The nature of species prepared by photolysis of half-reduced, fully reduced and fully reduced carbonmonoxy-cytochrome *c*-551 peroxidase from *Pseudomonas aeruginosa*

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The half-reduced, fully reduced and fully reduced CO-bound forms of the enzyme cytochrome *c*-551 peroxidase isolated from *Pseudomonas aeruginosa* were examined by a combination of low-temperature absorption and magnetic-circular-dichroism spectroscopy. Deliberate low-temperature (4.2 K) photolysis of these forms of the enzyme, in all of which the high-potential haem is in the ferrous state, revealed that this haem group, assigned to have a histidine–methionine ligand set, is photosensitive. The photolabile ligand is most likely to be the methionine residue, and the product of photolysis, namely the high-spin ($S = 2$) ferrous form, is stable at low temperature (4.2 K). Warming to approx. 20 K allows thermal recombination to occur, restoring the low-spin ($S = 0$) state. The low-potential haem (bis-histidine ligation) is photoinert in both ferric and ferrous states; however, the photosensitive CO adduct of this centre cannot be maintained as the photolysed ($S = 2$) product at 4.2 K. This surprising observation may be due to quantum-mechanical tunnelling of the CO through the activation barrier even at 4.2 K, implying that the activation barrier to thermal recombination is both narrow and low. Low-temperature absorption spectroscopy reveals that the high-potential haem has a very characteristic low-spin ferrous spectrum with intense highly structured β - and split α -bands, whereas the spectrum of the low-potential ferrous haem contains α - and β -bands devoid of fine structure.

Pseudomonas cytochrome *c*-551 peroxidase (ferrocytochrome *c*-551 : H_2O_2 oxidoreductase, EC 1.11.1.5) catalyses the oxidation of ferrocytochrome *c*-551 by H_2O_2 in a reaction essentially identical with that catalysed by the peroxidases isolated from yeast and horseradish root tissue. The bacterial enzyme differs from other peroxidases in having two haem moieties covalently attached to a single polypeptide chain (Ellfolk & Soininen, 1971). A number of studies have clearly demonstrated the inequivalence of the haem centres in both structure (Rönnberg *et al.*, 1980; Aasa *et al.*, 1981) and reactivity (Rönnberg & Ellfolk, 1979; Rönnberg *et al.*, 1981). Ellfolk *et al.*

(1983) have determined the oxidation–reduction potentials to be widely different, at about +320 and –330 mV respectively. The HP haem centre can be reduced by the physiological electron donors azurin (Cu^{I}) and cytochrome *c*-551 (Fe^{II}) as well as by such chemical reductants as ascorbate, ferrocyanide and NADH. This half-reduced form of the enzyme does not bind CO, it being necessary to reduce the enzyme completely with a powerful chemical reductant such as $\text{Na}_2\text{S}_2\text{O}_4$ in order to observe CO-reactivity. The rate of combination of CO with the fully reduced enzyme is $1.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 6.0 (Foote *et al.*, 1983), and because of the very high affinity it has been possible to establish the stoichiometry of CO binding as one per two haem groups (Rönnberg & Ellfolk, 1979). The conclusion from CO-binding studies therefore is that only one haem, presumed to be the LP one, reacts with this ligand, and, from the lack of CO-sensitivity of the peroxidative oxidation,

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.

that this haem centre is never reduced during the catalytic cycle.

Photodissociation of ferrous haemoproteins has been recognized since the observation by Haldane & Lorrain-Smith (1896) of the photodissociation of CO from carbonmonoxyhaemoglobin. The list of photodissociable ligands now includes cyanide from ferroperoxidase cyanide and ferromyoglobin cyanide (Keilin & Hartree, 1955) and O₂, NO and isocyanide from ferrohaem complexes (Gibson & Ainsworth, 1957). This photolability contrasts with the photoinert character of liganded ferrihaems. However, there is, to our knowledge, no report of the photodissociation from a ferrous haem of an axial ligand supplied by the side chain of an amino acid residue of the protein.

In the present paper we report the use of low-temperature absorption and m.c.d. spectroscopy to study the nature of photolysed and thermally recombined forms of the enzyme cytochrome *c*-551 peroxidase. This work demonstrates, we believe for the first time, the photolytic removal of an endogenous (protein) haem ligand and, further, that the photolabile group, most probably a methionine residue, is associated with the HP haem.

Materials and methods

Sodium dithionite (Na₂S₂O₄ + H₂O, 85% min. assay) and glycerol (AnalaR) were obtained from BDH Chemicals. NADH (grade III), phenazine methosulphate, Tween 80, Mes (4-morpholine-ethanesulphonic acid) and horse heart cytochrome *c* (type III) were obtained from Sigma Chemical Co. ²H₂O (99.8 atom% ²H) was purchased from Aldrich Chemical Co., and CO from British Oxygen Co. (London S.W.19, U.K.).

