Activation of the Cytochrome \( cd_1 \) Nitrite Reductase from \( P.\) pantotrophus

**REACTION OF OXIDIZED ENZYME WITH SUBSTRATE DRIVES A LIGAND SWITCH AT HEME \( c^* \)**

Received for publication, February 9, 2007, and in revised form, July 5, 2007 Published, JBC Papers in Press, July 10, 2007, DOI 10.1074/jbc.M701242200

Jessica H. van Wonderen⁷, Christopher Knight⁷, Vasily S. Oganesyan⁷, Simon J. George⁶, Walter G. Zumft⁴, and Myles R. Cheesman¹

From the ¹Centre for Metalloprotein Spectroscopy and Biology, School of Chemical Sciences and Pharmacy, University of East Anglia, Norwich NR4 7TT, United Kingdom, ²Institute of Applied Biosciences, Division of Molecular Microbiology, University of Karlsruhe, PF 6980, 76128 Karlsruhe, Germany, and ³Physical Biosciences Division, Lawrence Berkeley Laboratory, Berkeley, California 94720

Cytochromes \( cd_1 \) are dimeric bacterial nitrite reductases, which contain two hemes per monomer. On reduction of both hemes, the distal ligand of heme \( d_1 \) dissociates, creating a vacant coordination site accessible to substrate. Heme \( c \), which transfers electrons from donor proteins into the active site, has histidine/methionine ligands except in the oxidized enzyme from \( P.\) pantotrophus where both ligands are histidine. During reduction of this enzyme, Tyr⁴ dissipates from the distal side of heme \( d_1 \), and one heme \( c \) ligand is replaced by methionine. Activity is associated with histidine/methionine coordination at heme \( c \), and it is believed that \( P.\) pantotrophus cytochrome \( cd_1 \) is unreactive toward substrate without reductive activation. However, we report here that the oxidized enzyme will react with nitrite to yield a novel species in which heme \( d_1 \) is EPR-silent. Magnetic circular dichroism studies indicate that heme \( d_1 \) is low-spin FeIII but EPR-silent as a result of spin coupling to a radical species formed during the reaction with nitrite. This reaction drives the switch to histidine/methionine ligation at FeII heme \( c \). Thus the enzyme is activated by exposure to its physiological substrate without the necessity of passing through the reduced state. This reactivity toward nitrite is also observed for oxidized cytochrome \( cd_1 \) from \( P.\) stutzeri sug¬

[\( \text{NO}_2^- + 2\text{H}^+ + \text{e}^- \rightarrow \text{NO} + \text{H}_2\text{O} \) (Eq. 1)]

The enzyme monomer comprises two distinct domains, one of which binds heme \( c \), the other heme \( d_1 \) (4). Heme \( c \) transfers electrons from donor proteins to the active site heme \( d_1 \), an iron-dioxoisobacteriochlorin unique to this class of enzyme. In the crystal structure of the aerobically isolated (“oxidized”) \( cd_1 \) from \( P.\) pantotrophus (\( Pp \)), each heme is in the FeIII state with two protein-derived axial ligands, His¹⁷ and His²⁵⁹ to heme \( c \) and His²⁰⁰ and Tyr²⁵⁵ to heme \( d_1 \) (see Scheme 1). MCD and EPR spectroscopy confirmed that these are the ligands in solution (5). Crystallography further showed that reduction of both hemes to the FeII state triggers a conformational change, which results in the replacement of the heme \( c \) ligand His¹⁷ with Met¹⁰⁶ and the dissociation of Tyr²⁵⁵ from heme \( d_1 \) (6), thus creating a vacant coordination site at which substrate can bind (shown in top line of Scheme 1). Both dissociating heme ligands are provided by a 7-residue c-domain loop, which extends into the \( d_1 \) domain (6, 7). The switch to His/Met ligation significantly raises the reduction potential of heme \( c \), more closely matching the donor proteins cytochrome \( c_550 \) and pseudooaza¬

[\( \text{H}_2\text{N}-\text{OH} + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{NH}_3 + \text{H}_2\text{O} \) (Eq. 2)]

[¹ To whom correspondence should be addressed: School of Chemical Sciences and Pharmacy, University of East Anglia, Norwich NR4 7TT, United Kingdom. Tel.: 01603592028; Fax: 01603582023; E-mail: m.cheesman@uea.ac.uk.

² The abbreviations used are: \( cd_1 \), cytochrome \( cd_1 \); \( Pp \), \( P.\) pantotrophus; \( Pa \), \( P.\) aeruginosa; \( Ps \), \( P.\) stutzeri strain ZoBell (ATCC 14405); CT, charge-transfer; MCD, magnetic circular dichroism; NIR, near-infrared; BTP, 1,3-bis(tris(hydroxymethyl)methylamine)propane; RD-MCD, ratio data method of MCD spectroscopy; H, magnetic field; T, tesla.]

