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A comparison by magnetic circular dichroism of compound X and compound II of horseradish peroxidase

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The chlorite product of horseradish peroxidase, compound X, is shown by magnetic circular dichroism (MCD) spectroscopy in the temperature range 1.6–50 K to have a very similar haem structure to compound II under the same conditions (pH 10.7). Both are concluded to contain the Fe(IV)=O group. The MCD spectrum also detects an unusual species, absorbing at wavelengths between 600 and 750 nm, that has magnetic properties different from those of the ferryl haem group. It is suggested that this is a species at the same oxidation level as ferryl haem but with the porphyrin ring having suffered a one-electron oxidation, i.e. [Fe(III) $P^{\cdot+}$].

Horseradish peroxidase; Compound II; Compound X; Magnetic circular dichroism

1. INTRODUCTION

Certain enzymes, such as chloroperoxidase from *Caldariomyces fumago* [1] and bromoperoxidase from *Penicillus capitatus* [2] are known to be capable of the two-electron oxidation of halide ions to form electrophilic halogenating species. Thus, in the presence of peroxide, halide ion and a halogen acceptor they will catalyse formation of a carbon-halogen bond:

$$AH + X^- + H_2O_2 \longrightarrow AX + H_2O + OH^- \quad (1)$$

Although HRP does not catalyse this process, it will promote an analogous chlorination reaction when chlorite (ClO_2^-) acts as both oxidant and halogen donor [3].

There has been much confusion in the literature concerning the mechanism of HRP-mediated chlorination and two contrasting views have consistently been expressed. One concept is that the process occurs in two stages. In the first step the reaction between HRP and chlorite produces another inorganic oxidised chlorine species; subsequently this species, unlike chlorite itself, can chlorinate an acceptor molecule by a non-enzymatic pathway. The opposing view is that there exists a 'chlorinating intermediate' of HRP following reaction with chlorite. This species would contain an enzyme-bound activated, electrophilic form of chlorine and be capable of passing it directly to a suitable nucleophilic acceptor.

The name compound X was given to an unstable intermediate formed rapidly at pH 4.5 on addition of chlorite to ferric HRP [4,5]. This was equated to the postulated electrophilic chlorinating form of HRP, and a ferric haem with an -OCl ligand was suggested as the structure. Later it was discovered that the reaction at pH 10.7 produces a stable species. Shahangian and Hager [6] demonstrated that up to 93% of the molecules contained chlorine derived from the added chlorite and that subsequent acidification to pH 4 allowed transfer of the chlorine to an acceptor.

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Abbreviations: HRP, horseradish peroxidase; $P^{\cdot+}$, the protoporphyrin IX radical cation

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This, the alkaline product of HRP and chlorite, is the compound X examined by various spectroscopic techniques. It has an absorption spectrum similar to that of compound II [7] which is the ferryl catalytic intermediate of HRP containing an Fe(IV) = O centre [8], and Fe(IV)-OCl has been proposed as a likely structure for compound X [6]. The Mössbauer spectra showed that compound X is indeed an Fe(IV), $S = 1$ haem species, probably with an axially coordinated oxygen [9], but the authors considered that small differences in the parameters measured for compounds II and X might indicate chlorine attachment to the ferryl oxygen of the latter. On the other hand, resonance Raman spectra of the two derivatives detected no differences in haem structure [10].

Much information is present in the MCD spectrum of a haemoprotein. The form and temperature dependence of the spectrum allow precise identification of the iron oxidation state and spin state, and band positions are sensitive to the nature of the axial ligands [11,12]. We have therefore undertaken variable temperature MCD measurements of compounds II and X in order to search for differences in their haem environments, especially with regard to the proposed -OCl ligand of the latter.

