Reactive Intermediates

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Driving Force for Oxygen-Atom Transfer by Heme-Thiolate Enzymes**

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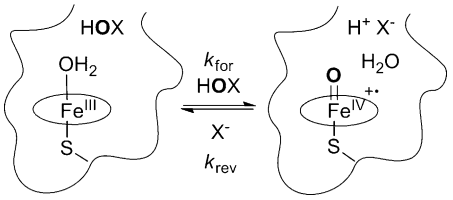
The heme-thiolate peroxygenase from *Agrocybe aegerita* (*Aae*APO, EC 1.11.2.1) is a versatile biocatalyst and cytochrome P450 analogue which catalyzes a variety of oxygenation reactions with high efficiency and selectivity.[1] Our recent kinetic characterization of *Aae*APO-catalyzed reactions has shown that *Aae*APO compound I is an oxo-Fe^{IV} porphyrin radical cation.[2] The reactivity of *Aae*APO-I toward a panel of substrates showed very fast C–H hydroxylation rates, similar to those of cytochrome P450 (CYP119-I),[3] and much faster than chloroperoxidase compound I (CPO-I).[4] Mechanistic probes have revealed a large hydrogen isotope effect for aliphatic C–H hydroxylation and rearranged products from the hydroxylation of norcarane.[1b] There is, however, very little information available regarding the thermodynamic properties of such highly reactive oxo-Fe species for any heme-thiolate proteins.

For hydrogen abstraction reactions, the redox potential of the oxidant is correlated with the rates of C–H activation.[5] Yet these values are often not accessible, especially for highly reactive oxidants. We have developed a method to measure redox potentials for oxometalloporphyrin model compounds and it takes advantage of the rapid, reversible oxygen-atom transfer between oxo-metal complexes and halide ions.[6] By using rapid-mixing stopped-flow spectroscopy, rate constants of both forward and reverse reactions are measured. Thus, the driving force of the unknown oxo-transfer redox couple ($M^{n+2}=O/M^{n}$) is obtained from the equilibrium constants for the reaction and the known potentials of the HOX/X^- couples. Using this method, the oxo-transfer driving force for several heme enzyme model complexes have been measured, such as [oxo-Mn^{V}TDMImP][6,7] and [oxo-Fe^{IV}-4-TMPyP]$^{+}$.[8]

Herein, we describe measurements of the driving force for oxygen-atom transfer by the heme-thiolate proteins *Aae*APO and CPO. We have found that oxo-transfer between *Aae*APO-I and chloride or bromide ions is fast and reversible (Scheme 1). The redox potential of the couple *Aae*APO-I/ferric-*Aae*APO has been obtained over a wide pH range from the rate constants of the forward and reverse reactions. Thus, the highly reactive *Aae*APO-I can be placed on an absolute energy scale and compared with those of CPO and horseradish peroxidase (HRP) for the first time.



Scheme 1. Reversible oxygen-atom transfer between ferric *Aae*APO and HOBr or HOCl (k_{for}), and *Aae*APO-I with halide ions (k_{rev}).

*Aae*APO-I was generated by the stoichiometric reaction of Fe^{III}/*Aae*APO with HOCl or HOBr and characterized by rapid-mixing, stopped-flow spectroscopy. The UV/Vis spectral features of *Aae*APO-I generated with these hypohalous acids (Figure 1) are the same as those we recently reported for peroxyacid oxidations.[2] The Soret band of the ferric enzyme at $\lambda=417$ nm diminished over the first 50 ms after mixing while new absorbances, characteristic of the formation of an oxo-Fe^{IV} porphyrin radical cation, appeared at $\lambda=361$ and 694 nm. *Aae*APO-I subsequently decayed in a second, slower phase. Singular value decomposition (SVD) analysis of these transient spectra indicated that only two species were present in significant amounts during this transformation. The

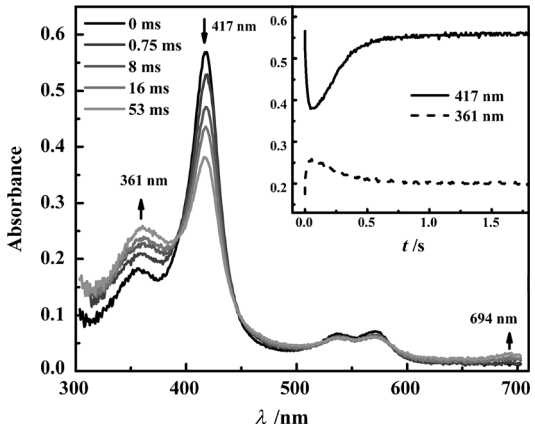


Figure 1. UV/Vis spectra observed upon 1:1 mixing of 5 μM *Aae*APO with 15 μM NaOBr at pH 5.0, 4 °C. Inset: Time courses of data obtained at $\lambda=417$ nm (ferric *Aae*APO) and $\lambda=361$ nm (*Aae*APO-I).