---

\( \text{NO}_2^- \) thermodynamically favorable (13, 14). The purpose of the ligand switch is therefore unclear. Other cytochromes \( cd_1 \) have His/Met liganded hemes \( c \) and lack a residue corresponding to His¹⁷. In these species, reduction appears sufficient to trigger dissociation of the distal heme \( d_1 \) ligand without a coor¬

Reoxidation of reduced cytochromes \( cd_1 \) using \( \text{NO}_2^- \) yields a mixture of half-reduced species containing stable FeII-nitrosyl heme \( d_1 \) (18–21), although a recent report described rapid NO dissociation from the fully reduced enzyme (22). Reoxidation of fully reduced \( cd_1 \) using dioxygen gives rise to oxoferryl (Fe(IV)=O) heme \( d_1 \) and a protein radical (23). Hydroxylamine, however, is a convenient two-electron non-physiological sub¬

\( \text{H}_2\text{N}-\text{OH} + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{NH}_3 + \text{H}_2\text{O} \) (Eq. 2)
**Activation of cd₁ by Substrate**

With *Pp* cd₁, the product of this reaction, freeze-trapped after ~10 ms, displays FeIII heme EPR g values different from those of the oxidized enzyme (11). Because these g values are almost identical to those observed for the His/Met liganded heme c in other cytochromes cd₁ (see Table 1), it was suggested that the *Pp* enzyme has been trapped in a transient form (cd₁* in Scheme 1) in which heme c retains the coordination normally associated with the reduced state (11, 12). Unambiguous identification of a His/Met ligand pair by MCD spectroscopy confirmed this to be the case (12). The transient cd₁* reverts to the oxidized conformation with His/His heme c over a period of ~20 min (11). Until this reversion has occurred, the enzyme is capable of NO₂⁻ reduction using the physiological electron donor pseudoazurin (11). It is reported that for full activity, oxidized *Pp* cd₁ requires preactivation by chemical reduction (11, 13). Consequently, His/Met ligated heme c is presumed to be a prerequisite of activity, and it has been proposed that the oxidized enzyme is a resting form bypassed in catalysis as the enzyme cycles rapidly compared with the rate of reversion to the His/His form (13, 26). The oxidized state would therefore not exist in vivo without an appropriate reactivation mechanism (8, 12, 26). The only candidate suggested to date is reducible equine cytochrome c as electron donor.

**EXPERIMENTAL PROCEDURES**

**Purification of cd₁**—Samples of cd₁ from *Pp* and *Pseudomonas stutzeri* (Ps) strain Zobell (ATCC 14405) were purified according to published methods (10, 31). Initially, samples of the *Pp* cd₁*-X were prepared according to reported methods (11, 29): oxidized *Pp* cd₁ was reduced in an anaerobic hood (Faircrest Engineering; operating at <1 ppm O₂ in an N₂ atmosphere) using aliquots of buffered solutions of sodium dithionite; excess reductant was removed by chromatography on a PD-10 desalting column; the enzyme was reoxidized using aliquots of buffered hydroxylamine solution. Assays of nitrite reductase activity were performed following published methods (13) using reduced equine cytochrome c as electron donor.

EPR spectra were measured with a Bruker ER300D spectrometer fitted with a dual mode cavity type ER4116DM interfaced to an ELEXSYS computer control system (Bruker Analytische Messtechnik GmbH) and equipped with a variable temperature cryostat and liquid helium transfer line (Oxford Instruments). EPR simulations were performed using the Bruker program WINP Ryan EPR SimFonia (v1.25). MCD spectra were recorded on Jasco circular dichrographs models J810 and J730 for the UV-visible and near-infrared (NIR) regions, respectively, used in conjunction with an Oxford Instruments SM4 split-coil superconducting solenoid capable of generating magnetic fields of up to 5 tesla.

The intensities of spectra presented are referred to concentrations of cd₁ monomer, calculated using the Soret absorbance intensity of the oxidized enzymes and extinction coefficients of ε = 141 mM⁻¹ cm⁻¹ for the *Ps* enzyme (32) and ε = 148 mM⁻¹ cm⁻¹ for the *Pp* enzyme (10). Samples for MCD spectroscopy were prepared in deuterium oxide solutions containing 50 mM BTP buffer at pH* = 6.5 to which equivalent volumes of glyc erol had been added as glassing agent (33). pH* is the apparent pH of the D₂O-based solutions measured using a standard glass pH electrode.

**RESULTS**

**Preparation and Absorption Spectra of cd₁*-X**—Samples of the *Pp* cd₁*-X species were prepared anaerobically at pH = 7.0 according to the methods described by Allen et al. (29). After 2-h incubation, the same type of absorption and EPR spectroscopic changes were observed. However, identical results were obtained during control experiments in which oxidized cd₁ was incubated with the same concentration (5 mM) of NO₂⁻. This was the case whether the incubation was performed anaerobically or aerobically, but at pH 7.0, the reaction still required several hours to go to completion. Further control experiments
revealed that the rate of this reaction of resting enzyme with NO$_2^-$ was strongly pH-dependent, increasing significantly at lower pH. Although there are some variations in the rate between preparations, at pH 6.5, the product is fully formed in 5–10 min but at pH 9.0 negligible reaction was observed over 24 h. The substrate itself (pK$_a$ 3.35) is fully deprotonated over the pH range investigated, and the pH dependence may be a characteristic of NO$_2^-$ binding and/or the subsequent reaction. No significant changes in the absorption spectrum of the oxidized enzyme were observed over the pH range 5.5–9.0. Assays of NO$_2^-$ reductase activity using published methods (13) confirmed that this state does not interact with NO$_2^-$ (34). The heme c ligand switch of the Pp enzyme is unique among known cytochromes cd$_1$, and this raises the question as to whether the ability to react with NO$_2^-$ in this manner is also unique. We found that the cd$_1$ from Ps also reacts with NO$_2^-$ at pH 6.5. When the enzyme is treated with NO$_2^-$ under identical conditions, the heme d$_1$ absorption band undergoes a small shift from 643 to 640 nm (Fig. 1b). However, in this enzyme, formation of a cd$_1$*-X species, which contained low-spin Fe$^{III}$ heme d$_1$, would involve no change of spin-state at either heme. Less significant absorption changes would therefore result. That, these small changes do not indicate the formation of a cd$_1$*-X, species is confirmed by EPR and MCD spectroscopic studies described below.