Cytochrome *c*-551 peroxidase was isolated from *Pseudomonas aeruginosa* (N.C.T.C. 6750) as described previously (Foote *et al.*, 1983, 1984). The purity ratio A_{407}/A_{280} was 4.65:1. Enzyme concentrations were calculated by using $\epsilon_{407}^{1\%} = 237 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ (Soininen & Ellfolk, 1973). The samples for low-temperature spectroscopy were prepared anaerobically in a Faircrest Engineering glovebox operating with a purified N₂ atmosphere containing less than 1 p.p.m. O₂. Half-reduction was achieved by using a slight excess of NADH in the presence of the mediator phenazine methosulphate, and full reduction by the addition of excess solid Na₂S₂O₄. Fully reduced CO-bound enzyme was formed by equilibrating fully reduced peroxidase with 0.1 MPa (1 atm) CO. Peroxidase samples were in 50 mM-Mes buffer at either pD 5.6 or pH 5.2, containing 0.01% Tween 80, which is required for full stability of the enzyme during degassing (Foote *et al.*, 1983). Cytochrome *c* was pre-

pared in 50 mM-Mes buffer, pD 6.0, and was reduced with excess solid Na₂S₂O₄.

The samples were subsequently mixed anaerobically with an equal volume of glycerol in order to produce transparent glasses at low temperature, and introduced into sealed cells of approx. 1.2 mm pathlength. These were plunged into liquid He contained in an Oxford Instruments SM-4 split-coil magnet, capable of generating fields of up to 5 T. Optical-absorption spectra were recorded with either a Cary 14 or a Cary 17D spectrophotometer, both possessing specially modified sample compartments. M.c.d. spectra in the region 250–1050 nm were measured with a Jasco J-500D spectropolarimeter, and in the region 800–2000 nm (when required) with a dichograph constructed in these laboratories and described previously (Eglington *et al.*, 1980). Temperatures were measured with a carbon/glass resistor calibrated by Cryogenic Calibrations (Aylesbury, Bucks., U.K.). The temperature was controlled by using a cryogenic linear temperature sensor and heater mounted on the sample probe, in conjunction with an Oxford Instruments DTC-2 temperature controller.

Photolysis was carried out *in situ* with the sample remaining immersed in liquid He at 4.2 K. The unfocused output of a 100 W slide projector was used to irradiate samples for 15–20 min until no further spectral changes were evident, and absorption and/or m.c.d. spectra at 4.2 K were recorded. Subsequently, the temperature was raised to at least 50 K for 1–2 min in the dark to allow thermal recombination. The temperature was then returned to 4.2 K and further absorption and/or m.c.d. spectra were recorded. Only in the Soret region (fully reduced CO-bound peroxidase) did the spectropolarimeter beam cause measurable photolysis of a thermally recombined sample during scanning at 4.2 K. This was used to advantage (Fig. 7), as it allowed gradual photolysis of this sample, either by repeated scanning or by leaving the sample in the monochromatic beam at 420 nm wavelength for 1 min.

Results

Half-reduced enzyme

The half-reduced enzyme, produced by NADH/phenazine methosulphate reduction, contains the HP haem in the reduced state and the LP haem in the ferric form. The e.p.r. spectrum of this state has previously been reported (Aasa *et al.*, 1981) to contain one low-spin ferric haem with *g*-values of 2.83, 2.35 and 1.54. The e.p.r. spectrum of the sample here had a *g_z* value of 3.00. We do not currently understand the reasons for the differences between the two types of half-reduced enzyme. However, a previous sample with e.p.r. *g*-values of 2.85, 2.37

and 1.55 had a 4.2K near-i.r. m.c.d. spectrum similar to the one reported here.

The 4.2K near-i.r. m.c.d. spectra of the half-reduced form of the enzyme, after photolysis and subsequent to thermal recombination, are given as Fig. 1(a). The visible-region-absorption and m.c.d. spectra of the same form of the enzyme, also after photolysis and subsequent to thermal recombination, are compared in Fig. 2.

Extensive changes in all the spectra occur on photolysis. We begin with an examination of Fig. 1(a) which clearly shows what is taking place. The long-wavelength feature, with a peak at 1450nm and a marked shoulder at 1250nm, is characteristic of a low-spin ferric porphyrin, being the porphyrin-to-ferric iron charge-transfer band. This band belongs to the species observed in the e.p.r. spectrum, and arises from the LP haem. The *g*-values suggest bis-histidine as the co-ordination pattern of this haem. Near-i.r. m.c.d. spectra of

various haemoproteins known to possess either histidine-histidine or imidazole-histidine ligation show a charge-transfer band varying in wavelength between 1500 and 1620nm (Sievers *et al.*, 1983a). Thus the wavelength of this band in the half-reduced peroxidase is shorter than has been recorded previously for bis-histidine systems.

