Formation and Peroxidatic Properties of Coprohaem Compound I

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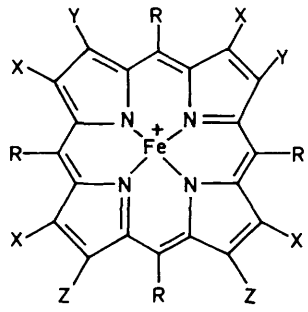
Coproferrihaem (cfh) reacts very rapidly with peroxybenzoic acids at pH = 6.0 in aqueous solution, in processes of 1 : 1 stoicheiometry, to form a spectroscopically distinct oxidised derivative coprohaem Compound I (ch-I), which is stable on the stopped-flow time-scale. The rate constants for ch-I formation are comparable to those for the formation of horse radish peroxidase Compound I with peroxybenzoic acids. The Soret band absorption spectrum of ch-I is independent of initial [cfh] and [peroxyacid] with R, the ratio [peroxyacid] : [cfh], in the range $1 < R < 4$ and independent of the nature of the peroxyacid. Although cfh exists as >99% monomer under the conditions employed, the absorption spectra at $R < 1$ suggest limited dimer formation between ch-I and unoxidised cfh. Coprohaem Compound I exhibits powerful peroxidatic oxidant properties. In particular ch-I is reduced by methanol, ethanol, and propargyl alcohol (prop-2-yn-1-ol) with rate constants that parallel in sequence those with catalase Compound I, although the enzymatic intermediate reactions are 10^2—10^4 times faster than those with ch-I.

Catalyses involving the ferrihaem-containing hydroperoxidase enzymes of sub-group E.C.1.11.1 (catalases and peroxidases) proceed *via* ordered mechanisms[1] in two stages: (*i*) oxidative activation of the native, Fe^{III}, enzyme by hydroperoxide (ROOH), leading to the formation of an oxidised enzyme intermediate, Compound I, and release of ROH; (*ii*) reduction of the Compound I intermediate back to the native enzyme concomitant with the oxidation of a donor (reductant) substrate. The Compound I intermediates are thus, formally, 'Fe^{V}' species and extensive structural studies have shown that, in most cases, they contain ferryl ($Fe^{IV}=O$) complexes of porphyrin π-cation radical ligands.[2] In the reduction of Compound I by donors the formation of a half-reduced, Fe^{IV}, intermediate (Compound II) is sometimes, but not always, observed. In the case of catalases, H_2O_2 can act both as oxidant and reductant substrate and consequently catalases cannot be completely converted into Compound I by H_2O_2. This can, however, be achieved using hydroperoxides which are good oxidant substrates but poor reductant substrates (*e.g.* peroxyacetic acid). With almost all peroxidases, hydroperoxides (including H_2O_2) are ineffectual reductant substrates, so that complete conversion of the native enzyme to the Compound I intermediate is achievable with a variety of hydroperoxides.

Thus, although a series of enzymes containing the same type of redox-active prosthetic group [most commonly protoferrihaem, (**1**), Figure 1] acts *via* qualitatively similar catalytic mechanisms, the enzymes in the series show a remarkable diversity in their activities and selectivities with both oxidant and reductant substrates. Protein-free ferrihaem complexes display both catalase-like and peroxidase-like properties in aqueous solution[3] and the concept that enzymatic proteins exert selection and amplification of intrinsic catalytic properties of the prosthetic group finds considerable experimental support. Although the formation of protein-free analogues of Compound I intermediates has been demonstrated in non-aqueous solvents at low temperature,[4] using appropriate ferrihaems, such systems do not permit a quantitative comparison of catalytic functions of enzymes and ferrihaem complexes under physiological conditions. In aqueous solutions, however, the redox chemistry of ferrihaems may be severely complicated by haem–haem interactions,[3] leading to the formation of dimeric species involving haems in either like or unlike oxidation states and also to the occurrence of electron-transfer reactions between unlike oxidation states. An oxidised derivative of deuteroferrihaem (**2**), which can be formed using a variety of oxidants,[5] has been shown to be a species at the Fe^{IV} oxidation level and is considered to arise *via* a rapid electron-transfer reaction between an initially formed (but unobserved) Compound I intermediate and unoxidised Fe^{III}–porphyrin, *i.e.* equations (i) (r.d. = rate determining) and (ii). This species can also be formed[6] by direct one-electron oxidation with radiolytically generated $Br_2^{\bullet -}$.



