate and assist in lowering its pK_a. Another structural component of the active site that may act to lower the Tyr pK_a is the His-Tyr cross-link, which can increase π -delocalization and inductive effects to stabilize the tyrosinate anion.³⁵ Based on synthetic modelling with substituted phenols, the cross-link is estimated to lower the Tyr pK_a by ~1.5 units, or 2 kcal/mol.^{36,37}

Also from Figure 8, starting from the H structures there is a lower barrier from O-O cleavage (i.e. **H** → **TS_{PI}**) in {1F} (7.5 kcal/mol) compared to {LS-AN + PhOH} (12 kcal/mol). This difference derives from a greater extent of electron donation from Fe into the peroxo (via backbonding from the Fe d _{π} orbital into the O₂ σ^*) in {1F}-**TS_{PI}** compared to LS-AN. The proton on O_{cu} enhances ET from Fe d _{π} by lowering the energy of the peroxo σ^* orbital (therefore activating the O-O bond for cleavage). Comparing {1F} to LS-AN, the H-bond from phenolate to the H⁺ on O_{cu} in LS-AN weakens the H-O_{cu} bond and lessens this effect.
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We also considered whether the cross-linked tyrosine participates in ET before the barrier, as this would both increase the negative charge on the peroxo (raising its proton affinity and lowering the energetic cost of PT from Tyr), and increase <DI>O₂²⁻ a^* </DI> occupation (therefore assisting O-O cleavage). However, our calculations (see also Section 3.2) indicate that electron transfer from Tyr⁻ does not occur until after the transition state, and therefore does not impact the barrier. Taken together the above results demonstrate the active site in CcO lowers the barrier for Proton-Initiated O-O cleavage relative to the synthetic model {LS-AN + PhOH} by providing both a more acidic proton source (due to H-bond stabilization of the resultant Tyr⁻), and enhanced backbonding from Fe into the peroxo σ^* LUMO (due to the stronger H-O_{cu} bond) before the barrier.

3.1.2.3 Alternative Proton-Initiated O–O Cleavage: Since the e^- from Tyr $^-$ transfers after the transition state, a key implication is that the Tyr is only impacting the barrier via the H^+ . This suggests that the H^+ bound to O_{Cu} could derive from a donor other than the active site Tyr, such that Tyr is not deprotonated until after the barrier.^{12,17,39,40} This would require direct H^+ transfer to the peroxide O_{Cu} and avoid the energy cost of going through a protonated O_{Fe} intermediate ($I_P \rightarrow H_{Fe2}$, Figure 7). Given the presence of alternative H^+ donors near the active site in CcO (such as Tyr129 and two Arg located ~ 10 Å from Cu), a H^+ could alternatively be shuttled to the O_{Cu} atom via 4 or more waters in a Grotthuss mechanism.^{41,42} In this case, the PT pathway would include a water molecule H-bonded to O_{Cu} , generating the active site $Fe^{III}\text{-OO(H)-Cu}^{II}$ species shown in **{1C+}-H** (Figure 9, the (+) signifies a proton supplied by a nearby donor not included in the model). After PT, the water molecule in **{1C+}-H** is weakly bound to the $O_{Cu}H$ ($H_{\text{binding}} = -7.3$ kcal/mol, $G_{\text{binding}} = +3$ kcal/mol) and may freely dissociate.

An externally derived H^+ shuttled directly to O_{Cu} can enable rapid O–O cleavage provided three criteria are met: 1) the barrier to Grotthuss-mediated PT to O_{Cu} is < 12.4 kcal/mol, 2) the thermodynamic cost of PT to the peroxo is ~ 5 kcal/mol, and 3) the water molecule involved in PT to O_{Cu} dissociates early in O–O cleavage (H-bonding to Cu–OH adds 5 kcal/mol to the barrier). Studies of Grotthuss mechanisms have reported barriers to proton migration that are typically 10 kcal/mol or lower, depending on the local environment and separation between water molecules.^{34,43,44} In CcO, the endergonicity of PT will add to the barrier (see SI). Nevertheless, using the same thermodynamics for PT from the active site Tyr (4 kcal/mol) and an upper estimate of the intrinsic barrier for Grotthuss mechanisms (10 kcal/mol), the effective barrier for PT would still be only 12.1 kcal/mol.^{45,46} If the pKa of the “alternative” amino acid donor is 2 log units higher than the active site Tyr (resulting in a G for PT of 6.7 kcal/mol), the PT barrier would raise to 13.6 kcal/mol, comparable to PT from the active site Tyr (Section 3.1.2.1).

Taken together, the above results show that a H^+ donor outside the active site could provide a low barrier for Proton-Initiated O–O cleavage that is comparable to the barrier found for PT from Tyr (11.5 kcal/mol, Figure 8). After the barrier, since oxidation of Tyr $^-$ is required by thermodynamics (vide supra), the Tyr must deprotonate (e.g. into bulk via the farnesyl hydroxyl group) and transfer its electron to the cleaving O–OH, generating the $Fe^{IV}=O/Cu^{II}\text{-OH/Tyr}^*$ products (which may have the water molecule bound to the Cu–OH, see **{1C}-P_M**, see Section 3.3). In all, this presents an alternative mechanism wherein the cross-linked Tyr does not actively contribute until after the barrier, yet still provides the tyrosinate electron necessitated by thermodynamics to generate the O–O cleaved product, **P_M**.

