# Probing the Unusual Oxidation/Reduction Behavior of *Paracoccus pantotrophus* Cytochrome $cd_1$ Nitrite Reductase by Replacing a Switchable Methionine Heme Iron Ligand with Histidine†

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*Received March 13, 2006; Revised Manuscript Received July 13, 2006*

ABSTRACT: A M106H variant, where M106 is a *c*-type heme iron axial ligand, of cytochrome $cd_1$ nitrite reductase is an inactive protein in vivo. Expression of the holoprotein in *Paracoccus pantotrophus* required generation of nitric oxide during cell growth through simultaneous expression of an exogenous copper nitrite reductase from *Alcaligenes faecalis*. In the absence of the latter protein, only a semi-apo form of M106H cytochrome $cd_1$ was formed. Thus it was demonstrated that expression of the chromosomal *nir* genes for $d_1$ heme biosynthesis in *P. pantotrophus* is NO-dependent, probably mediated by the transcription factor NNR, and a route to low or zero activity mutants had been established. The value of such variants for mechanistic studies on cytochrome $cd_1$ is illustrated by the use of M106H to demonstrate that the $d_1$ heme potential can be resolved and measured at approximately +175 mV with the *c* heme shifted to −60 mV, consistent with its bishistidinyl coordination. The unusual highly cooperative and strongly hysteretic redox titration of the wild type is lost in the M106H protein. The same *c* heme midpoint potential was observed in a M106H variant of a *c*-domain construct. The difference between $d_1$ heme and *c* heme redox potentials has allowed preparation of a M106H protein with oxidized *c* heme and reduced $d_1$ heme. This one electron reduced form will reduce nitrite to nitric oxide, but the latter remains bound to the resulting fully oxidized enzyme.

Denitrification is the set of *N*-oxide (nitrate, nitrite, nitric oxide, and nitrous oxide) reductions used by some anaerobically respiring bacteria during energy transduction. In *Paracoccus pantotrophus* and many other bacteria the one-electron reduction of nitrite to nitric oxide and water is carried out by cytochrome $cd_1$ nitrite reductase (NirS). This is not the case in all denitrifying bacteria as a second copper-containing nitrite reductase (NirK) exists. Neither form of nitrite reductase has been found in the same species of bacteria as a functional enzyme, and no significant evolutionary advantage can be attributed to either protein. However, it is clear that the production of cytochrome $cd_1$ is biosynthetically more demanding because it involves the insertion of the unique $d_1$ heme cofactor. Studies using polyclonal antibodies have shown that bacterial species utilizing cytochrome $cd_1$ are more abundant than those utilizing CuNIR;[1] however, CuNIRs are found in bacteria exhibiting great physiological diversity, e.g., extreme halophiles and symbiotic nitrogen fixers (*1*). There appears to be no genus-specific enzyme; for example, *Pseudomonas* species can have either cytochrome $cd_1$ or CuNIR (*2*).

Cytochrome $cd_1$ is a periplasmic homodimer which contains one covalently bound *c* heme and one noncovalently bound $d_1$ heme per monomer. The *c* heme is bound through thioether bonds to the cysteine residues of the CXXCH motif that is in the N-terminal domain. The $d_1$ heme sits in a C-terminal eight-bladed $\beta$-propeller domain. The *c* heme is the site of electron entry, and the $d_1$ heme is the site of nitrite binding and reduction to NO. In the oxidized "as isolated" form of *P. pantotrophus* cytochrome $cd_1$ the axial ligands to the *c* heme and $d_1$ heme are His17/His69 and Tyr25/His200, respectively (*3*). Upon reduction of the cytochrome $cd_1$ both hemes undergo structural rearrangement; the *c* heme loses His17 to be replaced with Met106 whereas the $d_1$ heme loses Tyr25, thus becoming pentacoordinate (*4*). This change in heme ligands leaves the $d_1$ heme iron available to bind substrate; it persists in the activated form of the oxidized enzyme (*5*). The $d_1$ heme is unique to this class of enzyme and is manufactured from uroporphyrinogen III, via a precorrin-2 intermediate, using specialized biosynthetic proteins encoded by the genes other than *nirS* in the *nir* cluster (*2*). In contrast, *c* heme production and incorporation into protein are regular occurrences in *P. pantotrophus* and many other bacteria (*6*).

In addition to anaerobosis, nitric oxide is required for the expression of both *nirS* (the cytochrome $cd_1$ structural gene) and the genes encoding nitric oxide reductase (*7*−*10*). This

† This work was funded by Grants C19340 and BBS/B/10323 from the BBSRC to S.J.F.

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[1] Abbreviations: CuNIR, copper-containing nitrite reductase; NO, nitric oxide; NNR, nitrite reductase and nitric oxide reductase regulator; FNR, fumarate and nitrate reductase regulator.

