Mössbauer Characterization of *Paracoccus denitrificans* Cytochrome *c* Peroxidase

FURTHER EVIDENCE FOR REDOX AND CALCIUM BINDING-INDUCED HEME-HEME INTERACTION*

Susana Prazeres, José J. G. Moura, and Isabel Moura‡

From the Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2825 Monte de Caparica, Portugal

Raymond Gilmour, Celia F. Goodhew, and Graham W. Pettigrew‡

From the Department of Preclinical Veterinary Sciences, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Summerhall, Edinburgh EH9 1QH, United Kingdom

Natarajan Ravi§, and Boi Hanh Huynh‡

From the Department of Physics, Emory University, Atlanta, Georgia 30322

Mössbauer and electron paramagnetic resonance (EPR) spectroscopies were used to characterize the diheme cytochrome *c* peroxidase from *Paracoccus denitrificans* (L.M.D. 52.44). The spectra of the oxidized enzyme show two distinct spectral components characteristic of low spin ferric hemes ($S = 1/2$), revealing different heme environments for the two heme groups. The *Paracoccus* peroxidase can be non-physiologically reduced by ascorbate. Mössbauer investigation of the ascorbate-reduced peroxidase shows that only one heme (the high potential heme) is reduced and that the reduced heme is diamagnetic ($S = 0$). The other heme (the low potential heme) remains oxidized, indicating that the enzyme is in a mixed valence, half-reduced state. The EPR spectrum of the half-reduced peroxidase, however, shows two low spin ferric species with $g_{max} = 2.89$ (species I) and $g_{max} = 2.78$ (species II). This EPR observation, together with the Mössbauer result, suggests that both species are arising from the low potential heme. More interestingly, the spectroscopic properties of these two species are distinct from that of the low potential heme in the oxidized enzyme, providing evidence for heme-heme interaction induced by the reduction of the high potential heme. Addition of calcium ions to the half-reduced enzyme converts species II to species I. Since calcium has been found to promote peroxidase activity, species I may represent the active form of the peroxidatic heme.

A cytochrome *c* peroxidase has recently been isolated from *Paracoccus denitrificans* (1), and initial characterization has been performed using UV-visible, nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) spectroscopies (2–4). The

Paracoccus peroxidase is a periplasmic protein with a molecular mass of 40 kDa (2). The physiological electron donor is most probably the soluble cytochrome c_{550} from the same organism (5). In many respects, this enzyme appears to be closely related to the peroxidase isolated from *Pseudomonas aeruginosa*, which has been extensively studied both spectroscopically and kinetically (6–16). Similar to the *Pseudomonas* enzyme (11), the *Paracoccus* peroxidase contains two *c*-type hemes with well separated midpoint redox potentials (2). For the high potential heme of the *P. denitrificans* enzyme, the redox potential was determined to be +176 mV. For the low potential heme, the redox potential was estimated to be within the range of −200 to −100 mV (2). Based on the spectroscopic and biochemical results (2), together with analogies drawn from the studies of *P. aeruginosa* peroxidase, it is concluded that the low potential heme is the peroxidatic center and the high potential heme functions as an electron transfer site.

Spectroscopic investigations of the *Paracoccus* peroxidase have revealed a complex activation mechanism that involves changes in the redox and spin state of the heme groups (2–4). As purified, the peroxidase is in the oxidized form where both hemes are in the ferric state. At room temperature, the high potential heme is at least partly high spin ($S = 5/2$) and the low potential heme is low spin ($S = 1/2$). At low temperatures, both hemes are low spin exhibiting typical low spin ferric EPR signals (2). A NMR peak at 90 ppm, assignable to the ϵ-CH$_3$ group of an axial methionine ligand, was observed and suggested to be associated with the high potential heme, indicating that the high potential heme has a methionine-histidine axial coordination. The coordination of the low potential heme is not yet confirmed but has been proposed to be either bis-histidine or histidine-lysine by analogy to the *Pseudomonas* peroxidase. The oxidized form of the enzyme is catalytically inactive (17) and does not bind exogenous ligands such as cyanide. Addition of ascorbate reduces the high potential heme to the ferrous state, and a half-reduced mixed valence form of the enzyme is attained. The reduced high potential heme is low spin ($S = 0$) (4). This half-reduced form of the enzyme is catalytically active for the reduction of hydrogen peroxide (17) and readily binds exogenous ligands such as cyanide (2). Addition of sodium dithionite generates a fully reduced all ferrous form of the enzyme, which is a catalytically irrelevant species. Similar characteristics have also been found for other diheme bacterial peroxidases such as the peroxidases from *P. aeruginosa* (6–16) and *Pseudomonas stutzeri* (18). In addition the *Paracoccus*

enzyme was found to bind calcium ions (2, 3, 17). Other peroxidases, such as lignin (19), manganese (20), and horseradish peroxidases (21–23), have also been found to contain bound calcium ions. Binding of calcium to the *Paracoccus* peroxidase promotes its activation (17) and appears to induce conformational changes surrounding the immediate environment of the low potential heme (2, 3). In the presence of calcium, the low potential heme of the half-reduced enzyme becomes high spin at room temperature, suggesting a more open configuration for the heme site. The low potential heme is therefore ready for substrate binding. The binding of calcium also increases the midpoint redox potential of the high potential heme by approximately 50 mV, which should facilitate its reduction (2).

