Spectroscopic characterization of cytochrome *c* peroxidase from *Paracoccus denitrificans*

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The cytochrome *c* peroxidase of *Paracoccus denitrificans* is similar to the well-studied enzyme from *Pseudomonas aeruginosa*. Like the *Pseudomonas* enzyme, the *Paracoccus* peroxidase contains two haem *c* groups, one high potential and one low potential. The high-potential haem acts as a source of the second electron for H_2O_2 reduction, and the low-potential haem acts as a peroxidatic centre. Reduction with ascorbate of the high-potential haem of the *Paracoccus* enzyme results in a switch of the low-potential haem to a high-spin state, as shown by visible and n.m.r. spectroscopy. This high-spin haem of the mixed-valence enzyme is accessible to ligands and binds CN^- with a K_D of 5 μM . The *Paracoccus* enzyme is significantly different from that from *Pseudomonas* in the time course of high-spin formation after reduction of the high-potential haem, and in the requirement for

bivalent cations. Reduction with 1 mM ascorbate at pH 6 is complete within 2 min, and this is followed by a slow appearance of the high-spin state with a half-time of 10 min. Thus the process of reduction and spin state change can be easily separated in time and the intermediate form obtained. This separation is also evident in e.p.r. spectra, although the slow change involves an alteration in the low-spin ligation at this temperature rather than a change in spin state. The separation is even more striking at pH 7.5, where no high-spin form is obtained until 1 mM Ca^{2+} is added to the mixed-valence enzyme. The spin-state switch of the low-potential haem shifts the midpoint redox potential of the high-potential haem by 50 mV, a further indication of haem–haem interaction.

INTRODUCTION

H_2O_2 is formed as a result of incomplete reduction of O_2 to water. The toxicity of H_2O_2 arises from its ability to form free radicals, e.g. hydroxyl radicals, which are highly reactive and can result in cell damage or cell death (Halliwell and Gutteridge, 1989). H_2O_2 may be removed by catalase in a dismutation reaction or by peroxidase in a process of reduction to water.

The bacterial cytochrome *c* peroxidase of *Pseudomonas aeruginosa* has been extensively studied both spectrally and kinetically. The enzyme contains two haem *c* moieties covalently attached to a single polypeptide chain (Ellfolk and Soininen, 1970; Soininen et al. 1970). The midpoint oxidation–reduction potentials of the two haems are +320 mV and –330 mV (Ellfolk et al., 1983). The high-potential haem acts as an electron-transferring pole and can be reduced physiologically by azurin or cytochrome *c*-551, or non-physiologically by ascorbate. The low-potential haem acts as the peroxidatic centre. The fully oxidized enzyme does not bind added ligands (Ellfolk et al., 1984a) and only slowly reacts with H_2O_2 (Araiso et al., 1980). In contrast, the low-potential haem of the ascorbate-reduced enzyme will readily bind added ligands, including H_2O_2 (Ellfolk et al., 1984a; Araiso et al., 1980). Thus a haem–haem interaction appears to be important in this enzyme, by which reduction of the high-potential haem results in the low-potential haem adopting a more open conformation, allowing access of the substrate.

Various spectroscopic studies have shown the low-potential haem of the *Pseudomonas aeruginosa* peroxidase to be high spin in the mixed-valence enzyme (Ellfolk et al., 1983, 1984a; Ronnberg et al., 1980; Ellfolk et al., 1984b; Foote et al., 1984,

1985; Villalain et al., 1984). Ellfolk and co-workers concluded that this haem was also high-spin in the fully oxidized enzyme (Ronnberg et al., 1980; Ellfolk et al., 1984b). Similarly, a study of the spin states of a cytochrome *c* peroxidase from *Pseudomonas stutzeri* led Villalain and co-workers to assign the high-spin signal to the low-potential haem in both the fully oxidized and ascorbate-reduced enzyme (Villalain et al., 1984). In contrast, Foote and co-workers, using m.c.d. spectroscopy, proposed that the high-spin signal of the fully oxidized enzyme of the *Pseudomonas aeruginosa* peroxidase was due to a labile methionine co-ordination of the high-potential haem (Foote et al., 1984, 1985).