Clearly, this band is unchanged by photolysis, showing that the LP haem remains in the six-coordinate ferric state. However, on photolysis new bands arise between 600 and 1100nm. These bands belong to a high-spin ferrous haem and are due to *d-d* transitions within the *d*-configuration of the iron. Room-temperature m.c.d. spectra of reduced cytochrome *c*' and deoxymyoglobin (Rawlings *et al.*, 1977) show a positive peak at approx. 750nm followed by a broader, weaker, peak at approx. 950nm. M.c.d. spectra of deoxymyoglobin, deoxyhaemoglobin and ferropoxidase (horseradish) have been reported by Sharonov *et al.* (1983) at

Fig. 1. Near-i.r. m.c.d. spectra of derivatives of *Ps. aeruginosa* cytochrome *c*-551 peroxidase showing the effect of photolysis (a) Half-reduced enzyme (290 μ M), containing 225 μ M-NADH and 12.5 μ M-phenazine methosulphate. (b) Fully reduced enzyme (200 μ M), containing excess dithionite. (c) Fully reduced CO-bound enzyme (110 μ M), containing excess dithionite and 500 μ M-CO. Broken lines represent photolysed samples, and continuous lines the same samples thermally recombined by warming to at least 50K. Conditions: temperature 4.2K, magnetic field 4.9T.

Fig. 2. Visible-region optical-absorption and m.c.d. spectra of half-reduced *Ps. aeruginosa* cytochrome *c*-551 peroxidase showing the effect of photolysis

-----, Photolysed; —, thermally recombined. Enzyme concentration was 290 μM , containing 225 μM -NADH and 12.5 μM -phenazine methosulphate. Conditions: temperature 4.2K, magnetic field 4.9T.

temperatures no lower than 10K and only at wavelengths below 800nm. Counterparts of the negative trough at 620nm and the positive peaks at 680 and 750nm are observed in these spectra. These results leave no doubt that the bands that appear on photolysis are due to the high-spin ferrous state of the HP haem.

Interpretation of the visible-region-absorption and m.c.d. spectra is made clear by comparison

with the spectra of horse heart cytochrome *c* recorded at 4.2K and 4.9T (Fig. 3). Cytochrome *c* is in the low-spin ferrous form, giving a highly structured temperature-independent m.c.d. spectrum, very similar to that observed at room temperature (Sutherland & Klein, 1972). At low temperature the absorption spectrum sharpens and reveals a great deal of fine structure. The absorption spectrum of cytochrome *c* at 77K also shows a clearly

Fig. 3. Visible-region optical-absorption and m.c.d. spectra of reduced horse heart cytochrome *c*. Protein concentration was $49\ \mu\text{M}$, containing excess dithionite. Conditions: temperature 4.2K, magnetic field 4.9T. Irradiation of the sample with white light did not affect the spectra.

resolved splitting of the α -band (Estabrook, 1961), resulting from a lifting of the x, y degeneracy of the excited state of the porphyrin ring. We have been unable to photolyse horse heart cytochrome *c* at 4.2K by using the same procedure as for the peroxidase enzyme.

The visible-region-absorption and m.c.d. spectra of the half-reduced enzyme in the thermally recombined state (Fig. 2) show clearly the presence of a low-spin ferrous haem. The fine structure in both the absorption and the m.c.d. spectrum is remarkably similar to that in the spectra of cytochrome *c*, and the HP haem is unambiguously assigned as methionine-histidine-ligated. The bands are shifted to longer wavelength in the enzyme by 4–7 nm, and the α -band splitting is 6 nm compared with 3 nm in the case of cytochrome *c*.

The spectral features of the ferrous HP haem are built upon broader underlying bands in both the

absorption and the m.c.d. spectrum. These features belong to the low-spin ferric haem revealed by the positive m.c.d. peak at 1450 nm (Fig. 1). They are most obvious as positive m.c.d. peaks at approx. 480 and 580 nm. These features are unaffected by photolysis, as expected. The α/β -region m.c.d. peaks of a low-spin ferric haem are rather broad and merely supply a sinusoidal base-line for the HP haem spectrum between 500 and 550 nm. The m.c.d. spectrum at 4.2K of a bis-histidine-coordinated haem is given in the preceding paper (Foote *et al.*, 1984).

After photolysis of the half-reduced enzyme (Fig. 2), the highly resolved features of the reduced haem are almost entirely lost from the absorption and the m.c.d. spectra. Only a small vestige is visible in the absorption spectrum at 548 and 555 nm. The formation of high-spin ferrous haem is seen in the absorption spectrum by the develop-

ment of the shoulder at approx. 570 nm. The m.c.d. spectrum of the photolysed form is the convolution of that of a low-spin ferric haem and that of a high-spin ferrous haem. Low-temperature visible-region m.c.d. spectra of deoxymyoglobin and ferroperoxidase have been reported (Sharonov *et al.*, 1983).