In this paper, a Mössbauer study of the *P. denitrificans* cytochrome *c* peroxidase is described. Both the oxidized and ascorbate-reduced forms of the enzyme were examined. Effects of the binding of calcium to the heme sites were investigated by both Mössbauer and EPR techniques. Intricate conformational changes induced by calcium binding to promote enzymatic activation are also suggested by this study.

MATERIALS AND METHODS

Cells of *P. denitrificans* (L.M.D. 52.44) were grown under low oxygen tension, and the periplasmic cytochrome *c* peroxidase was purified as described previously (1). The enzyme is isolated in the oxidized state. The growth medium was either enriched with the Mössbauer isotope ^{57}Fe (95% plus enrichment) or contained natural abundance ^{57}Fe (2.2%). The protein concentration of the purified enzyme solutions was determined by using $\epsilon = 250$ mM^{-1} cm^{-1} at 409 nm (oxidized enzyme) (1). The half-reduced form of the enzyme was achieved by anaerobic addition of a concentrated solution of sodium ascorbate. The enzyme was incubated under argon atmosphere for 60 min prior to calcium addition.

Mössbauer spectra of samples with a protein concentration of 1 mM in 10 mM Hepes (pH 6.4) and a volume of 350–400 μl were recorded, either on a weak field Mössbauer spectrometer equipped with a Janis 8DT variable temperature cryostat or on a strong field Mössbauer spectrometer equipped with a Janis 12 CNDT/SC SuperVaritemp cryostat containing an 8-tesla superconducting magnet. Standard transmission Mössbauer measurements were made with a 50-mCi $^{57}Co(Rd)$ source driven by a Doppler velocity transducer operating in the constant acceleration mode. The velocity scale was calibrated using room temperature spectra of a metallic iron foil, and the zero velocity of the Mössbauer spectra refers to the centroid of these spectra. EPR measurements were performed on a Bruker ER 200-SRC spectrometer with an Oxford Instrument ESR-9 continuous flow cryostat. For the EPR samples, the protein concentration was about 200 μM in 10 mM Hepes buffer (pH 6.4), and the volume was 200–250 μl.

RESULTS AND DISCUSSION

Fig. 1*A* shows the EPR spectrum of the oxidized cytochrome *c* peroxidase from *P. denitrificans* recorded at 8 K. This spectrum is presented for the reason of clarity and for the purpose of comparison with those of the half-reduced state but is similar to the earlier published spectrum (2) in that two sets of resonances corresponding to the two *c*-type hemes were detected. The resonances at $g_{max} = 3.00$, $g_{med} = 2.27$, and $g_{min} = 1.44$ (not shown) were assigned to the low potential heme and the signal at $g_{max} = 3.41$ to the high potential heme (2). As with other low spin ferric hemes with a large g_{max}, the other two resonances for the high potential heme are not detected. It was assumed that g_{med} was ~2.0, and the corresponding g_{min} was estimated to be ~0.6 according to the equation, $g_{max}^2 + g_{med}^2 + g_{min}^2 = 16$ (24). The weak signal observed at $g = 6$ represents a minor high spin ferric heme component, possibly associated with the high potential heme, which is in a high spin-low spin equilibrium at room temperature (7).