The amino acid sequence of the *Pseudomonas aeruginosa* peroxidase is known (Ronnberg et al., 1989). There is faint similarity (26% identity) to *Pseudomonas aeruginosa* cytochrome *c*-551 in the C-terminal region of the molecule, and this area may form a class I domain with a Cys-Xaa-Yaa-Cys-His haem-binding site and extraplanar iron co-ordination by His-181 and Met-254. Ronnberg et al. (1989) have proposed that His-240 is the distal ligand of the peroxidatic haem which controls the access of ligands to the haem group. The close proximity of Met-254 to His-240 may provide the link through which the high-potential haem is able to alter the structure around the low-potential haem. Indeed, the residue in cytochrome *c*-551 (Gly-51) corresponding to His-240 in the peroxidase lies very close behind the methionine ligand at the left hand side of the molecule.

A cytochrome *c* peroxidase from *Paracoccus denitrificans* has recently been identified (Goodhew et al., 1990). The enzyme is a dihaem *c*-type cytochrome of M_r 42000 on SDS/PAGE. The physiological electron donor to the peroxidase is probably the

Table 1 Amino acid composition of *P. denitrificans* cytochrome *c* peroxidase

The amino acid composition was determined as described in the Materials and methods section. The 'Average' is the average of 20 and 70 h values, except that 70 h values were taken for Ile, Val and Leu.

Amino acid	Composition (mol/2 mol of haem)			Nearest integer
	20 h	70 h	Average	
Asp	33.6	35.9	34.8	(35)
Thr	29.7	33.4	31.6	(32)
Ser	13.7	14.5	14.1	(14)
Glu	46.3	52.7	49.5	(50)
Pro	27.8	30.7	29.3	(29)
Gly	28.7	32.3	30.5	(31)
Ala	40.3	44.2	42.3	(42)
Val	13.1	19.5	19.5	(20)
Met	8.9	9.6	9.3	(9)
Ile	10.1	16.3	16.3	(16)
Leu	18.3	23.2	23.2	(23)
Tyr	4.1	5.1	4.6	(5)
Phe	16.3	20.1	18.2	(18)
His	7.1	7.1	7.1	(7)
Lys	10.7	12.7	11.7	(12)
Arg	10.0	11.3	10.7	(11)
Cys			[4]*	
Trp			[1]†	
Haem			[2]	
Total M_r ...			39565	

* Two haem-binding sites assumed, each containing two cysteine residues.

† Assumed value of 1 for tryptophan by analogy with *Ps. aeruginosa* cytochrome *c* peroxidase.

soluble cytochrome *c*-550 (Pettigrew, 1991). The present paper describes a spectroscopic characterization of the enzyme and proposes a mechanism for the haem-haem interaction.

MATERIALS AND METHODS

Growth of cells

Paracoccus denitrificans (A.T.C.C. 19367, N.C.I.B. 8944, L.M.D. 52.44) was grown as described by Goodhew et al. (1990).

Enzyme purification

Cytochrome *c* peroxidase was purified by the method of Goodhew et al. (1990). The pure enzyme had an A_{408}/A_{280} ratio of 5.6 and gave a single band on SDS/PAGE when converted into the apoprotein (see Goodhew et al., 1990).

Redox titration

Reductive and oxidative titrations were performed in an anaerobic cuvette constantly flushed with argon and magnetically stirred. The cuvette contained approx. 6 μ M cytochrome *c* peroxidase in 5 mM Mes/5 mM Hepes (sodium salts), adjusted to pH 7.5 with HCl, and 17 μ M phenazine methosulphate (PMS), phenazine ethosulphate (PES), diaminodurrol (DAD), 2-hydroxy-1,4-naphthoquinone (HNQ) and FMN as redox mediators. Higher buffer concentrations were avoided in order to prevent Ca^{2+} contamination from buffer salts. The pH at the end of the redox titration was measured, and these values are given in the Figure legends.

Figure 1 Visible absorption spectra of *P. denitrificans* cytochrome *c* peroxidase

The spectrum of oxidized cytochrome *c* peroxidase (i) was obtained from an untreated, 8 μ M sample of enzyme in 5 mM Mes/5 mM Hepes, pH 6. Mixed-valence spectra were obtained by addition of 1 mM ascorbate and 5 μ M DAD. (ii) Shows a spectrum of the peroxidase 2 min after ascorbate addition. The same sample is shown in (iii), 60 min after ascorbate addition in the presence of 1 mM CaCl_2 .

