Redox-linked spin-state changes in the di-haem cytochrome *c*-551 peroxidase from *Pseudomonas aeruginosa*

Nicholas FOOTE,* Jim PETERSON,† 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.

(Received 20 February 1985/12 April 1985; accepted 29 April 1985)

Magnetic-c.d., e.p.r. and optical-absorption spectra are reported for the half-reduced form of *Pseudomonas aeruginosa* cytochrome *c*-551 peroxidase, a di-haem protein, and its fluoride derivative. Comparison of this enzyme species with oxidized peroxidase shows the occurrence of spin-state changes at both haem sites. The high-potential haem changes its state from partially high-spin to low-spin upon reduction. This is linked to a structural alteration at the ferric low-potential haem group, causing it to change from low-spin to high-spin. Low-temperature spectra demonstrate photolysis of an endogenous ligand of the high-potential haem. In addition, an inactive form of enzyme is examined in which the structural change at the ferric low-potential haem does not occur on reduction of the high-potential haem.

The cytochrome *c*-551 peroxidase enzyme isolated from *Pseudomonas aeruginosa* comprises a single polypeptide chain, to which are covalently bound two haem groups (Ellfolk & Soininen, 1971). These have widely separated oxidation-reduction potentials, estimated as –330 and +320 mV (Ellfolk *et al.*, 1983), the former value being close to the redox potentials of other peroxidases. Thus the LP haem centre is probably the site of H₂O₂ binding.

In the oxidized enzyme state neither haem group will readily bind added ligands. Reduction of the HP haem group alone can be accomplished with NADH, ascorbate, ferrocyanide or the physiological electron donors azurin and cytochrome *c*-551, and this allows the binding of ligands such as fluoride and cyanide to the LP haem (Ellfolk *et al.*, 1984). This is clear evidence for an interaction between the two haem groups. Kinetic studies (Ronnberg & Ellfolk, 1978; Ronnberg *et al.*, 1981) have demonstrated that the half-reduced enzyme reacts rapidly and productively with H₂O₂ and is therefore presumed to be involved in the catalytic cycle. Thus the haem–haem interaction appears to have an important functional role in this enzyme.

This effect is of considerable interest, as the di-

haem *Ps. aeruginosa* cytochrome *c*-551 peroxidase may prove to be a model for the study of haem–haem interactions in electron-transfer proteins. In particular, information about the mechanism of the interaction can be provided by monitoring the haem iron spin states and axial ligands.

We have previously established certain properties of the two ferric haem centres in the oxidized form of the enzyme (Foote *et al.*, 1984), using mainly m.c.d. spectroscopy. In oxidized *Ps. aeruginosa* peroxidase the spin state of one haem group, assigned to be the HP centre, was found to vary with temperature: at 4.2 K it is wholly low-spin with histidine–methionine axial ligation, but at room temperature a proportion (estimated to be 40–50%) of this haem iron assumes a high-spin configuration. On the other hand the LP haem is low-spin at both room temperature and 4.2 K in the oxidized enzyme and has histidine–histidine axial co-ordination (Foote *et al.*, 1984). This accounts presumably for the lack of reactivity of this haem group towards added ligands in the oxidized enzyme. It is at first sight a surprising property, since this haem group is the site of peroxide binding, which in other peroxidases is found to be high-spin. However, since the half-reduced form of the enzyme is probably the catalytically active state, we have carried out an investigation of the spin states and axial co-ordination of the haem groups in this form of the enzyme, with both e.p.r.

Abbreviations used: m.c.d., magnetic circular dichroism; HP haem and LP haem, high-potential and low-potential haem groups respectively in cytochrome *c*-551 peroxidase.

and m.c.d. spectroscopy (especially in the near-i.r. wavelength region, which is most useful for assignment of haem axial ligation). We have also made a comparative study of a form of the enzyme that is inactive towards reduction of H_2O_2 and appears to lack the haem-haem interaction.

Materials and methods

Materials

Phenazine methosulphate, Tween 80, NADH (grade III) and Mes were obtained from Sigma Chemical Co. Glycerol and NaF were of AnalaR grade and were purchased from BDH Chemicals. $^2\text{H}_2\text{O}$ (99.8 atom% ^2H) was obtained from Aldrich Chemical Co.