Incubation of the oxidized sample with ascorbate for 60 min under an argon atmosphere yielded a half-reduced peroxidase, which exhibits the EPR spectrum shown in Fig. 1*B*.[1] In this
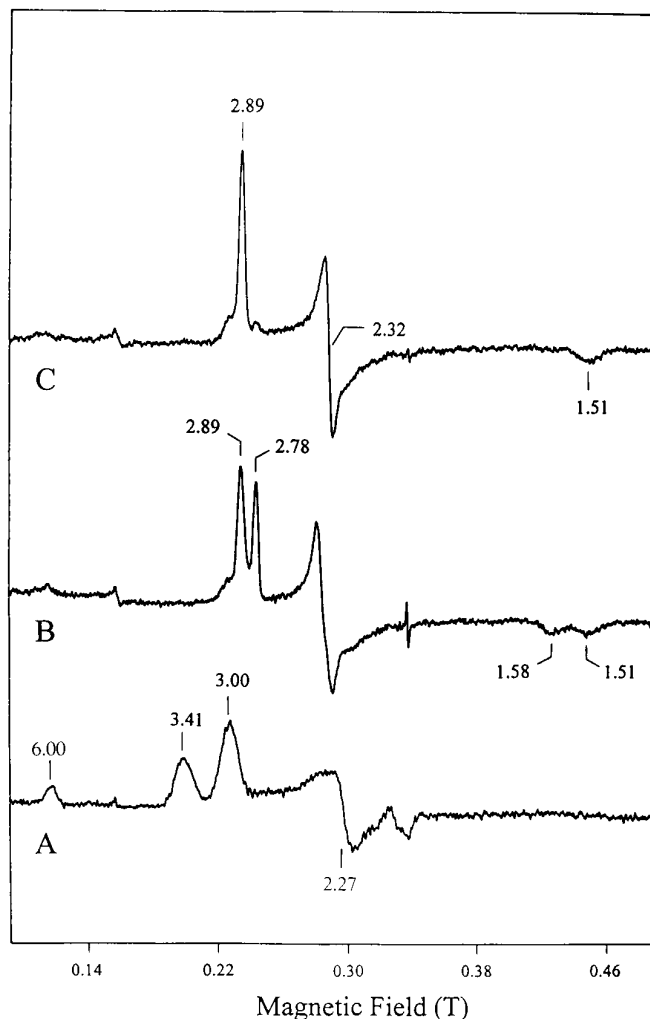
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[1] The spectra of Ref. 2 also contained a prominent peak at $g_{max} = 3.3$,

FIG. 1. **EPR spectra of cytochrome *c* peroxidase from *P. denitrificans.*** *A*, oxidized enzyme; *B*, half-reduced enzyme (0.4 mM ascorbate); *C*, half-reduced enzyme in the presence of 20 mM Ca^{2+}. Experimental conditions: temperature, 8 K; microwave frequency, 9.43 GHz; microwave power, 2 milliwatts; modulation amplitude, 1 mT; receiver gain, 8×10^4.

half-reduced enzyme, the high potential heme is reduced, as expected from its redox behavior and as supported by the absence of the $g_{max} = 3.41$ signal. Most interestingly, the low potential heme in this half-reduced enzyme displays two sets of EPR signals corresponding to two different species, both of which are distinct from that of the low potential heme in the oxidized enzyme. The observed *g* values are 2.89, 2.32, and 1.51 for species I and 2.78, 2.40, and 1.58 for species II. Also, these signals are sharp in comparison with that of the low potential heme in the oxidized enzyme. These observations indicate a subtle heme-heme interaction in which the reduction of the high potential heme induces a structural modification at and around the low potential heme. The relative proportion of species I and II was found to be dependent on the enzyme preparation and is probably due to the different amounts of residual calcium bound to the purified enzyme (see below). For the sample shown in Fig. 1*B*, the relative concentration of species I to species II was estimated to be approximately 3 to 2 from the relative intensity of the peaks at $g_{max} = 2.89$ and 2.78, using the method of Aasa and Vänngård (25). Addition of calcium ions to this half-reduced state converts species II ($g_{max} = 2.78$)

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which is absent in Fig. 1*B*. We are unsure of the basis for this, but it may be due to differences in enzyme preparations.

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into species I (g_{max} = 2.89), resulting in an EPR spectrum showing a majority of species I (Fig. 1*C*). Consequently, both the reduction of the high potential heme and the binding of calcium ions to the protein have effects on the low potential heme. These effects could include protein conformation changes or changes in the coordination structure such as an increase in histidinate character of the proximal ligand, which is observed in some eukaryotic peroxidases (19, 23). It is interesting to note that, for peroxidases isolated from *P. aeruginosa* and *P. stutzeri*, the half-reduced enzymes display EPR spectra showing only one low spin ferric heme species. Either these proteins do not require Ca^{2+} or they bind Ca^{2+} so strongly that it is not lost during purification.

To further characterize the heme groups in the *Paracoccus* peroxidase, Mössbauer measurements were performed. We will first present the data recorded at 200 K, where the electronic relaxation time is fast in comparison with the nuclear precession, resulting in the observation of quadrupole doublets for the heme irons regardless of their oxidation states. Under such
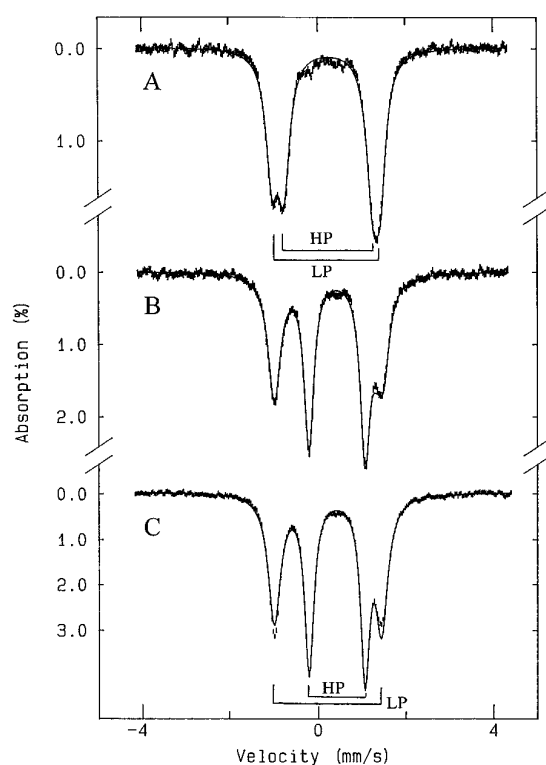