(**1**) R = H; X = CH_3; Y = $-CH=CH_2$; Z = $-CH_2CH_2COO^-$
(**2**) R = H; X = CH_3; Y = H; Z = $-CH_2CH_2COO^-$
(**3**) R = H; X = CH_3; Y = $-SO_3^-$; Z = $-CH_2CH_2COO^-$
(**4**) R = H; X = CH_3; Y = Z = $-CH_2CH_2COO^-$
(**5**)

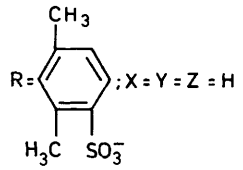


Figure 1. Structures of ferrihaems; overall charge depends on peripheral porphyrin substituents

$$Fe^{III} + ROOH \xrightarrow{r.d.} Fe^{V} + ROH \quad (i)$$

$$Fe^{V} + Fe^{III} \xrightarrow{fast} 2Fe^{IV} \quad (ii)$$

These results suggest that attempts to stabilize a protein-free Compound I species in aqueous solution require a porphyrin

structure which, as far as possible, inhibits haem–haem interactions. Sulphonation of deuteroferrihaem as in (**3**), results in a marked reduction in dimerisation constant so that substantially monomeric ferrihaem solutions can be obtained at pH *ca.* 6. However, on addition of H_2O_2, no oxidised intermediate was observed spectrophotometrically,[7] although catalytic coupled oxidation of added donor occurs effectively.[8] Moreover, in the presence of H_2O_2 alone a marked oxidative degradation of the porphyrin ligand occurred. Upon reaction with peroxyacids partial formation of the oxidised intermediate was observed but oxidative destruction of the ligand interfered with characterisation. It was concluded that porphyrin sulphonation in the β-pyrrolic positions, although effective in electrostatically blocking haem–haem interactions, resulted in a relative enhancement of the rate of oxidation of H_2O_2 by the oxidised intermediate (and hence a very low steady-state concentration of intermediate) and also in an enhanced rate of ferrihaem degradation. It seems probable that these effects could arise from the electron-withdrawing properties of the sulphonate groups and, in the present work, we have therefore employed coproferrihaem (**4**) in which a similar level of electrostatic blocking results in a dimerization constant[9] comparable to that of (**3**). A characterisation of coprohaem Compound I (ch-I) formed by reaction of coproferrihaem (cfh) with peroxyacids is reported.

Experimental

Materials.—Coproporphyrin III * was isolated from cultures of *rhodopseudomonas spheroides* according to the procedure of Lascelles[10,11] as modified by Brown and Hatzikonstantinou.[9,12] The absorption spectrum of the product was in accord with literature values[13] and paper chromatography[14,15] indicated a single component. Under conditions of heavy loading, thin-layer chromatograms[16] showed traces of other porphyrins. Lascelles[10] has reported that this procedure yields *ca.* 97% coproporphyrin III and 3% uroporphyrin I and III † and 3, 5, and 7 carboxylic acid-containing porphyrins. Iron was inserted into the porphyrin using the iron(II) sulphate method,[9] the progress of the reaction being monitored spectrophotometrically[17] and the final reaction solution exposed to air to effect oxidation to the ferrihaem. After extraction with diethyl ether and washing with HCl to remove any excess porphyrin, coproferrihaem (**4**) was precipitated as haematin at the diethyl ether–aqueous layer interface by raising the pH of the aqueous layer to 4—5 by addition of sodium acetate solution. The coproferrihaem precipitate was collected by centrifugation, washed with cold acetate buffer and finally triply-distilled water and dried in a vacuum desiccator. The material was characterised by paper chromatography;[18,15] no fluorescent porphyrin contaminants were visible nor were other haem species apparent. The purity of two independently prepared samples, assessed by pyridine haemochrome spectrophotometry[9] and by elemental analysis was 95 ± 5%. Stock solutions were prepared by dissolving accurately weighed amounts of cfh in a small volume of 1.0 mol dm^{-3} NaOH and diluting to 80—100 μmol dm^{-3}. The solutions were stored refrigerated in the dark. No change in the Soret band absorption coefficient of buffered (pH = 6.0, phosphate) solutions was observed for up to one year after preparation.