Pseudomonas aeruginosa (N.C.T.C. 6750) was cultivated as described previously (Foote *et al.*, 1983), but the method of purifying cytochrome *c*-551 peroxidase has been improved as follows. Preparation of a cell-free extract, the acid precipitation step and batch chromatography on CM-Sephadex C-50 were as described by Foote *et al.* (1983). The equilibration buffer for the subsequent Sephadex G-100 column was 20 mM-sodium phosphate, pH 6.5. The peroxidase-containing fractions from this column were, without further treatment, passed through a column (3 cm $\times$ 14 cm) of DE-52 DEAE-cellulose equilibrated with the same buffer, and the coloured fractions from this step were pooled. The pool was then concentrated to approx. 5 ml and equilibrated with 25 mM-Mes/NaOH buffer, pH 5.3, in an Amicon ultrafiltration apparatus equipped with a PM10 membrane. Final purification was performed by using a Fast Protein Liquid Chromatography system from Pharmacia Fine Chemicals. Portions (1 ml) of the protein solution were injected on to a Mono S cation-exchange column HR 5/5 (1 ml capacity) equilibrated with 25 mM-Mes buffer, pH 5.3. The peroxidase, which bound tightly, was eluted with a linear gradient of NaCl in the same buffer, and was removed at approx. 0.1 M-NaCl. The pooled peroxidase-containing fractions had a spectral purity ratio A_{407}/A_{280} of 4.72:1 at pH 6.0.

Cytochrome *c*-551 peroxidase was assayed as described previously (Foote *et al.*, 1983), and enzyme concentrations were calculated by using $\epsilon_{407}^{ox} = 237 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Soininen & Ellfolk, 1973). The turnover number (mol of ferrocycytochrome *c*-551 oxidized/mol of enzyme per s) of this preparation was 121 s^{-1} with $14 \mu\text{M}$ *Ps. aeruginosa* ferrocycytochrome *c*-551 and $80 \mu\text{M}$ - H_2O_2 at 25°C in 10 mM-sodium phosphate buffer, pH 6.0. This is higher than values (determined under similar conditions) calculated from the literature, e.g. 84.0 s^{-1} (Table 1 of Ronnberg & Ellfolk, 1975), 58.8 s^{-1} [Table 1 of Ronnberg & Ellfolk (1978):

$13.3 \mu\text{M}$ -cytochrome *c*-551, $84 \mu\text{M}$ - H_2O_2], 101.5 s^{-1} [Foote *et al.* (1983): $12 \mu\text{M}$ -cytochrome *c*-551, $78 \mu\text{M}$ - H_2O_2]. Since the measurement of m.c.d. spectra from 250 nm to 2000 nm takes several hours, the samples of active oxidized and half-reduced peroxidase that were scanned at room temperature were assayed for peroxidatic activity both before and after the experiments. In no case was there a measurable decrease in specific activity.

In a preliminary experiment, maximal peroxidatic activity in Mes buffer was found to occur at pH 5.2 (pD 5.6), and this value was therefore used throughout. The optimum pH in phosphate and acetate buffers has been reported to be 6.0 (Soininen & Ellfolk, 1972). Samples that required degassing were stabilized by the addition of 0.01% (v/v) Tween 80 (Foote *et al.*, 1983).

The inactive enzyme samples used in this work derived mainly from earlier preparations that sometimes tended to lose activity even when spectrally pure ($A_{407}/A_{280} > 4.6:1$). Other samples came from a batch that lost activity at an early stage of a purification procedure when the temperature of the crude enzyme may have risen above 2°C for 12 h. In both cases it is suspected that peptide-bond cleavage may be involved, as a neutral proteinase tends to be co-purified with the peroxidase (Soininen & Ellfolk, 1975). Ion-exchange chromatography, with the Fast Protein Liquid Chromatography system, of an inactive peroxidase sample resolved three major protein peaks. All of these were coloured and appeared still to contain both haem groups, as judged by the optical-absorption spectra of their dithionite-reduced forms.

Half-reduced enzyme samples were prepared anaerobically in a glovebox (Faircrest Engineering) operating with a purified N_2 atmosphere containing less than 1 p.p.m. of O_2 . A slight excess of NADH was used as the reductant, in the presence of the mediator phenazine methosulphate. Even in the presence of a large (350-fold) molar excess of NADH it was demonstrated that no fully reduced peroxidase was formed, since the absorption spectra, and low-temperature photolysis and thermal recombination, were performed as described previously (Foote *et al.*, 1984; Greenspectroscopy were introduced into stoppered cells, and samples for low-temperature spectroscopy were mixed anaerobically with an equal volume of glycerol before being injected into sealed cells.

Measurements of m.c.d., e.p.r. and optical-absorption spectra, and low-temperature photolysis and thermal recombination, were performed as described previously (Foote *et al.*, 1984; Greenwood *et al.*, 1984) except that the magnet used for room temperature m.c.d. spectroscopy was capable of generating a field of 6 T.

Results and discussion

It is now well established that thermal spin equilibria as well as thermally induced changes in axial ligation can take place at the haem sites of cytochrome *c*-551 peroxidase (Ellfolk *et al.*, 1983; Foote *et al.*, 1984). It is therefore necessary to make separate investigations of the axial ligand co-ordination at both liquid-helium and room temperature. The first section describes the results obtained from e.p.r. spectroscopy at 12 K, and m.c.d. and optical-absorption spectroscopy at 4.2 K. The second section refers to data measured with samples at room temperature.