The ambient redox potential (E_{obs}) was monitored by a Pt pin electrode in combination with an Ag/AgCl reference (Russell pH Ltd.) and the potential, with reference to the standard hydrogen electrode, was obtained by adding 196 mV to E_{obs} .

Reductive titrations were carried out by addition of anaerobic sodium dithionite (30 mM). Sodium dithionite stock solutions were prepared in anaerobic 100 mM Mes/100 mM Hepes (sodium salts) adjusted to pH 7.5 with HCl. Oxidative titrations were carried out using 30 mM potassium ferricyanide in the same buffer.

Amino acid composition and determination of haem content

The amino acid composition of the protein was determined on a Locarte analyser after hydrolysis in 6 M HCl/0.1% phenol *in vacuo* for 20 or 70 h. The haem content of the same protein

Figure 2 Appearance of a high-spin state after ascorbate reduction of cytochrome *c* peroxidase

A 4 μM solution of cytochrome *c* peroxidase in 5 mM Mes/5 mM Hepes, pH 6, was reduced with 1 mM ascorbate/5 μM DAD. (a) shows the difference spectrum of the enzyme 2 min after ascorbate addition minus that of the oxidized enzyme. Ascorbate reduction is complete within 2 min and is followed by the slow appearance of ferric high-spin signals. (b) Shows difference spectra with increasing time after ascorbate addition minus that of the enzyme 2 min after ascorbate addition. The high-spin state forms slowly over 30 min in the absence of added Ca^{2+} and reaches an initial end point (1). After addition of 1 mM CaCl_2 an enhanced high-spin state (2) forms within 15 min. The high-spin formation is temperature-dependent and can be reversed by cooling on ice. (c) Shows the difference spectrum of the ascorbate-reduced high-spin enzyme put on ice for 20 min minus that of the same sample at room temperature.

solution was determined by the pyridine ferrohaemochrome method (ϵ_{550} 29.1 $\text{mM}^{-1}\cdot\text{cm}^{-1}$) (Morton, 1958).

RESULTS AND DISCUSSION

Haem content and amino acid analysis

The amino acid composition of the *Paracoccus denitrificans* cytochrome *c* peroxidase is shown in Table 1, expressed as mol of amino acid per 2 mol of haem. Summation of this composition yields a relative molecular mass of 39 565, in reasonable agreement with the value of 42 000 obtained from SDS/PAGE (Goodhew et al., 1990). We conclude that the protein has two haem groups, as observed for the *Pseudomonas aeruginosa* peroxidase (Ellfolk and Soininen, 1970; Soininen et al., 1970).

Optical spectra of oxidized and ascorbate-reduced cytochrome *c* peroxidase

Figure 1 shows optical spectra of the oxidized and ascorbate-reduced peroxidase. The oxidized enzyme has a Soret maximum at 409 nm and possesses a weak absorption band at 640 nm [Figure 1, trace (i)]. The 640 nm signal indicates the presence of a ferric high-spin haem (Moore and Pettigrew, 1990). This signal is also observed in the *Pseudomonas* enzyme, but there is disagreement as to its origin (Ellfolk et al., 1984b; Foote et al., 1984). Reduction of the high-potential haem by ascorbate/DAD is complete within 2 min and results in a shift to 419 nm of the high-potential haem Soret and disappearance of the 640 nm

band [Figure 1, trace (ii)]. The shoulder on the u.v. side of the high-potential haem Soret arises from the oxidized low-potential haem. The loss of the 640 nm band suggests that the high-spin signals of the fully oxidized enzyme arise from the high-potential haem. This assignment is in agreement with that of Foote et al. (1984) for the *Pseudomonas* enzyme. If the mixed-valence enzyme is aged for 60 min at room temperature in the presence of 1 mM CaCl_2 , marked changes are seen to occur in the spectrum [Figure 1, trace (iii)]. The gain of absorbance at 380 nm with concomitant loss at 410 nm represents a blue shift in the Soret of the oxidized low-potential haem, which, in conjunction with the appearance of a band at 640 nm, represents a low-spin to high-spin transition in that haem.

