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A study of the oxidized form of *Pseudomonas aeruginosa* cytochrome *c*-551 peroxidase with the use of magnetic circular dichroism

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The magnetic properties at different temperatures of oxidized *Pseudomonas aeruginosa* cytochrome *c*-551 peroxidase were studied, with the use of the technique of magnetic-circular-dichroism spectroscopy. At 4.2K, both constituent haems were found to be low-spin, and the axial ligand pairs were identified as histidine–histidine and histidine–methionine. At room temperature high-spin signals were observed, amounting to less than 25% of the total haem present. These signals are concluded to arise mainly from a temperature-dependent spin-state equilibrium in the methionine-ligated haem.

Pseudomonas aeruginosa cytochrome *c*-155 peroxidase (EC 1.11.1.5) is a monomeric protein containing two protohaem IX groups, both covalently attached to the polypeptide chain (Ellfolk & Soininen, 1971). In the protein, the two haem groups are not equivalent optically, magnetically or in reactivity. However, most notable is the large difference between their redox potentials, which are estimated to be +320 and −330mV respectively (Ellfolk *et al.*, 1983). At room temperature, the presence of a high-spin haem in the native (oxidized) enzyme has been deduced from resonance Raman (Rönnberg *et al.*, 1980) and optical-absorption (Rönnberg & Ellfolk, 1979) spectra. However, e.p.r. spectra measured below 77K showed only a small amount of high-spin haem, and two sets of signals characteristic of low-spin ferric haem groups (Aasa *et al.*, 1981). The observed g_z values, 3.26 and 2.94, were taken to indicate axial co-ordination of the two haem iron atoms by, respectively, histidine–methionine and histidine–histidine. It is the latter haem centre that Ellfolk *et al.* (1983) suggest is in the high-spin form at room temperature, and undergoes a spin-state conversion when the temperature is lowered.

In order to test the conclusions drawn from optical-absorption spectra and e.p.r. spectroscopy by Ellfolk's group, we have carried out a combined m.c.d. and e.p.r. spectroscopy study of the enzyme.

Abbreviation used: m.c.d., magnetic circular dichroism.

M.c.d. spectroscopy probes the magnetic properties of a metal centre via its optical-absorption bands, and most importantly for the present work the wavelength range available extends to 2000nm, enabling low-energy electronic states to be detected even in the presence of vibrational overtone absorption from solvent and protein alike. Ferric protohaem IX proteins possess transitions in the region 600–2000nm arising from charge-transfer either between the porphyrin π-orbitals and the iron *d*-orbitals or between the axial ligand methionine and the iron levels (Smith & Williams, 1970). As a consequence the energies of these transitions are sensitive to the spin-state and to the ligation of the ferric ion. If the two haem groups have different spin-states or co-ordination shells, then they can be distinguished readily in the near-i.r. m.c.d. spectrum. Furthermore, such experiments have been carried out at both 4.2 and 290K, enabling temperature-dependent changes in spin-state and axial ligation to be monitored.

In the present paper we report the results of such a study of the oxidized form of *Pseudomonas aeruginosa* cytochrome *c*-551 peroxidase, with the use of both visible and near-i.r. m.c.d. at temperatures of 4.2 and 290K and e.p.r. spectroscopy at low temperature. We have now compiled sets of m.c.d. spectra over this wavelength range and at these temperatures for a wide variety of haemoproteins involving a range of axial ligation. This enables secure assignments of the spectra of *Pseudomonas aeruginosa* cytochrome *c*-551 peroxidase to be made. We present the m.c.d. spectra of *Desulfo-*

vibrio vulgaris cytochrome c_3, an example of a haemoprotein with bis-histidine axial ligation (Hase *et al.*, 1979; Bando *et al.*, 1979), and *Pseudomonas aeruginosa* *c*-551, a haemoprotein with histidine–methionine co-ordination (Almassey & Dickerson, 1978; Adman, 1979). Both proteins are crystallographically defined and so provide models of well-defined structure.

Materials and methods

All reagents were of AnalaR grade and were purchased from BDH Chemicals, with the following exceptions: Mes (4-morpholine-ethanesulphonic acid), Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] and Tween 80 from Sigma Chemical Co.; ^2H_2O (99.8 atom% ^2H) from Aldrich Chemical Co.; DE-52 DEAE-cellulose from Whatman Chemical Separation.

Cultivation of *Pseudomonas aeruginosa* (N.C.T.C. 6750) and isolation of cytochrome *c*-551 peroxidase were as described in Foote *et al.* (1983), except that an additional ion-exchange column was employed as follows. The pooled fractions from the second CM-cellulose C-50 column were adjusted, with NaH_2PO_4 and distilled water, to the same pH and conductivity as 20mM-sodium phosphate, pH6.5. The preparation was then passed down a column (4.5cm × 19cm) of DE-52 DEAE-cellulose equilibrated with the buffer, and coloured fractions were collected and pooled. This step increased the purity ratio A_{407}/A_{280} from 4.1:1 to 4.65:1. Concentrations of the enzyme were calculated by using $\varepsilon_{407} = 237\,mM^{-1}\cdot cm^{-1}$ (Soininen & Ellfolk, 1973). Cytochrome *c*-551 was prepared from *Pseudomonas aeruginosa* by the method of Parr *et al.* (1976), and assayed spectrophotometrically by using $\Delta\varepsilon_{550(red.-ox.)} = 20.0\,mM^{-1}\cdot cm^{-1}$ (Ambler, 1963). Cytochrome c_3 isolated from *Desulfovibrio vulgaris* was kindly given by Dr. J. LeGall (Athens, GA, U.S.A.) and Dr. I. Moura, Dr. J. J. G. Moura and Professor A. V. Xavier (Lisbon, Portugal). pD values are the measured pH-meter reading +0.4 (Perrin & Dempsey, 1974).