E.p.r. spectra

Oxidized *Pseudomonas aeruginosa* cytochrome *c*-551 peroxidase gives rise to two sets of low-spin e.p.r. signals, shown in Fig. 1(a), with g_z values of 3.00 and 3.27. The half-reduced enzyme species has a single set of low-spin signals arising from the ferric LP haem, but two forms of the e.p.r. spectrum have been observed. One form (Fig. 1c) has a sharp g_z peak at $g = 2.85$, with $g_y = 2.36$ and $g_x = 1.54$. A small e.p.r. signal is also present at $g = 3.28$, the origin of which is unknown and the size of which varied from sample to sample. It is diminished in the presence of 50% glycerol, which is added as a glassing agent for low-temperature optical experiments, but the spectrum is otherwise unaffected (Fig. 1b). The other form of the e.p.r. spectrum of half-reduced peroxidase exhibits a g_z value of 3.00 and $g_y = 2.26$ (Fig. 1e). These closely resemble signals observed in the spectrum of the oxidized enzyme. Steady-state assays demonstrated that samples with the $g_z = 2.85$ e.p.r. signal were fully active in the peroxidatic reaction, whereas those with the $g_z = 3.00$ signal had little or no activity. We therefore designate these respectively the active and the inactive half-reduced enzyme.

Interaction between the two sites in the active form of the enzyme causes the LP-haem e.p.r. signals to shift in g -value when the HP haem is reduced. It is therefore unclear which of the two g_z values of the oxidized enzyme arises from the same haem group as the $g = 2.85$ peak of active half-reduced peroxidase. However, the inactive enzyme form is helpful for the assignment of e.p.r. signals due to the HP and LP haem groups. The e.p.r. signals of inactive half-reduced peroxidase correspond closely to the set of g -values of the oxidized enzyme with $g_z = 3.00$, and these can be assigned with confidence to the LP haem centre.

Addition of 1 M-NaF to the active half-reduced peroxidase produced a large high-spin e.p.r. signal at $g = 6.0$ (Fig. 1d). The small peak remaining at $g = 2.85$ in this spectrum represents a fraction of

the peroxidase molecules that have not bound fluoride. In contrast, fluoride had no effect on the e.p.r. spectrum of inactive half-reduced enzyme.

Low-temperature m.c.d. and absorption spectra

Fig. 2 compares the near-i.r. m.c.d. spectra at 4.2 K and 4.9 T of the active and inactive forms of the half-reduced enzyme. The HP haem is photosensitive when in its low-spin ferrous state, leading to ligand displacement (Greenwood *et al.*, 1984). Low temperatures (below 20 K) inhibit recombination. Therefore, after cooling of the sample to 4.2 K, it was warmed in the magnet sample space to approx. 70 K in the dark, so that thermal recombination could take place, and then re-cooled to 4.2 K, still in the dark, so that a low-spin ferrous form of the HP haem was obtained before measurement of the m.c.d. spectra. The data in Fig. 2 show the spectra obtained after this thermal re-combination procedure and also after prolonged deliberate photolysis of the sample at 4.2 K. It is evident that the active and inactive half-reduced enzyme species yield different near-i.r. m.c.d. spectra in both their photolysed and thermally recombined forms. However, in neither case is there a significant effect of photolysis of the HP haem on the spectrum in the 1200–1800 nm region, which we associate with the LP haem. Low-spin ferric haems are expected to exhibit charge-transfer bands in this part of the spectrum.

The 1450 nm peak in the spectrum of the inactive form is close to the positions of the charge-transfer bands found in bis-histidine-co-ordinated haem groups (Sievers *et al.*, 1983a). The g_z value at 3.00 (Fig. 1c) must belong to the same haem group. This, in addition to the m.c.d. evidence, unambiguously assigns the species to be a bis-histidine-co-ordinated haem. A feature of similar intensity at 1500 nm is observed in the 4.2 K m.c.d. spectrum of the oxidized enzyme (Foote *et al.*, 1984), which has also been assigned to bis-histidine ligation of the LP haem.

The 4.2 K m.c.d. spectrum of the active form of half-reduced peroxidase possesses a maximum in the same wavelength region, but it is a broad band, with the intensity apparently spread over a wider wavelength range. The species that gives rise to this spectrum also generates the rather sharp e.p.r. spectrum (Fig. 1b), with $g_z = 2.85$. The minor peak at $g_z = 3.28$ is too weak to contribute significantly to the m.c.d. spectrum. Therefore we assign the broad m.c.d. peak at approx. 1450 nm to the species that gives the g_z at 2.85, i.e. a low-spin ferric haem, which is the LP moiety. The sharpness of the e.p.r. signal suggests that there is no structural heterogeneity at the site of this haem. We do not fully understand the reasons for the breadth of the charge-transfer band in the m.c.d.

