pH-dependent forms of the ferryl haem in myoglobin peroxide analysed by variable-temperature magnetic circular dichroism

Nicholas FOOTE,* Paul M. A. GADSBY,† Colin GREENWOOD† and Andrew J. THOMSON†‡

*School of Biological Sciences and †School of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.

The reaction product of myoglobin and H_2O_2 exists in two different forms according to the external pH. Varied-temperature magnetic-circular dichroism (m.c.d.) spectroscopy demonstrates that both contain the oxyferryl ion Fe(IV)=O . Alkaline myoglobin peroxide has often been used as a model for oxidized intermediates in the catalytic cycles of haem-containing peroxidases, but absorption and m.c.d. spectra show that the acid form is much more closely related to species such as horseradish peroxidase Compound II. The differences are tentatively ascribed to ionization of the proximal histidine ligand in alkaline myoglobin peroxide. It is also shown that the m.c.d. method allows an estimate of the zero-field splitting parameter of both forms, values of $D = 28.0 \pm 3 \text{ cm}^{-1}$ and $35.0 \pm 5 \text{ cm}^{-1}$ being obtained for the alkaline and acid forms respectively.

INTRODUCTION

Myoglobin reacts with H_2O_2 to form an oxidized compound, termed myoglobin peroxide, which is 1 oxidizing equivalent above the ferric state. It is of interest as a model of the intermediates formed in the enzymic cycle of haem peroxidases, even though myoglobin itself does not follow the same catalytic cycle and in this sense it is not a true peroxidase (George & Irvine, 1959). Nevertheless, a comparison between the electronic and molecular structures of the various peroxide compounds is likely to be of help in indicating the features that are important for effective catalysis.

The relevant peroxidase intermediates for comparison are those in which the haem iron is 1 oxidizing equivalent above the ferric state, namely Compound II of horseradish peroxidase (HRP) and Compound I (or Compound ES) of cytochrome *c* peroxidase (CCP) from yeast. It is now well established by a variety of spectroscopic methods that both contain the oxyferryl ion Fe(IV)=O (Turner *et al.*, 1985; Hashimoto *et al.*, 1986; Penner-Hahn *et al.*, 1986; Chance *et al.* 1986b), which has the low-spin d^4 configuration yielding a ground state with electronic spin $S = 1$ subject to a large positive axial zero-field splitting (Schulz *et al.*, 1979, 1984).

A parallel set of studies has been carried out on myoglobin peroxide. For example, e.x.a.f.s. measurements of horse myoglobin peroxide have suggested an oxyferryl structure and indicated a short Fe(IV)=O bond of 0.169 nm (1.69 Å) (Chance *et al.*, 1986a). Mössbauer studies have demonstrated the electronic structure of the Fe(IV) ion in myoglobin peroxide to be the low-spin (d^4 , $S = 1$) configuration with a positive axial zero-field splitting of approx. 22 cm^{-1} (Schulz *et al.*, 1979). Resonance Raman spectroscopy of the sperm-whale myoglobin peroxide compound at pH 8.6 has detected the Fe(IV)=O stretching frequency at 797 cm^{-1} (Sitter *et al.*, 1985a,b).

However, myoglobin peroxide can exist in two forms, with different absorption spectra, according to the

external pH. The alkaline form is produced when the reaction is carried out at pH values above 6 (King & Winfield, 1963) and is stable for several hours. The acid form is fully developed only at pH values below 5 and is very short-lived at room temperature (King & Winfield, 1966). The studies outlined above have all been carried out on the alkaline form of myoglobin peroxide. They demonstrate unambiguously that alkaline myoglobin peroxide contains the Fe(IV)=O ion. However, Wittenberg (1978) performed careful optical-absorption studies on the pH-dependent peroxide derivatives of kangaroo, horse and sperm-whale myoglobins and showed that it is the acid form of myoglobin peroxide that most resembles HRP Compound II and yeast CCP Compound I. The absorption spectra of the alkaline form of myoglobin peroxide (King & Winfield, 1963; Wittenberg, 1978) and of leghaemoglobin peroxide (Aviram *et al.*, 1978) are unlike those of any peroxidase intermediates so far reported. This important point appears to have been overlooked by the body of literature that seeks to make comparison between the structures of alkaline myoglobin peroxide and the oxyferryl intermediates of peroxidases.