10.1021/bi0604983 CCC: $33.50 
Published on Web 08/24/2006

Table 1: Strains and Plasmids

| | characteristics | source/ref |
|---|---|---|
| strain | | |
| *P. pantotrophus* | | |
| GB17 | Strep resistant | *22* |
| EG6202 | Δ*nirS*::*aph* Km$^r$ | *32* |
| *E. coli* | | |
| DH5α | *supE44* Δ*lacU169* Δ*M15 hsdR17 recA1 endA1 gyrA96 thi-1 relA1* | Invitrogen |
| S17-1 | *pro thi hsdR hdsM*$^+$ *recA* with integrated RP4-2 (Tet::Mu Kan::Tn7) | *24* |
| SM10 | C600, recA, Mu$_c^+$ with integrated RP4 2-tet::Mu | *24* |
| plasmids | | |
| pEG400 | broad host vector | *42* |
| pLACsol | broad host NapC expression vector | *28* |
| pNIR501 | *A. faecalis nirK* amplified from genomic DNA and inserted into pUC19 with *Hin*dIII *Pst*I | *26* |
| pEG276 | broad host expression vector, *rhn* promoter, Gent$^r$ | *32* |
| pEG105 | pTZ19R, carrying *Eco*RI-*Hin*dIII 1.8 kb *nirS* fragment from pEG10 | *32* |
| pRZ020 | *Eco*RI-*Hin*dIII 1.8 kb *nirS* fragment from pEG105 inserted into pEG276 | this work |
| pRZ014 | M106H mutation introduced into pEG105 | this work |
| pRZ024 | *Eco*RI-*Hin*dIII 1.8 kb *nirS* fragment from pRZ014 inserted into pEG276 | this work |
| pRZ203 | *Eco*RI-*Hin*dIII 1.5 kb *nirK* fragment amplified from pNIR501 and inserted into pEG276 | this work |
| pRZ209 | 1.5 kb *nirK* fragment amplified from pNIR501 inserted into pEG400 with upstream *cycA* promoter | this work |

method of transcriptional control means the machinery for both the production and catabolism of the highly toxic NO radical is always coexpressed, although it is counterintuitive that the toxic NO should enhance its own synthesis. These processes are mediated via an unknown mechanism by the transcriptional activator NNR (*10*). It is clear that for the expression of holocytochrome $cd_1$ not only must all the $d_1$ heme biosynthesis genes be expressed, along with the adjacent *nirS*, but also there must be a functional nitrite reductase in order to catalyze the production of NO and consequently initiate expression of *nirS*. Any dependence on NO of the transcription of the $d_1$ heme biogenesis genes has not been established for *Paracoccus* species in which monocistronic mRNA for *nirS* has been reported (*11*); in *Pseudomonads* the transcript is polycistronic, implying that a single NNR binding site activates transcription of not only *nirS* but also some or all of the $d_1$ heme biogenesis genes (*12*, *13*).

There are many mechanistic uncertainties surrounding cytochrome $cd_1$. In vitro the enzyme readily forms an inactive complex in which the product, NO, is tightly bound to ferrous $d_1$ heme (*14*). A study of the fully reduced *P. pantotrophus* enzyme, but in the absence of excess reductant, showed that upon addition of nitrite the expected oxidation of the two heme centers with concomitant production of 2 molecules of NO per polypeptide chain did not occur (*15*). These and other observations illustrate that we do not yet understand how the enzyme binds nitrite, reduces it to NO, and releases this product during steady-state turnover. A related uncertainty concerns the reduction potential for the $d_1$ heme. For the *Pseudomonas aeruginosa* enzyme several values have been reported in the range +190 − +280 mV (*16*). The potential appears to be close to that for the *c* heme, which complicates the analysis. For the enzyme from *Pseudomonas nautica*, the $d_1$ heme potential was reported as +199 mV, surprisingly less positive than the redox potential determined for the *c* heme (+234 mV) (*17*). The potential for the latter is in line with expectation for a normal His/Met-coordinated *c*-type cytochrome and is supported by studies on the isolated *c*-domain of the *P. pantotrophus* protein (*18*). However, the intact *P. pantotrophus* wild-type cytochrome $cd_1$ behaves in a remarkable way during oxidative and reductive titrations. Hysteresis and cooperativity were exhibited such that for both hemes a single potential of +60 mV was found for the reductive direction and +210 mV for the oxidative (*19*).