m-Chloroperoxybenzoic acid was supplied by BDH Chemicals Ltd.; all other peroxyacids were supplied by Laporte Industries Ltd. These materials were used as supplied and contained *ca.* 85% peroxyacid, the only significant impurity being the respective parent carboxylic acid. Stock solutions of peroxyacids were prepared in triply-distilled water immediately before use, stored in a refrigerator, and assayed iodometrically. No hydrogen peroxide could be detected cerimetrically in stored stock solutions within 24 h of preparation. Reaction solutions were prepared by quantitative dilution of stock solutions with triply-distilled water.

Both 'AnalaR' and 'Spectrosol' grade ethanol (BDH) were used and gave indistinguishable kinetic results. Methanol was 'AnalaR' material (BDH). Propargyl alcohol (prop-2-yn-1-ol) (Aldrich) was repeatedly distilled until almost colourless (residual absorbance at 390 nm was $\varepsilon = 0.004\ dm^3\ mol^{-1}\ cm^{-1}$). Alcohol solutions were prepared using deoxygenated triply-distilled water. Other materials used were 'AnalaR' grade reagents.

Methods.—Spectrophotometric measurements employed a Pye-Unicam SP8-150 instrument and an HP8541 diode-array instrument fitted with a stopped-flow attachment. For rapid reaction measurements a Durrum D-110 stopped-flow spectrophotometer was used. Unless otherwise stated measurements were made at 25 °C, pH = 6.0 (10 mmol dm^{-3} phosphate buffer), and ionic strength 0.1 mol dm^{-3} (adjusted with NaCl).

Results and Discussion

Formation of Coprohaem Compound I (ch-I).—Upon reaction of cfh with H_2O_2 in equimolar concentrations (*ca.* 1 μmol dm^{-3}) no change in the cfh Soret band absorption was observed. Increasing the relative $[H_2O_2]$ served only irreversibly to diminish the Soret absorption, *i.e.* destroy the haem. Nevertheless in cfh–H_2O_2 systems effective catalase-like[19] and peroxidase-like[8] catalysis can occur. If a Compound I intermediate is formed the results imply an undetectably low steady-state concentration and hence that reaction of the intermediate in H_2O_2 oxidation is much faster than the reaction of cfh in H_2O_2 reduction.

Preliminary studies of the reaction of cfh with peroxyacids employed the HP8541 instrument so that the Soret band spectrum could be frequently sampled. Upon admixture of cfh with equimolar peroxyacid the absorbance at the Soret maximum (390 nm) was more than halved within the mixing time. This was followed during a period of *ca.* 100 s by a regeneration of the cfh spectrum although the final spectrum intensity corresponded to the presence of *ca.* 75% of the initial cfh. These results imply the rapid formation of an intermediate of reasonable stability on the stopped-flow time-scale and the D-110 instrument was used in more detailed studies of the kinetics and stoicheiometry of intermediate formation. Studies of the 'spontaneous' decomposition of the intermediate are described in the following paper.[20]

Figure 2 presents the results of a series of stopped-flow studies of the reaction of cfh with *m*-chloroperoxybenzoic acid as spectrophotometric titrations, plotting the maximum decrease in absorbance at 390 nm (expressed as changes, $\Delta\varepsilon_{obs.}$, in the apparent haem molar absorptivity) against R, the initial ratio [peroxyacid]:[cfh]. When $R > 1$ the values of $\Delta\varepsilon_{obs.}$ ($\Delta\varepsilon_{max.}$) are constant, independent of increasing R in the range $1 < R < 4$ and $\Delta\varepsilon_{max.}$ is independent of [cfh]. Closely similar results were obtained with independently prepared cfh samples. These results are consistent with reaction of cfh with peroxyacid in a 1:1 stoicheiometry to form a Compound I intermediate (ch-I), equation (1). When $R < 1$ the values of $\Delta\varepsilon_{obs.}$ are somewhat larger than expected on the basis of the relationship (2) and the deviations tend to increase with increasing [cfh] and are a maximum at R *ca.* 0.5. These results imply a more complex

* 3,8,13,17-Tetramethylporphyrin-2,7,12,18-tetrapropionic acid.
† 3,8,13,18-Tetrakis(carboxymethyl)porphyrin-2,7,12,17-tetrapropionic acid and 3,8,13,17-tetrakis(carboxymethyl)porphyrin-2,7,12,18-tetrapropionic acid respectively.