FIG. 2. **High temperature Mössbauer spectra of cytochrome *c* peroxidase from *P. denitrificans*.** *A*, oxidized enzyme; *B*, half-reduced enzyme (6 mM ascorbate); *C*, half-reduced enzyme in the presence of 17 mM Ca^{2+}. The data were recorded at 200 K in the absence of an applied field. The *solid lines* are least-squares fits of the experimental spectra (see text). The quadrupole doublets originating from the high potential (*HP*) and low potential (*LP*) hemes are indicated by *brackets*.

conditions, data analysis is simpler. Fig. 2 shows the 200 K spectra of the *Paracoccus* peroxidase in its oxidized (spectrum *A*), ascorbate-reduced (spectrum *B*), and ascorbate-reduced-plus-calcium (spectrum *C*) states. The oxidized peroxidase shows two partially resolved quadrupole doublets (Fig. 2*A*) corresponding to the two inequivalent *c*-type hemes. The data were least-squares fitted assuming two doublets with equal intensity and line width. For the half-reduced samples, the relative proportion of the two species of the low potential heme determined from the EPR data was used for the analysis of the Mössbauer data (Fig. 2, *B* and *C*). The results of the analysis are plotted as *solid lines* in Fig. 2, and the parameters obtained are listed in Table I. The good agreement between the data and the fits indicates that the Mössbauer data are consistent with the EPR findings. Also, this analysis allows unambiguous assignments of the parameters to the two different hemes. For the high potential heme, the parameters (ΔE_Q = 2.01 mm/s and δ = 0.24 mm/s for the oxidized $S = 1/2$ state and ΔE_Q = 1.27 mm/s and δ = 0.42 mm/s for the reduced $S = 0$ state) are common for low spin heme compounds and are consistent with a methionine-histidine coordination (26–32) (see also Table II). The quadrupole splittings for the low potential heme (2.45 mm/s in the oxidized enzyme and 2.46 mm/s in the calcium-bound enzyme), however, are atypically large (see Table II). The origin and significance of these unusually large values are currently unclear.

At 4.2 K, the electronic relaxation is slow and the oxidized peroxidase exhibits Mössbauer spectrum with magnetic hyperfine structures as expected for low spin ferric heme compounds. Fig. 3 shows the Mössbauer spectra of the oxidized *P. denitrificans* peroxidase recorded with the presence of a 50-mT [2] field applied parallel (spectrum *A*) and perpendicular (spectrum *B*) to the γ-beam and with an 8-T parallel field (spectrum *C*). Similar to the high temperature data, these low temperature spectra can also be analyzed as a superposition of two components of equal intensity corresponding to the two low spin ferric hemes. The electronic *g* values have been determined from the EPR measurements, and the parameters, ΔE_Q and δ, at 4.2 K can be extrapolated from the high temperature spectra. The only unknown parameters for the analysis of the low temperature data are the magnetic hyperfine coupling tensor A, which can be estimated from a ligand field theory commonly applied to low spin ferric heme complexes (33). In this theory the t_{2g} orbitals are assumed to be well isolated from the e_g orbitals and are split by the tetragonal and rhombic crystal fields, Δ and V, respectively. The spin-orbit interaction mixes the t_{2g} orbitals, resulting in three Krammers doublets. The observed *g* values and the low temperature Mössbauer spectra are originating from the ground doublet. If the *g* values of a low spin ferric compound are known, this theory can be used to estimate the corresponding A tensor (34) and the crystal field parameters Δ

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[2] The abbreviations used are: mT, millitesla; T, tesla.

TABLE I

Least-squares fit parameters for the different oxidation states of the P. denitrificans cytochrome c peroxidase at 200 K

Oxidation state	Heme group	Spin	ΔE_Q	δ	Γ^a	Absorption^b
			mm/s	*mm/s*	*mm/s*	*%*
Oxidized	High potential	1/2	2.01 ± 0.03	0.24 ± 0.02	0.33	50
	Low potential	1/2	2.45 ± 0.03	0.20 ± 0.02	0.31	50
Half-reduced	High potential	0	1.27 ± 0.03	0.42 ± 0.02	0.26	50
	Low potential, I	1/2	2.46 ± 0.03	0.25 ± 0.02	0.32	30
	Low potential, II	1/2	2.31 ± 0.04	0.17 ± 0.03	0.36	20
Half-reduced + Ca^{2+}	High potential	0	1.27 ± 0.03	0.43 ± 0.02	0.25	50
	Low potential, I	1/2	2.43 ± 0.03	0.23 ± 0.02	0.33	50

^{a} Full width at half-maximum.
^{b} Fixed parameter.