**EPR Spectra of cd$_1$*-X**—Fig. 2 shows the 10 K X-band perpendicular mode EPR spectra of the oxidized cytochromes cd$_1$ from Pp and Ps, and of the species formed following treatment of each enzyme with NO$_2^-$, No signals were detected in the parallel mode for any of these samples. Heme c in oxidized Pp cd$_1$ (Fig. 2a) gives rise to a “large $g_{max}$” spectrum with a $g_s$ feature at $-3.06$. This is one of two limiting types of EPR observed for low-spin Fe$^{III}$ c-type hemes (5, 30). The other two $g$ values are often difficult to detect but spectral simulations, presented below, show that broad $g_s$ features are present in the $g = 2.2 – 2.3$ range.
region. Heme c of oxidized Ps cd\textsubscript{1} gives rise to an EPR spectrum of the second limiting type. This “rhombic” spectrum (Fig. 2c) comprises three detectable features at g = 2.99, 2.29, 1.61. Note that, although the hemes c in these two enzymes have different axial ligation, it is not this which leads to the different EPR spectra. The occurrence of a large \( g_{\text{max}} \) or rhombic spectrum is dictated primarily by ligand orientation and not ligand identity. Thus both ligand sets could give rise to either type of EPR spectrum. In the case of the His/His-coordinated heme c of oxidized Pp cd\textsubscript{1}, the observation of a large \( g_{\text{max}} \) spectrum is consistent with the perpendicular ligand arrangement observed in the crystal structure (4, 30).

At 10 K, only the low-spin state of Fe\textsuperscript{III} heme d\textsubscript{1} in oxidized Pp cd\textsubscript{1} is significantly populated, and minor traces of the high-spin state are observed at g values of 6.86 and 4.99 (5). The low-spin Fe\textsuperscript{III} state of heme d\textsubscript{1} gives rise to EPR spectra, which are unlike either of the limiting cases described for heme c, having narrow features and low g value anisotropy (5). These are observed in the oxidized cd\textsubscript{1} spectra with g values of 2.52, 2.19, 1.84 for Pp and 2.58, 2.44, 1.87 for Ps. After addition of NO\textsubscript{2}\textsuperscript{-}, the EPR spectra (Fig. 2b) are devoid of the characteristic low-spin Fe\textsuperscript{III} heme d\textsubscript{1} features, although this state of d\textsubscript{1} is apparent in the absorption spectra (Fig. 1). The Ps cd\textsubscript{1} \textsuperscript{*-} X EPR spectrum (Fig. 2d) retains the signals which were assigned to heme c in the oxidized state, and it is reasonable to assume that these g values do indicate His/Met ligation because the cd\textsubscript{1} from this species does not exhibit ligand switching. For NO\textsubscript{2}\textsuperscript{-}-treated Pp cd\textsubscript{1} (Fig. 2b), the large \( g_{\text{max}} \) spectrum of heme c has been replaced by a rhombic spectrum, with g values of 2.93, 2.32, 1.42 as reported by Allen et al. (29), who proposed that these features originate from Fe\textsuperscript{III} heme c with His/Met ligation. The validity of this assignment will be addressed below using MCD spectroscopy. It has not previously been shown that this EPR spectrum accounts for a single heme, although, given its unusual electronic ground state, low-spin Fe\textsuperscript{III} heme d\textsubscript{1} is unlikely to give rise to these signals (5, 30, 35). The EPR signals assigned to heme c in the cd\textsubscript{1} \textsuperscript{*-} X forms (Fig. 2, b and d) were quantitated by double integration of the spectra and comparison to a Cu\textsuperscript{II}-EDTA spin standard using established methods (36). Each represents a single low-spin Fe\textsuperscript{III} heme per monomer confirming that heme d\textsubscript{1} is EPR-silent.