3.1.2.4. Kinetics and Isotope Effects in Proton-Initiated O–O Cleavage: The above results describe two possible mechanisms for O–O cleavage in CcO. While both are Proton-Initiated in nature, they differ by the active proton donor. In both cases, proton transfer and O–O cleavage occur in a sequential manner, and the barriers for both steps are comparable (either could be rate-limiting). However, kinetic data on CcO have shown that the decay of **A** to **P_M** has a deuterium kinetic isotope effect (KIE) of 1.4–1.9.³¹ We therefore calculated KIEs (see SI) for the Proton-Initiated mechanisms above (with models **{1C}** and **{1F}**), evaluating both the PT and O–O cleavage processes, as it is not clear from experiment which

step is rate-limiting. The results are summarized in Scheme 1. In the mechanism initiated by PT from Tyr to O_{Fe} (model {1F}), the KIE calculated for PT via a Grotthuss mechanism with one water molecule ($\{1F\}\text{-I}_P \rightarrow \{1F\}\text{-H}_{Fe1}$, Figure 7) is $k_H/k_D = 8.3$. The subsequent step, in which the proton rearranges onto the O_{cu} atom, $\{1F\}\text{-H}_{Fe1} \rightarrow \{1F\}\text{-H}_{Fe2}$ (the $G^\ddagger$ is comparable to the preceding PT step), has a calculated k_H/k_D is 1.4. Note that the results obtained with a one-water bridge are similar to those with a two-water bridge (see SI). In the mechanism involving PT from an external donor (model {1C}) a KIE for Grotthuss-mediated PT can be estimated from literature on biological Grotthuss mechanisms, which are generally on the order of 1.4–3 for transfer over several water molecules^{47,48} (although KIEs can increase to 5 or greater for transfer through only one or two water molecules).³³ Finally, regardless of the mechanism of proton transfer, the KIE for FeO–O(H)Cu cleavage (using the transition state {1F}-TS_{PT}) is calculated to be $k_H/k_D = 0.95$, an inverse KIE. Given that the experimental KIE is in the range of 1.4–1.9, these results suggest that proton transfer, rather than O–O cleavage, is rate-limiting in HCOs. If the proton that initiates O–O cleavage derives from the cross-linked Tyr, the rate-limiting step involves proton migration from the O_{Fe} atom to the O_{cu} atom.

In all, the calculations presented above and the HCO literature to-date have contributed several possible mechanisms for O–O cleavage, varying in: 1) the identity of the active proton donor, 2) participation and orientation of water molecules, 3) timing and involvement of PT and ET from Tyr, and 4) overall spin state. While the literature has reached a consensus that the O_{cu} atom is protonated when the O–O bond is broken, the rate-limiting step (PT or O–O(H) cleavage) remains an open issue.^{9,21} The calculations herein evaluate both processes, and suggest that the barrier to O–O cleavage in HCOs (A to P_m) is proton transfer to O_{cu}. This is due to sterics within the active site, which require that PT must involve either a higher-energy intermediate where the O_{Fe} atom is protonated (model {1F}), or PT over a large (~10 Å) distance to go directly to the O_{cu} atom (model {1C}). Nevertheless, these results describe two possible mechanisms for O–O cleavage in HCOs, where both could provide a barrier (< 12.4 kcal/mol) and KIE (1.4–1.9) in agreement with experimental estimates.

3.2 Electron Flow from Tyr[−]

While the results above considered multiple possible origins for the H⁺ involved in lowering the barrier to O–O cleavage, our calculations have shown that given the available electron sources near the active site (tryptophan and tyrosine), tyrosinate is the only electron donor able to afford thermodynamically favorable O–O cleavage. Although the literature has widely proposed that Tyr provides the fourth electron, we have examined the active ET process to understand the connection between the structure (Cu-His-Tyr linkage) and function (electron transfer) from the cross-linked tyrosine.

Examining the electronic structure over the reaction coordinate for O–O cleavage from transition state ($r(\text{O}=\text{O}) = 1.83 \text{ \AA}$) to product (TS_{pi} to P_M, Figure 8), the electron flow from Tyr[−] to the peroxo is observed as a rapid transfer at an O–O distance of ~2.0 Å. The calculations show that as the O–O bond elongates, the O₂ σ* progressively lowers in energy, eventually becoming isoenergetic with the Tyr[−] HOMO. This enables ET from Tyr[−], where

the transferring e^- has an alpha spin to pair with the beta e^- transferring from LS Fe^{III} , which completes the 4-electron reduction of O_2 . Prior to transfer, the Tyr^- electron resides in an MO that is localized primarily on the Tyr ring (α HOMO at $\text{O}-\text{O} = 2.0 \text{ \AA}$ in Figure 10), although it mixes with the π HOMO on the His, which has efficient π overlap. To examine the orbitals involved in this process, we have evaluated both the singlet and triplet surfaces, using the simplified model without water molecules (**{0}**).