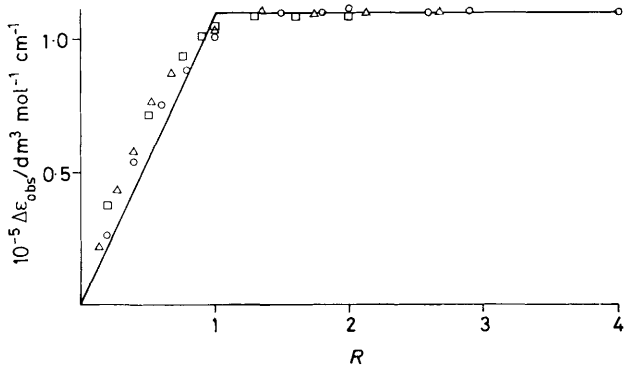


Figure 2. Stopped-flow spectrophotometric titrations of cfh with *m*-chloroperoxybenzoic acid. Dependence of $\Delta\varepsilon_{obs.}$ on R (λ = 390 nm, pH = 6.0). [cfh] = 0.69 (○), 1.03 (△), or 1.38 μmol dm^{-3} (□)

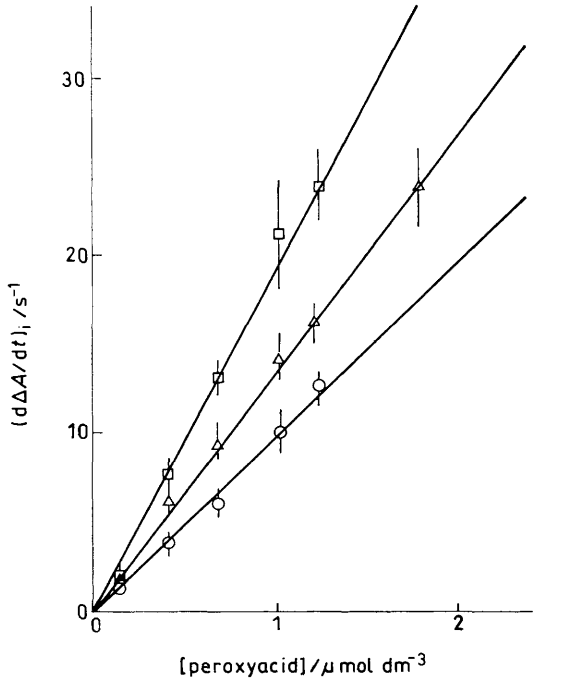


Figure 3. Dependence of $(d\Delta A/dt)_i$ on [*p*-methylperoxybenzoic acid] (λ = 390 nm, pH = 6.0). [cfh] = 0.69 (○), 1.03 (△), or 1.38 μmol dm^{-3} (□)

behaviour in systems where unoxidised cfh [on the basis of equation (1)] is present and this is discussed further below.

$$\text{cfh} + \text{ROOH} \longrightarrow \text{ch-I} + \text{ROH} \quad (1)$$

$$\Delta\varepsilon_{obs.} = R{\cdot}\Delta\varepsilon_{max.} \quad (2)$$

Further series of stopped-flow spectrophotometric titrations were carried out using a range of substituted peroxybenzoic acids (*o*- and *p*-chloro, *m*- and *p*-nitro, and *p*-methyl) as oxidants. These studies again yielded titration curves indicating reactions of 1 : 1 stoicheiometry between cfh and peroxyacid and $\Delta\varepsilon_{max.}$ was identical within experimental error to the value obtained with *m*-chloroperoxybenzoic acid. The results are consistent with the formation of a ch-I species from cfh with a spectrum independent of the nature of its oxidant progenitor.

$$k = (d\Delta A/dt)_i/[\text{cfh}][\text{ROOH}]\Delta\varepsilon_{max.}l \quad (3)$$

Table 1. Second-order rate constants for the formation of ch-I and horse radish peroxidase Compound I (hrp-I)

	$10^{-7}\,k$/dm^3 mol^{-1} s^{-1}	
Peroxybenzoic acid	ch-I[a]	hrp[b]
m-Chloro	8.4	11.8
m-Nitro	10.6	1.3
p-Methyl	5.8	9.5

[a] pH = 6.0, I = 0.1 mol dm^{-3}, 25 °C, this work. [b] pH = 5.0, I = 0.1 mol dm^{-3}, 25 °C, ref. 26.