M.c.d. measurements in the region 250–1050nm were made with a Jasco J-500D spectropolarimeter, and in the region 800–2000nm were recorded with a laboratory-built instrument described elsewhere (Eglinton *et al.*, 1980). The magnetic field was generated for room-temperature samples by a superconducting magnet with a maximum field of 4.7T, and for low-temperature samples by an Oxford Instruments SM-4 split-coil magnet with a maximum field of 5T. E.p.r. spectra were recorded with a Bruker ER 200D-SRC spectrometer, interfaced with an Aspect 2000 computer. Temperature regulation of e.p.r. samples was by an Oxford Instruments ESR-900 Continuous Flow Cryostat, with a DTC-2 temperature controller and a gold+0.03%-iron/chromel temperature sensor.

Results

Low-temperature m.c.d. spectra

Fig. 1 shows the m.c.d. spectrum of oxidized cytochrome *c*-551 peroxidase at 4.2K and 4.9T between 600 and 2000nm. There are two prominent positive peaks at 1500 and 1870nm with $\Delta\varepsilon$ values of 0.48 and $0.29\,mM^{-1}\cdot cm^{-1}$ respectively. These bands belong to two low-spin ferric haem groups with different states of axial ligation. The sharp negative trough at 708nm followed by a positive peak at 655nm is also an important feature for axial ligand assignment, since these are

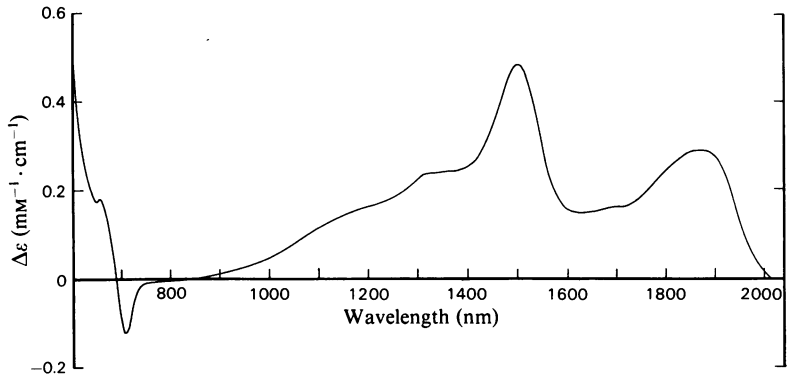

Fig. 1. *Near-i.r. m.c.d. spectrum of oxidized cytochrome c-551 peroxidase at 4.2K and 4.9T*
The enzyme concentration was 200 μM, in 25mM-Mes buffer, pD6.4, containing 50% (v/v) glycerol. The pathlength was 1.17mm.

the m.c.d. counterparts of the methionine–ferric-charge-transfer band often referred to as the '695nm' band. In Hepes buffer at pH8.0 the m.c.d. spectrum is similar in form to that at pH6.4, although slightly less intense.

Fig. 2 presents the m.c.d. spectra at 4.2K and 4.9T of cytochrome c_3 from *Desulfovibrio vulgaris* and cytochrome *c*-551 from *Pseudomonas aeruginosa*. The former is a tetrahaem protein, the haem groups of which are each co-ordinated by a pair of histidine ligands. The m.c.d. spectrum shows the characteristic low-spin ferric haem band at 1505nm and no further spectral features until 650nm. The latter has a positive m.c.d. peak at 1800nm and a sharp negative trough at 697nm. Also shown in the Figure is the result of adding together the m.c.d. spectra of the two cytochromes in an attempt to simulate the near-i.r. m.c.d. spectrum of cytochrome *c*-551 peroxidase (Fig. 1). The peak at 1505nm has a contribution underlying it from the tail of the band at 1800nm. This increases the apparent intensity of the m.c.d. peak at 1505nm from 0.21 to 0.31 $mM^{-1}\cdot cm^{-1}$ at 4.9T.

Fig. 3 shows the near-u.v. and visible-region m.c.d. spectra at 4.2K and 4.9T of the oxidized enzyme and of the two cytochromes c_3 and *c*-551. These m.c.d. spectra are very similar to one another and do not provide a good basis on which to distinguish the states of axial ligation of the haems. However, it is important to note that the m.c.d. spectrum of the enzyme corresponds well to the overlap of the two sets of spectra (*a*) and (*b*) and that the intensities of the peaks and troughs are approximately the sum of the two.

E.p.r. spectra

E.p.r. spectra of the oxidized enzyme, recorded below 15K, show two sets of low-spin signals (Fig. 4). The values of g_z, which are approx. 3.3 and 3.0, vary slightly from sample to sample. Another feature that can vary between samples is the size of the peak at $g = 5.8$ arising from high-spin ferric haem. There appears to be an inverse relationship between the intensity of the signal at $g = 5.8$ and the $g = 3.3$ peak. Samples with added glassing agent (glycerol or ethanediol) are usually devoid of the $g = 5.8$ signal, whereas change of the buffer system from Mes to sodium phosphate causes an increase in the $g = 5.8$ e.p.r. intensity. At pD8.0 (in Hepes buffer) the e.p.r. spectrum of oxidized enzyme was found to be similar to that measured at pD6.4 in Mes.