On the singlet surface ($\text{Fe } ^1/\text{Cu } ^1$), the beta-spin Cu^{II} has an alpha hole in the $\text{dx}^2\text{-y}^2$ orbital. The Cu hole is tilted and has overlap with the occupied n valence orbital on the His, delocalizing onto the Tyr via a superexchange pathway through the His π orbital (Figure 10). This results in partial occupation of the Cu $\text{dx}^2\text{-y}^2$ (α) and oxidation of the Tyr HOMO. Elongation of the $\text{O}-\text{O}$ past the TS brings its σ^* down in energy so that the α e^- propagates from the Tyr HOMO into the $\text{O}_2^{2-} \sigma^*$ via the Cu and His. Therefore, the His-Tyr cross-link provides a superexchange pathway for the Cu $\text{dx}^2\text{-y}^2$ α unoccupied orbital to gain occupied character from the Tyr, such that its e^- can readily transfer into the $\text{O}_2^{2-} \sigma^*$ as ET becomes energetically favorable. The tilting of the Cu $\text{dx}^2\text{-y}^2$ orbital off the $\text{Cu}-\text{N}_{\text{His}}$ axis, and therefore the formation of the superexchange pathway occurs after the transition state (but before complete $\text{O}-\text{O}$ cleavage) and ET is triggered by the driving force at the elongated $\text{O}-\text{O}(\text{H})$ distance.

On the overall triplet surface ($\text{Fe } ^1/\text{Cu } ^1$), all alpha d-orbitals on Cu^{II} are occupied, resulting in an ET mechanism that involves e^- hole transfer (Figure 11) through filled valence orbitals. Following the α LUMO orbital character over $\text{O}-\text{O}$ elongation (top half of Figure 11), the hole propagates from the unoccupied $\text{O}_2^{2-} \sigma^*$ orbital, through the occupied α d_{yz} (π^*) orbital on Cu and the occupied π orbital on the His, terminating with the Tyr HOMO. Thus, on the triplet surface, the cross-link also provides a superexchange pathway between the Tyr^- HOMO and the $\text{O}_2 \sigma^*$ LUMO, but via a doubly occupied Cu orbital.

Taken together, these results highlight several key aspects of electron flow from Tyr. First, the electron transfers after the barrier to $\text{O}-\text{O}(\text{H})$ cleavage, occurring as a barrierless ET process driven by the $\text{O}_2 \sigma^*$ energy decreasing below the Tyr HOMO. Second, the spin and ground state of Cu are unchanged during charge flow into the $\text{O}_2 \sigma^*$. Third, the charge propagates through an occupied orbital on the His (as well as the Cu, on the triplet surface), and is therefore best described as hole transfer through the cross-link. Fourth, the observed ET mechanism differs fundamentally for the singlet and triplet surfaces, primarily due to the hole on Cu having either the same (triplet) or opposite (singlet) spin as the e^- transferring into the $\text{O}_2^{2-} \sigma^*$ orbital. Thus, in addition to keeping the $\text{Tyr}^- > 5 \text{ \AA}$ from the peroxide, the Cu-His-Tyr cross-link provides a means for electron transfer from the Tyr^- into the $\text{O}_2^{2-} \sigma^*$, regardless of overall spin state.

3.3. Geometric and Electronic Structure of P_m

While multiple possible structures for P_m have been presented in the literature, it is generally considered to be comprised of three common components: an $\text{S}=1 \text{ Fe}^{\text{IV}}=\text{O}$, an $\text{S}=\frac{1}{2} \text{ Cu}^{\text{II}}$, and an $\text{S}=\frac{1}{2} \text{ Tyr}^\bullet$.^{20,25,26,49–51} The P_m product generated in the DFT calculations depends on the reaction mechanism, including interactions with nearby water molecules and spin state. The results in sections 3.1 and 3.2 above demonstrate how PT from the active site Tyr (or

alternatively another nearby donor) initiates O–O cleavage with a low barrier that is consistent with kinetic data, which can be accomplished on either the singlet or triplet spin surfaces. Therefore, we have evaluated the possible structures for P_m deriving from these models in terms of their geometric and electronic characteristics.

The structural information presently available for P_m derives from rR and EPR. The rR data indicate that P_m has a strong Fe–O bond ($\nu_{\text{Fe–O}} = 804 \text{ cm}^{-1}$) that is insensitive to both H/D substitution and changes in pH,²⁵ suggesting an $\text{Fe}^{\text{IV}}=\text{O}$ with minimal H-bonding interactions. Indirect insights have also drawn from EPR data on P_m , where neither the Cu^{II} nor the Tyr radical signal has been observed, yet both are $S=1/2$ and proposed to be present.⁵² This has often been assumed to result from antiferromagnetic (AF) coupling between the Cu^{II} and the Tyr $^{\bullet}$.^{53–56} However, this could also result from rapid relaxation associated with the close proximity of three paramagnetic centers (Fe^{IV} , Cu^{II} , Tyr $^{\bullet}$), or strong ferromagnetic coupling between the Cu^{II} and Tyr $^{\bullet}$ to yield a triplet. As the overall spin state in P_m (and therefore O–O cleavage) remains an open issue, our calculations explore P_m derived on both the singlet and triplet spin surfaces.⁵⁷ Thus, we have evaluated the possible structures of P_m with respect to the nature of the $\text{Fe}^{\text{IV}}=\text{O}$ bond and the magnetic coupling in the active site. The results for each model are compiled in Table 2.