Fig. 1. *E.p.r. spectra of oxidized cytochrome c-551 peroxidase and some derivatives*

(a) Oxidized enzyme, concentration $410\text{ }\mu\text{M}$ in 50 mM -Mes buffer, pD5.6. Gain: 6.3×10^5 . (b) Active half-reduced enzyme, concentration $205\text{ }\mu\text{M}$ in 25 mM -Mes buffer, pD5.6, containing 50% (v/v) glycerol, $400\text{ }\mu\text{M}$ -NADH, $4\text{ }\mu\text{M}$ -phenazine methosulphate and 0.005% (v/v) Tween 80. Gain: 4×10^5 . (c) Active half-reduced enzyme, concentration $410\text{ }\mu\text{M}$ in 50 mM -Mes buffer, pD5.6, containing $800\text{ }\mu\text{M}$ -NADH, $8\text{ }\mu\text{M}$ -phenazine methosulphate and 0.01% (v/v) Tween 80. Gain: 8×10^5 . (d) Active half-reduced fluoride-bound enzyme, concentration $185\text{ }\mu\text{M}$ in 50 mM -Mes buffer, pD5.6, containing $350\text{ }\mu\text{M}$ -NADH, $13\text{ }\mu\text{M}$ -phenazine methosulphate, 0.01% (v/v) Tween 80 and 1 M -NaF. Gain: 2×10^5 . (e) Inactive half-reduced enzyme, concentration $310\text{ }\mu\text{M}$ in 50 mM -Mes buffer, pD5.6, containing $600\text{ }\mu\text{M}$ -NADH, $6\text{ }\mu\text{M}$ -phenazine methosulphate and 0.01% (v/v) Tween 80. Gain: 8×10^5 . Conditions: temperature, 12 K ; microwave frequency, 9.42 GHz ; power, 2.01 mW . The sample-tube diameter was not constant for all samples.

Fig. 2. Near-i.r. m.c.d. spectra of active and inactive half-reduced cytochrome c-551 peroxidase at 4.2 K and 4.9 T, showing the effect of photolysis

—, Photolysed; —, thermally recombined. (a) Active half-reduced peroxidase, concentration 205 μM , containing 400 μM -NADH and 4 μM -phenazine methosulphate. The pathlength was 1.04 mm. (b) Inactive half-reduced peroxidase, concentration 290 μM , containing 225 μM -NADH and 12.5 μM -phenazine methosulphate. The pathlength was 1.07 mm. The buffer was 25 mM-Mes, pH 5.6, containing 50% (v/v) glycerol and 0.005% (v/v) Tween 80.

spectrum. Two porphyrin-to-ferric charge-transfer transitions are thought to occur in this wavelength region, being the transitions from the two highest filled porphyrin molecular orbitals (Rawlings *et al.*, 1977; Schejter & Eaton, 1984). It may be that there is an unusual orientation of the distal ligand that binds the haem at low temperature, causing these two transitions to separate and give rise to an apparently broad charge-transfer band. We are exploring these ideas with the aid of model haem compounds liganded by sterically hindered histidine ligands (P. M. A. Gadsby & A. J. Thomson, unpublished work). Although we have no systems that reproduce the broad m.c.d. band of the active half-reduced enzyme, there is little doubt that the co-ordination of the haem is by two histidine groups. The m.c.d. spectra do not support the suggestion by Aasa *et al.* (1981) that the change in the g_z value to 2.85 on half-reduction is caused by deprotonation of one of the histidine ligands. The charge-transfer band of half-reduced peroxidase at 4.2 K lies at approx. 1450 nm irrespective of whether the enzyme is in the active, $g_z = 2.85$, or

inactive, $g_z = 3.00$, state. Deprotonation of a co-ordinated histidine or imidazole ligand has been shown to cause the charge-transfer peak to shift to higher energy by 1000–1100 cm^{-1} (Gadsby & Thomson, 1982; Sievers *et al.*, 1983b). This would represent a wavelength change approximately from 1500 to 1300 nm.

