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Kinetic Isotope Effects on the Rate-Limiting Step of Heme Oxygenase Catalysis Indicate Concerted Proton Transfer/Heme Hydroxylation

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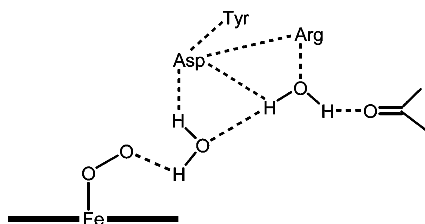
Heme oxygenase (HO) catalyzes the O_2 and NADPH/cytochrome P450 reductase-dependent conversion of heme to biliverdin, free iron ion, and CO through a process in which the heme participates as both dioxygen-activating prosthetic group and substrate.[1] We confirmed recently[2,3] that the first step of HO catalysis is a monooxygenation in which the addition of one electron and two protons to the HO oxy-ferroheme produces ferric-α-*meso*-hydroxyheme (**h**). We did so through the use of cryoreduction/EPR and ENDOR spectroscopies to characterize enzymic intermediates. Oxy-HO, whose distal-pocket proton-delivery network[4] is modeled by Scheme 1, was subjected to one-electron 77 K radiolytic reduction and subsequent annealing at 200 K.[3,7−9] This generates a structurally relaxed hydroperoxo-ferri-HO species,[10] denoted **R**.

Scheme 1



We found that during annealing steps at 214 K, intermediate **R** converts in a single kinetic step to the high-spin, five-coordinate product, **h**, without formation of a Compound I.[3] However, there has been no detailed information about this rate-limiting step, neither about the actual O-atom transfer nor about the delivery of the second proton, assumed to activate the hydroperoxo group for reaction. We here report this information, acquired through the cryoreduction/annealing experiments that *directly* measure the solvent (solv) and secondary (sec) kinetic isotope effects (KIEs) of the **R** → **h** conversion in enzyme prepared in H_2O/D_2O buffers and with *meso*-deuterated heme. This approach is unique in that KIEs are measured by monitoring the rate-limiting step directly and are not susceptible to masking by KIEs of other processes. This report thus presents the first direct measurement of the KIEs of product formation by a kinetically competent reaction intermediate in *any* dioxygen-activating heme enzyme.[11,12]

Oxyferrous human HO was prepared in H_2O and D_2O buffers, and oxyferrous-HO (heme *meso*-D)[13] was prepared in H_2O buffer.[14] Cryoreduction[3,7,8] of oxyferrous-HO at 77 K and annealing to 200 K produces the reactive form (**R**; g = [2.37, 2.180, 1.917]) of the hydroperoxoferri-HO, along with a minority form that is unreactive (or slowly reactive) at this temperature (**R1**; $g \approx$ [2.393, 2.188, 1.917]).[3] Figure 1 (inset) shows EPR signals of **R/R1** (heme *meso*-H) in H_2O buffer taken during annealing at 215 K.[3,14] Figure 1 plots the time course of this reaction, overlaid with the fit to an exponential decay, $I = a + b[\exp(-t/\tau)]$.[15] The reactive **R** component, with b = 80% of the signal, decays with a half-time of τ = 2.0(1) min.

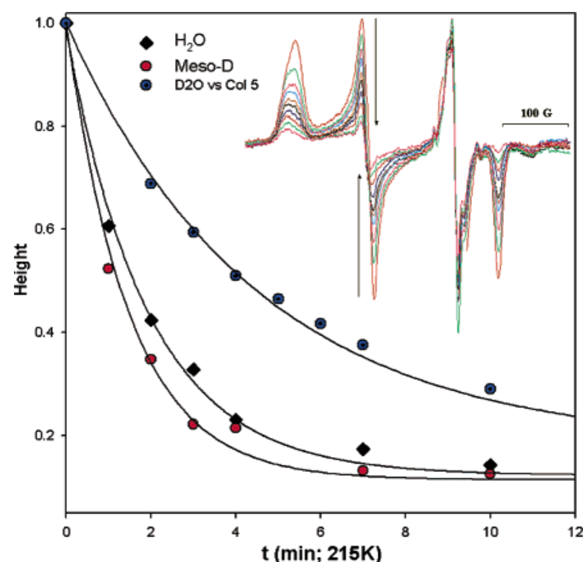


Figure 1. Inset: 77 K X-band EPR spectra of **R**/**R1** intermediates of HO in H_2O buffer during stepwise annealing at 215 K. Figure: Relative peak-trough heights of g_2 feature of **R**/**R1** spectra for HO in H_2O (◆) and D_2O (black ●) buffers, and for HO (heme *meso*-D) in H_2O buffer (red ●).