In summary, these results show that the reduced HP haem at 4.2 K has methionine-histidine coordination and is photolabile to generate a high-spin ferrous haem, presumed to be five-co-ordinate. The LP haem remains in the low-spin state throughout and is unaffected by irradiation. It is probably co-ordinated by two histidine residues.

Fully reduced enzyme

Treatment of either the fully oxidized or half-reduced enzyme with $\text{Na}_2\text{S}_2\text{O}_4$ results in the formation of a fully reduced form of the enzyme in which both HP and LP haem groups are ferrous. The visible-region-absorption and m.c.d. spectra at 4.2 K and 4.9 T after photolysis and after thermal recycling are given in Fig. 4. The m.c.d. spectra between 600 and 1050 nm are shown in Fig. 1(b). The latter reveal that both haem groups are low-spin ferrous before photolysis. The weak features arise from the $d-d$ bands of the low-spin ferrous configuration. They are clearly seen in the absorption, c.d. and m.c.d. spectra of reduced horse heart cytochrome *c* in the region 600–1000 nm (Sutherland & Klein, 1972). After photolysis of the fully reduced peroxidase a set of m.c.d. bands appears in the region 600–1050 nm, identical in form and magnitude with those observed for the half-reduced enzyme, suggesting that one haem only, the HP haem, is being photolysed.

These conclusions are fully borne out by the visible-region spectra (Fig. 4). The absorption spectrum before photolysis is highly resolved and almost identical with that in Fig. 2, except that the shorter-wavelength component of the split α -band is more intense and broader. After photolysis this structure is lost, leaving a peak at 548 nm due to the α -band of the low-spin ferrous LP haem, which remains unphotolysed. The m.c.d. spectrum of the thermally recombined form contains features only in the region 500–560 nm and results from the superposition of the m.c.d. spectra of two low-spin ferrous haem groups, one liganded by methionine-histidine and the other by two histidine residues. The m.c.d. spectrum at 4.2 K of reduced cytochrome *c*₃ from *Desulfovibrio vulgaris* was also measured (results not shown), as an example of a haemoprotein with bis-histidine ligation. The m.c.d. spectrum (Fig. 4) between 540 and 555 nm results from the overlap of two sharp A -terms: that arising from the LP haem is centred at 548 nm, under the shorter-wavelength component of the split α -band of the HP haem. On photolysis the

contribution of the HP haem is changed into broad features of the form seen in Fig. 2. The LP haem remains low-spin and gives the sharp m.c.d. trough at 548 nm and peak at 544 nm plus the fine structure down to 500 nm.

Therefore we conclude that in the fully reduced enzyme before photolysis both HP and LP haem groups are low-spin ferrous in form. Photolysis causes the HP haem only to become high-spin ferrous and presumably five-co-ordinate.

Fully reduced CO₂-bound enzyme

The fully reduced enzyme binds CO to form a complex in which the CO/Fe ratio is 0.5:1 (Rönnberg & Ellfolk, 1979). The complex is photosensitive at room temperature (Foote *et al.*, 1983), although the quantum yield appears to be substantially less than 1 (N. Foote, D. Barber & C. Greenwood, unpublished work). It was therefore decided to examine this derivative of the enzyme at liquid-He temperature in order to determine which haem combines with CO. Fig. 1(c) shows the near-i.r. m.c.d. spectra, and Figs. 5 and 6 show the visible- and Soret-region-absorption and m.c.d. spectra of the photolysed and thermally recombined forms of carbonmonoxy-cytochrome *c*-551 peroxidase.

We refer to the thermally recombined form of the enzyme first. The visible-region-absorption and near-i.r. m.c.d. spectra show only low-spin ferrous haem present. There are no temperature-dependent bands or intense features between 560 and 1000 nm. The absorption spectrum is dominated by the split α -band and fine structure of the HP haem in the low-spin ferrous state. This spectrum is almost identical with that of the half-reduced enzyme (Fig. 2). Similarly, the m.c.d. peaks and troughs in the region 500–560 nm all have counterparts in the spectrum of the half-reduced enzyme (Fig. 2). Therefore we conclude that the spectrum of the HP haem has been unaffected by the binding of CO and hence this haem is not the site of CO binding. Consequently the CO ligand must be bound to the LP haem.