M.c.d. and optical-absorption spectra in the Soret and visible regions of active half-reduced peroxidase at 4.2 K are set out in Fig. 3. The thermally recombined form shows evidence in the m.c.d. spectrum of a low-spin ferric haem, which dominates the Soret region and contributes further bands at 470 and 575 nm, and a low-spin ferrous haem group, which gives rise to the sharp bands between 500 and 560 nm. After photolysis, the main m.c.d. spectral features arising from the resulting high-spin ferrous haem group are a positive peak at 438 nm and an enlargement of the negative peak between 404 and 429 nm. The m.c.d. signals that develop with photolysis resemble those of deoxymyoglobin and deoxyhaemoglobin (Sprin gall *et al.*, 1976; Brittain *et al.*, 1982), and the Soret-

Fig. 3. Near-u.v.-visible-region m.c.d. (a) and optical-absorption (b) spectra of active half-reduced cytochrome c-551 peroxidase at 4.2 K and 4.9 T, showing the effect of photolysis
 —, Photolysed; —, thermally recombined. Enzyme concentration was 29 μM , in 25 mM-Mes buffer, pH 5.6, containing 50% (v/v) glycerol, 57 μM -NADH, 0.57 μM -phenazine methosulphate and 14 p.p.m. of Tween 80. The pathlength was 1.00 mm.

region spectrum is affected little by the disappearance of the low-spin ferrous haem contributions, which are relatively weak (Vickery *et al.*, 1976).

The absorption spectrum of the thermally recombined species (Fig. 3) gives further evidence for the presence of low-spin ferric haem, absorbing at 407 nm, and low-spin ferrous haem, to which are assigned the peak at 418 nm and the fine structure in the visible region. The latter includes a split α -band, the peaks of which lie at 548 and 555 nm. Loss of the low-spin ferrous features on photolysis

is accompanied by the development of a shoulder at approx. 435 nm due to the high-spin ferrous haem group.

The Soret-region and visible-region spectra therefore show clearly the spin-state change at the ferrous HP haem caused by low-temperature photolysis. However, this spectral region is not very sensitive to changes in the environment of a low-spin ferric haem. The visible-region spectra are very similar to those of inactive half-reduced peroxidase (Greenwood *et al.*, 1984), and do not

reflect the differences between the active and inactive enzyme forms that are revealed in the near-i.r. region.

Room-temperature m.c.d. and absorption spectra

Although little or no high-spin ferric haem can be detected at low temperature, both oxidized peroxidase and active half-reduced peroxidase give m.c.d. signals and absorption bands at room temperature in the wavelength region 600–1200 nm that can be assigned with confidence to the porphyrin-to-metal charge-transfer bands of high-spin ferric haem (Stephens *et al.*, 1976). Fig. 4(a) shows that the near-i.r. m.c.d. spectrum of oxidized peroxidase contains several bands, both positive and negative, between 600 and 1200 nm. In addition, peaks arising from two different low-spin ferric haem groups are evident at approx. 1500 and 1800 nm. Analysis of this spectrum (Foote *et al.*, 1984) showed that the 1500 nm peak represents one fully low-spin haem centre with histidine-histidine axial ligation, and that the band at 1800 nm and the high-spin signals indicate a spin-state equilibrium at the other, histidine-methionine-ligated, haem group. This equilibrium was estimated to lie slightly in favour of the low-spin form at room temperature.

Active half-reduced peroxidase shows no evidence of low-spin ferric haem in its room-temperature near-i.r. m.c.d. spectrum (Fig. 4b). Instead, high-spin ferric haem bands are present that are greater in size than those of the oxidized enzyme. The intensity difference between the maximum at 1030 nm and the minimum at 1230 nm is $0.51 \text{ M}^{-1} \cdot \text{cm}^{-1} \cdot \text{T}^{-1}$, which is only slightly below the range normally found in high-spin ferric haemoproteins (Foote *et al.*, 1984).

In contrast, inactive half-reduced peroxidase gives rise to the spectrum of Fig. 4(d), in which the dominant feature is the intense low-spin ferric haem band at 1450 nm. No features arising from high-spin ferric haem can be discerned in this spectrum. The weak peaks and troughs in the range 620–900 nm are probably due to the *d-d* states of the low-spin ferrous form of the HP haem.

The addition of fluoride to active half-reduced enzyme produces new high-spin ferric haem signals. The maximum and minimum in the near-i.r. m.c.d. spectrum are shifted to 830 and 910 nm respectively (Fig. 4c), and the intensity difference between these two wavelengths is $0.64 \text{ M}^{-1} \cdot \text{cm}^{-1} \cdot \text{T}^{-1}$. Similar bands have been reported in the spectra of the fluoride derivatives of methaemoglobin (Stephens *et al.*, 1976), metmyoglobin (Nozawa *et al.*, 1976) and lactoperoxidase (Sievers *et al.*, 1983b), and in each case the peak-to-trough intensity difference was approx. $1 \text{ M}^{-1} \cdot \text{cm}^{-1} \cdot \text{T}^{-1}$. Other signals are present in the

spectrum shown in Fig. 4(c): some of the features at wavelengths longer than 1000 nm arise from active half-reduced enzyme that has not bound fluoride. The corresponding e.p.r. spectrum of Fig. 1(d) contains, as well as signals from the fluoride-bound haem group, which remains high-spin at low temperature, a residual peak at $g = 2.85$ arising from ferric haem that has not bound fluoride and has become low-spin at 12 K. In addition, a small proportion of the sample may have become inactivated, in which state it does not bind fluoride.