In the reaction coordinate for O–O cleavage initiated by PT from Tyr (model **{1F}**), where a water molecule involved in PT bridges the Tyr and the O_{Fe} atom, geometry optimization of the product structure yields **{1F}- P_m** (Figure 12), in which the water is H-bonded to the $\text{Fe}^{\text{IV}}=\text{O}$ (H-bonding to the neutral Tyr $^{\bullet}$ is calculated to be 4 kcal/mol higher in energy). The water molecule in **{1F}- P_m** has a calculated binding energy of $G = -0.1 \text{ kcal/mol}$ ($H = -11.7 \text{ kcal/mol}$). This structure has two H-bonds to the $\text{Fe}^{\text{IV}}=\text{O}$ (from the $\text{Cu}^{\text{II}}\text{-OH}$ and the H_2O), and an Fe–O bond length of 1.642 Å. For a benchmark comparison, the heme fragment was also optimized separately, giving an Fe–O of 1.633 Å. The longer Fe–O in **{1F}- P_m** suggests that the H-bonds from Cu-OH and H_2O weaken the Fe–O bond. Applying Badger's rule, an empirically-derived method for correlating bond length to stretching frequency,⁵⁸ an elongation of the Fe–O bond from 1.633 Å in the isolated $\text{Fe}^{\text{IV}}=\text{O}$ fragment to 1.642 Å in **{1F}- P_m** would correspond to a shift down in frequency of 16 cm^{-1} ($\nu_{\text{Fe–O}} = -16 \text{ cm}^{-1}$). Based on heavy-atom distances, the H-bond from water ($\text{O}_{\text{Fe}}\cdots\text{O}_{\text{water}} = 2.56 \text{ Å}$) appears to be stronger than the H-bond from the Cu-OH ($\text{O}_{\text{Fe}}\cdots\text{O}_{\text{Cu}} = 2.92 \text{ Å}$). The Fe and Cu centers are predicted to be AF coupled ($J = -5.0 \text{ cm}^{-1}$), while the $\text{Cu-Tyr}^{\bullet}$ pair is predicted to be ferromagnetically coupled ($J = +4.4 \text{ cm}^{-1}$). Therefore, the lowest energy electronic state is an overall singlet (local spins are shown in Figure 12, right), consistent with the lack of an EPR signal.

Since the rR data on P_m suggest minimal H-bonding to the $\text{Fe}^{\text{IV}}=\text{O}$, the impact of two H-bonds in **{1F}- P_m** (predicted to lower the $\nu_{\text{Fe–O}}$ by $\sim 16 \text{ cm}^{-1}$) is greater than would be expected. Thus, we considered an alternative P_m structure, **{0}- P_m** , where the water molecule dissociates away from the active site (or is weakly bound to the Tyr $^{\bullet}$ and too far to interact with the $\text{Fe}^{\text{IV}}=\text{O}$, as suggested by Sharma et al.²⁰). In this case, only the Cu-OH is H-bonded to the $\text{Fe}^{\text{IV}}=\text{O}$, and the optimized Fe–O bond length is 1.634 Å. Based on a Badger's rule analysis, this would correspond to a $\nu_{\text{Fe–O}}$ that is only $\sim 2 \text{ cm}^{-1}$ lower than an isolated $\text{Fe}^{\text{IV}}=\text{O}$, suggesting that the H-bond from the Cu-OH has a minor impact on the Fe–O bond

strength. The magnetic coupling within the active site is similar to **{1F}-P_m**: the Fe and Cu centers are AF coupled ($J = -3.4 \text{ cm}^{-1}$), while the Cu-Tyr[•] pair is ferromagnetically coupled ($J = +4.9 \text{ cm}^{-1}$), yielding an overall singlet ground state (Figure 12).

Evaluating the P_M structure resulting from the reaction coordinate where a proton is shuttled directly to the O atom on Cu (as in model **{1C}**), O–O cleavage leads to the product shown as **{1C}-P_m** in Figure 12, which includes the water molecule near Cu involved in the initial proton transfer. The lowest energy structure has the water H-bonding to the O of the Cu-OH, with the latter oriented toward the Fe^{IV}=O. The binding energy for the water molecule in **{1C}-P_m** (taken from bulk solution) is calculated to be favorable, giving a binding ΔG of -4.1 kcal/mol ($\Delta H = -15.5 \text{ kcal/mol}$). The Fe–O bond length is $1.635 \text{ \AA}$, which is very close to the $1.633 \text{ \AA}$ for the isolated Fe^{IV}=O (from Badger's rule, an elongation of $0.002 \text{ \AA}$ corresponds to $\nu_{\text{Fe-O}} = -4 \text{ cm}^{-1}$). Relative to **{0}-P_m**, the H-bond between the Cu^{II}-OH and the Fe^{IV}=O is slightly stronger ($\text{O}_{\text{Fe}} \cdots \text{O}_{\text{Cu}} = 2.74 \text{ \AA}$). Comparable to the possible P_M structures above, in **{1C}-P_m** the Fe^{IV} and Cu^{II} are AF-coupled ($J = -5.7 \text{ cm}^{-1}$), while the Cu^{II} and Tyr[•] are ferromagnetically coupled ($J = +4.0 \text{ cm}^{-1}$), yielding an overall singlet ground state.