However, there are no features in the visible-region-absorption or m.c.d. spectra of the CO derivative that can be assigned to the CO-bound LP haems. Therefore the spectral bands must be much less intense. The visible-region-absorption spectra of CO-bound haem groups are rather broad, without a well-resolved and sharp α -band. For example, the absorption of CO-(horseradish peroxidase) has two broad peaks at 575 and 544 nm with ϵ values of about $11 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Nozawa *et al.*, 1976). The room-temperature m.c.d. spectra have been reported of CO-(horseradish peroxidase) (Nozawa *et al.*, 1976) and of CO-myoglobin (Vickery *et al.*, 1976). The spectra are expected to

Fig. 4. Visible-region optical-absorption and m.c.d. spectra of fully reduced *Ps. aeruginosa* cytochrome c-551 peroxidase showing the effect of photolysis

-----, Photolysed; —, thermally recombined. Enzyme concentration was 200 μM , containing excess dithionite. Conditions: temperature, 4.2K; magnetic field, 4.9T.

be little different at 4.2K, since the haem is diamagnetic. The m.c.d. spectra have relatively weak bands in the α/β -region with $\Delta\epsilon$ values of $0.2\text{mm}^{-1}\cdot\text{cm}^{-1}$ at 5T, showing a negative trough at 580nm and a positive peak at 565nm. There is no evidence for the expected counterparts of these bands in the m.c.d. spectrum of the thermally recombined form of the enzyme (Fig. 5).

After photolysis with white light at 4.2K, the highly structured features of the HP haem are lost from the visible-region-absorption spectrum and m.c.d. spectrum (Fig. 5), and the bands characteristic of high-spin ferrous haem appear between 600 and 1050nm (Fig. 1c). Clearly, the HP haem undergoes photolysis in the carbonmonoxy derivative of the enzyme, as it does in the half- and fully

Fig. 5. Visible-region optical-absorption and m.c.d. spectra of fully reduced CO-bound *Ps. aeruginosa* cytochrome c-551 peroxidase showing the effect of photolysis

----, Photolysed; —, thermally recombined. Enzyme concentration was $110\ \mu\text{M}$, containing excess dithionite and $500\ \mu\text{M}$ -CO. Conditions: temperature, 4.2K; magnetic field, 4.9T.

reduced forms of the enzyme. However, the magnitudes of the m.c.d. bands in the region 600–1050 nm (Fig. 1) remain virtually constant in all three photolysed species, namely, the half-reduced, the fully reduced and the CO-bound enzyme. Furthermore, the spectra are superimposable, no wavelength shifts having occurred. Similarly, the high-spin ferrous peak at 563 nm has the same magnitude in the spectra of both fully reduced and CO-bound enzyme after photolysis (Figs. 4 and 5).

Thus the conclusion we draw is that the CO-bound LP haem is not high-spin ferrous at 4.2K after photolysis but remains unphotolysed and effectively masked in the visible region of the m.c.d. spectrum by the relatively intense m.c.d. features of the HP haem, in both the photolysed and thermally recombined states.

These conclusions are nicely substantiated by the absorption and m.c.d. spectra in the Soret region (Fig. 6). The unphotolysed enzyme contains

Fig. 6. Soret-region optical-absorption and m.c.d. spectra of fully reduced CO-bound *Ps. aeruginosa* cytochrome c-551 peroxidase showing the effect of photolysis ———, Photolysed; —, thermally recombined. Enzyme concentration was $19\text{ }\mu\text{M}$, containing excess dithionite and $500\text{ }\mu\text{M}$ -CO. Conditions: temperature, 4.2 K; magnetic field, 4.9 T.

an intense Soret band at 415 nm at 4.2 K which shows some structure on its peak. After complete photolysis a new broad peak at 434 nm appears and the intensity of the sharp peak at 413 nm falls. The identity of these species is shown by the m.c.d. spectra (Fig. 6) and the time course of the evolution of the m.c.d. spectra during photolysis by the monochromatic-light beam of the Jasco dichrograph (Fig. 7). As photolysis proceeds, a broad spectrum with a positive peak at 436 nm and a negative trough between 419 and 406 nm grows in intensity. This is the m.c.d. spectrum of high-spin ferrous haem, as comparison with the m.c.d. spectra of deoxymyoglobin and deoxyhaemoglobin shows (Brittain *et al.*, 1982). However, the sharp peak at 410 nm and trough at 416 nm do not change in intensity as photolysis proceeds. This peak-to-trough distance remains constant, although these features are gradually submerged by the stronger

Fig. 7. Effect of gradual photolysis on the Soret-region m.c.d. spectrum of fully reduced CO-bound *Ps. aeruginosa* cytochrome c-551 peroxidase

—, Spectrum at 4.9 T showing increasing photolysis. ———, Spectrum at 4.9 T of fully photolysed sample. ·····, Spectrum in the absence of a magnetic field, showing the natural c.d. of the unphotolysed sample. The 4.9 T m.c.d. traces are not corrected for the natural c.d. contribution. Enzyme concentration was $19\text{ }\mu\text{M}$, containing excess dithionite and $500\text{ }\mu\text{M}$ -CO. The temperature was 4.2 K.

spectrum of the high-spin ferrous haem. Therefore we assign these features to the Soret band m.c.d. spectrum of CO-bound LP haem. They are similar to the Soret-band m.c.d. spectrum of CO-ferroperoxidase (Nozawa *et al.*, 1976) and CO-myoglobin (Vickery *et al.*, 1976).

