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Direct Examination of H_2O_2 Activation by a Heme Peroxidase

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Heavy atom isotope effects are powerful probes of mechanism, especially when analyzed using appropriate theoretical formalisms.[1] Interpreting kinetic isotope effects (KIEs) for enzymatic reactions, however, can be a particular challenge when there is uncertainty in the reactive species involved and/or the reaction coordinate.[2] Presented here is an examination of a classic heme peroxidase reaction; an approach based on oxygen isotope effects is outlined that should be generally applicable to the study of other H_2O_2-utilizing enzymes as well.

Though horseradish peroxidase (HRP) has been extensively studied,[3–5] the O−O bond cleavage step has eluded direct observation.[4b] Computational efforts have sought to model this step in HRP[6] as well as in other heme enzymes that consume cellular H_2O_2.[7] The experimental results afforded by the present work provide a critical calibration for these calculations.

At least two pathways are possible for peroxidase-mediated H_2O_2 activation (Scheme 1). It is widely accepted that HRP reacts by the Poulos-Kraut mechanism[8] in which O−O heterolysis leads to the Fe^{IV}=O porphyrin π-cation radical (compound I, denoted $Fe^{IV}O^{\bullet+}$) and H_2O. The reaction in HRP is facilitated by H^+ transfer from His42 to O_β of a $Fe^{III}O_\alpha O_\beta H$ intermediate.[3c,4a] Alternatively, O−O homolysis in enzymes that lack the H^+ donor can produce the Fe^{IV}=O porphyrin (compound II, $Fe^{IV}O$) and an associated $^{\bullet}OH$.[7,9] Proton-coupled electron transfer within this high-energy intermediate could subsequently form compound I.

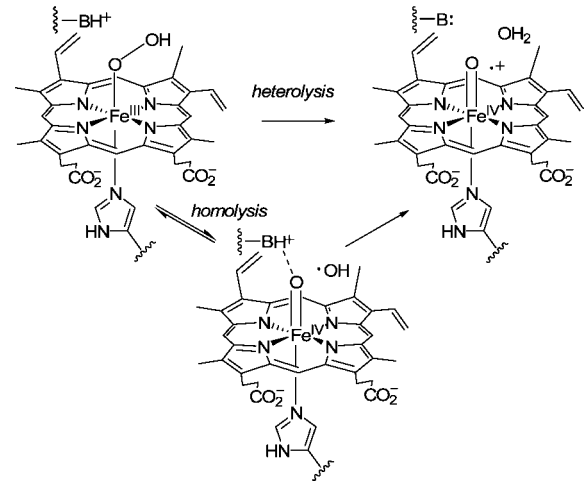
We applied oxygen isotope fractionation to examine the reaction of HRP[10] under steady-state conditions. Analyzing the change of $^{18}O/^{16}O$ in the H_2O_2 from natural abundance gives the competitive ^{18}O KIE, $[k_{cat}/K_M(H^{16}O^{16}OH)]/[k_{cat}/K_M(H^{16}O^{18}OH)]$, which measures all steps beginning with H_2O_2 encounter, leading up to and including the first irreversible step.[1c] Ground-state and transition-state structures from density functional theory (DFT) calculations were used to analyze the isotopic results.

The technique used to measure the ^{18}O KIE on the reaction of Fe^{III}HRP with H_2O_2 is modified from that originally developed to study reactions of O_2.[1c] The modifications include (i) the use of anaerobic solutions and (ii) the introduction of a step using acidic Ce^{IV} to quench the enzymatic reaction and convert all H_2O_2 to O_2.[11] The isolated O_2 is then completely combusted to CO_2 for analysis by isotope ratio mass spectrometry.[12]

Isotope fractionation plots (Figure 1) reveal ^{18}O enrichment of the unreacted H_2O_2 due to HRP turnover. R_0 is the $^{18}O/^{16}O$ of the H_2O_2 prior to adding HRP and R_f is the $^{18}O/^{16}O$ at fractional consumption, f, of H_2O_2. 2-Methoxyphenol (1−5 mM) was present (KP^i buffer pH 7.2, μ = 0.1 M, 22 °C) to reduce the oxidized HRP and prevent production of O_2 by H_2O_2 disproportionation. The ^{18}O-fractionation indicates an ^{18}O KIE = 1.0127 ± 0.0008.

A labeling experiment was conducted to test for ^{18}O exchange from H_2O into the unreacted H_2O_2 (Figure 1b). The results reveal ^{18}O scrambling, the R_f/R_0 falling within error of that predicted for a reaction where all of the ^{18}O in the enriched H_2O is rapidly distributed into the H_2O_2. In control experiments at low conversions where $f \approx 0$, R_f/R_0 is ∼1.00, indicating no ^{18}O exchange from H_2O in the absence of H_2O_2 consumption by HRP.