For comparison, we also evaluated a P_m structure with a water ligand on the Cu (rather than hydroxo), which has been invoked in past computational studies.⁴² Optimizing such a product in our model generated the structure shown in Figure S4 (denoted **{H₂O}-P_m**). In contrast to the three P_m structures (with a Cu-OH) presented above, this model exhibits a much stronger H-bond to the Fe^{IV}=O ($\text{O}_{\text{Fe}} \cdots \text{O}_{\text{Cu}} = 2.44 \text{ \AA}$), giving a correspondingly longer Fe=O bond ($1.657 \text{ \AA}$; $\nu_{\text{Fe-O}} = -41 \text{ cm}^{-1}$ versus the isolated Fe^{IV}=O by Badger's rule). In accordance with a stronger interaction between their oxo and aquo ligands, the AF coupling between Fe and Cu has increased to $J = -14.7 \text{ cm}^{-1}$. However, the coupling between Cu and Tyr[•] has switched from ferro- to antiferromagnetic ($J = -5.7 \text{ cm}^{-1}$), therefore favoring the overall triplet spin state.

To understand the nature of the magnetic coupling between the Fe^{IV}=O/Cu^{II} and Cu^{II}/Tyr[•] centers, the molecular orbitals involved in their exchange interactions were examined, specifically the overlap of magnetic orbitals. Depicted in Figure 13A,B the antiferromagnetic coupling between Fe and Cu arises from a superexchange pathway between the Fe d_{π} and Cu dx^2-y^2 , which involves overlap between a filled O p -orbital on both of the oxo(Fe) and hydroxo(Cu) ligands. This interaction results in the α -spin Fe d_{π} (+ $\text{O}_{\text{Fe}} p$) HOMO mixing into the Cu dx^2-y^2 (+ $\text{O}_{\text{Cu}} p$) LUMO, and the β -spin Cu dx^2-y^2 (+ $\text{O}_{\text{Cu}} p$) HOMO mixing into the Fe d_{π} (+ $\text{O}_{\text{Fe}} p$) LUMO. Therefore, the oxygenous ligands on Fe and Cu promote AF coupling between the metals by facilitating overlap of their magnetic orbitals.

In contrast, ferromagnetic coupling arises when magnetic orbitals involve orthogonal orbitals on a single atom center. Correlated to the magnetic coupling in P_m, this condition is observed in the calculations for the Cu-Tyr[•] pair in P_m structures containing a Cu^{II}-OH species (**{0}-P_m**, **{1F}-P_m**, and **{1C}-P_m**). While the Cu dx^2-y^2 LUMO has efficient overlap with the σ -donating orbital on His that is in the imidazole plane (Figure 13A), the Tyr[•] LUMO mixes with a π orbital on the His (Figure 13C, see also Section 3.3), which has lobes

perpendicular to the imidazole plane. This orthogonal magnetic orbital electronic structure results in ferromagnetic coupling, depicted schematically in the left side of Figure 14.

As illustrated by the results for $\{\text{H}_2\text{O}\}\text{-P}_\text{M}$ (Figure S3.3.1), changing the ligand on Cu from OH^- to H_2O also changes the ligand field, such that the Cu $\text{dx}^2\text{-y}^2$ LUMO now has His (π) character. The interaction between the Cu $\text{dx}^2\text{-y}^2$ and the His (π) arises from a rotation of the former off the Cu- N_{His} axis, shown schematically on the right in Figure 14. Since the half-occupied orbitals on Cu^{II} and $\text{Tyr}^\bullet$ both have His (π) character, the Cu^{II} and $\text{Tyr}^\bullet$ are antiferromagnetically coupled in $\{\text{H}_2\text{O}\}\text{-P}_\text{M}$. This contrasts with the behavior for $\text{Cu}^{\text{II}}\text{-OH}$ because the strong OH ligand dominates the ligand field (and therefore the orientation of the Cu $\text{dx}^2\text{-y}^2$ orbital).

In summary, the above calculations evaluate possible PM models, particularly involving the $\text{Fe}^{\text{IV}}=\text{O}$ bond and the magnetic couplings. Our results show that an H-bond between the $\text{Fe}^{\text{IV}}=\text{O}$ and a water molecule (either exogenous or as a ligand on Cu) would significantly weaken the Fe-O bond, inconsistent with existing rR data for P_M . However, an H-bond between the $\text{Cu}^{\text{II}}\text{-OH}$ and the $\text{Fe}^{\text{IV}}=\text{O}$ would have a minor impact on the Fe-O bond (in agreement with the rR data), yet it would provide a pathway for exchange coupling between the Fe and Cu, and may explain why a Cu^{II} EPR signal is not observed in P_M . The predicted ground state for P_M is a singlet (for the lowest energy structure), arising from antiferromagnetic coupling between the $\text{Fe}^{\text{IV}}=\text{O}$ and $\text{Cu}^{\text{II}}\text{-OH}$, and ferromagnetic coupling between the $\text{Cu}^{\text{II}}\text{-OH}$ and $\text{Tyr}^\bullet$. Thus, the geometric and electronic structure described above for $\{\mathbf{0}\}\text{-P}_\text{M}$ (followed closely by $\{\mathbf{1C}\}\text{-P}_\text{M}$, Figure 12) is consistent with all available experimental and computational data to-date.