The ascorbate reduction and subsequent spin-state changes can be seen more clearly using difference spectroscopy (Figure 2). At pH 6.0, the different spectrum of the enzyme 2 min after ascorbate addition versus the oxidized enzyme (Figure 2a) shows characteristic features of a reduced low-spin haem with maxima at 419, 525 and 557 nm. Difference spectra, recorded at various times after this, versus the enzyme 2 min after ascorbate addition (Figure 2b), show the slow appearance of ferric high-spin signals with maxima at 380, 500 and 640 nm. The level of high-spin state formed and the time for its formation varies from one enzyme preparation to the next and may be due to different amounts of residual CaCl_2 already on the enzyme. (After treatment of oxidized enzyme preparations with EGTA, no formation of the high-spin state is observed after ascorbate reduction.) Addition of 1 mM CaCl_2 after the partial high-spin state has formed leads

Figure 3 300 MHz ^1H n.m.r. spectroscopy of cytochrome *c* peroxidase

Spectra were recorded on a 1 mM sample of enzyme in 10 mM Mes/10 mM Hepes, pH 6 (99.8% $^2\text{H}_2\text{O}$). The temperature was 303 K, and number of scans was 5000. The oxidized spectrum (a) was obtained from an untreated peroxidase sample. The ascorbate-reduced spectrum (b) was obtained 60 min after addition of 5 mM ascorbate/5 μM DAD.

to an enhancement of the high-spin signals (from 1 to 2 in Figure 2b). The Ca^{2+} effect is rapid and complete within 15 min. The low-spin to high-spin transition can be reversed if the Ca^{2+} -treated, ascorbate-reduced sample is put on ice for 20 min (Figure 2c). Ca^{2+} ions can be replaced by Mg^{2+} ions with similar results being obtained (results not shown). The partial high-spin state in the absence of added Ca^{2+} is not observed at pH 7.5, although addition of 1 mM Ca^{2+} results in the full appearance of the high-spin form. Thus the residual Ca^{2+} on the enzyme seems to be more weakly bound at this higher pH. This convenient property was exploited in obtaining the spectra of Figure 4 (below) and in the redox titrations (Figure 5, below).

300 MHz ^1H n.m.r. spectroscopy of cytochrome *c* peroxidase

N.m.r. spectroscopy confirms the presence of a high-spin haem in the fully oxidized and mixed-valence enzyme. The fully oxidized enzyme shows two groups of haem methyl resonances (Figure 3a). The group of strongly downfield-shifted resonances at 58.2 (2 methyls), 55 and 51.5 p.p.m. arise from the partially high-spin, high-potential haem. The high-spin ferric cytochromes *c'* have been shown to have very large downfield-shifted haem methyl resonances, in the region 60–90 p.p.m. (Emptage et al., 1981). The group of resonances at 33.2 and 23.8 p.p.m. arise from the low-spin, low-potential haem. The observation of a broad resonance near 90 p.p.m., which we assign to the $\epsilon\text{-CH}_3$ group of an axial methionine, suggests that the partially high-spin haem has methionine–histidine co-ordination.

A spectrum taken 60 min after ascorbate addition shows the disappearance of the signals seen in the oxidized enzyme, with a new set of resonances occurring between 51 and 64 p.p.m. (Figure 3b). The strong downfield shifts of these resonances arise from the oxidized low-potential haem being in a high-spin state.

N.m.r. spectroscopy has been applied to both the *Ps. aeruginosa* and the *Ps. stutzeri* cytochrome *c* peroxidases (Ellfolk et al., 1983, 1984a; Villalain et al., 1984). The results are summarized in Table 2. The fully oxidized enzymes all show two sets of

Figure 4 Spectral changes in the low-potential haem after ascorbate reduction of cytochrome *c* peroxidase

