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# Peroxidase Activity of the Hemeoctapeptide *N*-Acetylmicroperoxidase-8

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The pH dependence of the peroxidase activity (guaiacol assay) of the ferric hemeoctapeptide *N*-acetylmicroperoxidase-8 (*N*-AcMP8) was studied under conditions where formation of the Compound I analogue of the peroxidase enzymes is rate limiting. The pH profile of the reaction rate is consistent with a mechanism where both $H_2O_2$ and $HO_2^-$ can displace $H_2O$ coordinated trans to neutral His but where the hydroxo complex of the hemepeptide ($OH^-$ trans to His) is kinetically inert. At pH > 11, where the proximal His ligand of Fe(III) ionizes to form a histidinate, the hydroxo complex ($OH^-$ trans to $His^-$) becomes kinetically labile. A comparison of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values for the reaction of $H_2O_2$ and $HO_2^-$, obtained from the temperature dependence of the rate constants, with values for $CN^-$ and cysteine reported previously, shows that the activation parameters depend on the identity of the incoming ligand. This suggests that ligand substitution at Fe(III) in *N*-AcMP8 proceeds through an interchange mechanism.

The ferric heme-containing peroxidase enzymes catalyze the oxidation of organic substrates by $H_2O_2$.[1−3] A typical example is horseradish peroxidase (HRP), which catalyzes the oxidative coupling of phenols.[4] $H_2O_2$ reacts with the enzyme to form the transient intermediate Compound 0 (Cmpd 0) in which $H_2O_2$ (but probably $HO_2^-$)[5] is coordinated to Fe(III).[6] The protein is then rapidly oxidized to two oxidizing equivalents above the ground state (Compound I, Cmpd I), forming, with release of $H_2O$, an oxyferryl species and a porphyrin radical. Cmpd I is reduced by the substrate (such as a phenol) in two one-electron-transfer steps to form, first, Compound II (Cmpd II), which contains the oxyferryl moiety, and then the resting enzyme.

Protoeolytic digestion of cytochrome *c* produces a series of hemepeptides called the microperoxidases (MPXs) that retain a number, *X*, of the amino acid residues of the parent protein, including the proximal His;[7] the other axial coordination site is occupied by $H_2O$. They have proved to be useful models for some hemoproteins including the peroxidases,[5,8−10] the cytochromes P450,[11,12] and the oxidative dehalogenating enzymes.[13,14]

Adams[15,16] studied the oxidation of ATBS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonate)) with $H_2O_2$ catalyzed by the hemeoctapeptide, MP8, under conditions where formation of Cmpd I was rate limiting and found that the kinetics were unaffected by addition of $Br^-$ or formate, both powerful scavengers of hydroxyl radical. This is consistent with a mechanism that proceeds through heterolytic cleavage of the O−O bond to produce a Cmpd I analogue and $H_2O$. However, there is a competitive pathway in which $H_2O_2$ irreversibly degrades the porphyrin, as previously reported.[5,17] The Cmpd 0 and Cmpd I analogues of MP8 have been characterized by rapid-scan UV−vis spectrophotometry.[18]

Under conditions where formation of Cmpd I is rate limiting,[5] the rate of oxidation of *o*-methoxyphenol (guaiacol, Gc) catalyzed by MP8 increases with pH between 6 and 9 which, it was suggested, is evidence that Fe(III) reacts with $HO_2^-$,[5,17] but no explanation was advanced for the apparent independence of the rate on pH below 6. A more comprehensive investigation of the peroxidase activity of MP8 has