In order to obtain a characterization of both acid and alkaline forms of the myoglobin peroxide compound we have carried out a varied-temperature m.c.d. study in the visible and near-i.r. region between 440 and 800 nm. The form of the m.c.d. spectrum provides an optical fingerprint of oxyferryl haem in different protein environments, and analysis of the field- and temperature-dependence of the m.c.d. intensity can give values of the ground-state electronic spin and the zero-field splitting parameters. An analysis of the temperature-dependence of the m.c.d. spectrum of oxyferryl haem is made in terms of an electronic-ground-state spin triplet, $S = 1$, and orbital singlet subject to an axial zero-field splitting, D . This has enabled values of D to be determined with reasonable precision. We show that, by the criterion of m.c.d. spectrum as well as absorption spectrum, the acid form of myoglobin peroxide is the more similar to HRP

Abbreviations used: m.c.d., magnetic circular dichroism; HRP, horseradish peroxidase; CCP, cytochrome *c* peroxidase.

‡ To whom correspondence should be addressed.

Compound II and yeast CCP Compound I and, as Wittenberg (1978) suggested, contains the oxyferryl ion. The alkaline form is spectrally unique in our experience of such complexes.

MATERIALS AND METHODS

Sample preparation

All reagents were of analytical quality and were obtained from Sigma Chemical Co. or from BDH Chemicals, except where stated.

Horse heart myoglobin (Sigma type II) was dissolved in 50 mM-Taps buffer, pH 8–9. The small amount of ferrous myoglobin present in the preparation was oxidized to the ferric level by treatment with a sub-stoichiometric amount of $K_3Fe(CN)_6$, which was then removed on a Sephadex G-25 column in the same buffer. The sample was then concentrated (and deuterated when desired) in an Amicon ultrafiltration apparatus equipped with a PM10 membrane, and 50% (v/v) ethanediol was added as a glassing agent. Alkaline myoglobin peroxide was generated by the addition of 2–4 molar equivalents of H_2O_2 . The mixture was allowed to react for 4 min at room temperature before being frozen by immersion in liquid He.

The unstable acid form was prepared by a pH-jump method. Alkaline myoglobin was prepared as above but in 5 mM-Taps buffer, pH 8.5, containing 50% ethanediol. After a reaction time of 4 min the myoglobin peroxide solution was rapidly mixed with 0.1 M-acetic acid, also containing 50% ethanediol, and the resulting mixture was frozen within 5 s. The home-built apparatus worked as follows. The plungers of two 0.5 ml syringes, filled with the two solutions, were driven simultaneously by a Perspex block to mix the reactants in a small glass T-piece and to force the resulting mixture through a plastic tube into a 1 mm-pathlength cuvette. As soon as this was filled it was plunged into liquid N_2 and then transferred, frozen, to the sample space of the split-coil magnet. The final pH of the sample was measured as 3.5.

The further purification of horseradish peroxidase (Sigma type VI) has been described elsewhere (Foote *et al.*, 1987). HRP Compound II was prepared by adding 2 molar equivalents of H_2O_2 to ferric HRP, mixed with 1 molar equivalent of *p*-cresol, in 5 mM-Taps buffer, pH 10, containing 50% (v/v) glycerol (Ultrapure grade; Bethesda Research Laboratories).

Instrumentation and data handling

Samples were held in an Oxford Instruments SM-4 split-coil magnet with a maximum field of 5 T. M.c.d. measurements were made with a JASCO J-500D spectropolarimeter and were recorded directly by a computer, which could also be used for the subtraction of spectra. A Cary 17 spectrophotometer was used to measure absorption spectra, which were then transferred manually on to computer files. Spectral data and computer simulations were drawn by use of a Hewlett-Packard plotter. The programs were written in this laboratory by S. J. George.