Room-temperature m.c.d. spectra

Fig. 5 shows the near-i.r. m.c.d. spectrum of oxidized cytochrome *c*-551 peroxidase at pD5.6. The positive bands at 1480 and 1740nm, with $\Delta\varepsilon$ values of 1.17 and 0.47 $M^{-1}\cdot cm^{-1}\cdot T^{-1}$, belong to low-spin ferric haem groups with different states of axial ligation. The spectral features between 700 and 1200nm have the form expected for high-spin ferric haem.

The room-temperature near-i.r. m.c.d. spectra of cytochromes *c*-551 and c_3 are given in Fig. 6. The low-spin ferric m.c.d. band of cytochrome *c*-551 is at 1690nm, having shifted from 1800nm at 4.2K (Fig. 2), although the band shape at room temperature is broad and asymmetric towards the long-wavelength side. The peak position of the longest-wavelength band in the near-i.r. m.c.d. spectrum of oxidized cytochrome *c*-551 peroxidase also shifts by approx. 400 cm^{-1} from 1870 to 1780nm on warming up from 4.2 to 290K. The m.c.d. spectrum of cytochrome c_3 at room temperature (Fig. 6) has the expected low-spin ferric haem band at 1500nm. There are no spectral features between 800 and 1000nm. Fig. 6 also shows the result of summing the m.c.d. spectra of cytochromes *c*-551 and c_3. The effect is to increase the intensity of the 1690nm peak from 0.68 to

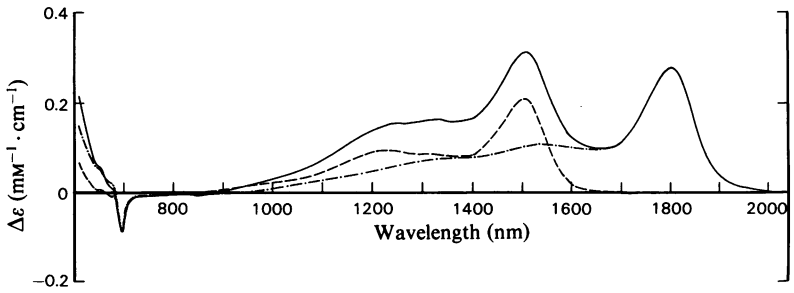

Fig. 2. *Near-i.r. m.c.d. spectra of Ps, aeruginosa cytochrome c-551 and D. vulgaris cytochrome c_3 at 4.2K and 4.9T*
—·—, Cytochrome *c*-551, 230 μM in 25mM-Mes buffer, pD7, containing 50% (v/v) ethanediol; ——, cytochrome c_3 at a haem concentration of 331 μM in 25mM-sodium phosphate buffer, pD6.5, containing 50% (v/v) glycerol; ——, the sum of these spectra. Both pathlengths were approx. 1.2mm. $\Delta\varepsilon$ values for cytochrome c_3 are expressed per haem.