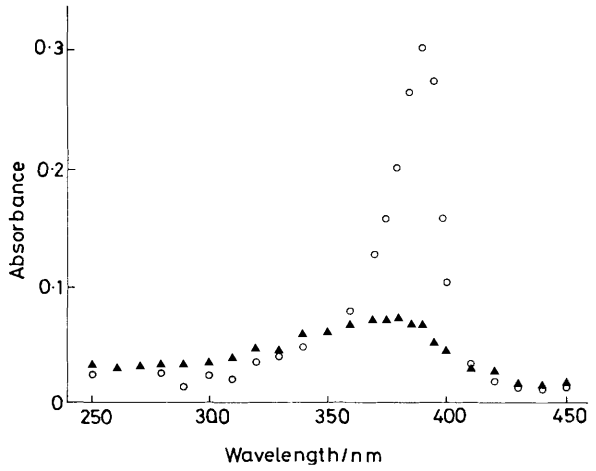


Figure 4. Comparison of the Soret band absorption spectra of 0.9 μmol dm^{-3} cfh (○) and ch-I (▲) (formed by reaction with 1.34 μmol dm^{-3} *m*-chloroperoxybenzoic acid); pH = 6.0

The kinetics of formation of ch-I were determined by measuring initial rates of decrease of absorbance, $(d\Delta A/dt)_i$, at 390 nm. The reactions were first order with respect to both [peroxyacid] and [cfh] as exemplified in Figure 3. Values of the second-order rate constants, k, at pH = 6.0, obtained using the relationship (3) (where l is the cuvette pathlength) are shown in Table 1. The Soret absorption spectrum of ch-I obtained from measurements of $\Delta\varepsilon_{max.}$ in the range 250—450 nm using *m*-chloroperoxybenzoic acid and R = 1.49 is shown in Figure 4. The spectrum at R = 1.99 was indistinguishable from that at R = 1.49. The deviations from equation (2) observed at $R < 1$ imply the presence of additional species in the system under these conditions. There are two possible processes that could account for the observed behaviour. (*a*) Formation of a coprohaem Compound II species (ch-II) by a comproportionation reaction between ch-I and unoxidised cfh: ch-I + cfh ⟶ 2ch-II. (*b*) Formation of a mixed-oxidation state dimer by reaction of ch-I with cfh: ch-I + cfh ⇌ ch-I·cfh. Both processes would lead to maximal deviation from equation (2) at R = 0.5. In principle the occurrence of the irreversible reaction (*a*) would yield a spectrophotometric titration curve in which the absorbance decrement is a discontinuous function of R in the range of $0 < R < 1$, whereas (*b*) would yield a continuous dependence on R in this range. In practice the deviations are so small that a distinction on this basis is not realistic. Studies of the decay of ch-I to cfh implicate ch-II as an intermediate in this process[20] [although not formed according to (*a*)]. These data imply that, if ch-II were formed according to (*a*) at $R < 1$, then $\Delta\varepsilon_{obs.} < R\Delta\varepsilon_{max.}$ would be observed, whereas, as shown in

Figure 2, $\Delta\varepsilon_{obs.} > R\Delta\varepsilon_{max.}$. On this basis it is suggested that some formation of a mixed-oxidation state dimer occurs according to (*b*) at $R < 1$. That the extent of this process is small is indicated by Figure 5 in which the experimental Soret band spectrum at $R = 0.5$ is compared with a spectrum calculated from the relationship (4). Significant differences between the observed and calculated spectra are evident only near the Soret band maximum. Although at pH = 6.0 with the [cfh] employed 99% cfh is present as monomeric ferrihaem, it is not surprising that detectable haem–haem interactions occur involving ch-I since the net negative charge is reduced in the oxidised intermediate. There is, however, no evidence that ch-I itself undergoes aggregation under the conditions of our experiments;

