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The Kinetics of Formation of Horseradish Peroxidase Compound I by Reaction with Peroxobenzoic Acids

pH AND PEROXO ACID SUBSTITUENT EFFECTS

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1. The kinetics of formation of horseradish peroxidase Compound I were studied by using peroxobenzoic acid and ten substituted peroxobenzoic acids as substrates. Kinetic data for the formation of Compound I with H_2O_2 and for the reaction of deuteroferrihaem with H_2O_2 and peroxobenzoic acids, to form a peroxidatically active intermediate, are included for comparison. 2. The observed second-order rate constants for the formation of Compound I with peroxobenzoic acids decrease with increasing pH, in the range pH 5–10, in contrast with the pH-independence of the reaction with H_2O_2. The results imply that the formation of Compound I involves a reaction between the enzyme and un-ionized hydroperoxide molecules. 3. The maximal rate constants for Compound I formation with unhindered peroxobenzoic acids exceed that for H_2O_2. Peroxobenzoic acids with bulky *ortho* substituents show marked adverse steric effects. The pattern of substituent effects does not agree with expectations for an electrophilic oxidation of the enzyme by peroxoacid molecules in aqueous solution, but is in agreement with that expected for a reaction involving nucleophilic attack by peroxo anions. 4. Possible reaction mechanisms are considered by which the apparent conflict between the pH-effect and substituent-effect data may be resolved. A model in which it is postulated that a negatively charged 'electrostatic gate' controls access of substrate to the active site and may also activate substrate within the active site, provides the most satisfactory explanation for both the present results and data from the literature.

The formation of the catalytic intermediate, Compound I, by reaction of the enzyme with alkyl and acyl hydroperoxide pseudo-substrates has been demonstrated for a number of ferrihaem hydroperoxidases. Schonbaum & Lo (1972) used studies of the reaction of horseradish peroxidase (EC 1.11.1.7) with *m*-nitroperoxobenzoic acid to establish the stoicheiometry of the reaction as:

Horseradish peroxidase + *m*-nitroperoxobenzoic acid → Compound I + *m*-nitrobenzoic acid

Thus horseradish peroxidase Compound I is not merely an enzyme–peroxide adduct but a derivative in which the active site is oxidized. Cotton & Dunford (1973) have shown that the Compound I species formed from a variety of peroxides exhibit identical reactivity.

Hager *et al.* (1973) have studied the reaction of chloroperoxidase (EC 1.11.1.10) with *m*-chloroperoxobenzoic acid. In this system, rapid catalytic dismutation of the peroxo acid occurs, and isotopic-labelling experiments imply that one oxygen atom of its parent peroxide molecule is retained in Compound I.

Jones & Middlemiss (1972, 1974) have studied the formation of the Compound I of catalase (EC 1.11.1.6) by reaction with peroxoacetic acid. For both the ox liver enzyme and the bacterial (*Micrococcus lysodeikticus*) enzyme, the effects of pH imply that the rate is essentially proportional to the concentration of undissociated peroxoacetic acid molecules.

A general reaction scheme consistent with these observations has the form:

$$\underset{\text{(Hydroperoxidase)}}{\text{Fe(+III)}} + \underset{\text{(Hydroperoxide)}}{\text{ROOH}} \rightarrow \underset{\text{(Compound I)}}{\text{Fe(+V)O}} + \text{ROH}$$

that is, the iron(III) centre of the ferrihaem prosthetic group of the enzyme is oxidized in Compound I to an oxy cation in which the formal oxidation state of iron is (+V).

In the present paper, studies are reported of the kinetics of formation of Compound I by using a series of peroxobenzoic acid species. The investigation had two objectives: (i) to examine the role of substrate protonation in the reaction; (ii) to analyse the influence of ring substituents on the reaction rate, with a view to establishing the nature of the redox process. For purposes of comparison, kinetic data are included

for the reaction of the enzyme with H_2O_2 and for the reaction of deuteroferrihaem with peroxides to form a peroxidatically active intermediate (Portsmouth & Beal, 1971; Jones *et al.*, 1974).