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(1) Welinder, K. G. *Curr. Opin. Struct. Biol.* **1992**, *2*, 388.
(2) Dunford, H. B. *Xenobiotica* **1995**, *25*, 725.
(3) Veitch, N. C. *Phytochemistry* **2004**, *65*, 249.
(4) Gajhede, M. In *Handbook of Metalloproteins*; Messerschmidt, A., Huber, R., Poulos, T. L., Wieghardt, K., Eds.; John Wiley and Sons: Chichester, 2001; Vol. 1, p 195.
(5) Baldwin, D. A.; Marques, H. M.; Pratt, J. M. *J. Inorg. Biochem.* **1987**, *30*, 203.
(6) Poulos, T. L.; Kraut, J. *J. Biol. Chem.* **1980**, *255*, 10322.
(7) Adams, P. A.; Baldwin, D. A.; Marques, H. M. In *Cytochrome c: A Multidisciplinary Approach*; Scott, R. A., Mauk, A. G., Eds.; University Science: Mill Valley, CA, 1996; p 635.
(8) Traylor, T. G.; Xu, F. *J. Am. Chem. Soc.* **1990**, *112*, 178.
(9) Adams, P. A.; Goold, R. D. *J. Chem. Soc., Chem. Commun.* **1990**, 97.
(10) Cunningham, I.; Bachelor, J.; Pratt, J. *J. Chem. Soc., Perkin Trans. 2* **1991**, 1839.
(11) Adams, P. A.; Milton, R. C.; Silver, J. *BioMetals* **1994**, *7*, 217.
(12) Spee, J. H.; Boersma, M. G.; Veeger, C.; Samyn, B.; van Beeumen, J.; Warmerdam, G.; Canters, G. W.; van Dongen, W. M.; Rietjens, I. M. *Eur. J. Biochem.* **1996**, *241*, 215.
(13) Osman, A. M.; Boeren, S.; Boersma, M. G.; Veeger, C.; Rietjens, I. M. *Proc. Natl. Acad. Sci., U.S.A.* **1997**, *94*, 4295.
(14) Osman, A. M.; Boeren, S.; Veeger, C.; Rietjens, I. M. *Chem. Biol. Interact.* **1997**, *104*, 147.
(15) Adams, P. A.; Goold, R. D. *J. Chem. Soc., Chem. Commun.* **1990**, 97.
(16) Adams, P. A. *J. Chem. Soc., Perkin Trans. 2* **1990**, 1407.
(17) Baldwin, D. A.; Marques, H. M.; Pratt, J. M. *FEBS Lett.* **1985**, *183*, 309.
(18) Primus, J. L.; Grunewald, S.; Hagedoorn, P.-L.; Albrecht-Gary, A.-M.; Mandon, D.; Veeger, C. *J. Am. Chem. Soc.* **2002**, *124*, 1214.



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been reported more recently.[19] Its dependence on pH presents as a bell-shaped curve, skewed to high pH, with an apparent maximum at pH 10. There are two apparent p$K_a$'s at 8.9 and 10.6, obtained by curve fitting; no attempt was made to suggest a plausible mechanism that would account for the pH dependence.

A problem with MP8 is its propensity to aggregate in aqueous solution, not only by $\pi-\pi$ aggregation of the porphyrins, but also by intermolecular coordination of Fe(III) by the N-terminal amino group of the peptide.[20,21] The interpretation of kinetic data may be problematic because of these aggregation effects.

Far better characterized is the solution behavior of *N*-Ac-MP8 where the terminal amino group of MP8 is protected by acetylation.[21] The species is monomeric up to 30 $\mu$M in aqueous solution at an ionic strength of 0.1 M. Increasing ionic strength promotes aggregation.[21]

We have now undertaken a study of the peroxidase activity of *N*-Ac-MP8 using the oxidation of guaiacol by $H_2O_2$ to produce a tetraguaiacol as assay[22] under conditions ([Gc] = 15 mM) where formation of Cmpd I is rate limiting.[5] Because the porphyrin is irreversibly oxidized by $H_2O_2$,[5,17] some curvature in the absorbance vs time trace at 470 nm occurs after some time; the rate of the reaction was therefore determined from the initial slope of the absorbance−time trace obtained with a HiTech SF51 stopped-flow spectrophotometer on mixing equal volumes of a solution containing *N*-Ac-MP8 (0.1−1.0 $\mu$M), buffer (TRIS/HCl, phosphate or CAPS), Gc, and ionic strength adjustor ($NaClO_4$, $\mu$ = 0.1 M) with one containing $H_2O_2$ only (to preclude decomposition at high pH[23]) prepared by dilution of a freshly prepared stock solution which was standardized spectrophotometrically ($\epsilon_{240}$ = 39.4 $M^{-1}$).[24] Under these conditions, rate = 4(d*A*/d*t*)/$\epsilon_{470}$, and it is known that $\epsilon_{470} = 26.6 \times 10^3$ $M^{-1}$ $cm^{-1}$.[22] The observed second-order rate constant, $k^{II}_{obs}$, was determined by dividing the slope of a plot of rate vs [$H_2O_2$] (which was varied by at least 10-fold) by [*N*-Ac-MP8].