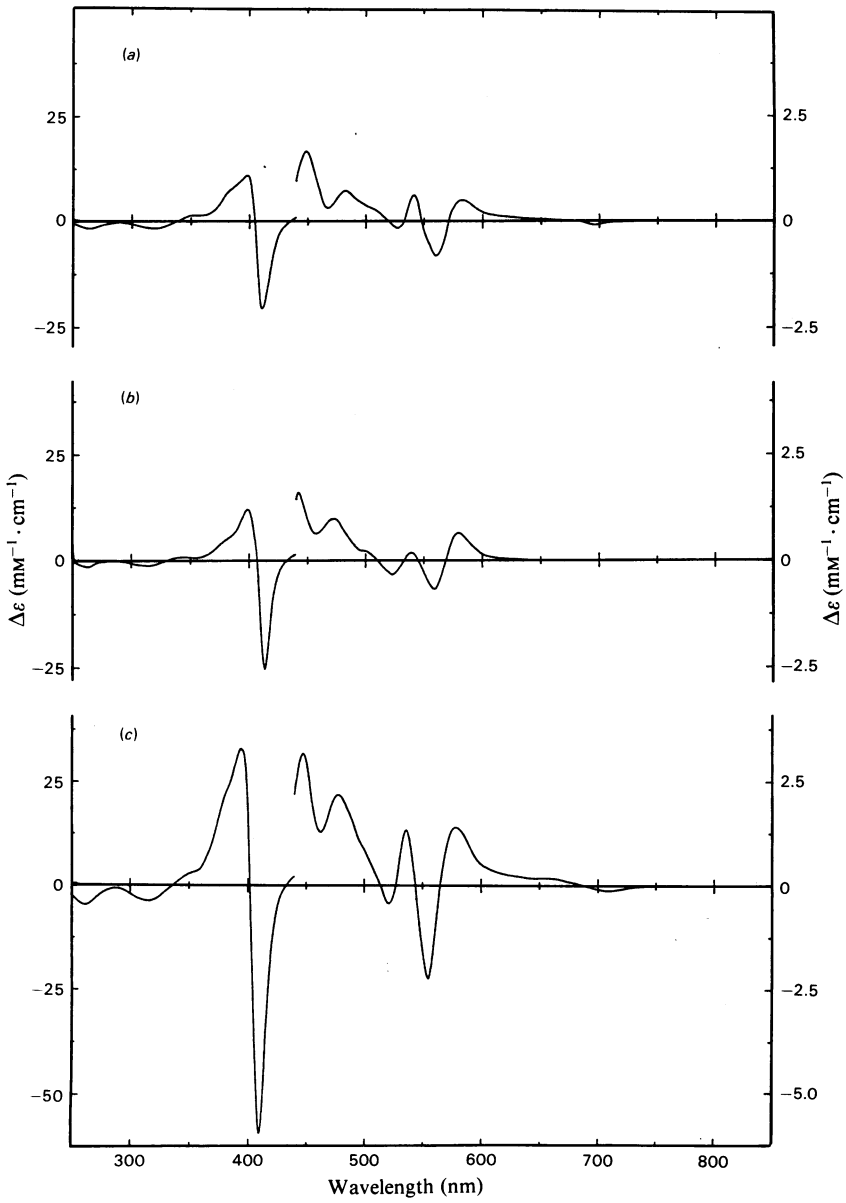

Fig. 3. *Near-u.v./visible-region m.c.d. spectra of Ps. aeruginosa cytochrome c-551, D. vulgaris cytochrome c_3 and cytochrome c-551 peroxidase at 4.2K and 4.9T*

(*a*) Cytochrome *c*-551 concentration was 230 μM (visible) and 20 μM (near u.v.) in 25mM-Mes buffer, pD7, containing 50% (v/v) ethanediol. (*b*) Cytochrome c_3 was at a haem concentration of 331 μM (visible) and 31 μM (near u.v.) in 25mM-sodium phosphate buffer, pD6.5, containing 50% (v/v) glycerol. (*c*) Cytochrome *c*-551 peroxidase concentration was 200 μM (visible) and 17.6 μM (near u.v.) in 25mM-Mes buffer, pD6.4, containing 50% (v/v) glycerol. Pathlengths were approx. 1.2mm. $\Delta\varepsilon$ values for cytochrome c_3 are expressed per haem.

0.72 $M^{-1}\cdot cm^{-1}\cdot T^{-1}$ and that of the 1500nm peak from 0.70 to 0.93 $M^{-1}\cdot cm^{-1}\cdot T^{-1}$, without altering the wavelength positions of the peak maxima.

The near-u.v. and visible-region m.c.d. spectrum of the enzyme at pD5.6 in Mes buffer is given in Fig. 7. Although high-spin and low-spin ferric haem groups have optical-absorption bands that overlap, the relative intensities of the m.c.d. signals of high-spin and low-spin ferric haem groups are sufficiently different, with the latter dominating, that the m.c.d. bands between 300 and 600nm can be assigned mainly to low-spin

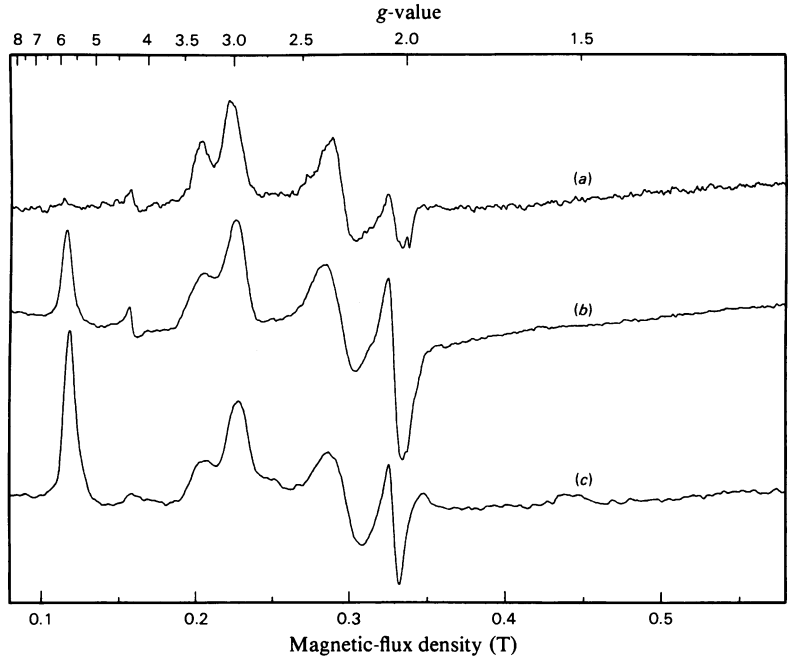

Fig. 4. *E.p.r. spectra of oxidized cytochrome c-551 peroxidase*
(*a*) Enzyme concentration was 100 μM in 25 mM-Mes buffer, pD 6.4, containing 50% (v/v) glycerol and 0.005% (v/v) Tween 80. Conditions: temperature 15 K, microwave frequency 9.44 GHz, power 2.01 mW and gain 5.01×10^5. (*b*) Enzyme concentration was 750 μM in 50 mM-Mes buffer, pD 6.4, containing 0.01% (v/v) Tween 80. Conditions: temperature 10 K, microwave frequency 9.41 GHz, power 2.01 mW and gain 3.2×10^5. (*c*) Enzyme concentration was 250 μM in 25 mM-sodium phosphate buffer, pH 6.0, containing 0.01% (v/v) Tween 80. Conditions: temperature, 10 K; microwave frequency, 9.43 GHz; power, 2.01 mW; gain, 6.3×10^5.