Experimental

Materials

Horseradish peroxidase (type VI; batch no. 44C-9570) was obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Concentrated solutions (approx. 10mg/ml) were made up in appropriate buffers and dialysed twice against the same buffer at 4°C. Dialysed solutions were diluted to the required concentrations with appropriate buffer solution and filtered through Millipore filters before use. The final preparation had a purity index $E_{403}/E_{280} = 3.0$ and the enzyme concentration was determined spectrophotometrically at 403nm by using $\varepsilon_{403} = 9.1 \times 10^4 M^{-1} \cdot cm^{-1}$ (Keilin & Hartree, 1951).

Chlorodeuteroferrihaem was prepared from chloroprotoferrihaem [haemin chloride (purum); Fluka A.G., Buchs, Switzerland] by the resorcinol-melt method (Falk, 1964).

m-Chloroperoxobenzoic acid was supplied by BDH Chemicals Ltd., Poole, Dorset, U.K. The material was recrystallized from light petroleum (b.p. 40–60°C)/diethyl ether (3:1, v/v) to give a purity greater than 99%. All other peroxo acids were used as supplied by Laporte Industries Ltd., General Chemicals Division, Widnes, Cheshire and were over 85% pure, the only significant impurities being the respective parent carboxylic acids. Peroxo acids (care: explosive!) were assayed iodometrically (Swern, 1970). Dissociation constants of the peroxo acids were determined, by using the procedure of Everett & Minkoff (1953), at 25°C and an ionic strength maintained as nearly as possible at 0.1M by using $NaNO_3$. The results are given in Table 1. Stock solutions of peroxo acids ($\approx$2mM) were made up immediately before use. In general, no detectable hydrolysis occurred for at least 8h after dissolution of the peroxo acid.

Buffer solutions (Na_2HPO_4/KH_2PO_4 or $Na_2CO_3/NaHCO_3$, as appropriate) were prepared from A.R. grade materials. In all final solutions the buffer concentration was 10mM and the ionic strength 0.1M (adjusted with A.R.-grade $NaNO_3$). All reaction solutions were prepared in triple-distilled water.

Kinetic measurements

Kinetic measurements were made at 25°C by using a Durrum-Gibson D110 stopped-flow spectrophotometer.

The rate constant for Compound I formation was measured at 403nm. To obtain maximal Compound I formation an initial [peroxide]:[peroxidase] ratio of 1.1:1 was used throughout, with initial [peroxidase] of 3–5μM, and the results were analysed by using a procedure suggested by Widequist (Frost & Pearson, 1961). Check experiments at 430nm indicated that Compound II formation was not significant on the time-scale for determination of the rate of Compound I formation. The results are presented in Table 1 and Fig. 1.

The rate constants for formation of the deuteroferrihaem peroxidatic intermediate were obtained from measurements of the initial rate of decrease of absorbance $(dE/dt)_0$ at 359nm, by using initial [peroxide]:[deuteroferrihaem] ratios in the range 1:1 to 10:1 and initial [deuteroferrihaem] = 5.53μM, at pH7.4 and 25°C. Under these conditions, maximal conversion into the intermediate was obtained and dE/dt was proportional to the initial [peroxide], so that apparent second-order rate constants ($k_{obs.}$) were calculated from the relation:

$$k_{obs.} = \frac{(dE/dt)_0}{[\text{deuteroferrihaem}][\text{peroxide}] \cdot \Delta\varepsilon \cdot l}$$

Table 1. *Second-order rate constants, for the formation of horseradish peroxidase Compound I with peroxobenzoic acids at 25°C, pH5, I = 0.1M*

Values in parentheses are the pK values of the peroxo acids measured at 25°C, $I = 0.1$M (see the text for details). N.A., value not available.

Substituent ...	$10^{-7}k_{+1}$ ($M^{-1} \cdot s^{-1}$)					
Position	MeO	Me	None	Cl	NO_2	CO_2^-
p	9.2 (7.91)	9.5 (7.80)	8.8 (7.64)	12.6 (7.60)	6.6 (7.14)	
m			8.8 (7.64)	11.8 (7.50)	1.3 (7.23)	
o		0.8 N.A.	8.8 (7.64)	10.4 (7.57)	0.03 (7.10)	v. slow (8.08)

where $\Delta\varepsilon$ is the decrease in extinction coefficient at 359nm obtained for maximal conversion into the intermediate, and l the path length of the cuvette. The results are presented in Table 2.