Anaerobic solutions of cytochrome *c* peroxidase (4 μM) in 5 mM Mes/5 mM Hepes, pH 7.5, in the absence (a) and presence (b) of 1 mM CaCl_2 were reduced by addition of 1 mM ascorbate and 5 μM DAD. Under these conditions the higher-potential haem is reduced (HP_{red}) and the lower-potential haem remains oxidized (LP_{ox}). This mixed-valence enzyme was incubated at room temperature for 30 min to allow any spin-state changes to occur. Small aliquots of a solution of anaerobic dithionite (50 mM) in 100 mM Mes/100 mM Hepes, pH 7.5, were added until full reduction of the enzyme was obtained ($\text{LP}_{\text{red}}\text{HP}_{\text{red}}$). The absolute spectra of $\text{LP}_{\text{red}}\text{HP}_{\text{red}}$ (—) and $\text{LP}_{\text{ox}}\text{HP}_{\text{red}}$ (---) forms are shown in (a)(i) and (b)(i). The difference spectra [(a)(ii), (b)(ii)] of the $\text{LP}_{\text{red}}\text{HP}_{\text{red}}$ minus $\text{LP}_{\text{ox}}\text{HP}_{\text{red}}$ is the redox difference spectrum of the low-potential haem and gives information on its spin state.

resonances, one set strongly downfield-shifted, representing a high-spin haem, and another set, less shifted and representing a low-spin haem. The ascorbate-reduced enzymes all show a set of strongly downfield-shifted resonances indicative of a high-spin haem.

Ellfolk et al. (1984a) assigned the strongly downfield-shifted resonances seen in the *Ps. aeruginosa* enzyme to the low-potential haem in both the oxidized and ascorbate-reduced states. Villalain et al. (1984) made the same assignments for the *Ps. stutzeri* peroxidase. In contrast, we propose the strongly downfield-shifted resonances of the oxidized *Paracoccus denitrificans* enzyme to be due to the high-potential haem in a high-spin state, whereas the downfield-shifted resonances of the mixed-valence enzyme are those of the high-spin, low-potential haem. This is consistent with m.c.d. spectroscopy of *Ps. aeruginosa* peroxidase, which led Foote et al. (1984) to conclude that, in the fully oxidized enzyme, it was the high-potential haem that was high-spin.

Spectral features of the reduced low-potential haem

Figure 4 shows the effect of fully reducing the enzyme after ascorbate reduction in the presence and absence of Ca^{2+} at pH 7.5.

In the absence of added Ca^{2+} , the fully reduced spectrum has an α - and a β -band at 551 and 525 nm respectively [Figure 4a(i)]. The α -band has a shoulder on the long-wavelength side,

Figure 5 Potentiometric redox titration of cytochrome *c* peroxidase in the absence and presence of Ca^{2+}

The redox titration was performed as described in the Materials and methods section (a)(i) and (b)(i) show reductive titrations of the peroxidase, as followed in the region of the α - and β -bands, in the absence and presence of Ca^{2+} respectively. (a)(ii) and (b)(ii) are Nernst analyses of the high-potential component in the absence and presence of Ca^{2+} respectively. Closed circles are for oxidative titration. Open circles are for reductive titration. The pH values measured after titration were 7.35 and 7.32 in (a) and (b) respectively.

at 557 nm. The spectrum of the reduced low-potential haem can be obtained by subtracting the ascorbate-reduced spectrum from the fully reduced spectrum [Figure 4a(ii)]. This difference spectrum shows fully resolved α - and β -bands, indicative of a ferrous low-spin haem. The absence of a trough at 640 nm shows that the low-potential haem was not high-spin in the ascorbate-reduced enzyme.

In the presence of Ca^{2+} , the fully reduced spectrum has an α - and a β -band at 557 and 525 nm respectively [Figure 4b(i)]. The α -band has a shoulder on the short-wavelength side (551 nm). A fully reduced minus ascorbate-reduced spectrum shows the reduced low-potential haem to have a fused α/β band similar to that seen in ferrous high-spin cytochromes *c'* [Figure 4b(ii)] (Meyer and Kamen, 1982). The trough at 640 nm suggests that this haem was also high-spin in the ascorbate-reduced enzyme.

These results show that the position and shape of the fully reduced α -band of the *Paracoccus* peroxidase is dependent on the spin state of the low-potential haem. In the absence of Ca^{2+} , the

fully reduced spectrum (with the low-potential haem in the low-spin state) has an α -band peak at 551 nm with a shoulder on the long-wavelength side. In the presence of Ca^{2+} (with the low-potential haem in the high-spin state) the α -band peak is at 557 nm with a shoulder at 551 nm.