In the EPR spectra of the oxidized enzymes (Fig. 2), several features appear to have incompletely resolved structure suggesting minor heterogeneities at each heme. These features are preparation-dependent and of unknown origin. Because cd\textsubscript{1} is a dimeric protein and we have identified a form in which both hemes are apparently in the low-spin Fe\textsuperscript{III} state but heme d\textsubscript{1} is EPR-silent, this raises the possibility that oxidized samples actually include substoichiometric levels of cd\textsubscript{1} \textsuperscript{*-} X so that in some dimers one monomer contains an EPR-silent heme d\textsubscript{1}. This would constitute a heterogeneity, which could be responsible for the EPR splitting. However, if this was so then the contribution of heme d\textsubscript{1} to the overall EPR envelope would be less than that of heme c. To the best of our knowledge, it has never been determined that the EPR signals arising from heme c and heme d\textsubscript{1} represent equivalent concentrations of heme. This has therefore been tested by simulating the EPR spectrum of each oxidized enzyme. The spectra for simulation were recorded under non-saturating conditions. This required recording at a temperature of 20 K, and because the spin-lattice relaxation of heme d\textsubscript{1} is slower than that of heme c, a microwave power of 0.64 milliwatt. The microwave power-dependence of the two hemes at 10 and 20 K is shown in Fig. 3. The spectrum of each oxidized enzyme was simulated using contributions from two low-spin Fe\textsuperscript{III} heme c species (c\textsubscript{a} and c\textsubscript{b}) and two low-spin Fe\textsuperscript{III} heme d\textsubscript{1} species (d\textsubscript{a} and d\textsubscript{b}). The position and g value for the low-field feature of each simulated species is also indicated on the simulations in Fig. 4. All the g values used in these simulations are included in Table 1, where the weighting for each species is shown in brackets as percentage figures cal-
The Electronic State of Heme $d_1$ in cd$_1$*-X—This work has established that the low-spin Fe$^{I}$I heme c in cd$_1$*-X has His/Met ligation and so, in the case of the Ps enzyme, a ligand switch has been triggered in the oxidized enzyme by the reaction with NO$_2^-$. The nature of heme $d_1$ in cd$_1$*-X is complex in that it appears to be low-spin Fe$^{I}$I on the basis of absorption spectra but is EPR-silent. The electronic and magnetic properties of this center have therefore been investigated using variable magnetic field variable temperature MCD spectroscopic methods. The UV-visible region MCD spectrum of a heme contains an extremely detailed pattern of electronic bands, and it is well established for b- and c-type hemes that this pattern is diagnostic of the spin and oxidation state of the iron, including the EPR-silent Fe$^{I}$I states (38). Although heme $d_1$ is unique to this class of enzyme, and the available data is limited, it is clear that heme $d_1$ MCD spectra are also sensitive to changes in spin and oxidation state (5, 32, 33–42). The UV region MCD spectra of this enzyme, with a magnetic field of 5 tesla.

### Table 1

| Heme $c$ $g_a$, $g_b$, $g_c$ | Heme $d_1$ $g_a$, $g_b$, $g_c$* | Refs. |
|-----------------------------|---------------------------------|-------|
| P. pantotrophus | 3.04 (2)$^a$, --, -- | 2.51 (1), 2.20 (1), 1.85 (1) | 5, 11, 23 |
| Simulations | $c_1$ 3.140, 2.235, 1.060 (84%)$^a$ | $d_1$ 2.588, 2.217, 1.863 (72%) | This work |
| cd$_1$*-X | $c_6$, 2.980, 2.280, 1.430 (16%) | $d_6$, 2.533, 2.217, 1.837 (29%) | This work |
| P. stutzeri | 2.94, 2.33, 1.40 | 2.67, 2.53 (1), -- | 11 |
| Oxidized | 2.95 (1), 2.32, 1.40 (2) | Not observed | 29, This work |
| Simulations | $c_6$, 2.841, 2.340, 1.560 (21%) | $d_6$, 2.466, 2.266, 1.867 (10%) | This work |
| P. aeruginosa | 2.99, 2.29, -- | 2.54 (4), 2.40 (5), 1.85 (2) | 5, 57 |
| Oxidized | 2.97 (5), 2.25 (1), 1.4 | 2.52 (1), 2.43, 1.7 (6) | 18, 32, 39, 45 |
| cd$_1$*-X | 2.95, 2.30, 1.42 | Not observed | 45 |
| T. denitrificans | 3.60, --, -- | 2.52, 2.44 (1), 1.70 | 58, 59 |
| Oxidized | --, --, -- | Not observed | -- |

---

* The order of g values is reversed in the unusual (d$_{xy}$)$^2$(d$_{yz}$)$^2$ ground state of low-spin Fe$^{III}$ heme cd$_1$ (5, 30).

* Numbers in parentheses represent the variation in the second decimal place in g values reported for each feature. This includes spread due to a 2-fold heterogeneity often observed for cd$_1$ EPR features.

* -- g-values not determined from the spectrum.

---

**Activation of cd$_1$ by Substrate**

The UV-visible region MCD spectra of a heme contains an extremely detailed pattern of electronic bands, and it is well established for b- and c-type hemes that this pattern is diagnostic of the spin and oxidation state of the iron, including the EPR-silent Fe$^{I}$I states (38). Although heme $d_1$ is unique to this class of enzyme, and the available data is limited, it is clear that heme $d_1$ MCD spectra are also sensitive to changes in spin and oxidation state (5, 32, 33–42). The UV region MCD spectra of this enzyme, with a magnetic field of 5 tesla.

---

**FIGURE 5. Low-temperature NIR-MCD spectra of cd$_1$ from Pp and Ps in the oxidized and cd$_1$*-X states.** Pp oxidized (---); Pp cd$_1$*-X (----); Ps oxidized (---); Ps oxidized cd$_1$*-X (——). Samples were in 1:1 (v/v) 50 mBTP, D$_2$O, pH 6.5/glycerol. Spectra were recorded at a temperature of 4.2 K with a magnetic field of 5 tesla.
tesla and at three temperatures, 1.7, 4.2, and 10 K. All bands are strongly temperature-dependent indicating that they arise from paramagnetic centers. The MCD band pattern of low-spin Fe\textsuperscript{III} c-type hemes is well known (38). The major transitions attributable to low-spin Fe\textsuperscript{III} heme \( c \) are therefore readily assigned and are indicated in the upper part of Fig. 6a by blocks, which are offset vertically to illustrate bands of positive and negative intensity. Thus, in the region 520–590 nm, the negative feature at 557 nm and the two positive bands to either side are solely due to low-spin Fe\textsuperscript{III} heme \( c \). Blocks in the lower part of Fig. 6a illustrate the positions and signs of transitions, which have previously been observed for heme \( d_1 \) in the low-spin Fe\textsuperscript{III} state (5, 32, 39, 40, 42). Transitions from both hemes overlap at 450–520 nm but features in the 590–700 nm region are pure heme \( d_1 \) transitions.\(^3\) The overall pattern of bands in the MCD spectrum here is virtually identical to that previously reported for oxidized \( cd \) from Ps, in which both hemes are already entirely low-spin Fe\textsuperscript{III} (5). These spectra therefore further support the conclusion that in \( cd \), heme \( d_1 \) is in the low-spin Fe\textsuperscript{III} state and is responsible for paramagnetic MCD intensity despite the fact that it does not give rise to a detectable EPR spectrum at X-band.