$k^{II}_{obs}$ (Table S1 of the Supplementary Information) varies with pH as shown in Figure 1. The results are in reasonable agreement with those reported by Yeh et al.[19] To explain the pH profile of $k^{II}_{obs}$, we took the following into consideration. Below pH 7, $k^{II}_{obs}$ is virtually independent of pH. It is known that the axial $H_2O$ ligand in aqua-*N*-AcMP8 (abbreviated as His−Fe−$H_2O$), which must be displaced by either $H_2O_2$ (or $HO_2^-$) in the first step of the reaction, ionizes with p$K_{Fe}$ = 9.59[21] to form the kinetically inert[25,26] hydroxo complex, His−Fe−$OH^-$. At high pH, the proximal His ligand of *N*-Ac-MP8 ionizes with p$K_{His}$ = 12.71[21] to form a histidinate, $His^-$−Fe−$OH^-$ (Scheme 1); this, by contrast, is kinetically labile.[25,26]

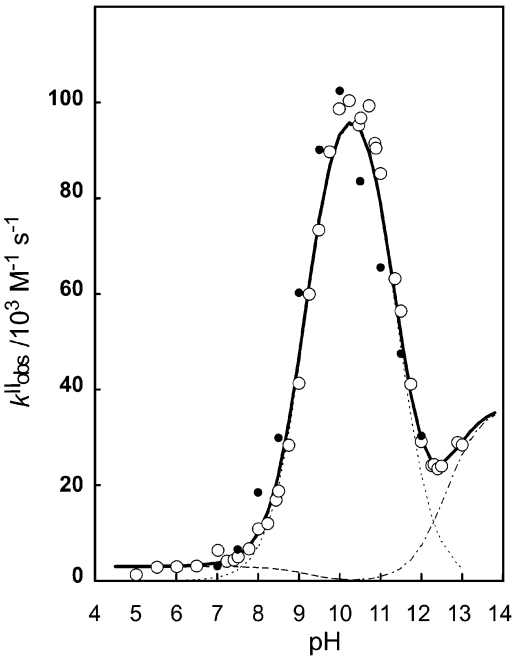
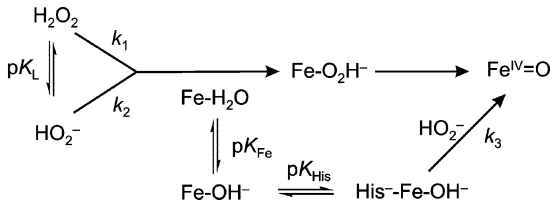



**Figure 1.** Dependence of the second-order rate constant for formation of Cmpd I on reaction of $H_2O_2$ during the oxidation of guaiacol catalyzed by *N*-Ac-MP8. Solid circles from ref 19. The mechanism proposed is shown in Scheme 1. The $k_1$ (− − −) and $k_2$ (···) pathways are for the reaction of the aqua-hemepeptide with $H_2O_2$ and $HO_2^-$, respectively; the $k_3$ pathway (− · − ·) is for the reaction of $HO_2^-$ with the hydroxo-histidinate hemepeptide.