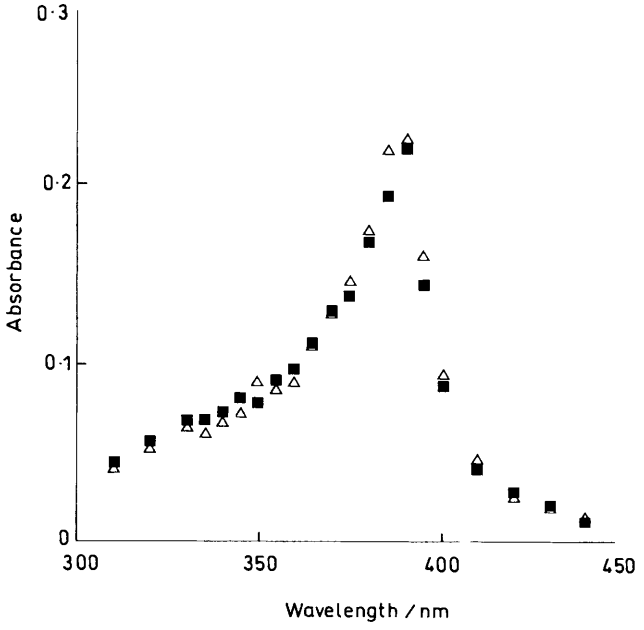


Figure 5. Experimental Soret band spectrum (■) obtained at [cfh] = 1.2 μmol dm^{-3}, $R = 0.5$ (*m* = chloroperoxybenzoic acid) compared with a spectrum (△) calculated from equation (4)

the values of $\Delta\varepsilon_{max.}$ are independent of [ch-I] in the range studied.

$$A = \tfrac{1}{2}[\text{cfh}](\varepsilon_{\text{cfh}} + \varepsilon_{\text{ch-I}})l \qquad (4)$$

Peroxidatic Properties of Coprohaem Compound I.—The limited stability of ch-I in the absence of added peroxidatic donor substrate affects the techniques that can be employed in studies of ch-I reduction by donors. The 'spontaneous' decomposition of ch-I, which is discussed in the following paper,[20] is a biphasic, consecutive first-order process (5). With $R = 1$ *ca.* 75% of the initial cfh is regenerated, the remainder being degraded to non-absorbing 'destruction products'. Multi-mixer stopped-flow techniques would be appropriate for the study of donor oxidation by ch-I but, in the absence of such equipment, studies are of necessity limited to donors which are present in the system before ch-I formation is initiated, *i.e.* to stopped-flow experiments in which a solution of cfh + donor is mixed with peroxyacid solution.

$$\text{ch-I} \xrightarrow{k_1} \text{ch-II} \xrightarrow{k_2} \text{cfh} \qquad (5)$$

With highly reactive peroxidase donors (ascorbate, phenol, hydroquinol) ch-I formation and donor oxidation are closely coupled so that, at $R = 1$, [cfh] = 1 μmol dm^{-3}, [donor] = 50 μmol dm^{-3}, no change in Soret band absorption was observed during the reactions. In these processes a 'sacrificial' oxidation of donor also acts to protect the haem against the destruction that ensues in the absence of donor. A similar completely protective effect was observed with iodide at a higher donor concentration (1 mmol dm^{-3}). With less reactive donors the formation of ch-I should be essentially complete before donor oxidation commences, so that reduction of ch-I by the donor would be observed as an enhanced rate of cfh regeneration over that observed in the absence of donor. If donor oxidation also proceeds *via* the ch-II intermediate the observed rate constants for the reduction of ch-I would be given by equations (6) and (7).