TABLE II
Mössbauer parameters of some low spin ferric heme proteins with His/His or Met/His axial coordination

Ligands	Protein (organism)[a]	ΔE_Q	δ	Γ	Temp. (K)	Ref.
		mm/s	mm/s	mm/s		
Met/His	Cyt. c_2 (*R. rubrum*)	2.26	0.31	0.5	4.2	26
		2.20	0.25	0.3	200	26
	Cyt. c_{551} (*P. aeruginosa*)	2.03	0.23	0.35	4.2	27
	Cyt. c (*Torula utilis*)	2.14	0.21		200	28
	Cyt. c (Tuna)	2.14	0.21		4.2	28
His/His	HAO (*N. europaer*)	2.1	0.24		4.2	29
	Cyt. b_5 (calf liver)	2.27	0.23		200	30
	Cyt. c_3 (*D. baculatus*)	2.06	0.24		200	31
	Nir (*D. desulfuricans*)	1.86–2.16	0.19–0.26	0.35	4.2	32

[a] Cyt., cytochrome; HAO, hydroxylamine oxidoreductase; *N*, *Nitrosomonas*; *D.*, *Desulfovibrio*; Nir, nitrite reductase.
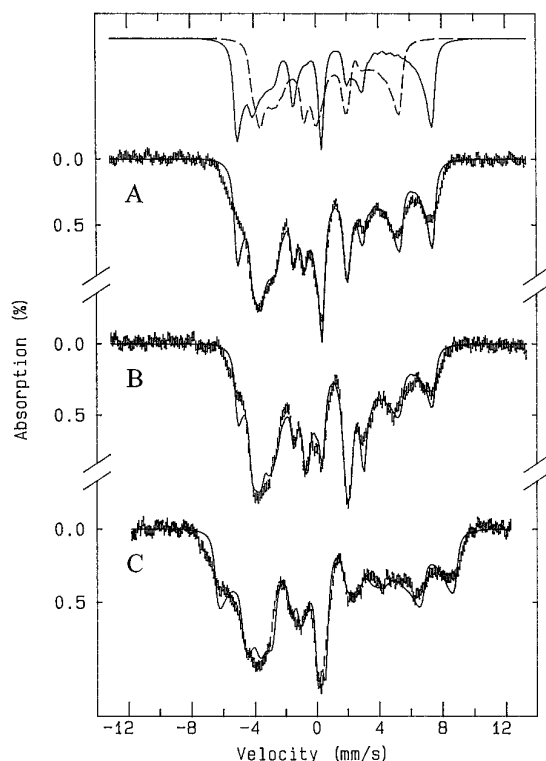

FIG. 3. **Low temperature Mössbauer spectra of oxidized cytochrome *c* peroxidase from *P. denitrificans*.** The data were recorded at 4.2 K with a 50-mT magnetic field applied parallel (*A*) and perpendicular (*B*) to the γ-beam and an 8-T magnetic field applied parallel to the γ-beam (*C*). The *solid lines* plotted over the experimental spectra are theoretical simulations using the parameters listed in Table III. The simulations of each spectral component in the presence of a 50-mT parallel field are also shown on top of spectrum *A* (*solid line*, high potential heme; *dashed line*, low potential heme).

TABLE III
Crystal field and hyperfine parameters for the oxidized P. denitrificans cytochrome c peroxidase at 4.2 K

	High potential heme		Low potential heme	
	Experiment	Theory	Experiment	Theory
Δ/λ		2.19		3.08
V/λ		0.84		1.76
E_{yz}/λ		1.15		1.91
g_x		0.61	1.44 ± 0.05	1.44
g_y		2.00	2.27 ± 0.01	2.27
g_z	3.41 ± 0.01	3.41	3.00 ± 0.01	3.00
$A_{xx}/g_n\beta_n$ (T)	-33.7 ± 2.5	-36.0	-40.2 ± 2.0	-40.2
$A_{yy}/g_n\beta_n$ (T)	$+28.5 \pm 1.0$	$+30.0$	$+19.3 \pm 1.0$	$+19.3$
$A_{zz}/g_n\beta_n$ (T)	$+78.7 \pm 0.3$	$+80.2$	$+53.4 \pm 0.5$	$+53.4$
ΔE_Q (mm/s)	2.10 ± 0.03		2.50 ± 0.05	
δ (mm/s)	0.30 ± 0.03		0.26 ± 0.02	
η	-1.0	-1.9	-2.0	-2.6
Line width (mm/s)	0.35		0.35	

the EPR and Mössbauer line shapes of low spin ferric complexes (37).