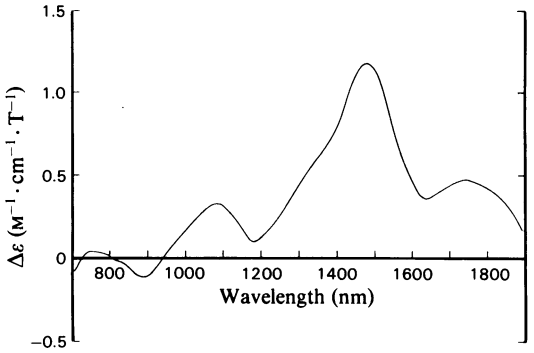

Fig. 5. *Near-i.r. m.c.d. spectrum of oxidized cytochrome c-551 peroxidase at room temperature*
The enzyme concentration was 590 μM in 50 mM-Mes buffer, pD 5.6, containing 0.01% (v/v) Tween 80. Conditions: temperature, 290 K; magnetic field, 4.7 T; Pathlength, 2 mm (above 1000 nm) and 5 mm (below 1000 nm).

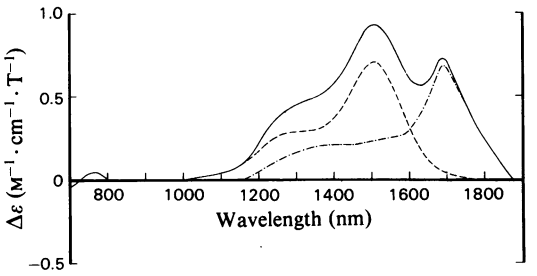

Fig. 6. *Near-i.r. m.c.d. spectra of Ps. aeruginosa cytochrome c-551 and D. vulgaris cytochrome c_3 at room temperature*
—·—, Cytochrome *c*-551, 920 μM in 50 mM-Mes buffer, pD 5.6, pathlength 2 mm; ——, cytochrome c_3 at a haem concentration of 685 μM in 50 mM-sodium phosphate buffer, pD 6.5, pathlength 5 mm; ——, sum of these spectra. Conditions: temperature, 290 K; magnetic field, 4.7 T. $\Delta\varepsilon$ values for cytochrome c_3 are expressed per haem.

ferric haem. The forms of the m.c.d. spectra of low-spin ferric haem groups ligated by two histidine residues and by histidine–methionine are not sufficiently different at room temperature for the relative contributions of each to be deconvoluted.

The m.c.d. bands between 600 and 800 nm in Fig. 7 are largely due to the high-spin ferric haem present. Room-temperature m.c.d. spectra of many high-spin ferric haemoproteins have been reported, and all show features similar to those of

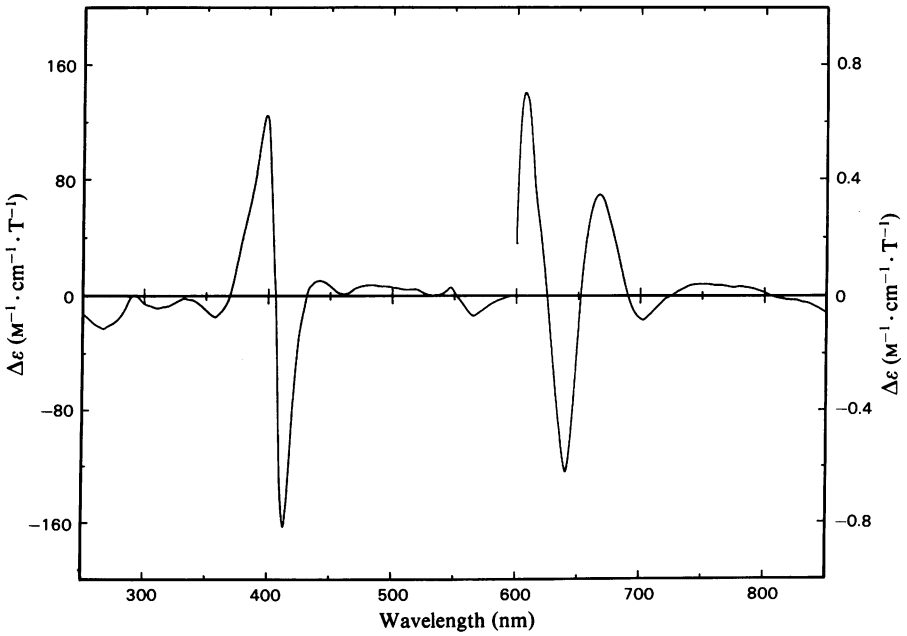

Fig. 7. *Near-u.v./visible-region m.c.d. spectrum of oxidized cytochrome c-551 peroxidase at room temperature*
The enzyme concentrations were 590 μM (visible) and 7 μM (near u.v.) in 50 mM-Mes buffer, pD 5.6, containing 0.01% (v/v) Tween 80. Conditions: temperature, 290 K; magnetic field, 4.7 T; pathlength, 5 mm.

Fig. 7. Low-spin ferric haem co-ordinated by methionine and histidine does show weak bands in the region 600–720 nm, the so-called '695 nm' band being diagnostic of this ligation. This band is very weak in the room-temperature m.c.d. spectrum of cytochrome *c* and is not much better resolved than in the absorption spectrum. Thus it is possible that the small negative trough at approx. 710 nm in Fig. 7 is the counterpart of this transition in the spectrum of cytochrome *c*-551 peroxidase. However, since this region is confused by the overlap of other bands, which certainly originate from high-spin haem, we cannot be certain of this assignment.