$$k_1(\text{obs.}) = k_1 + k'_1[\text{donor}] \qquad (6)$$

$$k_2(\text{obs.}) = k_2 + k'_2[\text{donor}] \qquad (7)$$

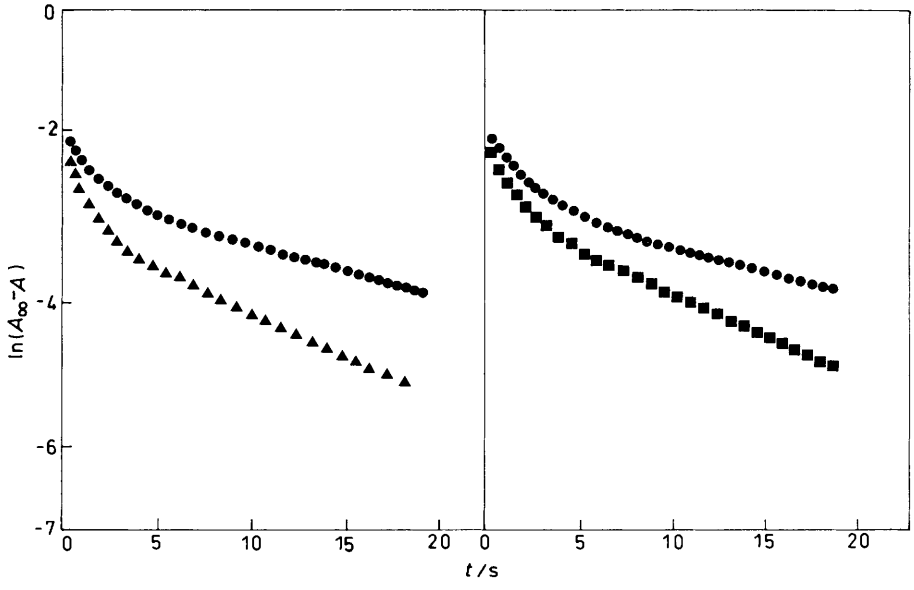


Figure 6. Influence of methanol and ethanol on the kinetics of decomposition of ch-I (formed by reaction of 1.0 μmol dm^{-3} cfh with 1.0 μmol dm^{-3} *m*-chloroperoxybenzoic acid): ●, no alcohol; ▲, 2.5 mol dm^{-3} ethanol; ■, 6.1 mol dm^{-3} methanol

Table 2. Kinetic data for the reduction of ch-I in the presence of propargyl alcohol. Conditions: $R = 1$, [cfh] = 1 μmol dm^{-3}, pH = 6.0, I = 0.1 mol dm^{-3}, 25 °C

[Propargyl alcohol]/mmol dm^{-3}	10^2k_1(obs.)/s^{-1}	10^2k_2(obs.)/s^{-1}
0	57.5 ± 0.5	7.4 ± 0.4
0.5	59.2 ± 2.8	7.9 ± 0.4
1.0	60.8 ± 3.5	9.2 ± 0.7
5.0	75.9 ± 8.7	12.7 ± 1.6
10.0	93.5 ± 7.2	18.9 ± 0.7

Table 3. Second-order rate constants for the reduction of oxidised haem intermediates by alcohols

	k/dm^3 mol^{-1} s^{-1}			
Alcohol	ch-I	ch-II	cat-I	cat-II
Methanol	0.13	0.014	830[a]	—
Ethanol	0.18	0.026	1 020[a] (180[b])	0.07[c]
Propargyl alcohol	36	11	2 500[a]	—

[a] Horse erythrocyte catalase, pH = 7.25, 25 °C, ref. 22. [b] Beef liver catalase, pH = 7.0, 24 °C, ref. 23. [c] Ref. 25.

On this basis the rate constants for thiosulphate oxidation under the initial conditions: $R = 1$, [cfh] = 1 μmol dm^{-3}, [thiosulphate] = 15 μmol dm^{-3}, were estimated as k'_1 *ca.* 1 × 10^5 dm^3 mol^{-1} s^{-1} and k'_2 *ca.* 1 × 10^4 dm^3 mol^{-1} s^{-1}. The computational procedures employed are described in detail elsewhere.[20] Although these estimates reflect orders of magnitude rather than precise data they imply that the cfh intermediates are appreciably more reactive than the oxidised deuteroferrihaem intermediates in this reaction.[21] No indications of enhanced ch-I reduction were observed in the presence of Br^- (1 mmol dm^{-3}), Cl^- (100 mmol dm^{-3}), or formate (100 mmol dm^{-3}).