Since the crystal field parameters Δ and V can be readily determined from the EPR g values and since these parameters are expected to be affected by the ligands, EPR spectroscopy has been used for axial ligand assignment for proteins containing low spin ferric protoheme IX. It was first demonstrated by Blumberg and Peisach (35) that proteins containing protoheme IX with the same axial ligands tend to have Δ and V values that are clustered in a plot of the rhombic (V/λ) *versus* the tetragonal (Δ/λ) fields. This method of axial ligand assignment, however, was later found to contain ambiguities, particularly in the case of bis-histidine heme proteins (38, 39). Another method that has been proposed for heme axial ligand identification uses magnetic circular dichroism spectroscopy to measure the near infrared π to d charge transfer bands, the frequencies of which are sensitive to the axial ligation (38). In a study of 34 low spin ferric heme proteins, Gadsby and Thomson (38) established a linear correlation between the charge transfer transition energy and the electronic hole energy of the t_{2g} orbital ($E_{yz} = \Delta/3 + V/2$). Consequently, E_{yz} can also be used for axial ligand assignment. Of the 34 heme proteins investigated, the Met/His ligation shows lower E_{yz} ($1.5\lambda - 1.9\lambda$) than that of the His/His ligation ($2.0\lambda - 2.3\lambda$). In Table III, the experimentally determined and the theoretically estimated A values agree very well, suggesting that the theory used in our analysis describes the electronic properties of the hemes in the oxidized *P. denitrificans* peroxidase quite well. Using the theoretical values of Δ and V, the energy E_{yz} for the high potential and the low potential heme was found to be 1.15λ and 1.91λ, respectively. Earlier NMR measurements (2, 3) have established methionine ligation to the high potential heme, and the value 1.15λ is therefore the smallest E_{yz} ever reported for a Met/His ligation. Using the formula derived by Gadsby and Thomson (38), 1.15λ for E_{yz}

and V (33, 35, 36) in the units of the spin-orbit coupling constant λ. Since the g values of the two hemes in the oxidized peroxidase are known from the EPR measurements, this theory was used to obtain initial guesses for the A values in the analysis of the low temperature Mössbauer spectra. These A values were then allowed to vary until a reasonable match between the theoretical simulations and experimental data was obtained. Results of such an analysis are shown as *solid lines* in Fig. 3, and the parameters obtained are listed in Table III along with the theoretical values. The good agreement between the simulated and the experimental spectra indicates that the data support the presence of two distinct low spin ferric hemes in the oxidized peroxidase. The less than desirable fit at the outer absorption region is due to a distribution of the crystal field strength, which is not included in the analysis. Distributions of Δ and V have generally been used to explain