4. DISCUSSION

While the HCO literature to-date has generally proposed that proton transfer from the active site Tyr precedes O–O bond cleavage, and that the Tyr supplies the fourth electron required for O_2 reduction, many details of the reaction mechanism remain unknown. This study evaluates: 1) how structural components of the active site (e.g. the cross-link and the farnesyl appendage on the heme) impact the mechanism and the barrier, 2) whether the proton transfer or O–O cleavage is rate-limiting, 3) how and when the fourth electron (from Tyr) transfers, and 4) the geometric and electronic structure of the product (P_M).

Drawing from the findings for O–O cleavage in a synthetic heme-peroxo-Cu model system employing phenolic substrates,¹⁰ the results in Section 3.1 reveal how the active site in HCOs lowers the barrier for Proton-Initiated O–O cleavage (where PT is early in O–O cleavage) to approximately half that of the synthetic model (12 kcal/mol vs. 25 kcal/mol). Thus, while the synthetic model favors a mechanism involving PT after the barrier, for which the calculated $G^\ddagger = 16.5$ kcal/mol, HCOs perform O–O cleavage involving early PT with a calculated $G^\ddagger$ of only 12 kcal/mol barrier. One primary reason for the lower barrier and different mechanism in HCOs relative to the previously studied synthetic model is the low pKa of the proton donor, Tyr. This derives from stabilization of the Tyr^- anion by H-bonding to the hydroxyl group of the farnesyl side-chain and the H_2O molecule involved in PT from Tyr to the bound O_2^{2-} , as well as the ortho-substitution to the cross-linked His-

Cu^{II} .³⁵ A second factor in the low O–O cleavage barrier is the separation between the Tyr^- anion and the Fe-OO(H)-Cu core, as H-bonding to the Cu-OH (as with an exogenous phenol in the synthetic model) would decrease the backbonding from the Fe into the $\text{O}_2^{2-} \sigma^*$, raising the O–O cleavage barrier.⁵⁹

It has been proposed in the literature (and in this study) that proton transfer from Tyr may be mediated by one or two water molecules in the active site via a Grotthuss mechanism.^{12,60–62} However, we have found that due to sterics within the active site (Section 3.1.2.1), the proton cannot transfer directly from the Tyr donor to the O_{Cu} acceptor without a high barrier, and must therefore transfer initially to the O_{Fe} atom. Protonation of the O_{Fe} yields a high-energy intermediate (calculated ~13 kcal/mol above the bridging peroxo structure), which causes the proton transfer to have a higher barrier than O–O cleavage, making the PT step rate-limiting. The KIE observed experimentally (1.4–1.9) is smaller than expected for PT between two sites with similar H^+ affinity (where the H^+ is in the middle in the transition state; thus KIEs are >5), while it is on the high side for secondary KIEs (a KIE of 1.4–1.9 would be expected for a change in H-bonding). It is larger than would be expected for the O–O(H) cleavage step, in which the $\text{O}_{\text{Cu}}\text{--H}$ bond is fully formed (the predicted KIE is 1). Thus, the calculations indicate that the observed barrier for A to P_{M} in HCOs involves proton transfer (where the H^+ is mostly transferred), which occurs *early* in O–O bond cleavage.

In section 3.1.2.3, we considered the possibility of the H^+ transferring directly to the O_{Cu} atom from a donor other than the active site Tyr. Examples in the literature of Grotthuss-mediated PT over similar distances (~10 Å) have reported barriers around 10 kcal/mol and KIEs of 1.4–3 (vide supra), in agreement with the data for A to P_{M} in HCOs. While such a mechanism may therefore be active in HCOs, the literature has produced a few key experimental results that can be correlated to the DFT, supporting that the proton bound to the O_{Cu} in P_{M} derives from the Tyr. First, recent crystallographic data by Rousseau et al. on the intermediate P_{r} (which is similar to P_{M} except the active site Tyr^- is reduced; P_{r} forms upon O–O cleavage from the fully reduced enzyme) have resolved a water molecule bound to the Tyr^- , positioned in the region between the Tyr and the O_{Fe} atom.²⁴ The authors suggested that this water molecule may be stabilizing the Tyr^- in P_{r} , identical to its role in O–O cleavage inferred from our DFT calculations (vide supra). Second, the rate observed for A to P_{M} is independent of pH from 6–9.5,³¹ and varies minimally between HCOs from different organisms (which differ in some of the outer sphere residues). Since PT over a large distance (~10 Å) would likely be impacted by changes in local electrostatics (different residues or protonation states), this suggests that the PT step occurs over a short distance that is highly conserved throughout HCOs, and can operate effectively in a range of pH environments.

In addition to PT, the timing and means of ET from Tyr were examined to understand the role and mechanism of the tyrosinate electron transfer during the reaction. It was found that the electron transfers from Tyr^- after the barrier to O–O cleavage, and therefore does not contribute in the barrier. While ET from Tyr early in O–O elongation would lower the O–O cleavage barrier by increasing $\text{O}_2^{2-} \sigma^*$ occupation, this would require a lower potential $\text{Tyr}^-/\text{Tyr}^\bullet$ couple (i.e. a less stable Tyr^-). Since the energetics of the PT step (which is rate-

limiting and precedes O–O elongation) are dictated by the thermodynamics of PT from Tyr to the peroxo O_{Fe} atom, lowering the stability of the Tyr^- anion would raise the PT barrier and consequently the overall reaction. Therefore, stabilizing the Tyr^- is key for yielding a low barrier for A to P_M in HCOs, although it prevents the electron from transferring until after the barrier.