Scheme 1. Alternative Mechanisms of Enzymatic H_2O_2 Activation



Results which suggest the possibility of reversible O−O bond formation involving electrophilic Fe oxo and peroxo species have been inferred from studies of synthetic model compounds.[13] Such reactivity relates to the proposal of nucleophilic attack by ^{-}OH upon a Mn oxo intermediate in the O_2 evolving complex of photosystem II.[16] To the best of our knowledge, reversible O−O cleavage/formation has not been proposed in a heme protein.

DFT calculations were performed to evaluate structures relevant to H_2O_2 activation by HRP.[14] Equilibrium isotope effects (EIEs) were derived from full sets of vibrational frequencies following Bigeleisen's formalism (eq 1).[1] Isotopic terms include zero point energy (ZPE), vibrationally excited states (EXC) and mass and moments of inertia (MMI).[14] The EIE_{calcd} in Table 1 is the average of columns 3 and 4 which differ with regard to the isotope attached to Fe; that is, the small isotope effect due to the preferred

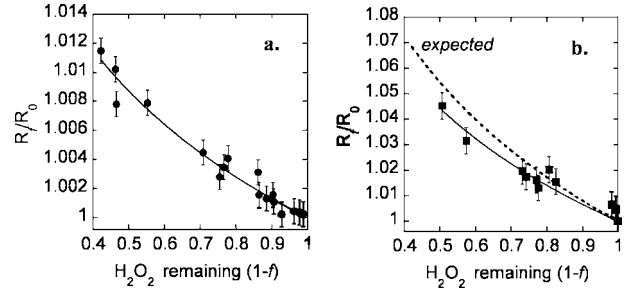


Figure 1. (a) Isotope fractionation due to Fe^{III}HRP + H_2O_2. Data are fitted to: ^{18}O KIE = $[1 + \ln(R_f/R_0)/\ln(1 - f)]^{-1}$.[1c] (b) The same reaction as in panel **a** performed in buffer containing 1.2% by volume ^{18}O-water, see ref 10. The dashed curve is based upon the ^{18}O KIE in panel **a** multiplied by the 6-fold enrichment of ^{18}O over its level at natural abundance (i.e., 0.2%).

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Table 1. Calculated ^{18}O EIEs for Reactions of HRP with H_2O_2[a]

	$Fe^{III}HRP + H_2O_2 \rightleftharpoons$	$Fe-{}^{16}O/{}^{18}O$	$Fe-{}^{18}O/{}^{16}O$	EIE_{calcd}
1	$Fe^{III}OOH$ ($\omega = 21.9°$)[b,c]	1.0031	1.0042	1.0037
2	$Fe^{III}OOH$ ($\omega = 161.3°$)[b]	1.0082	1.0073	1.0078
3	$Fe^{III}O_\alpha O_\beta H$, $MeImH^+-O_\alpha$[b,d]	1.0038	1.0033	1.0036
4	$Fe^{III}O_\alpha O_\beta H$,$MeImH^+- O_\beta$[b,e]	1.0058	1.0159	1.0109
5	$Fe^{IV}(O)$[f] + ·OH	1.0484	1.0115	1.0300
6	$Fe^{IV}(O)\cdot^{+}$[g]+ H_2O	1.0097	1.0112	1.0105

[a] Calculated using Gaussian 03[15] and the *m*PW functional.[14] [b] $S = 1/2$. [c] H-bonding occurs to a pyrrole N. [d] $\omega = 42.3°$. [e] $\omega = 24.8°$. [f] $S = 1$. [g] $S = 3/2$.

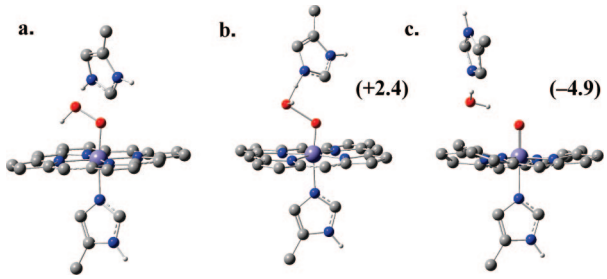

Figure 2. The reactant (a), TS (b), and product of O−O heterolysis in the gas phase. Potential energies are quoted as (kcal mol^{-1}) relative to structure **a**.