Small variations in the relative intensities of the m.c.d. bands throughout the spectrum have been observed when the pD was raised from 5.6 to 6.4, and when the buffering system was changed from Mes to sodium phosphate. However, the shape of the spectrum was unaltered and the relative proportions of high-spin and low-spin did not change significantly.

Discussion

Although *Pseudomonas aeruginosa* cytochrome *c*-551 peroxidase contains two haem groups of identical structure, there is a good deal of evidence to show that they are functionally distinct within the protein. The conclusions drawn from previous studies are that the enzyme possesses one haem group, the high-potential haem, liganded by methionine and histidine and low-spin ferric in the oxidized enzyme at all temperatures, and that the other haem group, the low-potential moiety, is high-spin at room temperature, but low-spin at low temperature and co-ordinated by two histidine ligands. The lack of reactivity of the latter haem group towards anionic ligands at room temperature is ascribed to a closed conformation of the haem pocket (Ellfolk *et al.*, 1984).

E.p.r. and low-temperature m.c.d. spectra

The room-temperature and low-temperature m.c.d. spectra obtained in the present work do not support all these conclusions. We start with discussion of the m.c.d. spectra recorded at 4.2 K. Both the visible and near-i.r. regions show only low-spin ferric haem groups present with the latter region, revealing that the two haem groups have different states of axial ligation, as demonstrated by peaks at 1500 and 1870 nm. The latter peak is assigned to haem co-ordinated by histidine and methionine. The near-i.r. m.c.d. spectrum at 4.2 K of cytochrome *c*-551 has a peak at 1800 nm, and horse heart cytochrome *c* at 1750 nm (J. Peterson, A. J. Thomson, C. Greenwood, N. Foote & B. C. Hill, unpublished work). Thus there is a variation of the energy of this charge-transfer band from protein to protein. However, the assignment can

be confirmed by the presence of a band in the region of 695nm. This band is characteristic of an intact methionine–histidine co-ordination of the haem (Dickerson & Timkovich, 1975). The band is very weak in the absorption spectrum, with an absolute molar absorption coefficient of approx. $198\,M^{-1}\cdot cm^{-1}$, in horse heart ferricytochrome *c* (Kaminsky *et al.*, 1973). It does give rise to an m.c.d. band, first noted in the room-temperature m.c.d. spectrum of horse heart cytochrome *c* (Wilson & Greenwood, 1971), but it is very prominent and easy to detect in the low-temperature spectrum. In the m.c.d. spectrum of horse cytochrome *c* at 4.2K there is a positive peak at 694nm followed by a negative trough at 668nm (J. Peterson, C. Greenwood, A. J. Thomson, N. Foote & B. C. Hill, unpublished work), whereas in that of cytochrome *c*-551 from *Ps. aeruginosa* there is a negative trough at 697nm followed by a positive peak at shorter wavelength (see Fig. 2). Thus the signs have inverted. It may be that the signs of the m.c.d. features arising from a cytochrome *c* in the region of 695nm are related to the chirality of the methionine group. It has been shown by n.m.r. experiments (Senn *et al.*, 1980) that there are two possible modes of attachment of the methionine group, corresponding to two chiralities, which give rise to c.d. peaks of opposite signs at 695nm. It is not clear how this change would affect the m.c.d. signals, and a wider range of examples is required before a definitive correlation can be established. However, we note the presence of a clear negative trough at 708nm in the m.c.d. spectrum of oxidized cytochrome *c*-551 peroxidase that can be assigned as the counterpart of the 695nm band in cytochrome *c*. It may be significant that this band is shifted to lower energy compared with that in horse heart cytochrome *c*, as in the long wavelength peak in the near-i.r. m.c.d. spectrum.

The other peak in the near-i.r. m.c.d. spectrum of the oxidized enzyme at 4.2K lies at 1500nm. The peak wavelengths of haem groups co-ordinated by imidazole–histidine or by two histidine ligands vary over the range 1500–1620nm (see Table 1 in Sievers *et al.*, 1983*a*). The spectrum of *D. vulgaris* c_3 reported in the present paper shows a peak at 1505nm and provides an excellent model of one of the haem groups in oxidized cytochrome *c*-551 peroxidase. An unambiguous assignment is not possible on the basis of the near-i.r. m.c.d. spectrum alone, however, since haem co-ordinated by histidine and an amino group, from lysine as in a lysine complex of haemoglobin (Rawlings *et al.*, 1977) or in the isobutylamine complex of leg-haemoglobin (P. M. A. Gadsby, A. J. Thomson & J. Peterson, unpublished work), also gives a peak at about 1500nm.

The g_z values obtained from the e.p.r. spectrum of the enzyme do not resolve this ambiguity. The value of approx. 3.0 lies within the range typical of haem groups co-ordinated by two histidine ligands, but the higher value of approx. 3.3 is close to the range 3.4–3.5 recorded for histidine–amino group co-ordination (Peisach *et al.*, 1973). The *g* values of haem groups co-ordinated by histidine and methionine vary from 3.01, 2.25 and 1.25 for horse heart cytochrome *c* to 3.2, 2.05 and 1.25 for cytochrome *c*-551 from *Ps. aeruginosa*. Therefore either of the g_z values measured from Fig. 4 might be assigned to the methionine-ligated haem centre clearly identified in the m.c.d. spectra. The magnetization properties of a m.c.d. band can often be used to relate that band to an e.p.r. signal (Thomson & Johnson, 1980), but the measured magnetization curves of the peaks at 1500nm and 1870nm are not sufficiently different in the case of this enzyme (results not shown). Although one haem ligand pair cannot firmly be established solely from the measured *g*-values and 4.2K m.c.d. spectra, it was observed that the intensities of the e.p.r. signals at $g = 3.3$ and $g = 5.8$ display an inverse correlation, and therefore that the minor high-spin species observed under some conditions of pH and buffer belongs to the haem centre with $g_z = 3.3$. In conjunction with the room-temperature m.c.d. spectrum (discussed later below), this provides evidence leading to a more secure assignment of the near-i.r. m.c.d. peaks to the e.p.r. *g*-values, and thereby to identification of the ligation pattern of both haem centres.