Potentiometric redox titration of cytochrome *c* peroxidase

Oxidative and reductive titrations were carried out in the absence and presence of Ca^{2+} at pH 7.5. The respective reductive titrations are shown in Figure 5(a)(i) and 5(b)(i). In both cases, because of the large separation of midpoint potential (E_m) values, the contribution of a higher-potential haem could be easily separated from that of a lower-potential haem, and, in each case, the high-potential contribution is fitted to a Nernst plot with a slope of 59 mV (25 °C) [Figures 5a(ii) and 5b(ii)].

The redox titration establishes the presence of a high-potential and a low-potential haem as found in the pseudomonad cyto-

Figure 6 E.p.r. spectroscopy of cytochrome *c* peroxidase

Spectra were recorded from a 337 μ M sample of enzyme in 10 mM NaCl/1 mM phosphate, pH 7. The temperature was 8 K, the microwave frequency was 9.45 GHz, the microwave power was 2 mW, the modulation was 10.39 and the gain was 1.6×10^5 . The oxidized spectrum was obtained from an untreated sample of enzyme. Ascorbate (asc) was used to obtain the mixed-valence spectrum. *g* values for selected resonances are shown.

chrome *c* peroxidases (Ellfolk et al., 1983; Villalain et al., 1984). The high-potential haem has a midpoint potential of 176 mV in the absence of Ca^{2+} and 226 mV in its presence. The latter value is consistent with an approximately isopotential electron transfer from the proposed electron donor, cytochrome *c*-550 [$E_m = 0.25$ V, pH 7 (Kamen and Vernon, 1955)]. Non-concordance of the midpoint potential of the high-potential haem in the absence and presence of Ca^{2+} may reflect an influence of the spin state of the low-potential haem. When the high-potential haem is titrated in the presence of Ca^{2+} , the low-potential haem is high spin, but in the absence of Ca^{2+} it is low spin.

The low-potential haem was difficult to titrate accurately, with

poor reversibility of spectroscopic changes and a midpoint potential in the range -100 to -200 mV. We suspect this difficulty may be due to spin-state changes in this haem during the titration, perhaps as a result of alternative haem ligands being generated from dithionite. The midpoint potentials for the peroxidatic haems of other peroxidases are: *Ps. aeruginosa* peroxidase, -330 mV (Ellfolk et al., 1983); horseradish peroxidase, -270 mV (Harbury, 1957); yeast cytochrome *c* peroxidase, -190 mV (Conroy et al., 1978).

E.p.r. spectroscopy of cytochrome *c* peroxidase

The high-to-low-spin transition seen upon cooling the enzyme from 20 to 0 °C (Figure 2c) means that, when the enzyme is subjected to e.p.r. spectroscopy, carried out at 8 K, very little in the way of high-spin features will be observed. The e.p.r. spectrum of the oxidized enzyme shown in Figure 6 indicates the presence of three spectral components. The signals at $g = 3.41$ and $g = 3.0$ are indicative of two haems in a low-spin state with the signal at $g = 6.0$ representing a minor high-spin component.

The relative intensity of the signal at $g = 3.41$ compared with that at $g = 3.0$ is less than 1. It is therefore possible that the signal at $g = 6.0$ represents a small proportion of the haem with $g = 3.41$ in a high-spin state even at very low temperature.

Ascorbate reduction of the enzyme results in the loss of the $g = 3.41$ signal with initially no change in the $g = 3.0$ signal. From this we conclude that the $g = 3.41$ signal is that of the high-potential haem, whereas the low-potential haem is represented by the $g = 3.0$ signal. This $g = 3.0$ signal disappears over a 60 min period as new spectral features appear at $g = 3.3$ (82%), $g = 2.9$ (16%) and $g = 2.79$ (2%). The alteration in the e.p.r. signal represents a change in the co-ordination structure around the haem. It is this same slow process that, at room temperature, gives rise to the high-spin state of that haem.

Interestingly, an inactive form of the *Ps. aeruginosa* peroxidase has been found in which no ferric high-spin state forms after ascorbate reduction (Foote et al., 1985). This inactive form has a $g = 3$ e.p.r. signal like the transient ascorbate-reduced form of the *Paracoccus* enzyme and may correspond in structure to this form.

