Peroxidase Activities of Ferrihaems: Kinetics of the Oxidation of Iodide by Peroxidatically Active Compounds formed by Reaction of Deuteroferrihaem with Hydrogen Peroxide, t-Butyl Hydroperoxide, and Peroxybenzoic Acids

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Deuteroferrihaem peroxide compounds (dpc) have been formed by reaction of deuteroferrihaem with H_2O_2, Bu^tOOH, and 10 peroxybenzoic acids, and the kinetics of oxidation of iodide by these species have been studied by stopped-flow spectrophotometry at 25 °C and I = 0.1 mol dm^{-3}. Under conditions where the reaction is first order with respect to both [dpc] and $[I^-]$, the second-order rate constant k = (1.6 ± 0.3) × 10^2 dm^3 mol^{-1} s^{-1} for all the hydroperoxides used at pH > 7.75. This result supports the concept that all the deuteroferrihaem peroxide compounds are the same oxidized form of deuterohaem, independent of the nature of the oxidant progenitor. The rate constant k is independent of pH in the range 7.75—10.0 but shows about a three-fold increase as the pH decreases in the range 7.75—6.5. In contrast, the rate constants for the oxidation of iodide by Compounds I and II of the ferrihaem hydroperoxidase enzyme, Horse Radish Peroxidase (E.C. 1.11.1.7), are directly proportional to $[H^+]$ over a wide range. Deuteroferrihaem peroxide compound is a much more effective oxidant (towards I^-) than Compound II over the whole pH range studied and has equal reactivity to Compound I at pH 8.5.

THE ability to catalyse the oxidation of iodide by hydrogen peroxide is ubiquitous among the ferrihaem peroxidase enzymes of sub-group E.C. 1.11. Extensive kinetic studies have been made of the redox reactions between iodide and pre-formed, peroxidatically active, intermediates (Compound I and Compound II) from Horse Radish Peroxidase, hrp (E.C. 1.11.1.7). Dunford and Stillman [1] recently reviewed the evidence in favour of the concept that iodide is oxidized by hrp Compound I [a formal iron(v) species] in a two-electron equivalent redox reaction. The mechanism of iodide oxidation by hrp Compound II [a formal iron(iv) species] is less clear. Dunford and his co-workers [2,3] determined the pH profiles of both reactions in an attempt to elucidate the role played by the protein in the enzymatic processes. The intermediates may be formed using hydroperoxides other than H_2O_2, and it has also been established that the rates of reaction of hrp Compound I and Compound II with reductants are not affected by changes in the oxidizing substrate used in their preparation. These results form part of the evidence that these species are oxidized forms of the enzyme and not conventional enzyme–substrate compounds.[1]

The present paper describes studies of the kinetics of oxidation of iodide by peroxidatically active compounds, formed by the oxidation of a protein-free ferrihaem complex with a variety of hydroperoxides. The objective of these ' model ' system studies was to provide information for comparison with the enzymatic processes. Similar studies have yielded interesting relations between the catalatic activities of the catalase enzymes (E.C. 1.11.1.6) and the ferrihaems.[4,5] Deuteroferrihaem,* dfh, has a number of advantages for this type of study compared with the native prosthetic group, protoferrihaem (pfh). It does not contain the oxidation-susceptible vinyl groups which complicate [6] studies of pfh and is much less dimerized in aqueous solution.[7] The latter characteristic has been shown to be a major determinant of both the steady-state catalatic and peroxidatic aspects of the hydroperoxidase activities of ferrihaems, in which ferrihaem monomers are the predominant catalytic species.[5,8] Recent work using a series of ferrihaems has shown that changes in the porphyrin substituents produce only minor effects on the reactivity of ferrihaem monomers.[8,9] Portsmouth and Beal [10] demonstrated the formation of a peroxidatically active compound, dpc, from dfh and H_2O_2, by means of the stopped-flow spectrophotometric techniques used much earlier to characterize the enzymatic intermediates, and surveyed the reactivity of pre-formed dpc towards a range of reducing substrates, including iodide. These workers considered that dpc is an analogue of peroxidase Compound I, but recent stoicheiometric studies [11] have shown that the situation is more complex. Although the formation of dpc species using peroxyacids yields dpc spectra which are independent of the peroxyacid used as oxidant, the reaction stoicheiometry corresponds to reaction of 1 mol of peroxyacid with 1.9 ± 0.2 mol of dfh. Furthermore, the absorption spectrum of dpc is dependent on [dpc] in a manner which suggests that the intermediate may involve both monomeric and dimeric haem components. The absorption spectrum of dpc, formed using H_2O_2,[10] is qualitatively similar to, but quantitatively different from, that obtained using per-