The above discussion describes how the Cu-His-Tyr cross-link impacts the barrier by lowering the Tyr pKa, preventing the Tyr^- from interacting with the Cu-OH during O–O cleavage, and placing the Tyr close enough to the bound peroxo that the H^+ can be shuttled through a single water molecule. However, this unique structural motif is also responsible for enabling barrierless ET from the Tyr^- to the peroxo σ^* orbital. As shown in Section 3.1.2, the molecular orbitals involved in the superexchange pathway through the bridging His and Cu depend on the spin state, most notably the unoccupied d -orbital on Cu. On the singlet surface, the electron propagates through this half-occupied Cu dx^2-y^2 orbital, while on the triplet surface it propagates as a hole transfer through the doubly occupied Cu d_{yz} orbital. Thus, the cross-link provides a means for efficient transfer of the fourth electron, regardless of the overall spin surface.

Taken together, the results above for O–O cleavage in HCOs provide a favored mechanism where a water molecule shuttles the proton from Tyr to the peroxo via the O_{Fe} atom, with subsequent O–O(H) cleavage yielding the product P_M . The water molecule involved in PT may be either H-bonded to the $Fe^{IV}=O$ or dissociated. The recent crystallographic data on P_R provide critical comparison, which indicate that the water molecule in the active site is bound to the Tyr^- and not the $Fe^{IV}=O$.²⁴ Since rR data have indicated that the P_m and P_r intermediates have identical $\nu(Fe-O)$ stretches,⁴⁹ these results suggest that the water molecule involved in PT is not H-bonded to the $Fe^{IV}=O$ in P_m . Thus, the geometric structure of P_m in closest agreement with experimental data is that of $\{0\}-P_m$ in Figure 12. Our calculations indicate that the electronic ground state is a singlet, arising from antiferromagnetic coupling between the Fe^{IV} and Cu^{II} , and ferromagnetic coupling between the Cu^{II} and Tyr^* , each on the order of $3-6\text{ cm}^{-1}$. This electronic structure is consistent with the Cu^{II} and Tyr^* being EPR-silent, yet it differs from models in the literature that have assumed these $S=1/2$ species are antiferromagnetically coupled.^{20,50,54,63} Experiments using variable-temperature, variable-field Magnetic Circular Dichroism (VTVH MCD) spectroscopy to probe this magnetic coupling are currently underway along with thier analysis that includes the effect of the zero field splitting of the $Fe(IV)=O\ S=1$ on this exchange coupled three spin ground state. Applying these insights to the mechanism of O–O cleavage, the overall singlet spin surface implies that the unobserved peroxo (I_p) species responsible for accepting a proton from Tyr to initiate O–O cleavage is a singlet (i.e. antiferromagnetically-coupled Fe^{III} and Cu^{II}), and the electron from Tyr transfers to the O–O σ^* via the cross-linked His and the magnetic (singly-occupied) orbital on Cu (which is tilted during ET to form the superexchange pathway).

While the combination of our DFT calculations and past experimental data offer insight into the mechanism of O–O cleavage in HCOs, as well as the geometric and electronic structure of P_m , our conclusions are limited by the experimental data available. Our understanding of the mechanism must draw heavily on the findings for a synthetic model that differs

mechanistically from HCOs in both proton transfer and electron transfer. Our understanding of P_m derives heavily from data²⁴ on an intermediate (P_P) that differs in the source of fourth electron (and therefore its electronic structure). Therefore, further studies involving synthetic models and spectroscopy on P_m will be critical for advancing our understanding of how HCOs perform efficient O–O bond cleavage, and how these enzymes use the resultant free energy gain to pump protons in 4 consecutive reductive steps after formation of P_m .

5. CONCLUSION

Applying past experimental and computational insights from synthetic models and enzyme intermediates, the results herein reveal how the active site in heme-copper oxidases enable an exceptionally low barrier for proton-initiated O–O bond cleavage. It is shown that a water molecule in the active site can assist in proton transfer from the cross-linked tyrosine, and that multiple H-bonding interactions help to lower the tyrosine's pK_a by stabilizing the tyrosinate. Proton transfer to the bound O_2 molecule precedes O–O elongation and is rate limiting. Due to sterically restricted access to the O_{Cu} atom (which is protonated in the product P_M), the proton must either transfer initially to the O atom bound to Fe (a high-energy intermediate), or transfer over a ~ 10 Å distance to reach the O_{Cu} atom directly. While the distance between the H^+ donor (Tyr) and acceptor (O_{Cu}) therefore causes a high barrier to PT, it is shown that this separation is critical for enabling a low barrier to O–O cleavage, as it enhances backbonding from Fe into the O–O σ^* orbital. Following the O–O cleavage reaction coordinate to completion, we evaluate possible geometric and electronic structures of the product, intermediate P_m . The geometric structure that is consistent with our computational results and all available experimental data contains a $Fe^{IV}=O$ that is H-bonded to the $Cu^{II}-OH$, without water molecules interacting with the $Fe^{IV}=O$. The ground state electronic structure of P_M is an overall singlet: the Fe^{IV} and Cu^{II} are antiferromagnetically coupled through the H-bond between the oxo(Fe) and hydroxo(Cu) ligands, while the Cu^{II} and Tyr $^{\bullet}$ are ferromagnetically coupled as their magnetic orbitals involve orthogonal orbitals on the cross-linked His residue. These findings provide critical insights into the mechanism by which HCOs carry out efficient O_2 reduction, and the nature of the P_M intermediate that connects this reduction to the proton pumping function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Structure of the heme/Cu active site in bovine CcO (PDB 3WG7).