There is no precedent for an isolated low-spin Fe\textsuperscript{III} heme not giving rise to a detectable EPR spectrum but there are cases of Fe\textsuperscript{III} hemes being rendered EPR-silent by a spin-spin interaction with a second paramagnetic center. For example, the heme at the active site of bacterial nitric oxide reductase gives rise to MCD bands characteristic of the high-spin Fe\textsuperscript{III} state but it is EPR-silent as a result of spin-coupling with a nearby non-heme Fe\textsuperscript{III} iron (43). Such an explanation that Fe\textsuperscript{III} heme \( d_1 \) in \( cd \) is spin-coupled to a second paramagnet would reconcile the EPR and MCD data, and this possibility was investigated by further MCD measurements.

The pattern of bands in the MCD spectrum reports fundamental properties local to the heme, such as spin and oxidation state, and this band pattern will not be significantly altered if the spin of the heme is spin-coupled to a second paramagnetic species. But even a relatively weak interaction of this nature could preclude the observation of EPR signals. Such an interaction could, however, be detected via the magnetic field and temperature dependence of the MCD intensity. These properties have, therefore, been studied for \( cd \) using the “ratio data” method of MCD spectroscopy (RD-MCD). This method was originally applied to cytochrome \( b_{59} \) to locate and analyze heme \( o_1 \) MCD bands from the active site Fe\textsuperscript{III} heme \( o_2-Cu_{59} \) pair against an intense background of low-spin Fe\textsuperscript{III} heme \( b \) bands (44). RD-MCD allows the immediate identification of transitions in the MCD spectrum, which arise from paramagnetic species for which the spin \( S \neq \frac{1}{2} \). Details of the method are given in Ref. 44, and only a brief summary will be presented here. For the case of an \( S = \frac{1}{2} \) chromophore, the MCD intensity varies as \( \tanh(-\beta H/2kT) \), where \( \beta \) is the Bohr magneton, \( k \) is Boltzmann constant, \( H \) is the magnetic field, and \( T \) the absolute temperature. \( \Gamma \) is a function of the \( g \) values, which determines the magnitude of the Zeeman splitting between the ground state components, \( m_s = \pm \frac{1}{2} \), at any orientation of the molecule to the external magnetic field expressed as the spherical polar angles \( \theta \) and \( \phi \).

\[
\Gamma = \frac{1}{2} \sin^2\theta \cos^2\phi + \frac{1}{2} \sin^2\phi \cos^2\theta \]  
(Eq. 3)

Except at extreme high magnetic field or low temperature, outside of those used in this work, \( \tanh(-\beta H/2kT) \approx \beta H^2/2kT \), and so the MCD intensities of \( S = \frac{1}{2} \) systems will be proportional to \( H/T \). Therefore if the MCD spectrum is recorded at different temperatures, but the magnetic field is adjusted so as to maintain the \( H/T \) ratio, then the contribution to the spectrum of \( S = \frac{1}{2} \) species remains constant. Variations in intensity will be observed only for bands arising from paramagnetic, which are not simple \( S = \frac{1}{2} \) species. Fig. 7 shows RD-MCD spectra of \( cd \) from Ps, recorded at four different combinations of \( H/T \). It is immediately apparent, for both enzymes, that the spectra are closely matched in intensity across the 520–590 nm region. This is entirely consistent with the previous assignment of these bands in Fig. 6 to the low-spin Fe\textsuperscript{III} heme \( c \). In the regions to either side, where heme \( d_1 \) bands occur, there are obvious variations in the MCD intensities. This divergence of the spectra is most pronounced in the 590–700 nm region where the bands are exclusively from heme \( d_1 \). These RD-MCD data therefore demonstrate that heme \( d_1 \) in \( cd \) is part of a paramagnetic but EPR-silent system. The \( S = \frac{1}{2} \) low-spin Fe\textsuperscript{III} heme \( d_1 \) must be interacting with another half-integer (Kramers) paramagnet. Heme \( c \) is clearly not involved; it remains EPR detectable and in the RD-MCD spectra gives rise to bands, which overlap as expected for an isolated \( S = \frac{1}{2} \) spe-

\(^3\) Fe\textsuperscript{III} hemes with His/Met ligation give rise to a CT transition near 695 nm. MCD spectra of the semi-apo form of \( cd \) show that the intensity of this band in the MCD is two orders of magnitude lower than these heme \( d_1 \) transitions. G. Kemp and M. R. Cheesman, unpublished.
is of sufficient strength to preclude observation of EPR signals at X-band, and so the magnitude of J, the spin-coupling constant, must therefore be several times that of the microwave photon energy (∼0.3 cm⁻¹). However, the two spins are not coupled sufficiently strongly to yield a diamagnetic system; the MCD intensifies significantly between 4.2 and 1.7 K (Fig. 6) placing an upper limit on J of ∼5 cm⁻¹ (our preliminary analysis⁴ of the RD-MCD suggests that J ∼ 3 cm⁻¹). This order of magnitude would require that the radical is located in close proximity to heme \( d_1 \). For example, in compound I of cytochrome c peroxidase, the exchange coupling between a tryptophan radical and an oxoferryl (FeIV=O) heme separated by ∼5 Å is <0.1 cm⁻¹ (46, 47).