Analysis of the temperature-dependence of m.c.d. intensity for oxyferryl haem groups

Analysis of the oxyferryl haem system is based on a ground electronic state with spin $S = 1$ that is subject to an axial zero-field distortion to give two states separated

in energy by a parameter D . The evidence from Mössbauer spectroscopy (Schulz *et al.*, 1979, 1984) is that the axial zero-field splitting parameter D of Fe(IV) ion in the ground electronic state lies in the range 20–40 cm^{-1} and is positive, i.e. the upper state has M_s values of ± 1 and the lower state $M_s = 0$. D values of this magnitude mean that the zero-field splitting energy is much greater than the first-order and second-order Zeeman interactions within the $M_s = \pm 1$ state and between this state and the $M_s = 0$ component. The experimental criterion of this is the fact that m.c.d. signal intensities are linearly proportional to magnetic field strength at all experimental temperatures. Analysis of this case, called the linear limit, was first used to determine the zero-field splitting of a ground electronic state by Collingwood *et al.* (1974) for $MoCl_6^{2-}$ doped into a crystal of Cs_2ZrCl_6 .

The intensity of the m.c.d. signal at any wavelength can be expressed in terms of three parameters, the so-called a , b and c terms (Stephens, 1976), only when the m.c.d. intensity is linearly dependent upon magnetic field and in a temperature range where Zeeman interactions are much less than kT , where k is Boltzmann's constant and T is the absolute temperature. In this case the m.c.d. intensity ΔA (where $\Delta A = A_L - A_R$, the absorbances for left- and right-circularly polarized light respectively) can be written in a simplified form as follows:

$$\Delta A = K(b + c/T)B \quad (1)$$

The b term arises from the field-induced mixing of two electronic states and c from the differential thermal population of the Zeeman components of a state that is degenerate in zero magnetic field. K contains a set of proportionality constants and includes terms such as line-widths, sample concentration and pathlength. Since we are concerned only with the relative variation of ΔA with T , we do not evaluate K . B is the applied magnetic field intensity. This simplified expression ignores contributions from a terms because these will be very small compared with the magnitudes of b and c in the case under consideration.

For the oxyferryl haem system, the contributions to ΔA from the $M_s = \pm 1$ and $M_s = 0$ states will be weighted by their respective Boltzmann populations. Hence ΔA can be written as follows:

$$\Delta A/B \cdot K = \alpha_1(b_1 + c_1/T) + \alpha_2(b_2 + c_2/T) \quad (2)$$

The subscripts '1' and '2' refer to the lower and upper states respectively (see Fig. 1) and values of α are the fractional populations of the states. α_1 and α_2 may be expressed in terms of D as follows:

$$\alpha_1 = 1 - \alpha_2 \quad (3)$$

and

$$\alpha_2 = 2 \exp(-D/kT) / [1 + 2 \exp(-D/kT)] \quad (4)$$

c_1 is zero because the lower state is non-degenerate and therefore has no first-order magnetic moment. The expression eqn. (2) simplifies to:

$$\Delta A/B \cdot K = b_1 + \alpha_2(b_2 - b_1) + \alpha_2 c_2/T \quad (5)$$

This expression forms the basis of a computer program in which the values of b_1 , b_2 , c_2 and D are varied in order to fit eqn. (5) to the experimental curves of m.c.d. intensity versus inverse temperature.

At very low temperature, when $kT \ll D$, α_2 tends to zero and $\Delta A/B \cdot K = b_1$. Thus the m.c.d. intensity at low temperature is independent of the temperature and its value determines b_1 . At high temperature the last term in

Fig. 1. Simulated plots of the intensity of the m.c.d. signal against the inverse of the absolute temperature (T) for a ground-state spin, $S = 1$, subject to a positive axial zero-field splitting (D)

The parameters, α_1 , α_2 , b_1 , b_2 and c_2 are defined in the text and the plots are obtained from eqn. (5). All curves have $B = 5$ T and $c_2 = 100$. b_1 is constrained equal to b_2 in this illustration only. The parameter values are as follows:

Plot	$b_1 (= b_2)$	D (cm $^{-1}$)	T at maximum of curve (K)
a	1.34	10	9.8
b	0.90	15	14.8
c	0.68	20	19.7
d	0.54	25	24.6
e	0.45	30	29.5
f	0.34	40	39.3
g	0.22	60	59.0

eqn. (5) tends to zero and, from eqn. (4), α_2 reaches a value of $\frac{2}{3}$. The intercept at $1/T = 0$ is therefore equal to $(b_1 + 2b_2)/3$, from which b_2 can be estimated. As the results of the simulations are normalized to '% of maximum intensity' the values of b_1 , b_2 and c_2 are all relative to one another. c_2 is therefore held constant and b_1 and b_2 are adjusted to fit the points at low temperature and the intercept at infinite temperature.

The major determinant of the temperature at which the maximum occurs in the simulated plots is the value of D , the zero-field splitting parameter. This is illustrated in Fig. 1, in which the value of D in a sample simulation is varied from 10 to 60 cm $^{-1}$. It is evident that the position of the maximum depends sensitively on this number but is not independent of the values of b_1 and b_2 .

It is significant that the intercepts at $1/T = 0$ in Figs. 4 and 7 all have high positive values. If b_1 and b_2 arose solely from field-induced mixing of the $M_s = 0$ and $M_s = \pm 1$ states, then it would be expected that $b_1 = -2b_2$, and the intercepts would be zero. In fact, the best fits to the experimental data occur with $b_1 \approx b_2$.

This finding arises for the following reasons. All the haem optical bands are likely to be polarized x - y , i.e. in the plane of the porphyrin ring. As a consequence the Zeeman interactions responsible for the b and c terms (as well as the a term) arise only from the component of the

applied magnetic field along the haem z -axis, the normal to the haem plane. This selection rule stems from the fact that the circularly polarized light-beam is propagating along the direction of the applied magnetic field and hence the electric-dipole components of the electromagnetic radiation are perpendicular to the direction of the applied magnetic field. If the electron-dipole transitions are polarized in the x - y molecular axis frame then the appropriate magnetic-field component must be parallel to the z -axis. There is no second-order Zeeman interaction between $M_s = 0$ and $M_s = \pm 1$ components of the ground electronic state because a magnetic field along the z -axis does not mix these two components. Alternatively stated, these components are eigenfunctions of the z component of the Zeeman operator. Thus no b terms between these two components will arise when the resulting optical transitions are x - y -polarized. The b terms that are observed must result from the second-order Zeeman effect between the two ground-state components, $M_s = 0$ and $M_s = \pm 1$, and excited electronic states.

RESULTS

Absorption spectra

Low-temperature optical-absorption spectra of the acid and alkaline forms of myoglobin peroxide are displayed in Fig. 2. These spectra were recorded from the same samples as were used in the m.c.d. study, since the split-coil magnet can be mounted in a specially enlarged sample compartment of a Cary 17 spectrophotometer.

The method employed by Wittenberg (1978) to generate the unstable acid species for examination at low temperature relied on the temperature-dependent pH

Fig. 2. Low-temperature absorption spectra of ferryl haem in (a) HRP Compound II, (b) acid ferryl myoglobin, pH 3.5, and (c) alkaline ferryl myoglobin, pH 9.0

Spectra (a) and (b) were recorded at 1.6 K and spectrum (c) at 4.2 K. For clarity the base-lines have been offset by 4 units of ϵ (mm $^{-1}$ ·cm $^{-1}$). See the text for preparation conditions of ferryl myoglobin. HRP Compound II (300 μ M-haem) in 1 mM-Mes/5 mM-Caps diluted to 50% (v/v) of glycerol, adjusted to pH 10 with NaOH in the presence of 600 μ M-*p*-cresol, was frozen within 30 s after addition of 3 molar equivalents of H $_2$ O $_2$.