FIG. 4. **Proposed models of the two domains of *P. denitrificans* cytochrome *c* peroxidase.** The cytochrome *c* peroxidase is represented as a two-domain structure. In model *A*, the heme domain with His (*H*)-Met (*M*) coordination of the heme accepts the incoming electron and can therefore be considered high potential. Entry of the electron results in dissociation of His-275 (*H**) from the heme of the second domain and allows access by hydrogen peroxide. *HP*, high potential; *LP*, low potential. In model *B*, both hemes are coordinated by methionine in the oxidized state. After reduction of one heme, the methionine of the second heme dissociates from the iron and allows access for hydrogen peroxide. In this model, His-275 (*H**) is not a heme ligand but still plays a role as a distal catalytic residue. In model *C*, the entry of an electron to center 1 causes a change in the properties of both hemes. Center 1 switches from high to low potential and reduces center 2, which becomes coordinated by a methionine. Center 1 itself becomes the peroxidatic center.
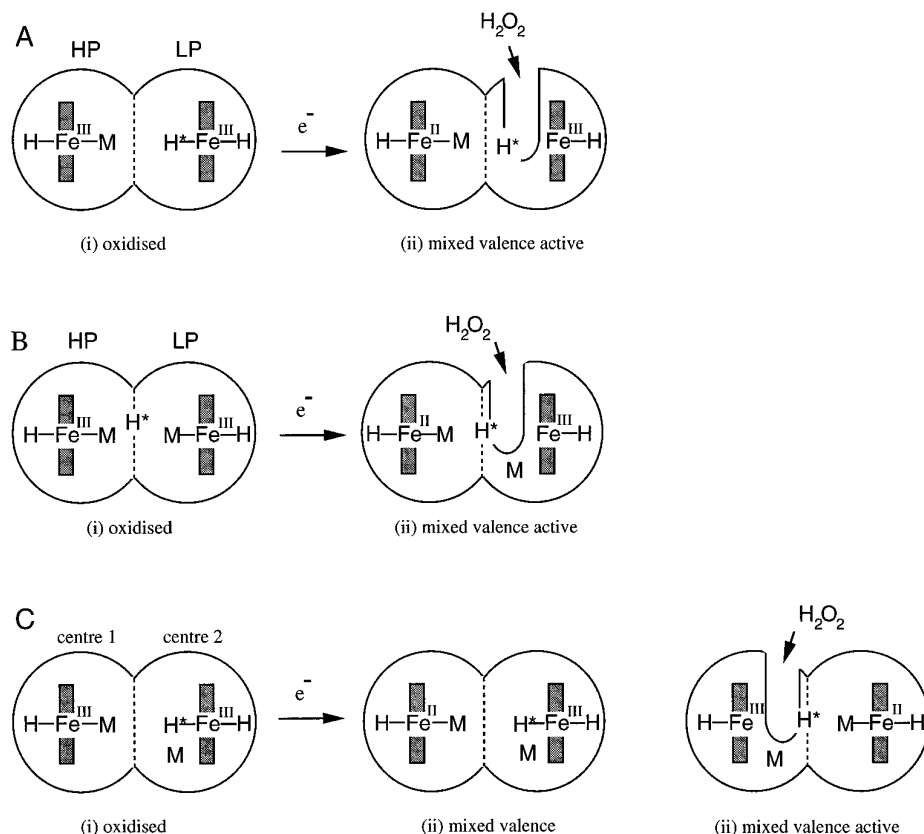
corresponds to a charge transfer energy in the order of 5300 cm^{-1}. It would be interesting to perform magnetic circular dichroism measurements to investigate whether the correlation between the E_{yz} and the charge transfer energy holds for the high potential heme. The value 1.91λ obtained for the low potential heme falls into the range of Met/His axial ligation and is slightly outside of the range reported for bis-histidine heme proteins. It is, however, important to point out that axial ligand assignments using EPR and Mössbauer spectroscopy contain large uncertainties and that methionine ligation to the low potential heme is not supported by NMR measurements (3, 4).

On the basis of their amino acid sequence work on the *P. aeruginosa* enzyme, Ellfolk *et al.* (40) proposed that a C-terminal domain contained a heme coordinated by a histidine and Met-254 (289 in *P. denitrificans* cytochrome *c* peroxidase numbering) and an N-terminal domain contained a heme coordinated by a histidine and either His-240 (His-275 in *P. denitrificans* cytochrome *c* peroxidase numbering) or a lysine. Extension of sequence analysis to the enzyme from *P. denitrificans*, the open reading frame f465 on the *Escherichia coli* chromosome and the Mau G proteins[3] revealed that Met-289 and His-275 were indeed conserved but also Met-129 was a conserved feature of the N-terminal domain. Although NMR evidence seems to preclude both the C- and N-terminal domains having methionine coordination, there is clearly potential for this in the sequence, and the presence of His-275 as a possible ligand leads to a number of permutations of coordination possibilities in the two redox states. Some of these possibilities are indicated in Fig. 4.

Fig. 5 shows the Mössbauer spectra of the half-reduced peroxidase in the absence (spectrum *A*) and presence (spectra *B* and *C*) of calcium. The data were recorded at 4.2 K with a
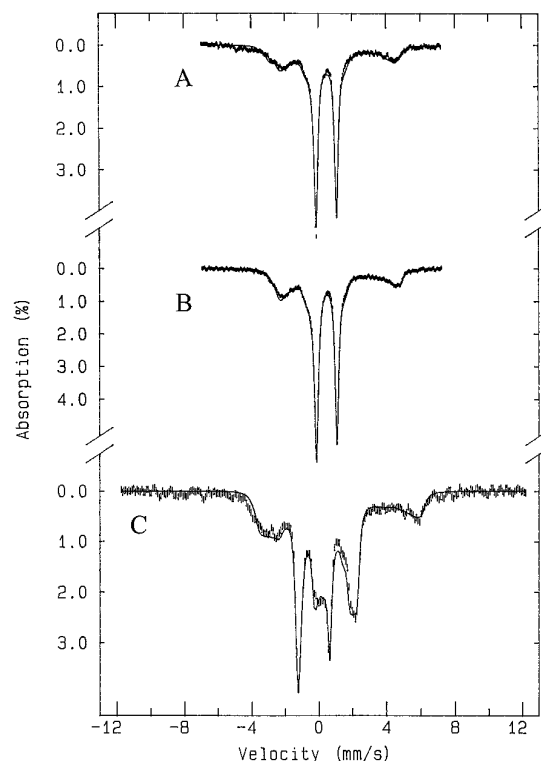

FIG. 5. **Mössbauer spectra of half-reduced *Paracoccus* cytochrome *c* peroxidase in the absence (*A*) and presence (*B* and *C*) of added calcium.** The data were recorded at 4.2 K with a 50-mT (*A* and *B*) and an 8-T (*C*) magnetic field applied parallel to the γ-beam. The *solid lines* are theoretical simulations using the parameters listed in Table III. In these simulations, the contributions from the oxidized and the reduced hemes are assumed to be equal. The relative proportions of species I and II for the oxidized low potential heme were taken from the EPR results. Diamagnetism was assumed for the reduced high potential heme.