Figure 2.

Current consensus mechanism in HCOs, including the proposed involvement of a peroxo intermediate (**I_P**) prior to O–O cleavage.

Figure 3. DFT-optimized structures constructed from the crystal structure of the bovine CcO active site (3WG7) without water molecules included (denoted model **{0}**), showing the A) peroxo (I_P), and B) ferryl/ Y^* (P_M) species (i.e. the reactant and product structures in O–O cleavage).

Figure 4. DFT-optimized structures for the reactant in the H-Bond Assisted mechanism in LS-AN (A) and CcO (B), where the H-bond donor is bound to the Ocu atom of the bridging peroxo ligand. Note that Val243 in {1C}-Ip has been removed for visual clarity.

Figure 5.

Calculated energetics (ΔG , in kcal/mol) in the reaction coordinate for the H-Bond Assisted mechanism in LS-AN (black) and CcO (blue). The experimental barrier in CcO ($G^\ddagger < 12.4$ kcal/mol) is given for reference.

Figure 6.

Side (left) and top-down (right) views of the active site, illustrating the two possible ways in which a water molecule may access the bound O_2 . The blue circle marks the water location in model {1C}, and the red circle marks the location occupied in model {1F} (note each contains only one H_2O molecule). The gray X in the left figure indicates the region that would provide a direct path from Tyr to the O_{Cu} atom, which is hindered by the His240 and Val243 residues.

Figure 7.

Process of H^+ transfer from the active site Tyr to the peroxo O_{Cu} atom, mediated by the interstitial water molecule in model **{1F}**. The upper half of each panel shows a side-on view of the active site, while the lower half shows a top-down view (down the $\text{O}_{\text{Fe}}\text{-Fe}$ bond). Calculated ΔG values (in kcal/mol, relative to **{1F}-I_p**) for each optimized species are shown in green, and barriers for each step given in red.

Figure 8.

Calculated energetics (ΔG , in kcal/mol) in the reaction coordinate for the Proton-Initiated mechanism in the synthetic model {LS-AN + PhOH} (black) and the CcO model {1F} (red), which has a water molecule bridging the Tyr and O_{Fe} atom of the peroxo. The $G^\ddagger < 12.4$ kcal/mol experimental barrier in CcO is shown for reference.

Figure 9. DFT-optimized structure with a H^+ transferred from an external donor to the O_{Cu} atom, where the Tyr remains protonated.

Figure 10.

MO contours along the singlet surface for the PI mechanism, showing the progressive transfer of an α electron from the Tyr HOMO (marked with a red label) into the O-O σ^* (marked with a green label) via the unoccupied α dx^2-y^2 as the O-O bond elongates, after the transition state.

Figure 11.

MO contours along the triplet surface for the PI mechanism, showing the progressive transfer of an α electron from the Tyr HOMO (marked with a red label) into the O-O σ^* (marked with a green label) as the O-O bond elongates, after the transition state.

Possible Geometric Structures

Possible Electronic Structures

Figure 12.

(left) DFT-optimized structures of **{0}-P_M**, **{1F}-P_M**, and **{1C}-P_M** (as labelled in the figure), representing the possible products generated following O-O cleavage via the Proton-Initiated mechanism. Selected bond distances (marked on the **{0}-P_M** structure) are given in the text. (right) Localized spins for the Fe^{IV}, Cu^{II}, and Tyr^{*} comprising the singlet and triplet ground states, with the magnetic couplings (*J*) labelled in the diagram for the singlet (top). The highlighted configurations (**{0}-P_M** and *S*=0) are those in closest agreement with available experimental and computational data. Note that Val243 has been removed in the **{1F}-P_M** structure shown for clarity.

Figure 13.

MOs involved in the superexchange pathway between Fe and Cu, illustrating (A) $\text{Fe}^{\text{IV}}=\text{O}$ singly occupied orbital character mixed into the Cu^{II} hole, and (B) Cu^{II} singly occupied orbital character mixed into a hole on $\text{Fe}^{\text{IV}}=\text{O}$. Orbitals labelled in parentheses are doubly occupied.

Figure 14.

Schematic representation of orbital overlap of the cross-linked His donor orbitals with the half-occupied orbitals on Cu^{II} and Tyr[•], which leads to either ferromagnetic (left) or antiferromagnetic (right) coupling, depending on whether His π character mixes into the Cu LUMO. Note that the orbitals depicted for ferromagnetic coupling represent those shown in Figure 13A and C.

Scheme 1.

Two possible reaction pathways for proton-initiated O–O cleavage, with calculated barriers (in kcal/mol) and H/D KIEs for each step. Energies in each model are relative to the optimized “I_P” structure. The barriers for PT from a nearby amino acid are calculated using transition state theory, assuming a pK_a that is 2 units higher than that of the active site Tyr (see text and SI).