Oxidized, active half-reduced and fluoride-bound peroxidase all have optical-absorption bands in the near-i.r. spectral region (Fig. 5) corresponding to the m.c.d. features, whereas the inactive half-reduced enzyme displays no distinct absorption in this region (results not shown). The spectrum of the oxidized enzyme rises from a minimum at 780 nm to a maximum at 1180 nm, with an absorption coefficient of $0.77 \text{ mM}^{-1} \cdot \text{cm}^{-1}$. In shape it resembles the spectrum of oxidized *Rhodospirillum rubrum* cytochrome *c'* at neutral pH, which has an absorption maximum close to 1200 nm (Rawlings *et al.*, 1977). This species is suggested to be penta-co-ordinate (Lanir & Schejter, 1975). The absorption band of active half-reduced peroxidase is rather more broad and has a peak at a shorter wavelength, 870 nm.

Fluoride-bound half-reduced peroxidase has the most prominent absorption band of the three. Almost identical signals arise from the fluoride complexes of other haemoproteins, such as metmyoglobin (Nozawa *et al.*, 1976) and lactoperoxidase (Sievers *et al.*, 1983b), which consistently give rise to an absorption peak in the region 850–870 nm with an absorption coefficient close to $1.2 \text{ mM}^{-1} \cdot \text{cm}^{-1}$. Such similarity suggests that, in common with these model haemoproteins, a histidine residue remains bound to the LP haem group in the proximal co-ordination position when this haem has become high-spin.

The m.c.d. spectra at room temperature given in Fig. 4 show clearly the changes that occur in haem ligation and spin state on partial reduction of the oxidized enzyme, and also reveal the difference between the haem states of the active and inactive forms of the half-reduced enzyme. We consider first the changes that take place on reduction of the HP haem to form the active half-reduced state. We have argued previously (Foote *et al.*, 1984) that the peak at 1800 nm is due to the low-spin ferric form of the HP haem when co-ordinated by methionine and histidine, but with an intensity corresponding to about 50% of one haem. We postulated (Foote *et al.*, 1984) that the other half of the HP haem is high-spin ferric and therefore contributing the high-spin charge-transfer bands in the range 600–

Fig. 4. Near-i.r. m.c.d. spectra of oxidized cytochrome *c*-551 peroxidase and some derivatives at room temperature (a) Oxidized enzyme, concentration $410\mu\text{M}$. The pathlength was 5 mm. (b) Active half-reduced enzyme, concentration $410\mu\text{M}$, containing $800\mu\text{M}$ -NADH, $8\mu\text{M}$ -phenazine methosulphate and 0.01% (v/v) Tween 80. The pathlength was 5 mm. (c) Active half-reduced fluoride-bound enzyme, concentration $185\mu\text{M}$, containing $350\mu\text{M}$ -NADH, $13\mu\text{M}$ -phenazine methosulphate, 0.01% (v/v) Tween 80 and 1 M-NaF. The pathlength was 10 mm. (d) Inactive half-reduced enzyme, concentration $580\mu\text{M}$, containing $450\mu\text{M}$ -NADH, $25\mu\text{M}$ -phenazine methosulphate and 0.01% (v/v) Tween 80. The pathlength was 2 mm. All samples were in 50 mM-Mes buffer, pD5.6. The temperature was 290 K; the magnetic field was 6 T. Note change of wavelength expansion at 680 nm.

Fig. 5. Near-i.r. optical absorption spectra of oxidized cytochrome *c*-551 peroxidase and some derivatives at room temperature —, Oxidized enzyme, concentration $410\mu\text{M}$. The pathlength was 5 mm. —, Active half-reduced enzyme, concentration $410\mu\text{M}$, containing $800\mu\text{M}$ -NADH, $8\mu\text{M}$ -phenazine methosulphate and 0.01% (v/v) Tween 80. The pathlength was 5 mm. - - - - , Active half-reduced fluoride-bound enzyme, concentration $185\mu\text{M}$, containing $350\mu\text{M}$ -NADH, $13\mu\text{M}$ -phenazine methosulphate, 0.01% (v/v) Tween 80 and 1 M-NaF. The pathlength was 10 mm. All samples were in 50 mM-Mes buffer, pH 5.6. The temperature was 290 K. Note change of wavelength expansion at 680 nm.