Discussion

Where direct comparison is possible the present data are generally in accord with the literature. For the reaction of horseradish peroxidase with H_2O_2, the observed pH-independence of the rate constant for Compound I formation (Fig. 1) is in agreement with that of Dolman *et al.* (1975) at pH > 5, and the absolute value of the rate constant ($2.0\times10^7\pm0.1\times10^7$ M$^{-1}\cdot$s^{-1}) compares well with their value (1.8×10^7 M$^{-1}\cdot$s^{-1}). Schonbaum & Lo (1972) reported a rate constant of 1.1×10^7, which is nearer to the value (1.15×10^7) obtained by Chance *et al.* (1967). These differences in the absolute values of the rate constant may reflect differences in the enzyme preparations. Although the present finding (Table 1), that the maximal rate constant for reaction with *m*-nitroperoxobenzoic acid is less than that for H_2O_2, is not in agreement with unpublished results of B. Chance, P. Fackre & G. R. Schonbaum (reported by Schonbaum & Lo, 1972), for a number of the peroxo acids studied the rate constant substantially exceeds that for H_2O_2.

The implications of the results presented in Table 1 and Fig. 1 may be considered under the following headings.

(*a*) *pH effects*

The pH-dependence of the peroxobenzoic acid reactions with horseradish peroxidase is strikingly similar to the behaviour of catalase with peroxoacetic acid (Jones & Middlemiss, 1972). In both cases the results are most simply interpreted as a reaction between the enzyme and un-ionized peroxo acid molecules, on which no enzyme ionizations have a significant influence over the pH range studied. It must be noted, however, that a rate law of this form may alternatively be derived from the model:

$$HP \overset{K_a}{\rightleftharpoons} H^+ + P^- \quad \text{(peroxo acid ionization)}$$

$$EH^+ \overset{K_e}{\rightleftharpoons} E + H^+ \quad \text{(enzyme ionization)}$$

$$EH^+ + P^- \xrightarrow{k_r} \text{Compound I}$$

Since it is readily shown that this model requires values of k_r which exceed the diffusion-controlled limit for a bimolecular rate constant, the alternative must be rejected, and it is therefore concluded that the attacking reagent is, indeed, the un-ionized H_2O_2 or peroxo acid molecule.

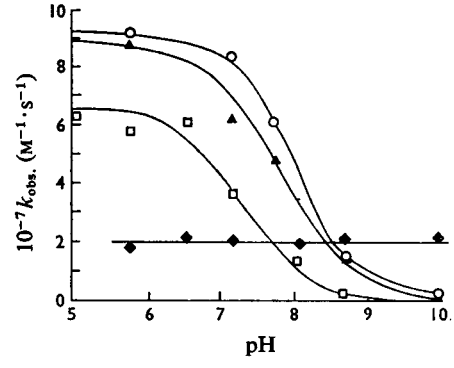


Fig. 1. *Influence of pH on the observed second-order rate constant ($k_{obs.}$) for the formation of horseradish peroxidase Compound I*

H_2O_2 (♦), peroxobenzoic acid (▲), *p*-methoxyperoxobenzoic acid (○) and *p*-nitroperoxobenzoic acid (□), at 25°C, $I = 0.1$ M. For details, see the text.

Table 2. *Second-order rate constants for the formation of the deuteroferrihaem peroxidatically active intermediate with peroxobenzoic acids at 25°C, pH7.4, $I = 0.1$ M*

Values are based on the stoicheiometric concentrations of deuteroferrihaem and peroxo acid. Numbers in parentheses are values of $10^{-7}k$ (M$^{-1}\cdot$s^{-1}), calculated by assuming the rate law: $v = k$ [deuteroferrihaem monomer][peroxo anion]. For H_2O_2 as substrate, $k = 8\times10^7\pm4\times10^7$ M$^{-1}\cdot$s^{-1}, calculated from the data of Jones *et al.* (1974).