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[3] J. Van Beeumen, W. Hu, C. F. Goodhew, R. Gilmour, D. McGinnity, N. Saunders, and G. W. Pettigrew, manuscript in preparation.

Table IV
Crystal field and hyperfine parameters for the half-reduced P. denitrificans cytochrome c peroxidase at 4.2 K

	Low potential heme		High potential heme (reduced ($S = 0$))
	Species I	Species II	
Δ/λ	2.90	2.58	
V/λ	1.99	2.34	
E_{yz}/λ	1.96	2.03	
g_x	1.51 ± 0.02	1.58 ± 0.02	
g_y	2.32 ± 0.01	2.40 ± 0.01	
g_z	2.89 ± 0.01	2.78 ± 0.01	
$A_{xx}/g_n\beta_n$ (T)	$-23.4 \pm 2.0\ (-41.2)$[a]	(-42.4)[a]	
$A_{yy}/g_n\beta_n$ (T)	$+2.2 \pm 1.0\ (+20.3)$[a]	$(+22.1)$[a]	
$A_{zz}/g_n\beta_n$ (T)	$+43.4 \pm 0.5\ (+48.0)$[a]	$(+41.0)$[a]	
ΔE_Q (mm/s)	2.52 ± 0.03	2.4 ± 0.1	1.23 ± 0.03
δ (mm/s)	0.31 ± 0.02	0.23 ± 0.05	0.46 ± 0.02
η	1.1	1.0	0
Line width (mm/s)	0.35	0.35	0.27

[a] The numbers in the parentheses are the theoretical values.

50-mT (spectra A and B) and a 8-T magnetic field (spectrum C) applied parallel to the γ-beam. These spectra clearly demonstrate that in the ascorbate-reduced peroxidase, one heme is reduced to a low spin ferrous ($S = 0$) state and the other remains oxidized. The reduced heme (presumably, the high potential heme) displays a quadrupole doublet with parameters ($\Delta E_Q = 1.23 \pm 0.03$ mm/s and $\delta = 0.46 \pm 0.02$ mm/s) typical of low spin ferrous hemes. Addition of calcium has no effect on this doublet. The oxidized heme (presumably, the low potential heme) exhibits a hyperfine split spectrum, which is distinguishable from that of the reduced heme. To analyze the spectral component of the oxidized heme, the contribution of the reduced heme was first removed, and the remaining spectral component was analyzed using the method described above. We began by analyzing the spectrum of the sample with calcium present, since it contains mostly species I ($g_{max} = 2.89$). The parameters obtained for species I were then used in the analysis of the spectra of the sample without added calcium where both species I and II are present. The spectral data for species I and II are not sufficiently resolved to allow reliable determination of the hyperfine parameters for species II. The theoretical *A* values for species II and the concentration ratio of species I to species II obtained from the EPR measurements were used in the analysis. The results are listed in Table IV. The *solid lines* plotted in Fig. 5 are simulations using a 50/50 oxidized/reduced heme ratio. Good agreement is observed between the experiments and the simulations, indicating that only one heme is reduced in these half-reduced protein samples with or without added calcium.

From these Mössbauer and EPR observations, the following conclusions can be drawn. The addition of ascorbate to the peroxidase reduces the high potential heme only. Reduction of the high potential heme affects the conformation surrounding the low potential heme, resulting in two low spin ferric states (species I and II) that are distinguishable from that in the oxidized form. The addition of Ca^{2+} converts species II into species I. Since calcium has been found to promote the peroxidase activity, the low spin species I probably represents the peroxidatic heme in its active form.

In summary, Mössbauer and EPR spectroscopy have been used to characterize the two heme groups of *P. denitrificans* cytochrome *c* peroxidase in its oxidized and half-reduced forms. It was found that the spectroscopic properties of the peroxidatic heme can be affected by the reduction of the high potential heme and by the binding of calcium to the peroxidase, providing further evidence for the long range heme-heme interactions observed in the bacterial cytochrome *c* peroxidases.

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Mössbauer Characterization of *Paracoccus denitrificans* Cytochrome *c* Peroxidase:
FURTHER EVIDENCE FOR REDOX AND CALCIUM BINDING-INDUCED
HEME-HEME INTERACTION

Susana Prazeres, José J. G. Moura, Isabel Moura, Raymond Gilmour, Celia F. Goodhew,
Graham W. Pettigrew, Natarajan Ravi and Boi Hanh Huynh

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