One further piece of evidence confirming that the CO-bound haem remains in the low-spin ferrous state after photolysis is given by the natural c.d. spectrum in the Soret region (represented by

the dotted line in Fig. 7). Rönnerberg *et al.* (1979) have shown that at room temperature the CO-bound enzyme gives an unusually prominent positive c.d. peak at 415 nm which is absent for the reduced form of the enzyme. This peak in the c.d. spectrum of the CO-bound enzyme sample at 4.2 K remained virtually unchanged throughout the photolysis procedure.

In summary, we have presented three lines of evidence that show conclusively that the CO-ligated LP haem remains in the low-spin ferrous state even after exposing the enzyme, held at 4.2 K, to white light. The m.c.d. peaks in the visible near-i.r. regions of the photolysed CO-bound enzyme have the same intensity as in the fully-reduced and half-reduced samples, showing that only one haem, namely the HP haem, has undergone photolysis. The absorption spectrum in the Soret region shows, after sample photolysis, two types of haem, one low-spin ferrous, CO-bound and one high-spin ferrous. Finally, the distinctive natural c.d. of the CO-bound haem remains unchanged in intensity after photolysis. This conclusion was unexpected because, at room temperature, the CO ligand can be flashed off the haem, although it rebinds with the highest recombination rate yet reported for CO binding, namely $1.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Foote *et al.*, 1983). The HP haem in the CO derivative does not have a CO molecule bound, and is photosensitive. This centre does not recombine with a detectable rate at 4.2 K.

Discussion

This study of the low-temperature absorption and m.c.d. spectra of some partially reduced and fully reduced derivatives of the cytochrome *c*-551 peroxidase from *Ps. aeruginosa* was originally undertaken in order to investigate the spin and ligand states of the two haem groups in their reduced, ferrous, states. However, during the work it became obvious that we were observing a quite unexpected photolytic reaction of the protein at 4.2 K.

Nature of the ferrous state of the HP haem

Our study of the visible-region-absorption and near-i.r. m.c.d. and e.p.r. spectra of the oxidized form of the enzyme (Foote *et al.*, 1984) shows quite conclusively that the HP haem is co-ordinated by methionine and histidine ligands, although involved in a thermal spin equilibrium between the high-spin and low-spin ferric states. The visible-region-absorption and m.c.d. spectra at 4.2 K of the half-reduced, fully reduced and CO-bound enzyme show that, in the absence of photolysis, the HP haem is in the low-spin state throughout. Spec-

tral features arising from this haem are almost identical with those of horse heart cytochrome *c*, and both show a split α -band. The m.c.d. spectrum is temperature-independent at low temperature but is intense, the α -band region peaks having $\Delta\epsilon$ values of approx. $2 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 4.9 T. Values of about $1.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at the same field have been reported for horse heart cytochrome *c* at room temperature (Sutherland & Klein, 1972). The increase in intensity on cooling can be attributed to band narrowing, although most of the intensity change due to this is complete by 77 K and low-temperature spectra are virtually independent of temperature over the range 1.5–77 K. Since m.c.d. spectroscopy is a form of difference spectroscopy, a great deal of intensity cancellation occurs. This is an important point to recognize for the present work, because the intensities of the visible-region m.c.d. bands of the CO-bound LP haem are an order of magnitude less intense than those of the HP haem, although both are diamagnetic.

Our results demonstrate clearly that the HP haem is photolabile whenever it is reduced, specifically in the half-reduced, fully reduced and CO-bound reduced forms. White light effected photolysis with the sample held at 4.2 K. Although no photolysis could be detected during the recording of the visible-region-absorption and near-i.r. spectra, photolysis readily takes place by irradiation with light in the Soret-band region. Whichever photolysis method is used, 100% of the HP haem is driven to the high-spin ferrous state. This state is stable indefinitely at 4.2 K, but re-warming to a minimum of 20 K promotes a rapid ligand recombination and the low-spin ferrous state results. The metastable photolysed state is high-spin ferrous ($S = 2$) and can be most clearly identified by means of the characteristic charge-transfer and $d-d$ bands in the region 600–1050 nm. It seems most probable that it is the methionine ligand that has been lost to give a five-co-ordinate haem with histidine remaining as the proximal ligand. The m.c.d. spectra between 500 and 800 nm down to 10 K have been reported for deoxymyoglobin, deoxyhaemoglobin and ferrous horseradish peroxidase (Sharonov *et al.*, 1983), all examples of high-spin ferrous haem groups with a histidine proximal ligand. The spectra are very similar to those of Figs. 1(a)–1(c). For example, the major positive band is at 757, 755 and 756 nm for deoxymyoglobin, deoxyhaemoglobin and horseradish peroxidase respectively. These compare with the value of 750 nm for the cytochrome *c*-551 peroxidase. The intensities have the same order of magnitude, but detailed comparison is not possible since the lowest temperatures of measurement in the Russian work were 17, 10 and 22 K respectively. Thus we conclude that the photolysed HP haem is very similar

to a high-spin ferrous haem co-ordinated by histidine.