	$10^{-7}k_{obs.}$ (M$^{-1}\cdot$s^{-1})					
Substituent ... / Position	MeO	Me	None	Cl	NO_2	CO_2^-
p	0.5 (4.9)	0.44 (4.0)	0.45 (3.8)	0.46 (3.7)	0.53 (3.4)	
m			0.45 (3.8)	0.66 (4.2)	0.61 (4.1)	
o			0.45 (3.8)	0.54 (4.3)	0.25 (1.6)	0.07 (0.73)

(*b*) *Steric effects*

The influence of substituents in the natural substrate, H_2O_2, on the rates of formation of Compound I is strikingly different, for horseradish peroxidase, from that with catalase. For the latter enzyme the results imply that steric constraints on access to the active site are very severe; thus only the lower members of the series of alkyl hydroperoxides are effective and, of the acyl hydroperoxides, only peroxoacetic acid is active (Jones & Middlemiss, 1972). In all cases the substituted peroxides react at markedly lower rates than the natural substrate.

With horseradish peroxidase, peroxobenzoic acids may react readily to form Compound I and, as Table 1 shows, the second-order rate constants in several cases are substantially greater than that with H_2O_2. Nevertheless the results also show significant constraints on access to the active site. This is particularly evident from comparison of the chloro- and nitro-substituted peroxobenzoic acids. For the former, the rate constant for the *o*-substituted acid is only 20% lower than that for the *p*-substituted species, whereas the rate for the *o*-nitro acid is only 0.45% of that for *p*-nitroperoxobenzoic acid. The idea that this latter behaviour results from adverse steric influence on species with bulky *o*-substituents is supported by the results obtained for *o*-methyl- and *o*-carboxyl-substituted acids.

In the formation of the peroxidatic intermediate from deuteroferrihaem the pattern of rate constants (Table 2) implies that steric factors are less acute than for horseradish peroxidase. Only for *o*-nitro- and *o*-carboxyl-substituted acids do the rate constants fall below a rather uniform level of reactivity across Table 2. This comparison implies that the apoprotein in the horseradish peroxidase provides the additional steric constraints.

(*c*) *Electronic effects of substituents*

Two types of two-electron-equivalent (non-radical) redox pathways are well established in substrate-oxidation reactions by peroxo acids in aqueous solutions (Robson, 1964).

I. *Bimolecular electrophilic attack of an un-ionized peroxo acid molecule on substrate.* This is a favourable pathway when, as is commonly the case, the reducing substrate is an electron-rich nucleophile [indeed this pathway is operative in the second-order thermal decomposition of peroxo acids, in which the peroxo acid molecule attacks peroxo anion nucleophile [for a review, see Jones *e al.* (1970)]. Electronic effects of substituents are straightforward; electron-withdrawing substituents in the peroxobenzoic acids increase the rate, relative to peroxobenzoic acid itself, whereas electron-donating groups decrease the rate. Studies in partially aqueous systems imply that water molecules may play important roles in the transition states for these reactions (Curci *et al.*, 1968).

II. *Attack of peroxo anion on the substrate to form a ligand adduct, which may either dissociate to regenerate the reactants or undergo* O–O *bond cleavage to yield products:*

$$\text{Peroxo anion} + \text{substrate} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{intermediate} \xrightarrow{k_{+2}} \text{products}$$

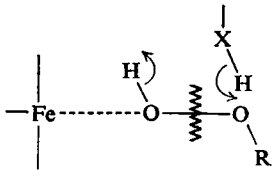
In this mechanism the pattern of substituent effects in the peroxo anion is less predictable. An electronegative substituent would lower the nucleophilicity of the peroxobenzoate ion, but the resultant intermediate would be less stable. With an electron-donating group k_{+1} would be increased but k_{+2} diminished. There is then the possibility of near-exact compensation and this behaviour was observed by Robson (1964) in a study of *o*-sulphobenzaldehyde oxidation. We have similarly found that the rates of oxidation by a variety of organic dyes in water by a series of peroxobenzoate anions are insensitive to substituent effects (Davies, 1975). The general reaction chemistry of high-spin Fe(III) complexes suggests that this could be a favoured pathway in these systems, because of both the electrophilicity of the iron(III) centre and the excellent nucleophilic properties (α-nucleophiles, Edwards & Pearson, 1962) of peroxo anions. Steady-state kinetic studies of the catalatic (Jones *et al.*, 1973) and peroxidatic (D. Mantle, unpublished work) reactions of ferrihaem complexes support a mechanism of this type, and the data of Table 2 illustrate an insensitivity to substituent effects in the formation of a peroxidatically active intermediate which is consistent with this model. The low observed rate constant for reaction of deuteroferrihaem with H_2O_2 becomes consistent with this pattern when the rate constant is recalculated on the basis of reaction via the HO_2^- anion.