* Chloro[3,7,12,17-tetramethyl-21*H*,23*H*-porphine-2,18-dipropanoato(2−)-N^{21},N^{22},N^{23},N^{24}]iron(III).

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oxyacids,[11] and titration studies with reductants indicate that, with H_2O_2, dpc is not completely formed. The situation closely resembles that with the catalase enzymes, where conversion into Compound I, using H_2O_2, is limited because H_2O_2 is also an effective reducing substrate for these enzymes.[12] Since dfh shows catalase activity,[5] the hypothesis that dpc may fulfil a role analogous to catalase Compound I is attractive. An important objective of the present work was to examine the reactivities towards a common reducing substrate (iodide) of dpc species pre-formed by oxidation of dfh with H_2O_2 and a wide range of hydroperoxides.

EXPERIMENTAL

Materials.—Chlorodeuteroferrihaem was prepared by the resorcinol melt method [13] from chromatographically pure chloroprotoferrihaem (Haemin Chloride, Fluka) and the preparation of solutions followed the previously described procedures.[4,14] The hydroperoxides used were supplied by Laporte Industries Ltd. Hydrogen peroxide was obtained as a 35% w/w unstabilized aqueous solution; the concentrations of more dilute stock solutions prepared from this material were determined iodimetrically. t-Butyl hydroperoxide was obtained as an 18% w/v aqueous alkaline solution (free from di-t-butyl peroxide); the concentrations of stock solutions prepared by dilution of this material were determined spectrophotometrically [15] using $\epsilon = 6.85$ dm^3 mol^{-1} cm^{-1} at 270 nm for Bu^{t}OOH. Peroxybenzoic acids were obtained as materials containing *ca.* 85% peroxyacid, with the residue as the respective parent carboxylic acid which greatly reduces the impact sensitivity. The concentrations of peroxyacid stock solutions were determined iodimetrically using the procedures described by Swern.[16] Potassium iodide was AnalaR grade material, as were $Na_2[HPO_4]$ and $K[H_2PO_4]$ (used for buffer solutions in the range pH 6.5—8.05) and $Na_2[CO_3]$ and $Na[HCO_3]$ (used for buffer solutions in the range pH 8.60—10.0). Except where otherwise indicated, the final reaction solutions had a total ionic strength of $I = 0.1$ mol dm^{-3}, which was maintained by addition of sodium chloride or sodium nitrate.

Kinetic Studies.—The kinetic measurements were made at 25 °C using a Durrum–Gibson D-110 stopped-flow spectrophotometer. A solution of KI was first introduced into one drive syringe of the instrument. The other drive syringe was then filled as rapidly as possible with a solution prepared by mixing solutions, at 25 °C, of dfh and hydroperoxide of appropriate concentration. For H_2O_2 and Bu^{t}OOH this procedure was relatively leisurely since the time required for maximal formation of dpc was 1—5 min (depending on concentration and pH). With peroxybenzoic acids (except monoperoxyphthalic acid) the formation of dpc is very rapid.[17] In the absence of deliberately added reductant, a ' spontaneous ' decay of dpc occurs, with the reformation of dfh. Although this decay is exponential under a particular set of conditions, the process is kinetically complex (I. Wilson, unpublished work). However, the decay process is sufficiently slow under the conditions employed (rate constants in the range 10^{-3}—10^{-2} s^{-1}) that it does not affect the measurements except that, in the case of peroxybenzoic acids, significant decay of dpc occurs during the introduction of the solution into the drive syringe of the stopped-flow instrument, with a consequent decrease in the signal amplitude during the subsequent stopped-flow experiment. Under the reaction conditions employed the irreversible oxidation of the ferrihaem porphyrin was $<5\%$.