We have tried and failed to photolyse at 4.2 K the haem in horse heart cytochrome *c*, but have succeeded in partially photolysing the haem in reduced cytochrome *c*-551 from *Ps. aeruginosa* (J. Peterson, N. Foote, C. Greenwood & A. J. Thomson, unpublished work). However, the resulting near-i.r. m.c.d. spectrum of the photoproduct is quite unlike that of the photolysed HP haem of cytochrome *c*-551 peroxidase.

These two examples appear to be the first report of the photolytic removal of an endogenous ligand of a ferrous haem. It seems unlikely that there are significant differences between the electronic structures of the various *c*-type cytochromes that we have tested for photolysis. It could be that the differences lie in the recombination rates at 4.2 K. It is noteworthy that, in the ferric state, the methionine ligand of the peroxidase HP haem is rather labile at room temperature (Foote *et al.*, 1984).

Nature of the LP haem

The LP haem has been observed in three different states, namely as a low-spin ferric haem (in the half-reduced form of the enzyme), as a low-spin ferrous haem (in the fully-reduced form) and as the CO derivative (in the carbonmonoxy form of the reduced enzyme). The m.c.d. spectra are different, as expected, in all three cases. In the half-reduced form of the enzyme at 4.2 K the LP haem is detectable by both m.c.d. and e.p.r. spectroscopy. The charge-transfer band at 1450 nm and the g_z value of 3.00 identify the state of ligation as bis-histidine. The spectra are independent of pD over the range so far examined, 5.6–6.4. The charge-transfer band has shifted with respect to its wavelength of 1500 nm in the fully oxidized enzyme (Foote *et al.*, 1984). It has been shown that the near-i.r. charge-transfer band of a low-spin ferric haem ligated by histidine and imidazole shifts to higher energy, approximately from 1500 to 1350 nm, on deprotonation at the N-1 of the co-ordinated imidazole (Gadsby & Thomson, 1982; Sievers *et al.*, 1983b). However, the shift in the charge-transfer band of the peroxidase LP haem on half-reduction of the enzyme is not sufficient to assign to this cause. The pH-independence of the spectrum of the half-reduced enzyme also argues against a deprotonation reaction. Rather, the shift can be ascribed to a haem–haem interaction, the shift resulting from the reduction of the HP haem. This work establishes the spin and ligand state of the LP haem only at temperatures below 77 K.

In the fully reduced form of the enzyme at 4.2 K the LP haem is low-spin ferrous by our results. There are no high-spin ferrous bands in the region 600–1050 nm in the m.c.d. spectrum of this form of

the enzyme before photolysis has occurred (Fig. 4). The state of ligation is almost certainly bis-histidine. M.c.d. spectra in the visible region have been reported for reduced pig liver cytochrome b_5 (Dolinger *et al.*, 1974; Vickery *et al.*, 1975) at room temperature. The haem is co-ordinated by two histidine residues. The m.c.d. spectrum between 500 and 570 nm is rather similar to that of reduced cytochrome *c*, having intense well-resolved peaks and troughs, the most intense of which have $\Delta\epsilon$ values close to $1\text{ mm}^{-1}\cdot\text{cm}^{-1}$ at 5 T. We have measured the 4.2 K m.c.d. spectrum of reduced *D. vulgaris* cytochrome c_3 , and confirmed that it has a spectrum similar to that of cytochrome b_5 . Thus the m.c.d. spectrum (Fig. 4) of the fully reduced enzyme consists of the overlap of the cytochrome *c* and c_3 types of spectra. This supports the conclusion of bis-histidine co-ordination for the LP haem.

We have been unable to detect photolysis in cytochrome c_3 at 4.2 K, and similarly the LP haem in cytochrome *c*-555 peroxidase does not appear to be photolabile in either the ferric or the ferrous state. It is possible that the ferrous–axial-ligand bond is photo-dissociated but that complete recombination takes place at 4.2 K. Only a time-resolved photolysis experiment would allow this possibility to be explored.

Finally, we consider the CO-bound form of the enzyme. There is no doubt that CO has bound to the LP haem. The absorption and m.c.d. spectra in the visible region (Fig. 5, thermally recombined) show the changes compared with those of the fully reduced form (Fig. 4). The Soret-region spectra (Fig. 6) are unambiguous. However, the CO-bound LP haem m.c.d. spectrum across the visible and near-i.r. regions is not observable in the presence of the intense spectrum of the reduced HP haem. The room-temperature m.c.d. of CO–(horseradish peroxidase) (Nozawa *et al.*, 1976) and CO–myoglobin (Vickery *et al.*, 1976) show that the visible-region spectra are about 10-fold less intense than the corresponding bands of cytochrome *c*.