No high-spin ferric haem signals are evident in the 4.2K near-i.r. m.c.d. spectrum (Fig. 1), even from samples that e.p.r. reveals contain a proportion of such a spin state. This is because the intensities of the m.c.d. bands of high-spin haem groups are weaker by a factor of about 50 than those of low-spin ferric haem groups (Eglinton *et al.*, 1983). Any such bands would have been close to the limits of detection under the experimental conditions used. However, the intensities of the low-spin ferric haem bands in Fig. 1, when compared, for example, with the protein model systems chosen (Fig. 2), support the contention that two low-spin ferric haem groups are present. The 1870nm band and the corresponding band of cytochrome *c*-551 have similar $\Delta\varepsilon$ values. The m.c.d. intensity of a histidine–lysine-co-ordinated haem group in the near-i.r. region at 4.2K is not known, but bis-histidine-co-ordinated haem groups have $\Delta\varepsilon$ values of $0.1–2.0\,mM^{-1}\cdot cm^{-1}$ at 4.9T. The 1500nm peak of the oxidized enzyme is consequently rather more intense than expected, even after allowance has been made for the contribution at this wavelength from the second haem group. The intensities of the m.c.d. signals in the visible region (Fig. 3) also support the

conclusion that two fully low-spin haem groups are present at 4.2K.

Neither the low-temperature m.c.d. spectrum nor the e.p.r. spectrum is affected when the pD is raised from 6.4 to 8.0. This shows that the axial ligations of the two haem groups remain unchanged in the 4.2K form over this pD range. It also eliminates any possibility of a histidine ligand losing a proton to form an imidazolate ligand. Such a change leads to a very pronounced shift to the blue of the near-i.r. charge-transfer band by about $1000\,cm^{-1}$ (Gadsby & Thomson, 1982).

Room-temperature m.c.d. spectra

The room-temperature spectra (Figs. 5 and 7) clearly show the presence of at least three types of haem structure. The near-i.r. region indicates that low-spin ferric haem groups similar to those detected at 4.2K are present also at room temperature. The bands at 1480 and 1740nm clearly belong to low-spin ferric haem groups. Again, the former could arise from either bis-histidine or histidine–amino group co-ordination of one of the haem moieties. The band at 1740nm is unambiguously assigned to co-ordination by methionine–histidine.

The additional features between 600 and 1200nm arise from a high-spin ferric haem. The room-temperature m.c.d. spectra of a number of high-spin ferric haem groups have been reported, and pairs of oppositely signed bands run through this wavelength range. Table 1 summarizes the wavelengths and intensities of the two longest-wavelength bands for various examples of high-spin haem groups with defined states of ligation. All have histidine as the proximal axial ligand. It seems a reasonable assumption that the fifth ligand will be histidine in the peroxidase enzyme. The m.c.d. spectrum of the enzyme has a positive peak at 1090nm and a trough at 1190nm, which, however, does not become negative. There is presumably an underlying positive m.c.d. contribution from the low-spin centres at these wavelengths, as would be expected from comparison with the model spectrum in Fig. 6. The sixth ligand, if present, of the high-spin haem cannot be identified from these data, although phenolate can be discounted because the leghaemoglobin–phenol bands lie at considerably shorter wavelengths (Sievers *et al.*, 1983*a*). Previously it has been suggested that the high-spin haem has a water molecule as the sixth ligand (Rönnberg & Ellfolk, 1979) or that a five-co-ordinate form is in equilibrium with a six-co-ordinate form possessing a carboxylate group as a ligand (Rönnberg *et al.*, 1980). We cannot at present rule out either of these possibilities.

The quantity of high-spin haem in the oxidized enzyme can be estimated only very approximately. The peak (1090nm)-to-trough (1190nm) height is $0.22\,M^{-1}\cdot cm^{-1}\cdot T^{-1}$: in the examples in Table 1 this quantity ranges from 0.6 to $2.0\,M^{-1}\cdot cm^{-1}\cdot T^{-1}$. Therefore the high-spin m.c.d. signals at room temperature represent rather less than 50% of one haem. This is consistent with the minimal increase in absorption intensity observed in the visible region on cooling from 290 to 4.2K (results not shown).

The data of Fig. 5 have interesting implications about the origins of the high-spin signals, i.e. whether they arise from one haem centre or from two. The 1480nm peak has a m.c.d. intensity of $1.17\,M^{-1}\cdot cm^{-1}\cdot T^{-1}$, which is slightly larger than the signal normally expected from a bis-histidine or histidine–amino group-co-ordinated haem

Table 1. *Near-i.r. m.c.d. bands of high-spin ferric haemoproteins and their derivatives at room temperature*
Key to references: 1, Nozawa *et al.* (1976); 2, Stephens *et al.* (1976); 3, Kobayashi *et al.* (1977); 4, Rawlings *et al.* (1977); 5, Sievers *et al.* (1983*a*); 6, Sievers *et al.* (1983*b*); 7, Vuk-Pavlović & Benko (1975).