Fig. 3. M.c.d. spectra of alkaline myoglobin peroxide at 5 T

Sample conditions were as indicated in Fig. 2 legend. Spectra were measured at various temperatures as indicated on the Figure.

properties of borate buffers. The more stable alkaline myoglobin peroxide compound was prepared in borate buffer at pH 8.3: on cooling, the solution became acidic and the compound was observed to adopt the characteristics of the acid form at 77 K. Unfortunately, we found that this procedure was not effective in the presence of glassing agent necessary for m.c.d. experiments. Therefore we developed an alternative technique (see the Materials and methods section) whereby solutions (containing 50% glycerol) of alkaline myoglobin peroxide and acetic acid were rapidly mixed, squirted into a cuvette and then frozen in liquid N₂ within a few seconds.

Comparison of the spectra of Fig. 2 with the 77 K data of Wittenberg (1978) shows that the same species have successfully been generated by our method. Both peak positions and absorption coefficients in the two sets of spectra are in good agreement. Fig. 2 also includes the spectrum of a sample of horseradish peroxidase Compound II, which clearly resembles the acid form of myoglobin peroxide more closely than it does the alkaline form.

The m.c.d. spectra of the two pH-dependent forms of myoglobin peroxide are given in Figs. 3 and 5. It is evident that they yield very different results. We discuss first the analysis of the alkaline form.

Myoglobin peroxide at high pH

The characteristics of the m.c.d. spectrum of alkaline myoglobin peroxide (Fig. 3) are signals of alternating sign that correspond to bands in the absorption spectrum (Fig. 2). For example, the absorption peaks at 578 and 592 nm are associated with m.c.d. bands at 573 nm (positive) and 595 nm (negative). The pattern is repeated in the signals at 525 and 548 nm, which correspond to the absorption peak at 545 nm. The polarized single-crystal absorption spectrum of sperm-whale myoglobin peroxide compound in the alkaline form, reported by Makinen & Chung (1983), shows that these absorption bands are polarized in the plane of the haem ring. They undoubtedly arise from the porphyrin π - π^* transitions corresponding

to the Q-bands normally seen in the visible region of the spectra of low-spin haemoproteins.

Of particular interest are the positive and negative m.c.d. bands between 700 and 760 nm. Corresponding absorption bands are difficult to detect. Indeed, Wittenberg (1978) lays some emphasis on the absence of absorption bands between 700 and 1300 nm. A small peak at approx. 720 nm can be seen in the absorption spectrum of alkaline myoglobin peroxide presented by Bagger & Williams (1971), but is not commented upon by the authors. An absorption band at 740 nm is, however, quite clear in the single-crystal spectra obtained by Makinen & Chung (1983). It is shown that the band is polarized in the haem plane with an underlying diffuse z-polarized component. These authors assign the band to a charge-transfer transition from the porphyrin filled π orbitals (a_{1u} and a_{2u}) to the Fe(IV) d orbitals d_{xz} and $d_{yz}(e_g)$.

The temperature-dependence at 5 T of some of the main features of the spectrum is plotted in Fig. 4. The graphs show the variation in peak-to-trough m.c.d. signal intensity as a function of $1/T$, the inverse of the absolute temperature. The spectra of Fig. 3 also contain a contribution from a minor species with different paramagnetic properties, which is most evident between 460 and 510 nm.

The plots of Fig. 4 all display the same overall shape, namely a region between 1.6 K and about 4.2 K in which the m.c.d. intensity is independent of temperature, followed by a rise in intensity to a peak at approx. 24 K and a subsequent diminution at higher temperatures. This behaviour is consistent with a ground magnetic state of spin $S = 1$ with a positive axial zero-field splitting, leading to a singlet component ($M_s = 0$) that lies below a doublet ($M_s = \pm 1$) (see Fig. 1). A plot (not shown) of the magnetic-field-dependence of the m.c.d. intensity at constant temperature was linear up to 5 T (at 4.2 K and above).

The simulation routine described in the Materials and methods section has been used to model this temperature-dependence. The best fit to the experimental data is obtained with $D = 28 \text{ cm}^{-1}$ with an estimated error of $\pm 3 \text{ cm}^{-1}$, which gives satisfactory fits to the intensity data measured at three different wavelengths in the m.c.d. spectrum of alkaline myoglobin peroxide (continuous lines in Fig. 4). The fitting parameters are given in the caption to Fig. 4.