1100 nm (Fig. 4a). As expected, on reduction of the HP haem the peak at approx. 1800 nm is lost. However, the sharp peak at 1500 nm arising from the LP haem co-ordinated by two histidine groups and in the low-spin ferric state is also lost on reduction of the HP haem. Clearly the LP haem has become high-spin, yielding the m.c.d. features between 600 and 1300 nm in Fig. 4(b) and the broad absorption bands at 630 nm and between 850 and 1200 nm (Fig. 5). The form of the m.c.d. band and wavelengths of the m.c.d. and absorption bands are different in the two states, showing that the nature of the high-spin ferric haem is indeed different in the two oxidation states of the enzyme. Therefore we conclude that in the active enzyme, on reduction of the HP haem, the LP haem switches from low-spin to high-spin. It must lose one of its histidine ligands, and either remain penta-co-ordinate or bind instead a different sixth ligand, which confers high-spin character. Presumably this opens up the LP haem to binding by anionic ligands such as F^- and CN^- , and also towards reactivity with H_2O_2 .

The data do not allow an unambiguous identification of the sixth axial ligand of the LP haem in the half-reduced enzyme, if indeed one is present. Many high-spin haemoproteins, such as metmyoglobin (Eglinton *et al.*, 1983) and horseradish peroxidase (Kobayashi *et al.*, 1977), display near-i.r. m.c.d. spectra similar to that of half-reduced peroxidase and broad optical-absorption bands centred around 1000 nm.

Comparison of the m.c.d. and absorption spectra of the active and the inactive forms of the half-reduced enzyme (Figs. 4b and 4d) shows clearly

that in the inactive form of the enzyme the LP haem remains low-spin ferric and co-ordinated by two histidine groups. Thus the haem-haem cooperativity that leads to the loss of the sixth axial ligand is lacking. However, the low-spin ferric charge-transfer band does shift slightly from 1500 to 1450 nm on half-reduction, revealing a small effect on the LP haem of reduction of the HP haem.

The difference between active and inactive peroxidase in the oxidized state is not obvious. No significant differences were found between the e.p.r., optical-absorption or m.c.d. spectra of enzyme samples that, on subsequent half-reduction, gave the two clearly different sets of results. In particular, the optical-absorption bands at 630 nm, which are evidence for the presence of a proportion of high-spin haem, were detected in both types of oxidized enzyme.

At room temperature, the ferrous HP haem of active half-reduced peroxidase appears to be fully low-spin. The high-spin ferrous state would be expected to possess a sharp positive m.c.d. peak close to 750 nm: for instance, that of deoxymyoglobin lies at 760 nm and has an intensity of approx. $0.6\text{M}^{-1}\cdot\text{cm}^{-1}\cdot\text{T}^{-1}$ (Nozawa *et al.*, 1976). No such peak is resolved in the room-temperature m.c.d. spectrum of half-reduced peroxidase (Fig. 4b). Furthermore, the $\Delta\epsilon$ values of the sharp diamagnetic m.c.d. signals in the visible region, shown in Fig. 6, are normal for a low-spin ferrous haem centre. Ferrocycytochrome *c* at room temperature has similar bands with almost identical sizes (Vickery *et al.*, 1976).

The axial ligands of the ferrous HP haem group

Fig. 6. Near-u.v.-visible-region m.c.d. spectrum of active half-reduced cytochrome c-551 peroxidase at room temperature. Enzyme concentration was $16.4\mu\text{M}$, in 50mM-Mes buffer, pH 5.6, containing $32\mu\text{M}$ -NADH, $0.32\mu\text{M}$ -phenazine methosulphate and 4p.p.m. of Tween 80. The pathlength was 5mm. The temperature was 290K; the magnetic field was 6T.

cannot firmly be identified by this work. Resonance Raman spectra of the fully reduced enzyme (Ronnberg *et al.*, 1980) were reported to show distal co-ordination of a low-spin haem group through a nitrogen atom. The m.c.d. spectra in the visible region, however, would also be consistent with histidine-methionine ligation of the ferrous HP haem.

Conclusion

This work has demonstrated that reduction of one of the two haem groups of *Ps. aeruginosa* cytochrome c-551 peroxidase causes spin-state changes at both groups, presumably by means of a protein conformational change.

The native ferric enzyme contains one low-spin haem group, the LP site, with bis-histidine axial ligation and one haem group, the HP site, in a spin equilibrium, with a distal methionine ligand only partly bound at room temperature but more firmly attached at low temperature. On reduction, the HP haem centre becomes fully low-spin. It appears that a structural change occurs at the HP haem on one-electron reduction that leads to complete binding of the sixth ligand, methionine. This process is linked via the protein to the movement of one histidine ligand away from the ferric LP-haem iron. This site takes up a high-spin configuration and becomes accessible to external ligands such as fluoride and H_2O_2 . A histidine ligand is still available nearby to bind at the sixth

co-ordination site of the LP haem when the temperature is lowered, although the haem environment at 4.2K is clearly different in the oxidized and half-reduced enzyme forms when the haem-haem interaction is intact. The rapid binding of CO to the ferrous LP haem in fully reduced peroxidase (Foote *et al.*, 1983) suggests that the LP haem remains high-spin when it too has been reduced.