**Scheme 1.** Mechanism Proposed to Explain the pH Dependence of the Second-Order Rate Constant for Formation of Cmpd I (Figure 1)




Both $H_2O_2$ and $HO_2^-$ react with His−Fe−$H_2O$ to form a Cmpd 0 analogue, His−Fe−$O_2H^-$ or His−Fe−$O_2H_2$ with rate constants, respectively, $k_1$ and $k_2$, but His-Fe−$OH^-$ is assumed to be kinetically inert. If $k_1 < k_2$ ($H_2O_2$ is a poorer nucleophile than $HO_2^-$ toward Fe(III)) at pH $\ll$ p$K_{H_2O_2}$ and pH $\ll$ p$K_{Fe}$, $k^{II}_{obs} = k_1$ and is independent of pH. As pH increases, $k^{II}_{obs}$ should increase and reach a maximum value = $k_2$. However, because His-Fe−$OH^-$ is inert, as we showed to be the case for the reaction of *N*-Ac-MP8 with cysteine[25] and with cyanide,[26] $k^{II}_{obs}$ should decrease as pH increases above p$K_{Fe}$. As the operating pH approaches p$K_{His}$, $k^{II}_{obs}$ should again increase as labile $His^-$−Fe−$OH^-$ is formed and the $k_3$ pathway becomes important. As shown in Figure 1, this is indeed the observed dependence of $k^{II}_{obs}$ on pH.

It is readily shown that the macroscopic rate constant $k^{II}_{obs}$ is related to the microscopic rate constants $k_i$, $i$ = 1, 2, or 3, and the various acid dissociation constants by eq 1. The experimental data were fitted, using

(19) Yeh, H.-C.; Wang, J.-S.; Su, Y. O.; Lin, W.-Y. *J. Biol. Inorg. Chem.* **2001**, *6*, 770.
(20) Aron, J.; Baldwin, D. A.; Marques, H. M.; Pratt, J. M.; Adams, P. A. *J. Inorg. Biochem.* **1986**, *27*, 227.
(21) Munro, O. Q.; Marques, H. M. *Inorg. Chem.* **1996**, *35*, 3752.
(22) Chance, B.; Maehly, A. C. *Methods Enzymol.* **1965**, *2*, 764.
(23) Cotton, F. A.; Wilkinson, G. *Advanced Inorganic Chemistry*; John Wiley and Sons: New York, 1988.
(24) Nelson, D. P.; Kiesow, L. A. *Anal. Biochem.* **1972**, *39*, 474.
(25) Marques, H. M.; Baldwin, D. A.; Pratt, J. M. *J. Inorg. Biochem.* **1987**, *29*, 77.
(26) Marques, H. M.; Rousseau, A. *Inorg. Chim. Acta* **1996**, *248*, 115.

$$k_{obs}^{II} = k_1/(1 + 10^{pH-pK_{H2O2}})(1 + 10^{pH-pK_{Fe}}) + k_2/(1 + 10^{pK_{H2O2}-pH})(1 + 10^{pH-pK_{Fe}}) + k_3/(1 + 10^{pK_{His}-pH}) \quad (1)$$

standard nonlinear least-squares methods, to eq 1 as objective function and $k_i$, $pK_{Fe}$, $pK_{H_2O_2}$, and $pK_{His}$ as variables. The solid line in Figure 1 is the best fit to the data; the contributions of the three pathways to $k_{obs}^{II}$ are shown as broken lines.

We found $k_1 = 3 \pm 1 \times 10^3$ $M^{-1}$ $s^{-1}$, $k_2 = 1.9 \pm 0.4 \times 10^7$ $M^{-1}$ $s^{-1}$, and $k_3 = 4 \pm 1 \times 10^4$ $M^{-1}$ $s^{-1}$, while $pK_{Fe} = 9.2 \pm 0.1$, $pK_{H_2O_2} = 11.38 \pm 0.06$, and $pK_{His} = 12.6 \pm 0.4$. These kinetically determined acid dissociation constants are in reasonable agreement with values of 9.59,[21] 11.8,[27] and 12.71,[21] respectively, determined previously either spectrophotometrically or potentiometrically.