2. MATERIALS AND METHODS

HRP (Sigma, type VI) was purified by cation-exchange fast protein liquid chromatography (Pharmacia). The details will be published elsewhere (Foote, N. et al., unpublished). The purified preparation was dialysed extensively against water deionised to 18 $M\Omega \cdot cm^{-1}$ by a Milli-Q system (Millipore), freeze-dried, and then redissolved in a mixture of Mops, Ches, Caps and Taps buffers (Sigma) at pH 7.3 containing 50% (v/v) glycerol (Ultrapure, Bethesda Research Laboratories). HRP concentrations were determined by using $\epsilon_{403} = 102\ mM^{-1} \cdot cm^{-1}$ [13]. The enzyme was pre-treated with a stoichiometric amount of hydrogen peroxide (Sigma) in order to minimise the amount of oxidisable impurities in the solution [14]. When the HRP had returned to the ferric state the pH was adjusted to 10.7 by the careful addition of 0.1 M NaOH.

Compound X was prepared by the addition of 1.0–1.5 molar equivalents of sodium chlorite (BDH, technical grade) that had been assayed iodometrically [15]. The reaction took 1 h or more to reach completion. Compound II was formed by the addition of 1.0–1.5 equivalents of hydrogen peroxide; impurities still present in the sample were sufficient to cause the slow decay of compound I to compound II. Once formed, both compound X and compound II samples were stable for several hours.

MCD measurements were made with a Jasco J-500D spectropolarimeter. The samples were held in an Oxford Instruments SM-4 split-coil magnet with a maximum field strength of 5 T. Absorption spectra were recorded with a Pye-Unicam SPB-200 spectrophotometer. Absorption and MCD spectra were re-plotted using a Hewlett-Packard plotter with programs written in this laboratory.

3. RESULTS AND DISCUSSION

Room-temperature absorption spectra of ferric HRP and its chlorite product, recorded at pH 10.7 in the presence of 50% (v/v) glycerol, are shown in fig.1. Identical spectra were recorded in the absence of glassing agent. No discernable differences were found between the spectra of compound X and compound II (not shown) under similar conditions.

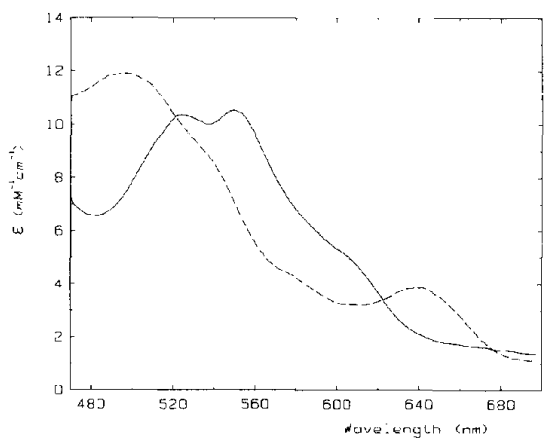

Fig.1. Room-temperature absorption spectra of ferric horseradish peroxidase and compound X. (– – –) Ferric HRP; (——) compound X. The enzyme concentration was 15.5 μM and the pH 10.7. The solution contained Ches, Mops, Caps and Taps buffers, each at 5 mM, and 50% (v/v) glycerol. Compound X was formed by the addition of 15.5 μM sodium chlorite. The path length was 1 cm.

MCD spectra exhibit variations in both sign and intensity as a function of wavelength. They therefore contain more information than corresponding absorption spectra. In fig.2 are compared the visible-region MCD spectra of compounds X (upper) and II (lower) at pH 10.7. The spectra were recorded over the temperature range 1.6–50 K.

It is immediately apparent in both sets of spectra that the temperature dependence of the MCD