Does this rate-limiting step for **R** → **h** conversion involve bond formation between the α-*meso*-carbon and the terminal hydroperoxo-oxygen? If the transition state for this conversion does involves O−C bond formation, and the associated O−O bond cleavage, then the associated rehybridization of the heme α-*meso*-carbon should introduce a secondary KIE (sec-KIE) upon deuteration of this heme position.[16,17] Indeed, the decay of **R** for oxy-ferrous-HO (heme α-D; Figure 1) is *faster* than that for the enzyme with α-H heme, τ(α-D) = 1.3 min, giving an *inverse* secondary isotope effect at 215K, sec-KIE(α-D) = k_H/k_D = τ(α-D)/τ(α-H) = 0.7(2). Using the exponential dependence of a sec-KIE on inverse temperature, this corresponds to sec-KIE(298 K) = 0.8(1). The sense (inverse) and magnitude of this value agrees with expectations[16,17] for rehybridization of the trigonal (sp^2) heme α-*meso* carbon as it becomes a tetrahedral (sp^3) hydroxylated intermediate during reaction with the terminal O.

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Is this process activated by delivery of the "second" proton of catalysis? The **R** → **h** conversion in fact is markedly slowed when oxyferrous-HO is prepared in D_2O buffer (Figure 1), $\tau(D_2O)$ = 4.7 min, giving a kinetic solvent isotope effect, solv-KIE(215 K) = $\tau(D_2O)/\tau(H_2O)$ = 2.3.[18] Using the exponential dependence of a solv-KIE on inverse temperature,[19,20] the value of 215 K is found to correspond to solv-KIE(298 K) = 1.8.

The solv-KIE(298 K) for a reaction can be estimated from eq 1,

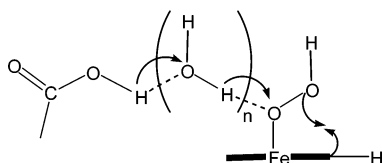
$$\text{solv-KIE} = \frac{k_H}{k_D} = \frac{\prod^{\text{react.}} \phi_i^r}{\prod^{\ddagger} \phi_i^{\ddagger}} = \left(\frac{\prod^{\text{react.}} \phi_i^r}{\prod^{\text{prod.}} \phi_j^p}\right)^{\delta} \left(\frac{1}{\phi_{if}^{\ddagger}}\right) = \left(\frac{K_H}{K_D}\right)^{\delta} \left(\frac{(\phi_{if}^p)^{\delta}}{\phi_{if}^{\ddagger}}\right)$$

where the φ are H/D fractionation factors for the solvent-exchangeable protons, with $\varphi_{if}^{\ddagger}$ being the value for the "in-flight" (if) proton being transferred, and K_H, K_D the equilibrium constants for the reaction in H_2O and D_2O; the parameter δ expresses the location of the transition state (TS) on the reaction coordinate, with values between 0 (reactant-like TS) and 1 (product-like).[17,20,21]

The spontaneous reaction of the hydroperoxo oxygen with the α-*meso* carbon would have solv-KIE = 1, while the reaction of an already-activated, protonated hydroperoxo would have an inverse effect (solv-KIE(298 K) = 0.69^{δ}); the experiment thus rules out both processes. Instead, it appears that the rate-limiting step for **R** → **h** conversion must involve not only O−C bond formation but proton delivery to the Fe^{3+}-OOH moiety as well. Proton transfer from an H_2O or H_3O^+ of the distal H-bond network would give solv-KIE values that are too large for the former donor (5 as $\delta \rightarrow 1$ $((2.5 \cdot 2^{\delta}))$ and too small for the latter (1.2, as $\delta \rightarrow 1$ $(2.5/2.1^{\delta})$). However, a carboxyl donor acting as a general acid catalyst, presumably Asp 140 with intervening water(s) (see Scheme 1) (or other donor with one exchangeable proton), would give solv-KIE-(298 K) = 2.5, in satisfactory accord with experiment.

The observation of *both* sec-KIE and solv-KIE indicates that the rate-limiting step for formation of five-coordinate, high-spin, ferric-α-*meso*-hydroxyheme (**h**) by HO is the concerted process of Scheme 2 (where arrows imply nuclear motions): activation by proton transfer to the hydroperoxo-ferri-heme through the distal-pocket H-bond network (Scheme 1), likely from a carboxyl group acting as a general acid catalyst, and synchronous bond formation between the distal O and the α-*meso* carbon, leading to a tetrahedral hydroxylated-heme intermediate. Subsequent rearrangement and loss of H_2O then generates **h**. Such behavior is consonant with theoretical predictions[22−25] of facile O−O bond breakage upon proton activation. This approach will be applied to other heme monoxygenases,[7,8] as well.

Scheme 2



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(15) A fit to a biexponential is not warranted here.
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(21) Rewritten from equations of Schowen. The φ are tabulated in Schowen and Isaacs. Schowen gives $\varphi_{if}^{\ddagger} = 0.4$; with a water bridge between donor/acceptor $\varphi_{if}^{\ddagger}$ is distributed among the transferring protons; thus, a bridge does not influence the computation.
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