# Hypervalent Iron-oxo Porphyrin Cation Radical Formation on Reaction of $H_2O_2$ with the Cytochrome-c-derived Haem Octapeptide Microperoxidase-8 (MP-8) in Aqueous Solution

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The reaction between microperoxidase-8 (MP-8) and $H_2O_2$ has been investigated in aqueous buffer solution using the UV-vis spectrophotometric probe 2,2′-azino-bis(3-ethylbenthiazoline-6-sulphonic acid) (ABTS); evidence is presented for the intermediate formation of a hypervalent iron-oxo radical species analogous to compound I of the peroxidase enzymes.

The reaction between $H_2O_2$ and the haem-peptides derived from cytochrome-c, the microperoxidases (MP), has been little studied, despite the potential of such systems as chemical models for the peroxidase enzymes. Reaction of the undecapeptide (MP-11) with $H_2O_2$ exhibited extremely complex kinetics, with concomitant and complete oxidative degradation of the porphyrin macrocycle;[1] this presumably occurred by attack of $H_2O_2$ directly on MP-11, or on the iron-oxo complex presumed to be formed in the reaction.

A comprehensive investigation of the aqueous/aqueous-organic solution chemistry (including aspects of the peroxidasic reaction) of the octapeptide (MP-8) has recently been carried out by Marques and co-workers.[2,3] They have demonstrated firstly, that MP-8 is >90% monomeric in aqueous solution at catalytic concentration levels ($5 \times 10^{-7}$ mol dm$^{-3}$), and secondly, that the fifth co-ordination position of the iron is occupied by the imidazole of His 18 (cytochrome-c sequence numbering), modelling the proximal histidine of the peroxidases. In the work reported here, we utilize the approach of Traylor *et al.*[4] and Bruice *et al.*[5] (whereby reactive oxo-radical intermediates are trapped as stable radical species) to demonstrate formation of a hypervalent iron-oxo complex, a peroxidase compound (cpd) I analogue, on reaction of $H_2O_2$ with MP-8.

When $H_2O_2$ ($0.02—3 \times 10^{-4}$ mol dm$^{-3}$) and MP-8 ($10^{-7}—10^{-6}$ mol dm$^{-3}$) are mixed at pH 7.00 in the presence of 2,2′-azino-bis(3-ethylbenthiazoline)-6-sulphonic acid (ABTS) ($0.1—5 \times 10^{-3}$ mol dm$^{-3}$) the emerald green $ABTS^{+\cdot}$ cation radical is formed. The kinetics of $ABTS^{+\cdot}$ formation were monitored at 660 nm and exhibited the following characteristics:

(a) In the $H_2O_2$ concentration range $2 \times 10^{-6}$ to $3 \times 10^{-4}$ mol dm$^{-3}$, $ABTS^{+\cdot}$ formation follows a pseudo-first order rate law (Figure 1).

(b) As $[H_2O_2]$ is varied from $3 \times 10^{-4}$ to $2 \times 10^{-6}$ mol dm$^{-3}$, $ABTS^{+\cdot}$ formation increases from 30% theoretical [calculated using absorbance coefficient (Abs) $ABTS^{+\cdot}$ = 14 000 mol$^{-1}$ dm$^3$ cm$^{-1}$ at 660 nm][6,7] and approaches 100% theoretical.

(c) Under conditions of constant [MP-8], variable $[H_2O_2]$ and *vice versa*, the initial velocity of $ABTS^{+\cdot}$ formation, $V_i$, varied in an accurately straight-line manner with $[H_2O_2]$ {or [MP-8]}. This implies a rate law of the form, $d(Abs)/dt = k[\text{MP-8}][H_2O_2]$; $k$ evaluated from the slope of the $V_i$ *vs.* $[H_2O_2]$ or [MP-8] plots was determined to be 1 700 (±30) mol$^{-1}$ dm$^3$ s$^{-1}$ and 1 720 (±50) mol$^{-1}$ dm$^3$ s$^{-1}$, respectively.

(d) The pseudo-first order rate constant for $ABTS^{+\cdot}$ formation at $[H_2O_2] = 1 \times 10^{-4}$ mol dm$^{-3}$ was directly

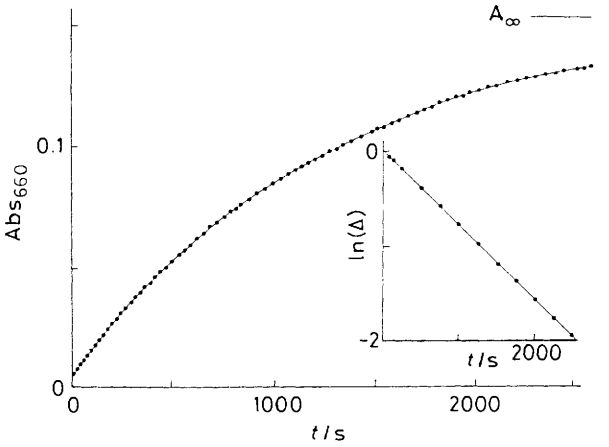


**Figure 1.** The kinetics of $ABTS^{+\cdot}$ formation at $[H_2O_2] = 1.0 \times 10^{-5}$ mol $dm^{-3}$. Increase in absorbance at 660 nm is shown, inset is the first-order kinetic plot of ln ($\Delta$) *vs.* time. Pseudo-first-order rate constant = 7.70 ($\pm$0.01) $\times 10^{-4}$ $s^{-1}$, pH = 7.00, $T$ = 25 °C $\pm$0.2 °C, [ABTS] = $3 \times 10^{-3}$ mol $dm^{-3}$, [MP-8] = $2.5 \times 10^{-7}$ mol $dm^{-3}$. $\Delta = (A_\infty - A_t)/A_\infty$; $A_\infty$ corrected for Abs at $t = 0$ s.

proportional to [MP-8] in the concentration range 0—8 $\times 10^{-7}$, *i.e.* doubling [MP-8] doubles $k_{obs}$.