In our samples of alkaline myoglobin peroxide there existed a minor component that was sensitive to irradiation at 4.2 K with the white light from a 200 W projector lamp. The form of the m.c.d. spectral change was characteristic of the conversion of low-spin into high-spin ferrous haem. Despite pretreatment with ferricyanide of the metmyoglobin used to prepare the samples, it seems that the subsequently formed myoglobin peroxide preparations contained a proportion of oxymyoglobin, which could be reversibly photolysed to the deoxy state at low temperature. Using the published differential absorption coefficient (Brittain *et al.*, 1982), we estimate that less than 1% of the sample was in this form. An earlier study by resonance Raman spectroscopy of photolysis with laser light at 80 K (Campbell *et al.*, 1980) suggested that the myoglobin peroxide compound undergoes two-electron photoreduction to give high-spin ferrous myoglobin. We have been unable to find any evidence of such a process. The more likely explanation

is the presence in the resonance Raman sample of significant amounts of the O₂ adduct of ferrous myoglobin, which underwent photolysis in the laser beam to the high-spin ferrous form.

Myoglobin peroxide at low pH

M.c.d. spectra (440–650 nm) of this sample, measured at 5 T and at temperatures from 1.6 to 100 K, are given

in Fig. 5. There appear to be two species present with quite different temperature-dependences to their m.c.d. intensities: one dominates the region between 440 and 500 nm, the other between 500 and 550 nm with a further band close to 620 nm. The magnetic-field-dependences of the two species are different as well, as shown in Fig. 6.

A plot of the variation with magnetic field of the m.c.d. intensity at 543 nm, measured at several different temperatures, is presented in Fig. 6(a). At all temperatures above 4.2 K the intensity varies linearly with field strength. The temperature variation at constant field (5 T) for the same peak is also shown (Fig. 7). The form of this plot is very similar to those of the signals arising from alkaline myoglobin peroxide (Fig. 4), indicating that the electronic ground state is very similar and identifying the species as oxyferryl haem. However, the maximum intensity of the signal at 543 nm occurs at 31 ± 3 K, rather higher than the alkaline myoglobin case. This is reflected in a higher value of D of 35.0 ± 5 cm⁻¹ obtained from the simulated curve (continuous line in Fig. 7). Although the m.c.d. spectrum of the low-pH form of myoglobin peroxidase has contributions from two paramagnetic species, the region of 543 nm is dominated by one of the species, namely the ferryl haem. This is clear from Fig. 7, which shows the signal to be temperature-independent over the range 1.6–10 K. The temperature-dependence of the other paramagnet is steep over this temperature range, as shown by the m.c.d. spectrum between 440 and 400 nm (Fig. 5).

In Fig. 6(b) are presented the magnetization properties of the peak at 451 nm, plotted according to the convention of Thomson & Johnson (1980). The m.c.d. intensity at 1.6 K at first rises linearly with increasing magnetic field, but becomes virtually independent of field strength above 4 T. Such behaviour is characteristic of an electronic ground state that is a doublet. There are two possible interpretations of the observed magnetic behaviour, between which we cannot distinguish with the present data.

The first is that the whole of the sample is in the Fe(IV) state, but that a fraction of the population is in an alternative conformation with an oppositely signed axial distortion relative to the predominant oxyferryl species. This could be caused by protonation or loss of an axial

Fig. 4. Temperature-dependence of the m.c.d. intensity at 5 T of alkaline myoglobin peroxide plotted as peak-to-trough intensity differences at (a) 532–550 nm, (b) 572–550 nm and (c) 720–745 nm

The data points are marked *. The continuous lines are computer simulations of the experimental curves using the following parameters in eqn. (5):

	(a)	(b)	(c)
B (T)	5.0	5.0	5.0
c_2	100	100	100
b_1	2.5	1.75	0.5
b_2	1.0	1.1	0.1
D (cm ⁻¹)	28.0	28.0	28.0
T_{max} , simulated (K)	21.2	24.5	25.6
T_{max} , experimental (K)	22.5 ± 1.5	23 ± 2	20–30

T_{max} is the temperature at which the m.c.d. exhibits a maximum intensity, experimentally and as simulated.