For further analysis of these issues, production of the M106H variant of *P. pantotrophus* cytochrome $cd_1$ was deemed essential because it would be expected that the *c* heme would be bishistidinyl coordinated in both redox states (as His17 or H106 would be ligands), a feature that could be expected to lower the potential of the *c* heme considerably, thus separating it from that of the $d_1$ heme. Realization of such an expectation would then allow mechanistic studies of the enzyme with the two types of heme in different oxidation states. It could be anticipated that the M106H enzyme would be inactive in vivo as the potential of the bishistidinyl *c* heme might not be compatible with accepting electrons from the physiological electron donors, pseudoazurin and cytochrome $c_{550}$, both of which have reduction potentials of around 250 mV (*20*). However, the theory of electron tunneling suggests that if the redox centers are close enough (<14 Å), then electron transfer from a donor to the $d_1$ heme via a bishistidinyl *c* heme might occur if the overall reaction is thermodynamically favorable (*21*). If the former were true, it was expected that a novel method would have to be devised for expression of an inactive cytochrome $cd_1$ because, as explained earlier, normal holoenzyme production was expected to depend on production of NO by cytochrome $cd_1$ itself.

## METHODS

*Escherichia coli* and *P. pantotrophus* strains and plasmids used are listed in Table 1. Luria broth and agar were routinely used for aerobic growth of these strains. Antibiotics were used at the following concentrations: ampicillin, 100 $\mu$g mL$^{-1}$; gentamycin, 20 $\mu$g mL$^{-1}$; kanamycin, 50 $\mu$g mL$^{-1}$; spectinomycin, 50 $\mu$g mL$^{-1}$ (20 $\mu$g mL$^{-1}$ in *E. coli*); streptomycin, 200 $\mu$g mL$^{-1}$ (in *P. pantotrophus* only). Anaerobic cultures of *P. pantotrophus* were grown on minimal media (*22*) supplemented with 20 mM succinate and 20 mM $KNO_3$ as carbon source and initial terminal electron acceptor, respectively. DNA was introduced into *E. coli* strains by routine transformation (*23*). DNA was introduced into *P. pantotrophus* strains using diparental matings using *E. coli* S17-1 and SM10 as the donor strains (*24*).

Site-directed mutagenesis was used to introduce the M106H mutation into the appropriate codon of the *nirS* gene in pEG105; this plasmid was then called pRZ014. The

primers used for this process were M106HF (5′-CGGTGCGCGTCTTCATGTCCTCCAGCGCG-3′) and M106HR (5′-CGCGCTGGAGGACATGAAGACGCGCACCG-3′). The mutated *nirS* gene was then excised from pRZ014 using *Eco*RI and *Hin*dIII and ligated into pEG276; the resultant plasmid was called pRZ024. This vector was then transformed into *E. coli* S17-1 and then mated into *P. pantotrophus* EG6202 (Δ*nirS*). Expression of the isolated *c*-domain of *nirS* in *E. coli* is reported elsewhere (*18*). Site-directed mutagenesis was used to make both the M106H and M106A mutations in this domain [M. Cartron (2003) D. Phil. Thesis, University of Oxford]. Variants of the *c*-domain protein were expressed and purified exactly as the native protein (*18*). Pseudoazurin was expressed and purified as described previously (*25*).

The internal *Eco*RI restriction site in the *Alcaligenes faecalis nirK* gene was deleted by silent mutation using the primers EcoDF (5′-CCCAAGGTGGTCGAGTTCACCATGGTG-3′) and EcoDR (5′-CACCATGGTGAATTCGACCACCTTGGG-3′). The *A. faecalis nirK* gene was amplified using PCR methodologies from pNIR501 (*26*) using primers AfnF (5′-AAAGAATTCATGGCCGAACAGATGCAAATCAG-3′) and AfnR (5′-TTTAAGCTTTACGTGCCAGATGGTGCGAGAACC-3′). These primers added 5′-*Eco*RI and 3′-*Hin*dIII restriction sites and were used to ligate the PCR fragment into pEG276 to create pRZ203. A second broad host expression vector needed to be used in order to coexpress two genes in the same strain of *P. pantotrophus*. The broad host plasmid pEG400 (*27*) was chosen to express *nirK* despite pEG400 not being designed as an expression vector. The primers Afn2F (5′-AAAAAGCTTATGGCCGAACAGATCAAATCAG-3′) and Afn2R (5′-TTTGAATTCTTACGTGCCAGATGGTGCGAGAACC-3′) were used to amplify *nirK* from pNIR501 using PCR. These primers inserted *Hin*dIII and *Eco*RI restriction sites onto the 5′ and 3′ ends of the PCR fragment, respectively. The *nirK* fragment was then ligated in pEG400 using these sites to form pRZ206. Next, a promoter had to be inserted upstream of the *nirK* gene to allow induction of expression. The *cycA* (*Paracoccus denitrificans* cytochrome $c_{550}$ gene) promoter was amplified from pLACsol (*28*) using CycAF (5-AAAGTCGACATGGGCCTGCCGGTCGGC-3′) and CycAR (5′-TTTAAGCTTCGCGTTTCCTCTTGGGTATCGC-3′) primers; this promoter is active under denitrifying conditions (*28*, *29*). These primers added *Sal*I and *Hin*dIII restriction sites to the 5′ and 3′ ends of the *cycA* PCR fragment, respectively. This fragment was then ligated into pRZ206 upstream of *nirK* in pEG206 to form pRZ209.