		Positive band		Negative band		
Sixth ligand	Example	λ (nm)	$\Delta\varepsilon$ ($M^{-1}\cdot cm^{-1}\cdot T^{-1}$)	λ (nm)	$\Delta\varepsilon$ ($M^{-1}\cdot cm^{-1}\cdot T^{-1}$)	Reference
H_2O	Metmyoglobin-^2H_2O	1010	0.3	1210	−0.7	1
	Methaemoglobin-H_2O	1030	0.3	1180	−0.3	2
H_2O/none (7)	Horseradish peroxidase, pD 6.86	1060	0.4	1270	−0.2	3
None	*Rhodospirillum rubrum* cytochrome *c'*, pD 5.3–7.1	1120	0.6	1360	−0.4	4
Carboxylate	Methaemoglobin-acetate	1050	0.8	1180	−0.7	4
	Methaemoglobin-formate	920	1.0	1040	−1.0	4
Phenolate	Leghaemoglobin-phenol	830	0.4	900	−0.5	5
Fluoride	Methaemoglobin-fluoride	810	0.5	900	−0.5	2
	Metmyoglobin-fluoride	810	0.4	890	−0.5	1
	Lactoperoxidase-fluoride	830	0.5	920	−0.5	6

centre (P. M. A. Gadsby, A. J. Thomson & J. Peterson, unpublished work), as indeed was observed at low temperature. It therefore appears to be fully low-spin at room temperature. The $\Delta\varepsilon$ value of the longer-wavelength peak is very close in magnitude to that of cytochrome *c*-551 in the 4.2K spectrum. It also shifts by a similar energy upon warming to 290K, yet the $\Delta\varepsilon$ value of this low-spin haem signal at room temperature is about 70% of that of the corresponding cytochrome *c*-551 m.c.d. signal intensity. The inference we draw is that most, and probably all, of the high-spin signals seen at room temperature belong to the high-potential haem and that there is a thermal spin equilibrium involving the haem with histidine–methionine co-ordination. This conclusion allows a fuller interpretation of the e.p.r. spectra (Fig. 5). Since samples of oxidized enzyme with a larger high-spin signal at $g = 5.8$ have the peak at $g = 3.3$ decreased in size, we assign the latter signal as the g_z value of the histidine–methionine-ligand–haem centre, as this is the one demonstrated by m.c.d. spectroscopy to have partial high-spin character. An amino group can now be ruled out as the sixth ligand of the other haem centre, because the remaining g_z value of 3.0 is too low. We therefore assign the $g = 3.0$ e.p.r. signal and the m.c.d. peak at approx. 1500nm to a haem group with histidine–histidine co-ordination. We note that this assignment of the two sets of low-spin e.p.r. signals is in agreement with that suggested by Aasa *et al.* (1981).

Nature of the high-potential haem

In summary, the data presented here reveal the presence of a high-potential haem with unusual properties for a methionine–histidine-ligated haem. The methionine ligand appears to be rather more loosely bound than is usual for a cytochrome of the *c*-class. We interpret our results in terms of a thermal equilibrium between low-spin and high-spin ferric forms at room temperature. The question arises whether this represents an equilibrium between two species that are respectively six- and five-co-ordinate, with and without an axial methionine, or an equilibrium between two six-co-ordinate haem groups, one with methionine and one with the methionine replaced by another weak-field ligand supplied by the protein or by solvent. We cannot decide between these possibilities. However, since in many preparations the haem goes fully low-spin on cooling, we suppose that the former explanation is the more likely. This view is also supported by the lack of reactivity of the haem with added anions in the oxidized state. It has been reported that in horse heart cytochrome *c* at room temperature and up to 60°C an increasing proportion of the haem becomes high-spin ferric (Moore & Williams, 1977). Thus the low-spin and high-spin states are relatively close in energy. It may be that there is a series of cytochromes *c* of increasing high-spin character at room temperature. It has been proposed that cytochrome *c'* represents one end of this scale, in which the methionine ligand has become so weakly attached that it readily leaves the haem and the protein becomes totally five-co-ordinate at room temperature (Akutsu *et al.*, 1983). Thus the high-potential haem of cytochrome *c*-551 peroxidase could be considered as intermediate between a fully low-spin cytochrome *c* and a cytochrome *c'*.

The other unusual features of the high-potential haem are the long wavelength of the band at 708nm, the counterpart of the '695nm' band of cytochrome *c*, and the long wavelength of the near-i.r. band compared with other species. We have also discovered that the high-potential haem is photosensitive in its ferrous state and can lose its methionine ligand, becoming high-spin ferrous on exposure to light. Again this may indicate a rather loose attachment of the methionine ligand.

Nature of the low-potential haem

We have been led to the conclusion that this haem remains predominantly in the low-spin ferric state at both room temperature and low temperature in the oxidized state of the enzyme. At low temperature it is co-ordinated by two histidine residues and is probably similarly liganded at room temperature. This accounts for the observation that the haem is unreactive towards anionic ligands. However, it poses an interesting mechanistic puzzle. Ellfolk *et al.* (1983) have identified this haem as the site of peroxidatic activity in the half-reduced state of the enzyme. It remains to be shown how the haem is activated towards reactivity with the substrate H_2O_2 in the half-reduced state.

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