Significant accelerations of the kinetics of ch-I reduction were observed in the presence of ethanol and methanol as shown in Figure 6. These donors are of particular interest since their oxidation is a notable feature of the peroxidatic activity of catalases.[22-24] Since propargyl alcohol (prop-2-yn-1-ol) is more reactive than ethanol towards catalase Compound I (cat-I) a more extensive study of this donor was undertaken. Preliminary studies of cfh + propargyl alcohol solutions in the absence of peroxyacid showed that the Soret absorbance of 1.8 μmol dm^{-3} cfh was constant for > 30 min in the presence of 0.25 mmol dm^{-3} propargyl alcohol but, in the presence of 12.5 mmol dm^{-3} propargyl alcohol the cfh absorbance decreases with a half-life of *ca.* 12 min. The cfh + propargyl alcohol solutions were therefore prepared immediately before use, although reproducible kinetic data were obtained during the first half-life of the alcohol–haem interaction. Kinetic data for the reduction of ch-I in the presence of propargyl alcohol are shown in Table 2.

In Table 3 values of k'_1 and k'_2 obtained by application of equations (6) and (7) to the results shown in Figure 6 and Table 2 are compared with literature values for the rate constants for reduction of cat-I by the alcohols. The rate constants follow the same sequence (methanol < ethanol < prop-2-yn-1-ol) for both ch-I and cat-I although the rate amplification achieved by the enzymatic intermediate is 10^2–10^4 fold. The relative reactivity of propargyl alcohol is much higher with ch-I than with cat-I and probably reflects the marked steric constraints on access of larger substrate molecules to the enzyme active site. An interesting difference between cfh and enzyme reactions is that catalase Compound II (cat-II) is not observed as an intermediate in the reduction of cat-I by ethanol. Indeed the rate constant for reaction of cat-II with ethanol[25] (Table 3) is comparable to that of cfh-II and is much too small for this process to contribute significantly to the coupled oxidation of ethanol by H_2O_2 catalysed by the enzyme. This does not necessarily imply that the reduction of cat-I by ethanol is a one-step two-electron equivalent redox process. Consecutive one-electron reduction of cat-I by ethanol is an equally tenable interpretation if the radical formed by one-electron oxidation of ethanol remains trapped in the active site so as to undergo a further rapid, one-electron oxidation to ethanol by cat-II before diffusing out of the active site. On this basis the consecutive one-electron reduction of ch-I that is observed implies the lack of a comparable constraint on diffusion away from the oxidation centre.

General Discussion.—Electrostatic blocking of haem–haem interaction in cfh has permitted the demonstration of the formation of a protein-free analogue of the enzymatic Compound I intermediates in aqueous solution. The rate constants for reaction of cfh with peroxybenzoic acids are comparable to those for horse radish peroxidase[26] (Table 1). In the latter case reaction with unionized *m*-chloroperoxybenzoic acid has been shown to be a diffusion-controlled process.[27] Unfortunately studies of the effects of pH on the cfh reactions up to pH values approaching the pK_a of the peroxyacids are complicated by the onset of cfh dimerization. Indeed stopped-flow studies of reaction of cfh with *m*-chloroperoxybenzoic acid at pH = 7 yield an apparent stoicheiometry of 1.47:1 rather than 1:1, indicative of increased complexity in the system. Thus the question as to whether monomeric cfh reacts with unionized peroxyacid, with peroxyanion or with both forms of the oxidant is not readily resolved.

A possible strategy for further development of iron–porphyrin models of hydroperoxidase enzymes is further to modify the ligand structure so as to add steric blocking to the electrostatic blocking of haem–haem interactions employed in the present work. This approach has been used[28] in studies of (**5**) which has been reported to form no detectable dimer in the pH range 3.25—9.71. At the present time no direct demonstration of the formation of a Compound I species has been reported for this system. Steady-state studies of the kinetics of coupled oxidations with H_2O_2 show that this approach presents additional problems in that the catalytic activity is much reduced and a more complex pattern of reactivity of ferrihaem with oxidant is observed.

The instability of ch-I in the absence of added donor contrasts with the stability of *e.g.* horse radish peroxidase Compound I. Reformation of cfh from ch-I under these conditions, together with oxidative degradation of some of the haem suggests that, in the absence of protective protein, self-destructive processes involving either haem–haem interactions or haem–solvent interactions occur. The nature of these processes is addressed in the following paper.[20]

Acknowledgements

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