One possible explanation is that a single turnover of NO₂ has taken place (Equation 1) using an electron abstracted from a nearby residue and so forming a radical. Displacement of the NO by a second NO₂ would yield a species \([R^+\cdot d_1^{III}\cdot NO_2^-]\) in which low-spin FeIII heme \( d_1 \) is coupled to the protein radical. Tyr263 is the only fully conserved residue in close proximity to heme \( d_1 \) and has already been proposed as the location of a radical species formed during the reaction of reduced \( cd_1 \) with oxygen (23).

However, although the visible region absorption and MCD spectra of \( cd_1^{\bullet-X} \) are characteristic of low-spin FeIII heme \( d_1 \), a band at this specific wavelength (∼631 nm, Fig. 1a) has previously been assigned to a FeIII heme \( d_1 \) nitrosoyl species (18, 48) formed in the reaction of NO₂ with FeIII heme \( d_1 \). The addition of NO to oxidized \( cd_1 \) from both \( Pa \) and \( Ps \) also results in absorption spectra similar to that of \( cd_1^{\bullet-X} \) (Fig. 1b) (19, 49). This would, at first, appear to contradict our data because ferric nitrosoyl hemes are best described as diamagnetic FeIII-NO⁺ species (50) and do not give rise to spectra characteristic of FeIII. The unpaired π⁺ electron of the NO is largely transferred to the FeIII dxy orbitals. But we have previously proposed that the fully occupied dxy orbitals in the unusual (dxy)²(dyz)³ ground state of low-spin FeIII heme \( d_1 \) could destabilize this mode of NO binding (5). Heme \( d_1 \) in \( cd_1^{\bullet-X} \) is clearly paramagnetic and may represent a genuine FeIII-NO species, in which the NO ligand itself is the radical species interacting with the spin of FeIII heme \( d_1 \). This interpretation would require that a transient protein radical is formed during the reaction but dissipates readily. A relatively weak coupling between a radical ligand and the spin of the metal ion to which it is bound is unusual but without precedent. In horseradish peroxidase compound I, the coupling between the oxoferryl (FeIV=O) iron and a radical on the porphyrin ligand is ∼4 cm⁻¹ (51, 52). In the case of low-spin FeIII heme \( d_1 \), the unpaired electron of metal is located not in dxy orbitals, which readily interact with NO, but in the dxz,dyz orbitals (5), which lies in the plane of the isobacteriochlorin and is potentially orthogonal to the NO π⁺ orbitals in a linear Fe-N-O conformation. However, these putative FeIII-NO species are stable, and so this property of heme \( d_1 \) alone is not sufficient to dissociate product. It was previously reported that addition of the chemical reductant ascorbate to as-prepared \( Pa cd_1 \) resulted in the appearance of the EPR sig-

⁴V. S. Oganesyan, M. R. Cheesman, and A. J. Thomson, manuscript in preparation.

**DISCUSSION**

This work has shown that \( cd_1 \) in the oxidized state will react directly with the physiological substrate NO₂ to form \( cd_1^{\bullet-X} \), a complex in which heme \( d_1 \) is paramagnetic but EPR-silent. This reaction drives a ligand switch at FeIII heme c from His/His to His/Met, the conformation associated with enzyme activity (11–13, 34). Hence, the enzyme has been activated without passing through the reduced state, something previously considered essential (6, 11, 34). Dissociation or displacement of Tyr25⁵ to allow NO₂⁻ binding does not therefore have to be driven by reduction of the two hemes, and the lack of appropriate \( P. pantotrophus \) cellular components with reduction potentials low enough to reduce \( cd_1 \) may not be an issue (27).

We have also shown that an equivalent \( cd_1^{\bullet-X} \) species is formed in the reaction of NO₂ with \( cd_1 \) from \( P. stutzeri \). The reactivity is not, therefore, unique to enzyme with the ability to switch heme c ligands but may be a property of cytochromes \( cd_1 \) in general. Over twenty years ago, Muhoberac and Wharton reported that the addition of NO₂ to \( Pseudomonas aeruginosa \) \( cd_1 \) results in the loss, from the EPR spectrum, of features associated with heme \( d_1 \) (45). The samples, at pH 7.0, were incubated for 24 h before spectra were recorded. There can be little doubt that this is the same reaction as has been described here in detail.

MCD showed that the EPR-silent FeIII heme \( d_1 \) is paramagnetic but that \( S \neq \frac{1}{2} \). Because heme c is magnetically isolated, and the enzyme contains no other cofactors, heme \( d_1 \) must be interacting with a second paramagnet, presumably a radical species formed in the reaction with NO₂. The spin interaction

![FIGURE 7. RD-MCD Spectra of \( cd_1 \) from \( Pp \) (a) and \( Ps \) (b) in the \( cd_1^{\bullet-X} \) form.](Image 60x505 to 288x733)
nature of Fe^{II}-NO (53). This was attributed to the presence of NO$_2^-$ in the enzyme, which was being reduced to NO. However, it cannot be excluded that the species responsible was equivalent to cd$^-$$^-X$ and contained Fe$^{III}$-NO. This would also yield ferrous nitrosyl EPR signals upon reduction. EPR spectra of the as-prepared enzyme are not shown (53), and where they are presented elsewhere (17, 32, 39, 45, 54), no integrations are reported. Consequently it is not possible to say whether or not these samples contained EPR-silent heme $d_1$. If they did, then it may be that Pa and Ps cytochromes cd$_1$ are prone to retaining product NO. The contrasting behavior of Pp cd$_1$ may be linked to an ability of Tyr$^{35}$ to displace distal heme $d_1$ ligands.