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*Aae*APO-I formation rate was directly measured by monitoring the conversion of the ferric enzyme into the oxo-Fe^{IV} radical cation. Binding of HOX to the heme iron is a rapid step and heterolytic FeO–X bond cleavage is rate limiting.[2] Plotting the initial absorbance change at $\lambda = 417$ nm against the HOX concentration afforded a linear relationship with no evidence of saturation. Second-order rate constants were obtained from the slopes (see Figure S1 in the Supporting Information).

The oxidation of *Aae*APO with HOCl or HOBr was examined over a range of pH values as shown in Table 1. At pH 3.0, HOCl was used because HOBr is not stable at this pH. The slightly milder oxidant, HOBr, was used to generate

Table 1: Data for oxygen-atom transfer between halide ions and *Aae*APO-I as a function of the pH value.

pH	k_{for} [$M^{-1}s^{-1}$]	k_{rev} [$M^{-1}s^{-1}$]	K_{equi}	$E'_{(cpd-I/ferric)}$[a] [V, vs.NHE]
3.0	7.7×10^5	1.0×10^2	7680	1.32[b]
4.0	1.8×10^5	4.3×10^5	0.4	1.25
5.0	7.1×10^5	2.6×10^5	2.8	1.19
6.0	5.7×10^5	1.9×10^5	3.0	1.17
7.0	7.5×10^5	5.1×10^4	15	1.12

[a] Based on the $HOBr/Br^-$ couple at 4 °C except as noted.[10] [b] Based on the $HOCl/Cl^-$ couple at 4 °C.[10]

*Aae*APO-I from pH 4.0–7.0 in good yield. We also measured the rates of CPO-I formation by the same method (see Table S1 in the Supporting Information). At pH 5.0 and 4 °C, the second-order rate constant for CPO-I formation was $2.3\times10^6\,M^{-1}s^{-1}$, which is three-fold faster than that of *Aae*APO. Although *Aae*APO and CPO share approximately a 30% sequence similarity, their active site environments, especially the acid-base residues, differ and CPO has a less accessible active site.[9]

We have found that *Aae*APO-I is also highly reactive toward halide ions. The formation of HOBr for the reaction of bromide ion with *Aae*APO-I was detected conveniently with the diagnostic indicator phenol red.[11] The rapid tetrabromination of phenol red was monitored by the characteristic red shift from $\lambda = 434$ nm to $\lambda = 592$ nm as shown in Scheme S1 and Figure S2 in the Supporting Information. The oxygenation of bromide by CPO-I was found to be much slower than that of *Aae*APO-I at the same pH value (see Figure S3 in the Supporting Information). The reaction of chloride ion with *Aae*APO-I to afford hypochlorous acid was also found to occur with high efficiency but only under acidic conditions.

The kinetic behavior of halide ion oxygenation by *Aae*APO-I was then investigated by double-mixing, stopped-flow spectroscopy. At each selected pH value, *Aae*APO-I was formed in the first push by mixing ferric enzyme with 3 equivalents of NaOBr or NaOCl. NaBr or NaCl solution was added in the second push after the peak amount of compound I had been achieved. Time-resolved, diode array spectra clearly showed the transformation of compound I back to the resting ferric state. Kinetic profiles were obtained by monitoring the return of the Soret band of ferric *Aae*APO at $\lambda = 417$ nm or ferric CPO at $\lambda = 399$ nm, and fitted to a single exponential equation (see Figure S4 in the Supporting Information). The observed pseudo-first-order rate constants (k_{obs}) were found to vary linearly with [NaBr] or [NaCl]. The apparent second-order rate constants (k_{rev}) were calculated from the slopes and are summarized in Table 1 and Table S1. The pH dependence of $\log k_{rev}$ is plotted in Figure 2. A slope of −1.0 was obtained over the pH range studied for CPO, and suggests that a single proton is involved in the reaction. However, for *Aae*APO, the $\log k_{rev}$/pH slope is only −0.3, thus suggesting that a protonation may not occur in the rate-determining step.

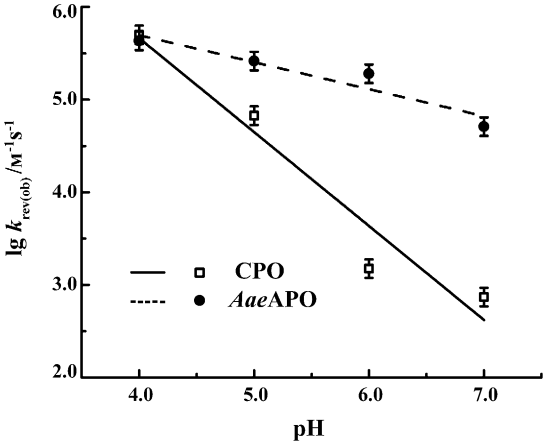

Figure 2. Plots of $\log k_{rev}$ as a function of pH for oxo transfer from *Aae*APO-I and CPO-I to the bromide ion.