After photolysis of the sample at 4.2 K no high-spin ferrous form of the LP haem can be detected. On the contrary, the Soret-band m.c.d. features of the CO–LP haem are present throughout the course of photolysis of the HP haem (Fig. 7). Therefore the CO ligand cannot be held off the haem thermally at 4.2 K.

The experiments of Foote *et al.* (1983) show that the CO-bound form of the enzyme can be photolysed at room temperature. These authors were not aware at that time of the photosensitivity of the reduced HP haem, reported for the first time in the present paper. However, it seems unlikely that the re-binding of the endogenous ligand of the HP haem was mistaken for the CO recombination, be-

cause the rate of CO combination with the fully reduced enzyme, as determined by stopped-flow measurements, was identical with the recombination rate measured after flash photolysis. The identity of these two rate processes is strong evidence that the experiments of Foote *et al.* (1983) have been correctly interpreted. In view of the discovery of the photolysis of the HP haem it will be of interest to attempt to detect the re-binding rate of the methionine ligand at room temperature.

Since CO can be photolytically removed from the LP haem at room temperature, we assume that it is also photolabile at 4.2 K, and that the recombination process at this temperature is rapid. Such a mechanism would prevent observation of a relatively long-lived photolysed high-spin ferrous LP haem at 4.2 K.

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After photodissociation of CO from ferrohaem, re-binding of the ligand may occur. The temperature-dependence of this process has been thoroughly investigated for the case of protohaem IX (Alberding *et al.*, 1976b), isolated haemoglobin chains (Alberding *et al.*, 1976a, 1978; Gerstman *et al.*, 1981) and myoglobin (Alberding *et al.*, 1978). The re-binding of CO to the haem iron takes place at temperatures below about 150 K in a process that is thermally activated and hence temperature-dependent. However, at temperatures below 20 K the process becomes temperature-independent and involves the tunnelling of the CO molecule through the thermal activation barrier (Alberding *et al.*, 1976a). The tunnelling rate depends on the width of the barrier and on the mass of the tunnelling particle (Atkins, 1982). The tunnelling rates reported for haem recombination with CO at 10 K vary over many orders of magnitude. A detailed analysis shows that the rate variation arises from the differences in the barrier height and hence its width. For example, in the case of myoglobin, which has a very low rate of recombination with CO, the barrier height is 10 kJ/mol and the barrier width at the tunnelling point is 0.12 ± 0.03 nm. By contrast, the barrier to tunnelling in protohaem IX by CO has a height of 1.0 kJ/mol and a width of 0.077 ± 0.02 nm (Alberding *et al.*, 1978). It is suggested that the barrier height is related to the energy needed to bring the iron atom from its out-of-plane position in the deoxy form to its position in the plane of the porphyrin in the carboxyhaem. Since this movement is linked in the protein to changes in the tertiary structure of the polypeptide

around the haem the barrier height is expected to be much greater in the protein than in the isolated protohaem IX. However, this ignores the possibility that the haem in a haemoprotein photolysed at 4.2 K may be frozen into a conformation closer to the ligand-bound conformation than to that of the deoxy form (Brittain *et al.*, 1982).

This discussion shows that there should be a relationship between the rate of CO re-binding at room temperature, insofar as it is determined by the height of the activation barrier, and the tunnelling rate at low temperature, which is governed by the width of the barrier. In the CO form of cytochrome *c*-551 peroxidase the tunnelling rate at 4.2 K has not been measured in the present work, but we can take it to be high, possibly comparable with the tunnelling rate of CO to protohaem IX. This implies a narrow barrier and hence a low activation barrier towards thermal recombination. The room-temperature recombination rate of CO after photolysis is $1.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Foote *et al.*, 1983), which should be compared with rates for myoglobin of 4.3×10^5 , yeast cytochrome *c* peroxidase of 4.6×10^3 and horseradish peroxidase of $5.7 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ (English *et al.*, 1984).

Therefore the two observations of efficient tunnelling at 4.2 K and of fast thermal recombination at room temperature are consistent with a low narrow barrier towards recombination. This would seem to imply a CO-binding process rather akin to that in isolated protohaem IX, presumably with little constraint placed upon the position of the proximal histidine group by the tertiary structure of the protein.

Finally, we consider the origin of the large difference between the low-temperature recombination rates of the CO ligand of the LP haem and the methionine ligand of the HP haem. The observations here raise the interesting question as to whether all cytochromes of the *c* type, and even the *b* class, have photo-labile iron-axial-ligand bonds but with very fast recombination rates that have effectively prevented observation of the lability so far. The HP haem of *Ps. aeruginosa* cytochrome *c*-551 peroxidase does not recombine with its methionine ligand at 4.2 K after photolytic cleavage. This may be a consequence either of a high barrier or of the large effective mass of the recombining ligand that is attached to the protein side chain. Further studies are required to determine the barrier height by thermal recombination experiments before this can be resolved.

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