The temperature dependence of $k_1$ and $k_2$ were determined at five temperatures at pH 6.0 (10−35 °C), where only the $k_1$ pathway is important, and at pH 10.0 (5−25 °C), where only the $k_2$ pathway is important (Table S2). A plot of $\ln(k_i h/k_B T)$, where $i = 1$ or 2, $h$ is the Planck constant, and $k_B$ is the Boltzman constant, against $T^{-1}$ gave good straight lines from which $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ could be determined from the slope and intercept, respectively (Figure S1). The values of $k_1$ are independent of pH at pH 6.0; those of $k_2$ were corrected for $pK_{Fe}$ and $pK_{H_2O_2}$ to give values that are independent of pH and refer specifically to the reaction of $HO_2^-$ with N−Fe−$H_2O$. We find $\Delta H^{\ddagger} = 35.7 \pm 1.0$ and $37.3 \pm 1.4$ kJ $mol^{-1}$ for $k_1$ and $k_2$, respectively, while the values of $\Delta S^{\ddagger}$ are $-59 \pm 3$ and $18 \pm 5$ J $K^{-1}$ $mol^{-1}$, respectively. Thus, the enthalpies of activation for reaction of $H_2O_2$ and $HO_2^-$ with N−Fe−$H_2O$ are similar (and not dissimilar to the values for the reaction of the hemepeptide with $CN^-$ anion, 39 kJ $mol^{-1}$,[28] or the zwitterionic (thiol) form of cysteine,[26] 34.6 kJ $mol^{-1}$) while the entropy of activation is negative for the reaction of the hemepeptide with neutral $H_2O_2$ but positive for its reaction with anionic $HO_2^-$. The values of $\Delta S^{\ddagger}$ for reaction with $CN^-$ and cysteine are 92 and −16.4 J $K^{-1}$ $mol^{-1}$, respectively.[26,28]

There are small but systematic differences in $\Delta H^{\ddagger}$ values and very significant differences in $\Delta S^{\ddagger}$ values. Values of $\Delta H^{\ddagger}$ are smaller for the neutral ligands $H_2O_2$ and cysteine than for the anionic ligands $HO_2^-$ and $CN^-$, while conversely, $\Delta S^{\ddagger}$ values are larger, and there appears to be a compensation effect between the two parameters (Figure S3). If the rate-determining step was dissociation of $H_2O$, then $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values should be independent of the identity of the incoming ligand, L. There therefore appears to be nucleophilic participation of L in the transition state.

We have noted such compensation effects in the ligand-substitution reactions at Co(III) in aquacobalamin where there is definitive evidence for a dissociative interchange mechanism.[29−31] We have interpreted this to mean that, as the extent of nucleophilic participation of L in the transition state increases, $\Delta H^{\ddagger}$ decreases as bond making between L and the metal compensates for bond breaking, but this is offset by a decrease in $\Delta S^{\ddagger}$ because of loss of freedom of L. Such behavior is characteristic of a weak interaction in solution.[32] However, it must be emphasized that this interpretation is tentative and further data with a wider variety of ligands are required before a more definitive conclusion can be reached.

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**Supporting Information Available:** Primary kinetic data, temperature dependence of rate constants, Eyring plots, and plot of the compensation effect between $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$. This material is available free of charge via the Internet at http://pubs.acs.org.

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(27) Smith, R. M.; Martell, A. E. *Critical Stability Constants*; Plenum: New York, 1976; Vol. 1.
(28) Smith, M. C.; McLendon, G. *J. Am. Chem. Soc.* **1980**, *102*, 5666.
(29) Marques, H. M.; Bradley, J. C.; Campbell, L. A. *J. Chem. Soc., Dalton Trans.* **1992**, 2019.
(30) Meier, M.; van Eldik, R. *Inorg. Chem.* **1993**, *32*, 2635.
(31) Marques, H. M.; Knapton, L.; Zou, X.; Brown, K. L. *J. Chem. Soc., Dalton Trans.* **2002**, 3195.
(32) Searl, M. S.; Westwell, M. S.; Williams, D. H. *J. Chem. Soc., Perkin Trans. 2* **1995**, *2*, 141.