Taking advantage of this reversible and kinetically well-behaved oxygen-atom-transfer reaction (Scheme 1), we determined a set of equilibrium constants, K_{equi}, from the ratios of the measured forward and reverse rate constants. Since the redox potentials for the couples $HOBr/Br^-$ and $HOCl/Cl^-$ are known,[12] the corresponding oxygen-atom-transfer driving force for *Aae*APO-I could be calculated at each pH value as shown in Equations (1) and (2) ($n = 2$, at 4 °C).

$$\Delta E' = \frac{2.3RT}{nF}\lg K_{equi} \quad (1)$$

$$E'_{cpd-I/Fe^{III}} = E'_{HOX/X^-} - 0.0275\lg K_{equi} \quad (2)$$

The derived compound I/ferric enzyme redox potentials for *Aae*APO and CPO are summarized in Table 1 and plotted in Figure 3. Fitting those points from pH 3.0 to 7.0 gave linear relationships with a slope of 0.048 for *Aae*APO and 0.056 for CPO, and is close to the theoretical value of 0.055 for the Nernst equation at 4 °C. This similarity supports a Nernst half-reaction involving two electrons and two protons as shown in Scheme 2.

As can be seen in Figure 3, the driving force for oxygen-atom transfer for *Aae*APO-I and CPO-I are similar to that of HOBr and about 200 mV less than that of HOCl. *Aae*APO-I and CPO-I are both significantly more oxidizing than HRP-I, while *Aae*APO-I has slightly larger redox potentials than those of CPO-I over the entire pH range. Thus, the ordering

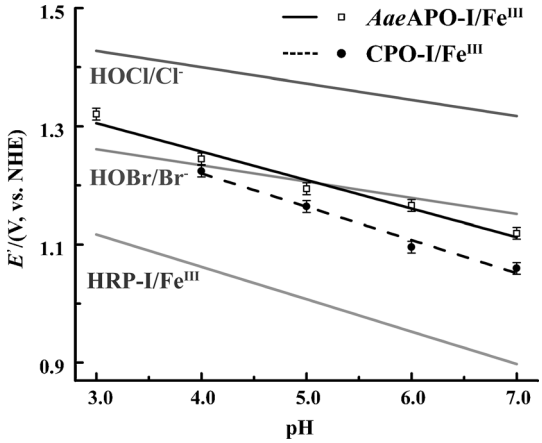

Figure 3. Calculated redox potentials, $E'_{(cpd-I/ferric)}$, as a function of pH value for *Aae*APO-I/Fe^{III} (open squares) and CPO-I/Fe^{III} (closed circles) at 4 °C. Nernst equations for HRP-I/Fe^{III},[13] $HOBr/Br^-$, and $HOCl/Cl^-$ are plotted in gray for comparison.

$$HOX + 2e^- + H^+ \rightleftharpoons X^- + H_2O$$
$$^{+\bullet}PorFe^{IV}{=}O + 2e^- + 2H^+ \rightleftharpoons PorFe^{III}(H_2O)$$

Scheme 2. Nernst half-reactions for HOX and $^{+\bullet}PorFe^{IV}{=}O$.

of the redox potentials parallels the reactivity of these heme proteins. CPO-I reacts slowly with even weak C–H bonds,[4,14] while HRP-I is barely able to oxidize C–H bonds at all. By contrast, *Aae*APO-I is highly reactive toward even very strong C–H bonds, so other active site factors may contribute to the greater facility of C–H hydroxylation than those of CPO. Similar halide oxidation data for cytochrome P450 is not available. However, by comparing the hydroxylation kinetics of *Aae*APO and CYP119 with similar aliphatic substrates,[3,15] the redox properties of P450-I and *Aae*APO-I appear to lie on a similar scale.

What factors contribute to the significantly higher driving force for ferryl oxygen-atom transfer by *Aae*APO-I and CPO-I reported here as compared to that of HRP-I? The axial ligand for *Aae*APO and CPO are both cysteine thiolate anions, while for HRP, it is a neutral, histidine nitrogen atom. The importance of hydrogen bonding to the cysteine thiolate of P450, CPO, and nitric oxide synthase (NOS) has been noted.[16] According to the Nernst half-reaction (Scheme 2), the driving force for the conversion of $^{+\bullet}PorFe^{IV}{=}O$ into $PorFe^{III}$ by oxygen-atom transfer has two contributions—the electron affinity and the proton affinity of the ferryl species. Although DFT calculations have indicated that the frontier orbitals of the heme-histidine compound I are at lower energy than the corresponding orbitals in heme-thiolate compound I,[17] the strong proton affinity of a thiolate-bound compound I may provide a large driving force resulting in a higher net redox potential and more reactive oxidants.[5k,18] The intrinsic basicity of the ferryl oxygen atom in Cys-S-$Fe^{IV}{=}O$ (compound II) in heme-thiolate enzymes has been established.[19] Since Cys-S-Fe^{III}-OH_2 is the resting state, Cys-S-Fe^{III}-OH is also basic, thus contributing additionally to the two-electron, two-proton oxo-transfer redox couples determined here.