coordination of ^{18}O to Fe or H was neglected.[1c,14] Entries 3 and 4 include H-bonding to protonated methylimidazole ($MeImH^+$) as a model for $His42H^+$, while entries 1, 2, 5, and 6 do not. The measured ^{18}O KIE $\ll$ ^{18}O EIE_{calcd} for entry 5 provides evidence against reversible O−O homolysis (as does the computed $E_{act} >$ 70 kcal mol^{-1}).[14] Neither reversible formation of $Fe^{III}OOH$ nor $Fe^{IV}O\cdot^{+}$ can be excluded on the basis of the size of the ^{18}O EIE_{calcd}. It follows that the first irreversible step which limits $k_{cat}/K_M(H_2O_2)$ occurs after formation of either intermediate.

$$^{18}O\ EIE = {}^{16,16}K/{}^{16,18}K = ZPE \times EXC \times MMI \quad (1)$$

$$^{18}O\ KIE = \left({}^{16,16}\nu_{RC}/{}^{16,18}\nu_{RC}\right)\left({}^{16,16}K_{TS}/{}^{16,18}K_{TS}\right) \quad (2)$$

Optimized structures for $MeImH^+$-assisted O−O heterolysis are shown in Figure 2. Transition-state theory permits calculation of the ^{18}O KIE from vibrational frequencies according to eq 2,[1] where K_{TS} is a pseudoequilibrium constant for converting the reactant to the TS, defined analogously to eq 1, and ν_{RC} is the imaginary mode corresponding to the reaction coordinate.[14] Isotope effects on $K_{TS} = 1.0117$ and $\nu_{RC} = 1.020$ indicate an ^{18}O $KIE_{calcd} = 1.032$. This value is significantly larger than the ^{18}O EIE_{calcd} (Table 1, entry 6) often invoked as the upper limit.[1c] The inflated KIE arises from a mass-dependent ν_{RC} which ranges from 226.5*i* cm^{-1} for $Fe^{16}O^{16}OH$ to 220.0*i* cm^{-1} for $Fe^{18}O^{16}OH$ due to a dominant contribution from the O−O stretch.[14] It remains to be seen whether similar effects upon ν_{RC} contribute to ^{18}O KIEs on metal-O_2 binding reactions,[17] underscoring the value of the DFT calculations in interpreting heavy atom KIEs.

That the measured ^{18}O KIE is significantly smaller than the ^{18}O KIE_{calcd} further argues that O−O heterolysis is not the rate-limiting step in H_2O_2 activation by HRP, as commonly assumed.[3−5] Shintaku et al. have also recently challenged this assumption on the basis of rapid kinetic studies.[4b] Reversible O−O cleavage prior to a rate-limiting transformation of compound I is consistent with ^{18}O incorporation from H_2O into the unreacted H_2O_2; this is because of the *competitive* nature of the isotope fractionation measurements which probe steps beginning with encounter up to and including the first irreversible step.[1c] Future studies of HRP mutants and other heme peroxidases may elucidate the mechanism of ^{18}O-scrambling in the context of the steady-state enzyme kinetics.

In summary, this first-of-a-kind study of enzymatic H_2O_2 activation has yielded two major findings: (1) O−O cleavage is not the rate-limiting step in the reaction of H_2O_2 with HRP. The observed ^{18}O scrambling from labeled H_2O into H_2O_2 indicates that reversible O−O heterolysis should be considered a possibility. (2) DFT calculations allow prediction of an ^{18}O KIE for O−O heterolysis that is larger than the ^{18}O EIE, not smaller as often assumed.[1c] This result has important ramifications for interpreting ^{18}O KIEs upon reactions of H_2O_2 and O_2-utilizing enzymes as well as synthetic compounds.[1c,2b] Continued application of the approach outlined here is needed to enhance our understanding of fundamental oxidative reactivity and to refine the methods used to computationally model essential steps in enzyme catalysis.

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Supporting Information Available: Computational details and vibrational frequencies. This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) HRP isozyme C (Type VI) and other chemicals were purchased from Sigma and used without purification. ^{18}O-water (>95.0 atom %) was obtained from Promy chemicals. H_2O_2 (30%) from Fisher was used as received; the concentration was determined from the $\varepsilon_{240\ nm} = 43.6\ M^{-1}\ cm^{-1}$.
(11) H_2O_2 solutions (0.5−2.0 mM) were stirred with >10 equiv $Ce^{IV}(SO_4)_2$ in 10−20% H_3PO_4 (20 min). The O_2 produced was collected under dynamic vacuum. Determination of the gas pressure, after converting to CO_2, agreed precisely with that expected based on the initial [H_2O_2]. As described in an early report (Cahill, A. E.; Taube, H. *J. Am. Chem. Soc.* **1952**, *74*, 2312.) the O_2 had the same isotope composition as the H_2O_2. $R_0 = 1.02196 \pm 0.00067$ vs. standard mean ocean water (SMOW) was determined from >20 independent measurements.
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