NO evolution is detected during cd$_1$ activity assays, which are performed in the presence of excess reductant (13, 55). Yet, in several studies of the reaction of NO$_2^-$ with the fully reduced enzymes from both Pp and Pa, the reaction stopped after a single turnover (18–21). Evidently the role of reductant in product release is complex and may well be related to redox-linked conformational changes at heme c.

The reaction with NO$_2^-$ constitutes a mechanism by which the oxidized Pp enzyme can be activated by substrate but at a rate which is several orders of magnitude slower than turnover. It is unlikely therefore that this species is directly involved in the catalytic cycle in this exact form. However, the involvement of a transient protein radical in NO$_2^-$ reduction would represent an additional redox center and raises the possibility that heme $d_1$ does not access the Fe$^{III}$ state during turnover of NO$_2^-$.

In summary, we have shown that cd$_1$ in the oxidized state reacts directly with its substrate NO$_2^-$ driving the heme c His/His to His/Met ligand switch required for activity. The reaction also occurs with cd$_1$s that do not undergo ligand switching and do not require activation. The resulting novel species contains low-spin Fe$^{III}$ heme $d_1$ that is rendered EPR-silent by spin-coupling to a radical that we propose is bound nitric oxide formed in the reaction.

Acknowledgments—We thank Dr. James Allen for valuable discussions and Prof. Andrew Thomson for critical reading of the manuscript.