After admixture of the iodide and dpc solutions in the stopped-flow spectrophotometer the reaction was followed by measuring the absorption increase either at 384 nm (the Soret band maximum for dfh) or at the pH-dependendent [18] isosbestic wavelength for the dfh monomer–dimer system. In all the experiments, iodide was present in considerable excess; total [haem] was in the range 2×10^{-6}—20×10^{-6} mol dm^{-3} and [I$^-$] was usually 2.5×10^{-3}—2.5×10^{-2} mol dm^{-3}. Under these conditions the reactions obeyed first-order kinetics and values of the pseudo-first-order rate constants, $k_{obs.}$, were determined from photographs of the stopped-flow oscillographic traces.

RESULTS AND DISCUSSION

Figure 1 illustrates the linear dependence of $k_{obs.}$ on [I$^-$] observed at different pH, for measurements at

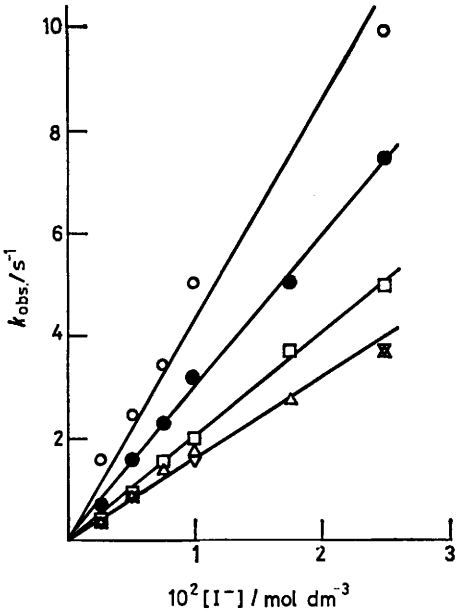

FIGURE 1 Dependence of $k_{obs.}$ on [I$^-$] at pH 6.5 (○), 6.8 (●), 7.4 (□), 7.75 (△), and 10.0 (▽). Measurements were made at 384 nm, 25 °C, $I = 0.1$ mol dm^{-3}, initial $[H_2O_2]$: [dfh] = 1 : 1, and total [haem] = 5×10^{-6} mol dm^{-3}

384 nm with total [haem] = 5×10^{-6} mol dm^{-3} and initial $[H_2O_2]$: [dfh] = 1 : 1. The second-order rate constant, k, obtained from the results shown in Figure 1 and those obtained under similar conditions at other pH values (Table 1, column 1), is independent of pH at pH > 7.75 but increases about three-fold as the pH decreases from 7.75 to 6.5. In Figure 2 the upper curve

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demonstrates that the values of k are insensitive to variation in total [haem] in the range 2.5×10^{-6}—22.5×10^{-6} mol dm^{-3}; the lower curve illustrates a similar

TABLE 1

Values of $10^{-2}\,k$(dm^3 mol^{-1} s^{-1}) for the oxidation of iodide by deuteroferrihaem peroxide compounds (dpc) at 25 °C and $I = 0.1$ mol dm^{-3}. dpc Solutions were prepared by reaction of equimolar deuteroferrihaem and hydroperoxide oxidant. The final total [haem] was 5×10^{-6} mol dm^{-3} in all the experiments

		Oxidant			
		H_2O_2		Bu^{t}OOH	mppa [a]
Buffer	pH	348 nm	I [b]	384 nm	384 nm
Phosphate	6.50	4.3			
	6.80	3.0	2.7		
	7.00	2.7	2.6	2.2	2.1
	7.40	2.1	2.1	1.5	1.8
	7.75	1.6	1.6		
	8.05	1.6	1.5	1.3	1.5
Carbonate	8.60	1.7	1.7	1.7	1.6
	9.00	1.8	1.6	1.6	1.6
	9.45	1.6	1.9	1.4	1.8
	10.00	1.6	1.8	1.9	1.6

[a] Monoperoxyphthalic acid. [b] The pH-dependent isosbestic wavelength for the deuteroferrihaem monomer–dimer system.[18]

insensitivity of k to variation in the initial $[H_2O_2]$: [dfh] ratio from 0.5 to 10 : 1, and that results obtained at 384 nm and at the dfh monomer–dimer isosbestic wavelength (366 nm at pH 8.05) are indistinguishable. A detailed comparison of the results obtained with H_2O_2, Bu^{t}OOH, and monoperoxyphthalic acid over a wide range of pH are presented in Table 1. Within experimental error (standard deviation *ca.* ±15% for each determination of k) the rate constant is independent of the hydroperoxide used in the formation of dpc.