For the reactions of horseradish peroxidase the reactions of *p*-substituted peroxobenzoic acids, which would be least susceptible to steric effects, also show no systematic substituent electronic effects. This insensitivity would not be expected for oxidation by electrophilic attack of un-ionized peroxo acid, and yet the pH effects demonstrate that it is the un-ionized species which is the attacking reagent. A satisfactory description of the nature of the reaction must resolve this apparent conflict.

(*d*) *Nature of the redox process*

The background of peroxide reaction chemistry so far considered has related to aqueous solution systems. Although this is appropriate for ferrihaem complexes in water, it may be quite inappropriate for the active-site environment of the ferrihaem complex

in the enzyme. If the immediate environment of the Fe(III) centre of the enzyme is a low-dielectric-constant hydrophobic region, then the preferential selection of un-ionized peroxide molecules as substrates may arise from the relatively favourable partition of these species between the aqueous environment and the hydrophobic region compared with the anionic conjugate bases. In this model the further reaction of bound hydroperoxide via ionization is unlikely because of the highly unfavourable energy requirements of charge separation. It is probable therefore that this model requires a concerted process for efficient formation of Compound I, for example:

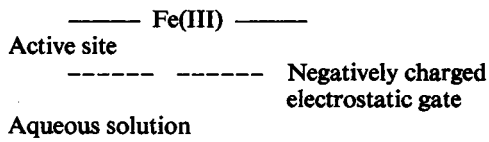


in which the group –X–H mediates the redox process by avoiding charge separation. Water molecules may act in this way in peroxo acid oxidations (Curci *et al.*, 1968) and it is possible that other appropriate species (protein residues) could act similarly. A process of this type in a hydrophobic medium could yield the insensitivity to substituent effects that we have observed (Ogata *et al.*, 1961). The relatively low rate of reaction of H_2O_2 with horseradish peroxidase compared with unhindered peroxobenzoic acids could be explained by preferential binding of the aromatic end-groups at the active site by hydrophobic interactions. Schonbaum (1973) has made a similar suggestion in comparing the binding of aromatic and aliphatic hydroxamic acids as ligands to the active site of horseradish peroxidase.

Lanir & Schejter (1975) have reported a low and pH-independent value for the molar relaxivity of water protons in horseradish peroxidase solutions and concluded that water does not become co-ordinated to the Fe(III) centre of the enzyme. Their results do not exclude slow exchange of water with the active site and the result is perhaps better interpreted as deriving from the operation of factors that inhibit either the ingress or effective binding of water molecules to the active site. Such a factor could be a hydrophobic environment of the ferrihaem group.

However, other work suggests that substrate selection and activation may be a more sophisticated process than that supposed in the 'hydrophobic-pocket' model above. Dolman *et al.* (1975) have investigated the rate of formation of Compound I by using H_2O_2 over a wide range of pH. At pH < 5 the rate constant markedly decreases and the behaviour is quantitatively described by supposing two consecutive protonations ($pK_1 = 3.9$ and $pK_2 = 3.2$, which were tentatively assigned to the porphyrin propionate side chains) which yield inactive forms of the enzyme. Tamura *et al.* (1972) found that the modified peroxidase formed by combining the apoprotein with protoferrihaem dimethyl ester had a very much lower reactivity (~0.01% of the native enzyme) towards hydroperoxides. Since protonation of both propionate groups could produce effects similar to those accompanying esterification, this result tends to re-inforce the above-mentioned assignments of pK_1 and pK_2. The situation is not quite clear-cut, however, since the re-constituted enzyme with protoferrihaem monomethyl ester has a reactivity of 18% of the native enzyme (Tamura *et al.*, 1972).