REFERENCES

1. Zumft, W. G. (1997) Microbiol. Mol. Biol. Rev. 61, 533–616
2. Berks, B. C., Ferguson, S. J., Moir, J. W. B., and Richardson, D. J. (1995) Biochim. Biophys. Acta 1232, 97–173
3. Ferguson, S. J. (1998) Curr. Opin. Chem. Biol. 2, 182–193
4. Fulop, V., Moir, J. W. B., Ferguson, S. J., and Hajdu, J. (1995) Cell 81, 369–377
5. Cheesman, M. R., Ferguson, S. J., Moir, J. W. B., Richardson, D. J., Zumft, W. G., and Thomson, A. J. (1997) Biochemistry 36, 16267–16276
6. Williams, P. A., Fulop, V., Garman, E. F., Saunders, N. F. W., Ferguson, S. J., and Hajdu, J. (1997) Nature 389, 406–412
7. Sjogren, T., and Hajdu, J. (2001) J. Biol. Chem. 276, 29450–29455
8. Koppenhofer, A., Turner, K. L., Allen, J. W. A., Chapman, S. K., and Ferguson, S. J. (2000) Biochemistry 39, 4243–4249
9. Samyn, B., Berks, B. C., Page, M. D., Ferguson, S. J., and Beeuman, J. (1994) Eur. J. Biochem. 219, 585–594
10. Moir, J. W. B., Baratta, D., Richardson, D. J., and Ferguson, S. J. (1993) Eur. J. Biochem. 212, 377–385
11. Allen, J. W. A., Watmough, N. J., and Ferguson, S. J. (2000) Nat. Struct. Biol. 7, 885–888
12. Allen, J. W. A., Cheesman, M. R., Higham, C. W., Ferguson, S. J., and Watmough, N. J. (2000) Biochem. Biophys. Res. Commun. 279, 674–677
13. Richter, C. D., Allen, J. W. A., Higham, C. W., Koppenhofer, A., Zajicek, R. S., Watmough, N. J., and Ferguson, S. J. (2002) J. Biol. Chem. 277, 3093–3100
14. Page, C. C., Moser, C. C., Chen, X., and Dutton, P. L. (1999) Nature 402, 47–52
15. Nurizzo, D., Cutruzzola, F., Arase, M., Bourgeois, D., Brunori, M., Camilli, C., and Tegoni, M. (1999) J. Biol. Chem. 274, 14997–15004
16. Nurizzo, D., Cutruzzola, F., Arase, M., Bourgeois, D., Brunori, M., Camilli, C., and Tegoni, M. (1998) Biochemistry 37, 13987–13996
17. Gudat, J. C., Singh, J., and Wharton, D. C. (1973) Biochim. Biophys. Acta 292, 376–390
18. George, S. J., Allen, I. W. A., Ferguson, S. J., and Thorneley, R. N. F. (2000) J. Biol. Chem. 275, 33331–33337
19. Silvestrini, M. C., Colosimo, A., Brunori, M., Walsh, T. A., Barber, D., and Greenwood, C. (1979) Biochem. J. 183, 701–709
20. Silvestrini, M. C., Tordi, M. G., Musci, G., and Brunori, M. (1990) J. Biol. Chem. 265, 11783–11787
21. Johnson, M. K., Thomson, A. J., Walsh, T. A., Barber, D., and Greenwood, C. (1980) Biochem. J. 189, 285–294
22. Rinaldo, S., Arcovito, A., Brunori, M., and Cutruzzola, F. (2007) J. Biol. Chem. 282, 14761–14767
23. Koppenhofer, A., Little, R. H., Lowe, D. J., Ferguson, S. J., and Watmough, N. J. (2000) Biochemistry 39, 4028–4036
24. Singh, J. (1973) Biochim. Biophys. Acta 333, 28–36
25. Yamanaka, T., and Okunuki, K. (1963) Biochim. Biophys. Acta 67, 394–406
26. Zajicek, R. S., and Ferguson, S. J. (2005) Biochem. Soc. Trans. 33, 147–148
27. Zajicek, R. S., Allen, J. W. A., Catron, M. L., Richardson, D. J., and Ferguson, S. J. (2004) FEBS Lett. 565, 48–52
28. Roldan, M. D., Sears, H. I., Cheesman, M. R., Ferguson, S. J., Thomson, A. J., Berks, B. C., and Richardson, D. J. (1998) J. Biol. Chem. 273, 28785–28790
29. Allen, J. W. A., Higham, C. W., Zajicek, R. S., Watmough, N. J., and Ferguson, S. J. (2002) Biochem. J. 366, 883–888
30. Walker, F. A. (1999) Coord. Chem. Rev. 186, 531–534
31. Coyle, C. L., Zumft, W. G., Körner, H., and Jakob, W. (1985) Eur. J. Biochem. 153, 459–467
32. Sutherland, I., Greenwood, C., Peterson, J., and Thomson, A. J. (1986) Biochem. J. 233, 893–898
33. Thomson, A. J., Cheesman, M. R., and George, S. J. (1993) Methods Enzymol. 226, 199–232
34. Fulop, V., Watmough, N. J., and Ferguson, S. J. (2001) Adv. Inorg. Chem. 51, 163–204
35. Cheesman, M. R., and Walker, F. A. (1996) J. Am. Chem. Soc. 118, 7737–7738
36. Aasa, R., and Vänngård, T. (1975) J. Magn. Reson. 19, 308–315
37. Gadsby, P. M. A., and Thomson, A. J. (1990) J. Am. Chem. Soc. 112, 5003–5011
38. Cheesman, M. R., Greenwood, C., and Thomson, A. J. (1991) Adv. Inorg. Chem. 36, 201–255
39. Walsh, T. A., Johnson, M. K., Greenwood, C., Barber, D., Springall, J. P., and Thomson, A. J. (1979) Biochem. J. 177, 29–39
40. Vickery, L. E., Palmer, G., and Wharton, D. C. (1978) Biochem. Biophys. Res. Commun. 80, 458–463
41. Walsh, T. A., Johnson, M. K., Barber, D., Thomson, A. J., and Greenwood, C. (1981) J. Inorg. Biochem. 14, 15–31
42. Walsh, T. A., Johnson, M. K., Thomson, A. J., Barber, D., and Greenwood, C. (1981) J. Inorg. Biochem. 14, 1–14
43. Cheesman, M. R., Zumft, W. G., and Thomson, A. J. (1998) *Biochemistry* 37, 3994–4000
44. Cheesman, M. R., Oganesyan, V. S., Watmough, N. J., Butler, C. S., and Thomson, A. J. (2004) *J. Am. Chem. Soc.* 126, 4157–4166
45. Muhoberac, B. B., and Wharton, D. C. (1983) *J. Biol. Chem.* 258, 3019–3027
46. Huyett, J. E., Doan, P. E., Gurbiel, R., Housemann, A. L. P., Sivaraja, M., Goodin, D. B., and Hoffman, B. M. (1995) *J. Am. Chem.* 117, 9033–9041
47. Housemann, A. L. P., Doan, P. E., Goodin, D. B., and Hoffman, B. M. (1995) *Biochemistry* 32, 4430–4443
48. Zajicek, R. S., Cartron, M. L., and Ferguson, S. J. (2006) *Biochemistry* 45, 11208–11216
49. Wang, Y. N., and Averill, B. A. (1996) *J. Am. Chem. Soc.* 118, 3972–3973
50. Enemark, J. H., and Feltham, R. D. (1974) *Coord. Chem. Rev.* 13, 339–406
51. Schulz, C. E., Devaney, P. W., Winkler, H., Debrunner, P. G., Doan, N., Chiang, R., Rutter, R., and Hager, L. P. (1979) *FEBS Lett.* 103, 102–105
52. Schulz, C. E., Rutter, R., Sage, J. T., Debrunner, P. G., and Hager, L. P. (1984) *Biochemistry* 23, 4743–4754
53. Muhoberac, B. B., and Wharton, D. C. (1980) *Fed. Proc.* 39, 2061–2061
54. Hill, K. E., and Wharton, D. C. (1978) *Biochem. J.* 153, 489–495
55. Cutruzzola, F., Brown, K., Wilson, E. K., Bellelli, A., Arese, M., Tegoni, M., Cambillau, C., and Brunori, M. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 2232–2237
56. Yamanaka, T., Ota, A., and Okunuki, K. (1961) *Biochim. Biophys. Acta* 53, 294–308
57. Besson, S., Carneiro, C., Moura, J. J. G., Moura, I., and Fauque, G. (1995) *Anaerobe* 1, 219–226
58. Legall, J., Payne, W. J., Morgan, T. V., and Dervartanian, D. (1979) *Biochem. Biophys. Res. Commun.* 87, 355–362
59. Huynh, B. H., Lui, M. C., Moura, J. J. G., Moura, I., Ljungdahl, P. O., Munck, E., Payne, W. J., Peck, H. D., Dervartanian, D., and Legall, J. (1982) *J. Biol. Chem.* 257, 9576–9581