Fig. 5. M.c.d. spectra of acid myoglobin peroxide at 5 T

Sample conditions were as indicated in Fig. 2 legend. Spectra were recorded at several temperatures as indicated.

Fig. 6. M.c.d. magnetization curves of acid myoglobin peroxide recorded at (a) 543 nm and (b) 451 nm

Each line gives the magnetic-field-dependence of the m.c.d. intensity at a constant temperature as indicated on the Figure. The sample conditions were as indicated in Fig. 2 and Fig. 5 legends.

Fig. 7. Temperature-dependence of the m.c.d. intensity at 5 T of acid myoglobin peroxide plotted at 543 nm

The data points are marked *. The continuous line is a computer simulation of the curve using eqn. (5) in the text and the following parameters: $B = 5$ T, $b_1 = 3.45$, $b_2 = 2.8$, $c_2 = 100$ and $D = 35.0$ cm⁻¹. The temperature of the maximum m.c.d. intensity of the data points is 31 ± 3 K and the temperature of the maximum in the simulated curve is 29.7 K.

ligand, giving rise to a change in the zero-field splitting parameter from a large positive value to a small negative value. The lower component of the state would be an effective doublet with $M_s = \pm 1$ and the upper a singlet with $M_s = 0$. The magnetization curves of Fig. 6 can indeed be simulated, by using a different computer program, by a ground state of $S = 1$ subject to an axial zero-field splitting of between -2 and -3 cm⁻¹. Without a suitable model system, we are unable to speculate about the quantity of such a species, if any, present in the sample.

The second explanation is partial decay of the myoglobin from ferryl to the low-spin ferric oxidation state, which has been reported to be the ultimate product of reaction between Fe(IV) myoglobin and excess peroxide at low pH (King & Winfield, 1966), and could be the aquo form of metmyoglobin. Magnetization curves measured for this species would be expected to overlay each other at all temperatures, but the nesting effect seen in Fig. 6(b) could arise from spectral contamination at 451 nm by a small negative contribution from the oxyferryl state. The strongly temperature-dependent signals in Fig. 5 can be resolved in a calculated temperature-difference spectrum (1.6 K minus 50 K; results not shown). This procedure virtually eliminates signals arising from the species with the temperature-dependence of Fig. 7, namely the 'normal' oxyferryl state, which gives approximately the same m.c.d. intensity at these two temperatures. The resulting spectrum is not dissimilar to that of low-spin ferric haem (Foote *et al.*, 1987). If this is the correct assignment, it is estimated that less than 5% of the sample of Fig. 5 was in the low-spin ferric state. No low-spin ferric e.p.r. signal could be detected from an acid myoglobin peroxide sample; however, the species might have been present at too low a concentration.

Fig. 8. M.c.d. spectra at 50 K and 5 T of ferryl haem in (a) alkaline myoglobin peroxide, (b) acid myoglobin peroxide and (c) HRP Compound II

The conditions of the samples were as indicated in Fig. 2 legend.

DISCUSSION

Features of the m.c.d. spectrum

The technique of m.c.d. spectroscopy has allowed, for the first time, magnetic measurements on the unstable acid form of myoglobin peroxide as well as the longer-lived alkaline form. Despite their rather different absorption and m.c.d. spectra, the two pH-dependent states of myoglobin are both $S = 1$ systems with large positive zero-field splitting parameters. Analysis of the temperature-dependence of the m.c.d. spectra of these two forms has given values of $D = 35 \pm 5 \text{ cm}^{-1}$ for the acid form and $D = 28 \pm 3 \text{ cm}^{-1}$ for the alkaline form. Simulation of the temperature-dependence of the high-field Mössbauer spectrum of 'metMb-H₂O₂' compound gave a value of $D = 22 \text{ cm}^{-1}$ (35 K) (Schulz *et al.*, 1979). No details of the sample pH or buffer are quoted by these workers. But it must be presumed that this analysis refers to the more readily prepared alkaline form of myoglobin peroxide. This value of D is considerably lower than the value obtained from the m.c.d. analysis. A similar difference in D values obtained by the two methods of m.c.d. and Mössbauer spectroscopy has emerged from an analysis of the temperature-dependence of the m.c.d. spectrum of HRP Compound II (Browett *et al.*, 1988), which gave a D value of $32 \pm 5 \text{ cm}^{-1}$ compared with a value of 22 cm^{-1} for the same compound from Mössbauer analysis (Schulz *et al.*, 1979).