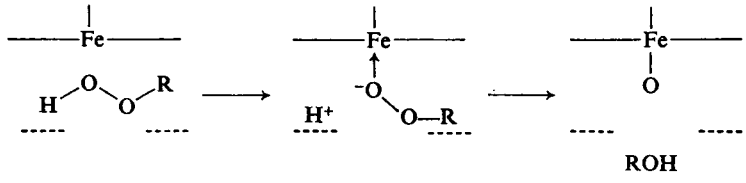
The available evidence (Ellis & Dunford, 1968) does not support the idea that protonation disrupts the active-site conformation of the enzyme. Although the postulate of steric effects might explain the observed behaviour, these results together with the present work suggest an alternative explanation of the nature of the substrate selection and activation process, as in the model:



It is supposed that negatively charged groups in the active site of horseradish peroxidase form an 'electrostatic gate', which controls access of substrate. In this situation un-ionized peroxidase molecules could diffuse through the 'gate' into the active site, but anions would be excluded by electrostatic repulsion. This argument should apply, not only to substrate, but to the wide variety of ligand species which react with the ferrihaem centre of horseradish peroxidase. It is a well established and unique aspect of the chemistry of horseradish peroxidase (and catalase) that these ligand-binding processes involve the un-ionized form of the ligand species (Brill, 1966; Schonbaum, 1973), e.g. the formation of the cyanide and fluoride complexes of horseradish peroxidase and catalase involves the binding of HCN and HF respectively. Generally in the ligand-addition reactions of Fe(III) species it is the ligand anions that are bound; indeed this is the case in metmyoglobin and methaemoglobin (Brill, 1966). It is therefore pertinent to enquire whether ionization within the active site may be involved. The 'electrostatic gate' that we have postulated would operate in both directions; that is, if ionization of a ligand molecule such as HCN occurs within the active site, the anion could not escape unless accompanied and neutralized by a proton. Thus in this model, ionization within the active site is 'de-coupled' from ionization in the

external environment. A kinetic or equilibrium study of ligand binding would 'see' the binding of un-ionized species, although it may well be the ligand anion which is attached to the Fe(III) centre, with the proton bound to another region of the active site.

This argument further suggests the possibility that substrate activation may occur by ionization within the active site:



The redox reaction could now proceed via nucleophilic attack of peroxo anion on the Fe(III) centre, a process which, though yielding a pattern of substituent effects consistent with the results of Table 1, would also provide a rate law indicating attack by un-ionized peroxide species. Comparison with Table 2 and with the results of steady-state catalytic activity of ferrihaem monomer species (Jones *et al.*, 1973) implies that, if peroxide activation via ionization within the active site is to be an effective catalytic pathway for horseradish peroxidase (and catalase), a considerable enhancement of the degree of ionization is required for the natural substrate, H_2O_2, compared with the equilibrium value in water at physiological pH. Such an enhancement is quite likely, for example the ionization constant of water is increased by more than 13 orders of magnitude ($pK \sim 16 \rightarrow pK < 3$) on co-ordination in Fe^{3+} (aq). Within the active site, the O-H dipole of a hydroperoxide molecule would orient preferentially in the electric field between the 'electrostatic gate' and the Fe(III) centre, so that ionization enhancement could be initiated via a microscopic analogue of the Second Wien Effect (the high-field dissociation effect; Harned and Owen, 1958). If ionization within the active site is essentially complete, this could account for the near identity of the overall maximal catalytic rate constant for catalase with that for the catalytic activity of ferrihaem monomer at high pH (Jones *et al.*, 1973).

The nuclear-magnetic-resonance results of Lanir & Schejter (1975) are less readily explicable on this model, although it is possible that the enzyme surface near the active site may influence the structure of water and the mobility of water molecules at the interface (Clifford & Pethica, 1968). On balance the available data appear to support the 'electrostatic-gate' model as the mechanism of substrate selection. The relative virtues of 'concerted' and 'ionization-activated' redox pathways are more evenly poised. It is clear, however, that, in this first phase of the reaction, a mechanism is operative which enables the enzyme to use un-ionized H_2O_2, and that this is desirable because of the high pK of H_2O_2. In the later phases of the catalytic cycle the controls exerted by the enzyme protein on substrate oxidation are much more complex (Dunford, 1974).

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