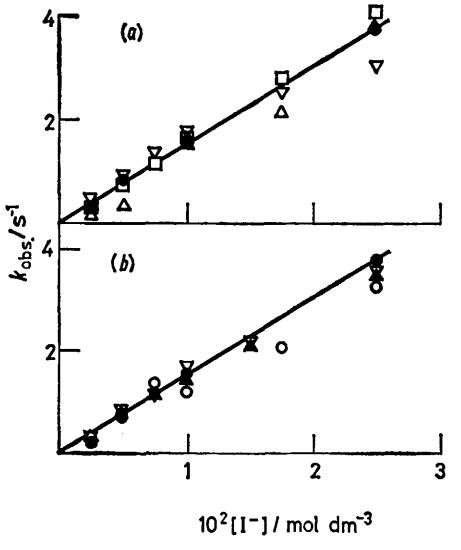

FIGURE 2 (*a*) Dependence of $k_{obs.}$ on [I$^-$] at total [haem] = 2.5×10^{-6} (▽), 5.0×10^{-6} (●), 1.1×10^{-5} (△), and 2.25×10^{-5} mol dm^{-3} (□). Measurements were made at 384 nm and initial $[H_2O_2]$: [dfh] = 1 : 1. (*b*) Dependence of $k_{obs.}$ on [I$^-$] at initial $[H_2O_2]$: [dfh] = 1 : 1 (●), 10 : 1 (○) (total [haem] = 5.0×10^{-6} mol dm^{-3}, measurements at 384 nm), 1 : 1 (▲), and 0.5 : 1 (▽) (total [haem] = 7.0×10^{-6} mol dm^{-3}, measurements at 366 nm)

Table 2 presents the results of a more limited study using nine other peroxybenzoic acids at pH 8.05. There is no significant influence of peroxyacid structure on the kinetics of the reaction and within the set of peroxyacids studied the rate constants are in close agreement with those obtained under similar conditions with H_2O_2 and Bu^{t}OOH (Table 1).

In summary, the reaction is described by an overall second-order rate law (first order with respect to both [dpc] and [I$^-$]) with a rate constant $k = 1.6 \pm 0.3 \times 10^2$ dm^3 mol^{-1} s^{-1}, which is independent of the hydroperoxide used as oxidant and independent of pH at pH > 7.75. No discontinuity is observed in the pH profile of k on passing from phosphate to carbonate buffers. It was not possible to extend the study of the effect of decreasing pH on k because of the insolubility of dfh at lower pH, but the phenomenon is now being examined using more widely soluble ferrihaem species. In one series of experiments the total ionic strength was increased to 0.25 mol dm^{-3} so that an extended range of [I$^-$] could be

TABLE 2

Values of $10^{-2}k$ (dm^3 mol^{-1} s^{-1}) for the oxidation of iodide at pH 8.05 by deuteroferrihaem peroxide compounds, prepared using peroxybenzoic acids as oxidants. Other conditions as in Table 1, except where indicated

	Peroxybenzoic acid substituent					
Substituent position	None	MeO	Cl	NO_2	CO_2^-	SO_3^-
para	1.6	1.7	1.4	1.7		1.3
meta	1.6		1.4 *	1.7		
ortho	1.6		1.4	1.8	1.5	

* [haem] = 7×10^{-6} mol dm^{-3}.

examined. At higher [I$^-$], concave downwards curvature of the plot of $k_{obs.}$ against [I$^-$] was observed and it seems possible that the first-order kinetics with respect to [I$^-$], observed at lower [I$^-$], represent the low-concentration limit of a first-order saturation kinetic dependence of $k_{obs.}$ on [I$^-$].