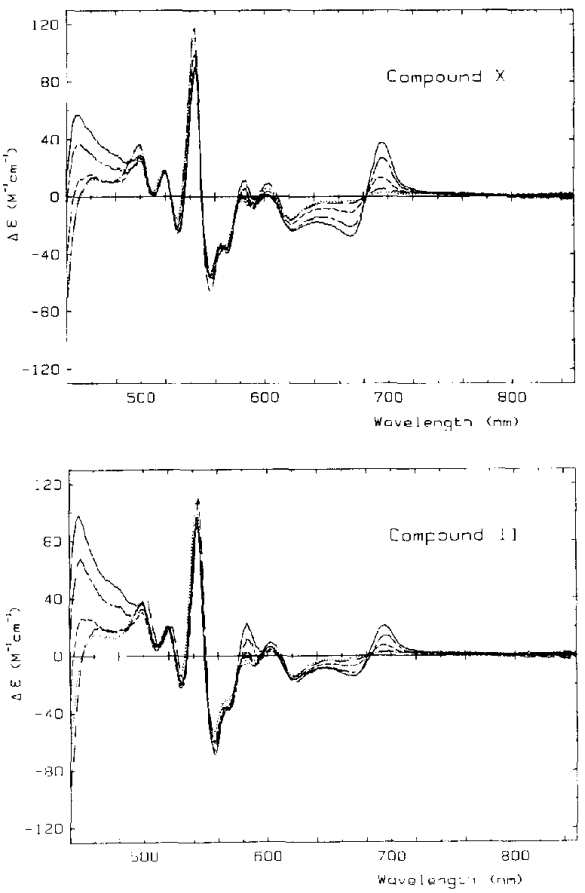

Fig.2. Temperature dependence of the visible-region MCD spectra of horseradish peroxidase compound X and compound II. (Upper) Compound X, (lower) compound II. (——) 1.6 K, (-----) 4.2 K, (---) 10 K, (– – –) 25 K, (···) 50 K. The enzyme concentration was 403 μM and the buffer composition the same as in fig.1. Compound X was formed by the addition of 600 μM sodium chlorite, and compound II by the addition of 600 μM hydrogen peroxide (see section 2). The path length was 1 mm and the magnetic field strength 5 T.

bands between approx. 500 and 570 nm is markedly different from that of the signals in the regions 440–500 and 570–750 nm. There must be at least two components in the samples.

The bands between 500 and 570 nm vary only slightly in intensity in the range 1.6–50 K. They have the general form previously measured for HRP compound II at room temperature [16] and at 208 and 127 K [17]. The peroxide compound of yeast cytochrome *c* peroxidase, which contains Fe(IV) = O haem [18], displays a similar MCD spectral form with a similar temperature dependence (Gadsby, P.M.A. et al., unpublished). Within the resolution of the spectropolarimeter (0.5 nm) the peaks and troughs in this wavelength region correspond exactly in the two sets of spectra of fig.2, with no detectable differences that might be ascribed to different axial ligands.

We take these results as evidence for Fe(IV) = O haem species, at approximately equal concentrations in the two samples of fig.2, giving rise to the MCD bands at 500–570 nm.

The other spectral features in fig.2 show marked dependence on temperature in the range employed. There are positive peaks at 448 and 584 nm, and a derivative-shaped feature centred around 682 nm. The MCD characteristics are quite unlike those of compound I [19], the ferrous-oxy compound III [17], or ferric peroxidase in either its neutral (high-spin) or alkaline (low-spin) form (Foote, N. et al., unpublished). The temperature-dependent bands do not therefore appear to arise from any of the HRP species that might be expected as minor contaminants.

The fact that there are optical bands at long wavelength (between 600 and 750 nm) raises the possibility that these spectral features arise from a form of HRP at the Fe(IV) oxidation level in which an electron has been removed from the porphyrin ring and passed to the metal, producing a π-cation radical containing ferric iron [20,21].

The two states, which can be represented as [Fe(IV) = O P] and [Fe(III) $P^{\cdot+}$], are at the same overall oxidation level and might well be in equilibrium with one another. We cannot at this stage estimate the concentration of either species without well-characterised model spectra to guide us. The two derivatives, compounds II and X, display some differences in intensity of the strongly temperature-dependent MCD signals. The

significance of this observation is not clear but the fact that these differences are not accompanied by noticeable variations in the other, ferryl, haem bands between 500 and 570 nm does imply that the Fe(IV) = O state is the majority species.

No EPR signal was detected from the paramagnetic component identified in the MCD spectra, but spin coupling between the ferric ion and the π-cation radical might abolish the EPR signals expected in the absence of interaction. It is quite possible that the species containing an oxidised porphyrin ring has not been detected in Mössbauer spectra [9] because it is present at a low concentration. Resonance Raman experiments [10] would detect positively only the Fe(IV) = O haem present.

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