The one-electron redox potential of *Aae*APO-I, $[E^o(I)]$, is a particularly important thermodynamic value because it is related to the bond strength $[D(O\text{-}H)]$ and the pK_a value, $[pK_a(II)]$ of $Fe^{IV}O$-H in *Aae*APO-II [Eq. (3)].[5a,b] For cases in

$$D(O{-}H) = n\,F\,E^o(I) + 2.3\,R\,T\,pK_a(II) + 57 \pm 2 \quad (3)$$

which $E^o(I)$ and $pK_a(II)$ cannot be measured independently, Equation (4) can be derived.[20] Since both $E^o(I)$ and $pK_a(II)$

$$D(O{-}H) = nFE'_{cpd-I/cpd-II} + 2.3\,R\,T\,pH + 57 \pm 2 \quad (4)$$

have not been measured independently for any heme-enzyme, the two-electron, two-proton redox potential of *Aae*APO-I measured here may be a good first approximation of $E^o(I)$. $E'_{(HRP-I/HRP-II)}$ and $E'_{(HRP-II/Ferric)}$ for HRP have been measured and were found to be similar (ca. 0.95 V at pH 6.0).[13,21] However, this result might be due to the fact that HRP-II is not basic and exists in the $Fe^{IV}{=}O$ form in the functional pH range. The situation is different if we consider that *Aae*APO compound II is protonated.[19] For example, if $D(O\text{-}H)$ is estimated to be in the range of 100 kcal mol^{-1},[2,22] the one-electron redox potential, $E'_{(cpd-I/cpd-II)}$, would be 1.4 V vs NHE at pH 7.0, which is significantly higher than the two-electron $E'_{(cpd-I/ferric)}$ potential of 1.1 V. Accordingly, from Equation (5), the reduction potential of *Aae*APO-II

$$2E'_{cpd-I/Fe^{III}} = E'_{cpd-I/cpd-II} + E'_{cpd-II/Fe^{III}} \quad (5)$$

$\left(E'_{(cpd-II/ferric)}\right)$ can be estimated to be approximately 0.8 V. This unsymmetrical partitioning of the two redox steps may be an important factor in facilitating homolytic C–H bond scission by heme-thiolate proteins.

In summary, the results show that chloride and bromide ions are readily oxidized by *Aae*APO-I to the corresponding hypohalous acids. The reversibility of this oxo-transfer reaction provides a rare opportunity to place ferryl oxo transfers by the highly reactive heme-thiolate *Aae*APO-I and that of CPO-I on an absolute energy scale. With an estimated bond dissociation energy for $Fe^{IV}O$-H in *Aae*APO-II we are able to obtain redox potentials of three redox couples interconnecting the resting ferric protein with its two oxidized forms, $^{+\bullet}Por\text{-}Fe^{IV}{=}O$ and $Fe^{IV}O$-H.

Experimental Section

Reagents: Wild-type extracellular peroxygenase of *A. aegerita* (isoform II, pI 5.6, 46 kDa) was produced in bioreactors with a soybean-flour suspension as the growth substrate and purified as described previously.[2,23] Kinetic experiments were performed as we have recently described.[2] Bromination of phenol red was detected by UV/Vis spectroscopy.[7a] At a chosen pH, 2 μL of 10 μM APO or CPO was added to a reaction mixture containing 20 μM of phenol red (sodium salt), 1 mM H_2O_2 and 10 mM NaBr.

The oxidation of ferric enzyme with NaOBr or NaOCl was performed by stopped-flow spectroscopy with the single-mixing mode under the diode-array or single-wavelength mode. The first syringe was filled with enzyme in a 100 mM buffer at a chosen pH. The second syringe was filled with the oxidant in slightly basic water solution.

Equal volumes of the two reactants were mixed quickly. The halide ion oxidation reactions were performed using the double-mixing mode. Native enzyme was mixed with an equal volume of oxidants (NaOCl or NaOBr) in the first push. After an aging time (optimized for each pH), the sodium halide solution was added in the second push. All concentrations reported are the final concentrations. All the experiments were carried out at 4 °C. The data were analyzed using Kinetic Studio from Hi-Tech.

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