The ferric haem group in the half-reduced peroxidase therefore resembles the haem groups of native yeast cytochrome c peroxidase and horseradish peroxidase. Ronnberg *et al.* (1981) have used kinetic methods to show similarities between the catalytic cycles of the *Pseudomonas* enzyme and other peroxidases, and e.p.r. signals thought to arise from intermediates in the peroxidatic cycle have been reported (Ellfolk *et al.*, 1983). It will be interesting to discover the influence of the additional haem group upon the catalytic mechanism.

References

- Aasa, R., Ellfolk, N., Ronnberg, M. & Vänngård, T. (1981) *Biochim. Biophys. Acta* **670**, 170-175
- Brittain, T., Greenwood, C., Springall, J. P. & Thomson, A. J. (1982) *Biochim. Biophys. Acta* **703**, 117-128
- Eglinton, D. G., Gadsby, P. M. A., Sievers, G., Peterson, J. & Thomson, A. J. (1983) *Biochim. Biophys. Acta* **742**, 648-658
- Ellfolk, N. & Soininen, R. (1971) *Acta Chem. Scand.* **25**, 1535-1540

- Ellfolk, N., Ronnberg, M., Aasa, R., Andréasson, L.-E. & Vänngård, T. (1983) *Biochim. Biophys. Acta* **743**, 23–30
- Ellfolk, N., Ronnberg, M., Aasa, R., Andréasson, L.-E. & Vänngård, T. (1984) *Biochim. Biophys. Acta* **784**, 62–67
- Foote, N., Thompson, A. C., Barber, D. & Greenwood, C. (1983) *Biochem. J.* **209**, 701–707
- Foote, N., Peterson, J., Gadsby, P. M. A., Greenwood, C. & Thomson, A. J. (1984) *Biochem. J.* **223**, 369–378
- Gadsby, P. M. A. & Thomson, A. J. (1982) *FEBS Lett.* **150**, 59–63
- Greenwood, C., Foote, N., Peterson, J. & Thomson, A. J. (1984) *Biochem. J.* **223**, 379–391
- Kobayashi, N., Nozawa, T. & Hatano, M. (1977) *Biochim. Biophys. Acta* **493**, 340–351
- Lanir, A. & Schejter, A. (1975) *Biochem. Biophys. Res. Commun.* **62**, 199–203
- Nozawa, T., Yamamoto, T. & Hatano, M. (1976) *Biochim. Biophys. Acta* **427**, 28–37
- Rawlings, J., Stephens, P. J., Nafie, L. A. & Kamen, M. D. (1977) *Biochemistry* **16**, 1725–1729
- Ronnberg, M. & Ellfolk, N. (1975) *Acta Chem. Scand. B* **29**, 719–727
- Ronnberg, M. & Ellfolk, N. (1978) *Biochim. Biophys. Acta* **504**, 60–66
- Ronnberg, M., Osterlund, K. & Ellfolk, N. (1980) *Biochim. Biophys. Acta* **626**, 23–30
- Ronnberg, M., Araiso, T., Ellfolk, N. & Dunford, H. B. (1981) *Arch. Biochem. Biophys.* **207**, 197–204
- Schejter, A. & Eaton, W. A. (1984) *Biochemistry* **23**, 1081–1084
- Sievers, G., Gadsby, P. M. A., Peterson, J. & Thomson, A. J. (1983a) *Biochim. Biophys. Acta* **742**, 637–647
- Sievers, G., Gadsby, P. M. A., Peterson, J. & Thomson, A. J. (1983b) *Biochim. Biophys. Acta* **742**, 659–668
- Soininen, R. & Ellfolk, N. (1972) *Acta Chem. Scand.* **26**, 861–872
- Soininen, R. & Ellfolk, N. (1973) *Acta Chem. Scand.* **27**, 35–46
- Soininen, R. & Ellfolk, N. (1975) *Acta Chem. Scand. B* **29**, 134–136
- Springall, J., Stillman, M. J. & Thomson, A. J. (1976) *Biochim. Biophys. Acta* **453**, 494–501
- Stephens, P. J., Sutherland, J. C., Cheng, J. C. & Eaton, W. A. (1976) in *Excited States of Biological Molecules* (Birks, J., ed.), pp. 434–442, John Wiley and Sons, New York
- Vickery, L., Nozawa, T. & Sauer, K. (1976) *J. Am. Chem. Soc.* **98**, 343–350