A summary of e.p.r. data for the bacterial peroxidases is shown in Table 2. The signals for the individual peroxidases are similar, except for the signal arising from the low-potential haem

Table 2. N.m.r. and e.p.r. data for bacterial cytochrome *c* peroxidases

Organism	Oxidized				Ascorbate-reduced‡		Spin-state assignments					Reference
	High-potential haem		Low-potential haem		Low-potential haem		Oxidized		Ascorbate-reduced			
	N.m.r.*	E.p.r.†	N.m.r.*	E.p.r.†	N.m.r.*	E.p.r.†	HP§ haem	LP haem	HP haem	LP haem		
<i>Ps. aeruginosa</i>	19.5, 21.5 p.p.m.	<i>g</i> = 3.24 <i>g</i> = 3.3	45–65 p.p.m.	<i>g</i> = 2.93 <i>g</i> = 3.0	45–65 p.p.m.	<i>g</i> = 2.84 <i>g</i> = 2.85 <i>g</i> = 2.94	Low High Low	High Low High	Low Low Low	High High High	Ellfolk et al. (1984) Foote et al. (1984) Villalain et al. (1984)	
<i>Ps. stutzeri</i>	22.7, 32.0 p.p.m.	<i>g</i> = 3.39	69.3, 64.1, 57.3, 55.9 p.p.m.	<i>g</i> = 3.01	62.0, 55.9, 55.1, 53.5 p.p.m.	<i>g</i> = 2.94	Low	High	Low	High	Villalain et al. (1984)	
<i>P. denitrificans</i>	58.2, 55.0, 51.5 p.p.m.	<i>g</i> = 3.41	23.8, 33.2 p.p.m.	<i>g</i> = 3.0	64.0, 57.0, 55.5, 51.0 p.p.m.	<i>g</i> = 3.3	High	Low	Low	High	The present study	

* Position of resolved downfield-shifted haem methyl groups.

† g_{max} values.

‡ Ascorbate-reduced high-potential haem is e.p.r.-invisible and, owing to being much less strongly downfield-shifted, is not resolved in n.m.r.

§ HP, high-potential.

|| LP, low-potential.

Figure 7 CN^- titration of the ascorbate-reduced form of cytochrome *c* peroxidase

A $1.76 \mu\text{M}$ solution of cytochrome *c* peroxidase (CCP) in 5 mM Mes/5 mM Hepes (pH 6)/1 mM CaCl_2 was reduced with 1 mM ascorbate/5 μM DAD. The sample was aged for 60 min at room temperature to allow formation of the high-spin state. A CN^- titration was performed using a stock neutralized NaCN solution. Selected spectra are shown at the following CN^- concentrations: 0, 0.5, 1.5, 3.5, 6.0, 10.0, 15.0, 40.0, 110.0, 150.0 μM (a). The dissociation constant for CN^- binding was calculated using the decrease in absorbance at 380 nm as an indicator of CN^- bound to the enzyme. (b) Shows a plot of CN^- -bound peroxidase versus $\log [\text{CN}^-]_{\text{free}}$. The experimental points are fitted to a theoretical curve for the titration of a single binding site with a K_D of 5 μM .

of the mixed-valence form of the *Paracoccus* enzyme. The g -value of 3.3 is a somewhat higher value than that observed for the other peroxidases.

CN^- titration of cytochrome *c* peroxidase

No change in the spectrum of the oxidized enzyme is observed upon addition of up to 150 μM CN^- (results not shown). This