Overall, the results support the view that dpc is an oxidized form of dfh which is independent of the nature of the hydroperoxide progenitor. Thus dpc fulfils one important criterion for assignment as an analogue of the enzymatic intermediates, although, as pointed out in the introduction, the detailed structure of dpc is not established, in particular with respect to the state of haem aggregation, so that, at this point, it is not obvious whether the properties of dpc should be compared with Compound I or Compound II. Nevertheless a comparison of the ability of dpc to oxidize iodide, with that displayed by both Compound I and Compound II, is not uninteresting, as shown in Figure 3. The most striking feature of Figure 3 is the difference in pH effect between the enzymatic intermediate reactions and that of dpc. Although only a small section of the curve [3] for hrp compound II is shown, the rate constant is accurately proportional to [H$^+$] in the range pH 2.7—9.0. Furthermore, application of the diffusion-controlled limit rule [19] implies that the reactants are I$^-$ (rather than HI) and a

[19] H. B. Dunford, *J. Theor. Biol.*, 1974, **46**, 467.

protonated form of Compound II, which must have p$K \sim 0$. The results [2] for Compound I also show a trend towards a limiting value at lower pH and the reaction

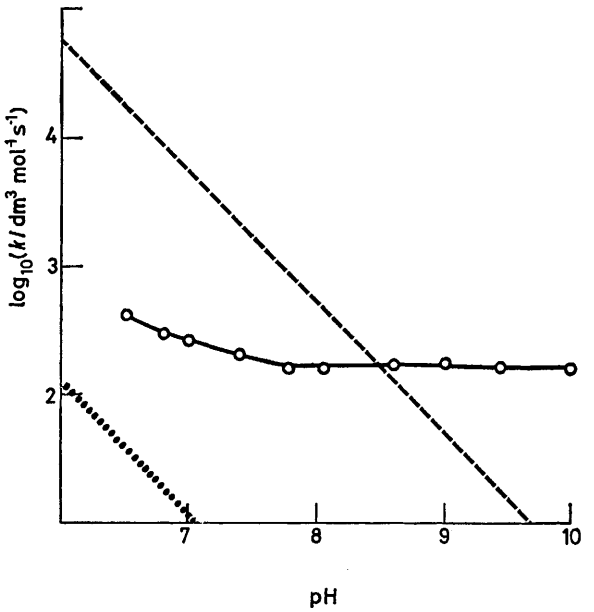

FIGURE 3 Comparison of the pH dependence of the second-order rate constants for iodide oxidation by: (———), Horse Radish Peroxidase Compound I, at 25 °C and $I = 0.11$ mol dm^{-3}, data of ref. 2; (· · · ·), Horse Radish Peroxidase Compound II, at 25 °C and $I = 0.11$ mol dm^{-3}, data of ref. 3; and (○), Deuteroferrihaem Peroxide Compound (dpc), at 25 °C and $I = 0.1$ mol dm^{-3}, data of Table 1 (column 1)

involves a protonated form of the intermediate with p$K \sim 4.6$.

The second interesting feature of Figure 3 is the high level of dpc reactivity; thus dpc is a more effective oxidant (towards I^-) than hrp Compound II over the whole accessible pH range. hrp Compound I and dpc have equal reactivity at pH 8.5, so that Compound I becomes progressively more effective than dpc as the pH is decreased. This behaviour could be biologically significant, perhaps more particularly in relation to peroxidatic coupled iodination reactions [20] and this type of process is now under investigation.

Comparison of Figure 3 with the results obtained in a previous comparison of the steady-state catalatic activities of the catalase enzymes and ferrihaems [5] reveals some features of interest. In the latter case the enzymatic reaction is pH independent and the ferrihaem monomer reaction rate varies with $1/[H^+]$ so that the enzymatic rate constant exceeds that of ferrihaem at all pH $<$ p$K(H_2O_2)$. It is, in this case, the ability of the enzyme to use the protonated form of the substrate, molecular H_2O_2, which is important, since paths involving $[HO_2]^-$ are necessarily restricted to relatively low overall catalytic efficiency.[21] In the case of hrp Compound I, protonation of the protein appears to be a major factor responsible for modifying the oxidizing power towards I^- of the oxidized ferrihaem prosthetic group in the protein environment of the enzyme.

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