The contaminating signals, noticeable throughout the range 440–610 nm in Fig. 5, have an m.c.d. intensity strongly dependent on the temperature (Fig. 6b), and at 50 K their contribution to the m.c.d. spectrum is virtually negligible. Therefore the 50 K m.c.d. spectra compared in Fig. 8 represent the m.c.d. features of $S = 1$ haem iron, with a large positive D , in three different protein environments, namely HRP Compound II and the two pH-dependent forms of myoglobin peroxide.

As in the absorption spectrum (Fig. 2) it is evident from Fig. 8 that the acid form of myoglobin peroxide closely resembles HRP Compound II whereas the alkaline form is rather different. In particular, the distinctive features at 700–760 nm are present only in alkaline myoglobin peroxide. We suggest that acid myoglobin peroxide is the more relevant model for oxyferryl peroxidase intermediates.

The other signals in the acid myoglobin peroxide spectrum, with the magnetization characteristics of Fig. 6(b), cannot be assigned with certainty. They arise either from a partial decay product, such as low-spin ferric haem at a level of less than 5%, or from an alternative structural form of the Fe(IV) state, in which case they cannot be quantified. Similar signals have been observed in the low-temperature m.c.d. spectra of HRP Compound II and yeast CCP Compound I (Thomson *et al.*, 1988), but further work is required to establish their origin and possible importance in reactivity of oxyferryl haem.

Difference between acid and alkaline myoglobin peroxide

Although acid and alkaline myoglobin peroxide contain the oxyferryl group with a ground state of $S = 1$ and slightly different D values, the absorption and m.c.d. spectra of the two are quite distinct from one another. The data here do not establish unambiguously the position of protonation responsible for the pH-dependence of the spectra. There are two clear possibilities. The presence of a hydrogen bond between the N-H group of the distal histidine residue and the oxygen atom of Fe(IV)=O has been suggested by resonance Raman studies of HRP Compound II, which show a pH-dependence of the Fe(IV)=O stretching frequency (Sitter *et al.*, 1985a,b). However, it seems unlikely that hydrogen-bonding to the ferryl oxygen atom could lead to such a major change in the optical spectrum of the haem group. It is known, again from resonance Raman studies, that the Fe(IV)=O stretch varies over a wide range from 776 cm^{-1} in HRP Compound II, pH 6.0, to over 850 cm^{-1} in model porphyrin ferryl complexes (Kean *et al.*, 1987). It is concluded that there is a *trans*-ligand effect on the ferryl bond strength. We suggest that deprotonation of the proximal histidine ligand may be occurring in the high-pH form of myoglobin peroxide and that it is the change in the nature of the axial ligation from histidine to histidinate that is responsible for the change in the form of the optical and m.c.d. spectra.

Loss of the N-1 proton of a histidine group coordinated to Fe(III) haem can occur with pK_a values varying between 7.0 (leghaemoglobin; Sievers *et al.*, 1983), 8.9 (*Escherichia coli* cytochrome *b-562*; Moore *et al.*, 1985) and 11.0 (metmyoglobin; Gadsby & Thomson, 1982). The pK_a depends upon the ability of neighbouring groups to stabilize histidinate, i.e. their basic strength. In this case both the protein residues and the polarizing power of the iron are important. Fe(IV)=O

is likely to be as polarizing as Fe(III), if not more so, and should therefore lead to considerable lowering of the pK_a of the N-1 of a co-ordinated histidine group. The pK_a in myoglobin peroxide has not been determined owing to the instability of the acid form, but must be in the region of 6.

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