(e) Addition of 0.1 mol $dm^{-3}$ bromide ion to the system at $[H_2O_2] = 1 \times 10^{-4}$ mol $dm^{-3}$ did not affect the kinetics of $ABTS^{+\cdot}$ formation. In particular the efficiency of $ABTS^{+\cdot}$ formation was not significantly affected {efficiency 50.7% at $[Br^+] = 0$; to 52.0% at $[Br^-] = 0.1$ mol $dm^{-3}$}. Formate ion, a powerful scavenger of the hydroxyl radical, also had no effect on the kinetics of the reaction at formate concentrations of 0.05 and 0.10 mol $dm^{-3}$.

(f) The pseudo-first-order rate constant for $ABTS^{+\cdot}$ formation was found to be independent of [ABTS] in the range 2—5 $\times 10^{-3}$ mol $dm^{-3}$, with constant [MP-8]: $2.5 \times 10^{-7}$ mol $dm^{-3}$ and $[H_2O_2]$: $2 \times 10^{-6}$ mol $dm^{-3}$.

These observations are consistent with the following. (i) The rate determining step is the reaction of $Fe^{3+}$MP-8 with $H_2O_2$ [from (c)], the ABTS being oxidised to $ABTS^{+\cdot}$ in a very rapid reaction, subsequent to the rate determining step [from (f)]. (ii) $Fe^{3+}$MP-8 is not saturated with $H_2O_2$ [from (a)]. (iii) The $Fe^{3+}$MP-8 is oxidised degradatively by $H_2O_2$ in a parallel non-catalytic reaction [from (b)].

Bruice *et al.*[5] have noted that O–O bond cleavage in the $Fe^{3+}$ (porphyrin)·($H_2O_2$) complex can proceed either by homolysis giving an oxo-iron(IV) porphyrin and a hydroxyl radical ($OH^\cdot$); or by heterolysis with formation of an oxo-iron(IV) porphyrin cation radical plus $H_2O$. The $OH^\cdot$ radical oxidises ABTS to $ABTS^{+\cdot}$ with 58% efficiency.[7] Thus, the argument in favour of a heterolytic O–O bond cleavage is supported by our observation (e), that carrying out the reaction in the presence of 0.1 mol $dm^{-3}$ bromide ion {at $[H_2O_2]$ which gives ~50% efficiency of $ABTS^+$ formation ($1 \times 10^{-4}$ mol $dm^{-3}$)} does not lead to increased efficiency of $ABTS^{+\cdot}$ formation. Rush and Koppenol[8] have argued that since the bromide ion in the presence of $OH^\cdot$ radicals forms the bromine radical ($Br^{\cdot -}$), and this species oxidises ABTS with an efficiency approaching 100%, the lack of effect of bromide ion in model systems is evidence against $OH^\cdot$ radical participation. Additionally, the absence of formate ion effect on the reaction kinetics provides strong supporting evidence against a reaction pathway involving significant $OH^\cdot$ involvement.[8] Our experimental observations thus strongly support heterolytic cleavage of the O–O bond in the obligatory $Fe^{3+}$MP-8 ($H_2O_2/HO_2^-$) complex to give an oxo-iron porphyrin $\pi$ cation radical, a direct chemical model for peroxidase cpd I.

The product of reaction between MP-8 ($1 \times 10^{-6}$ mol $dm^{-3}$) and stoicheiometric amounts of $H_2O_2$ in the absence of ABTS also exhibits spectral changes consistent with the formation of a peroxidase cpd I analogue, $\lambda_{max.(Soret)}$ showing a small, but significant, change from 396.6 nm (MP-8) to 395.2 nm (product) (*cf.* horseradish peroxidase: 406 nm, horseradish peroxidase cpd I: 405 nm),[9] while the intensity of the Soret peak decreases to about 60% of the value for MP-8. Changes of the latter magnitude have also been found on reaction of stoicheiometric amounts of $H_2O_2$ with monomeric deuteroferrihaem, and ascribed similarly to a peroxidatic intermediate which is preceded by formation of a Michaelian complex [designated $Fe^{3+}(H_2O_2/HO_2^-)$ MP-8 in this study].[10,11] Addition of higher concentrations of $H_2O_2$ result in increasingly rapid and irreversible oxidative haem destruction.

The system reported here provides a linkage between the non-aqueous and aqueous model system studies of Traylor[4] and Bruice,[5] respectively, in that the proximal histidine effect utilized in the former work is combined with the aqueous phase non-aggregation properties of the catalyst in the latter system. This allows the mechanism of peroxidase cpd I formation to be studied in aqueous solution with a structurally relevant catalyst possessing an axial (proximal) histidine. A further point of importance is that since a series of discrete microperoxidases can be prepared from MP-6 up to cytochrome-c itself, the system provides a means whereby the relative effect of protein and solvent on the reaction kinetics can be studied from essentially 'naked' active site (MP-6) to fully enfolded active site (cytochrome-c).

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