Scheme 1 Model of action for cytochrome *c* peroxidase of *P. denitrificans*

The diagram represents the cytochrome *c* peroxidase as a two-domain dihaem enzyme. We propose that the smaller domain is formed by a class I cytochrome *c* sequence with methionine-histidine co-ordination of one haem. This is based on the amino acid sequence of the *Ps. aeruginosa* enzyme (Ellfolk et al., 1991) and the same class I features are present in the amino acid sequence of the enzyme from *P. denitrificans* (J. van Beeumen, R. Gilmour, C. F. Goodhew and G. W. Pettigrew, unpublished work). In addition, the oxidized form has a weak absorption band at 695 nm indicative of methionine co-ordination (Moore and Pettigrew, 1990). Class I cytochromes tend to have positive redox potentials (Pettigrew and Moore, 1987) and we propose that this domain accepts the incoming electron to the oxidized enzyme (a). An intermediate mixed-valence state [(b)(i)] is formed in which the low-potential oxidized haem remains low-spin. Reduction of that haem with dithionite results in a low-spin bis-Fe^{II} enzyme [(b)(ii)]. In a slow phase, enhanced by Ca^{2+} the intermediate state [(b)(i)] is converted into a form [(c)(i)] which contains a high-spin low-potential Fe^{III} haem. A channel indicates ligand access to this haem. When this species [(c)(i)] is reduced with dithionite, a high-spin Fe^{II} haem is observed [(c)(ii)]. The enzyme cycle at the bottom of the diagram has not been studied directly in the present paper and is based on the model of Ellfolk et al. (1983) for the *Pseudomonas* enzyme. It shows the reduction of H_2O_2 using one electron from the methionine-histidine haem and a second from the Fe^{II} of the peroxidatic centre (Ellfolk et al., 1983). Although the amino acid sequence strongly suggests a methionine-histidine co-ordination for the high-potential domain, the co-ordination of the peroxidatic haem is speculative. The distal ligand to the peroxidatic haem (L) is thought to be either a histidine or lysine (Ellfolk et al., 1991). It should be noted that the evidence for the high-potential domain being the methionine-histidine haem is slight, and is based on the knowledge that the class I cytochromes tend to have positive potentials (Pettigrew and Moore, 1987). However, if the methionine is weakly bound, this haem could conceivably be low potential with the bis-histidine haem high potential. Thus an alternative model would have an electron entering the bis-histidine haem as a high-potential haem, followed by a fast switch in haem potentials so that the methionine-histidine haem becomes reduced by internal electron transfer. Such a model would retain the bis-histidine haem as the peroxidatic centre.

result indicates that the high-spin haem of the fully oxidized enzyme is not available for ligand binding, a result not surprising considering that the high-spin haem in this state is the high-potential, electron-transferring haem. The fully oxidized form of *Ps. aeruginosa* peroxidase is also unable to bind cyanide unless concentrations of over 4 mM are used, at which point reduction of the enzyme is also seen (Ellfolk et al., 1984a).

When the ascorbate-reduced aged enzyme (in 1 mM CaCl_2) is titrated with CN^- , marked spectral changes occur, representing a high-to-low-spin transition in the oxidized low-potential haem

(Figure 7a). The decrease in absorbance at 380 nm can be used as a measure of CN^- bound to the peroxidase. The experimental data fit a theoretical saturation curve created for the titration of a single binding site per protein molecule with a dissociation constant of $5 \mu\text{M}$ (Figure 7b), which is similar to the $23 \mu\text{M}$ found for the *Ps. aeruginosa* peroxidase (Ellfolk et al., 1984a). The Soret band of the low-potential haem with CN^- bound is more red-shifted than would be expected for a low-spin low-potential haem. This is a result of the CN^- ligation, and has also been observed in the CN^- adducts of *Ps. aeruginosa* (Ellfolk et al., 1984a), horseradish (Keilin and Hartree 1951) and yeast cytochrome *c* peroxidases (Sievers, 1978).

Conclusion

The cytochrome *c* peroxidase of *P. denitrificans* is a dihaem *c*-type cytochrome with high- and low-potential haems. The haems of the fully oxidized enzyme are unavailable for ligand binding, even though the high-potential haem is at least partly high-spin. Ascorbate reduction of the enzyme results in the rapid conversion of the high-potential haem from high-spin into low-spin, followed by a slow conversion of the low-potential haem from low-spin into high-spin. The high-spin formation in the low-potential haem is temperature-dependent and enhanced by the presence of bivalent cations. The high-spin low-potential haem will readily bind added ligands.

The spin state change seen in the high-potential haem when it becomes reduced may play a pivotal role in the haem-haem interaction in this enzyme. The stronger association of the methionine ligand to the reduced high-potential haem may be linked through the protein to the movement of a histidine away from the low-potential haem, ensuring that the substrate will only bind when the enzyme has the reducing power available to carry out the reaction. A model for the proposed activation of the enzyme is shown in Scheme 1. The reaction cycle shown is based upon the model proposed for the *Ps.* peroxidase (Ellfolk et al., 1983).

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