Optical densities and nitrite assays were recorded for all growths of *P. pantotrophus*. The optical densities of each sample were measured using absorption spectroscopy at 600 nm. The method of Nicholas and Nason (*30*) was modified to assay the amount of nitrite in the cell cultures. *N*-1-Naphthylethylenediamine dihydrochloride (0.02%) and sulfanilamide (1% w/v) were used in a colorimetric test assayed by absorption spectroscopy at 540 nm.

Potentiometric titrations were carried out essentially as in Koppenhöfer et al. (*19*) with some alterations. The reduction potential of the M106H cytochrome $cd_1$ preparation was measured using an Ag/AgCl electrode (Mettler Toledo) connected to a Hanna Instruments H19321 meter. The probe was calibrated using the $Fe^{III}/Fe^{II}$ EDTA couple, and all values reported are versus the standard hydrogen electrode. The protein spectra were recorded using a gastight cuvette into which the Ag/AgCl probe was inserted through a rubber septum. In addition, the cuvette was constantly flushed with humidified argon gas to ensure anoxic conditions. The protein solution was diluted from a concentrated stock with 100 mM phosphate buffer, pH 7.0. The following mediators were added to the protein solution to a final concentration of 15 $\mu$M: anthraquinone-2-sulfonic acid sodium salt ($E^{\circ\prime} = -225$mV), 2-hydroxy-1,4-naphthoquinone ($E^{\circ\prime} = -145$ mV), phenazine ethosulfate ($E^{\circ\prime} = +55$ mV), and phenazine methosulfate ($E^{\circ\prime} = +80$ mV). In addition, 15 $\mu$M DAD (2,3,5,6-tetramethyl-1,4-phenylenediamine) ($E^{\circ\prime} = +260$ mV) was added in 30% ethanol. During the reduction step sodium dithionite was titrated into the protein solution using a Hamilton syringe; potassium ferricyanide was added for the oxidative titration. All spectra were recorded using a Cary 50 instrument.

In order to add nitrite to one electron reduced M106H cytochrome $cd_1$, the above conditions were employed to reduce the $d_1$ heme only and leave the *c* heme oxidized. Upon full reduction of the $d_1$ heme potassium nitrite (Sigma) was injected using a Hamilton syringe to a final volume of 1 mM. Spectra were taken after 10 s and again after 5 and 30 min. Pseudoazurin was reduced and used in experiments as described in Zajicek et al. (*31*).

## RESULTS

Wild-type *P. pantotrophus* uses denitrification for anaerobic respiration when grown overnight on minimal media supplemented with 20 mM nitrate and a carbon source (30 mM succinate). When wild-type *P. pantotrophus* was cultivated under these conditions, it grew to an optical density ($OD_{600nm}$) of ~1.7 and showed no significant buildup of nitrite in the media (Figure 1A). In contrast, when *P. pantotrophus* strain EG6202 [with the gene encoding cytochrome $cd_1$ (*nirS*) knocked out with a kanamycin insertion cassette] was grown under the same conditions (Figure 1B), there was a significant buildup of nitrite and a cessation of growth at a much lower OD. The levels of nitrite approached 20 mM (Figure 1B), showing that all of the initial nitrate in the media had been converted to nitrite, which could no longer be reduced to nitric oxide owing to a lack of a functional nitrite reductase. *P. pantotrophus* EG6202 was complemented with a plasmid-encoded copy of wild-type cytochrome $cd_1$. Following overnight culture, this strain showed restored nitrite reduction and an increased final OD (Figure 1C) close to that of wild-type *P. pantotrophus*. It has been shown previously that the presence of a plasmid-borne copy of the gene encoding a Y25S variant of *P. pantotrophus* cytochrome $cd_1$ (*32*) had the same effect. When purified, both recombinant wild-type and Y25S cytochrome $cd_1$ contained a full complement of $d_1$ heme. However, complementation of EG6202 with a plasmid-borne gene encoding the M106H variant of cytochrome $cd_1$ did not confer nitrite reductase activity on the resulting cells (RZ009). The nitrite reduction and growth profiles for RZ009 were identical (not shown) to those of EG6202 (Figure 1B). M106H cytochrome $cd_1$ was purified from RZ009 cells, and the lack of any absorbance at 640 nm showed this variant of cytochrome $cd_1$ purified as a semi-apoprotein (containing no $d_1$ heme), the *c* heme being observable from its charac-

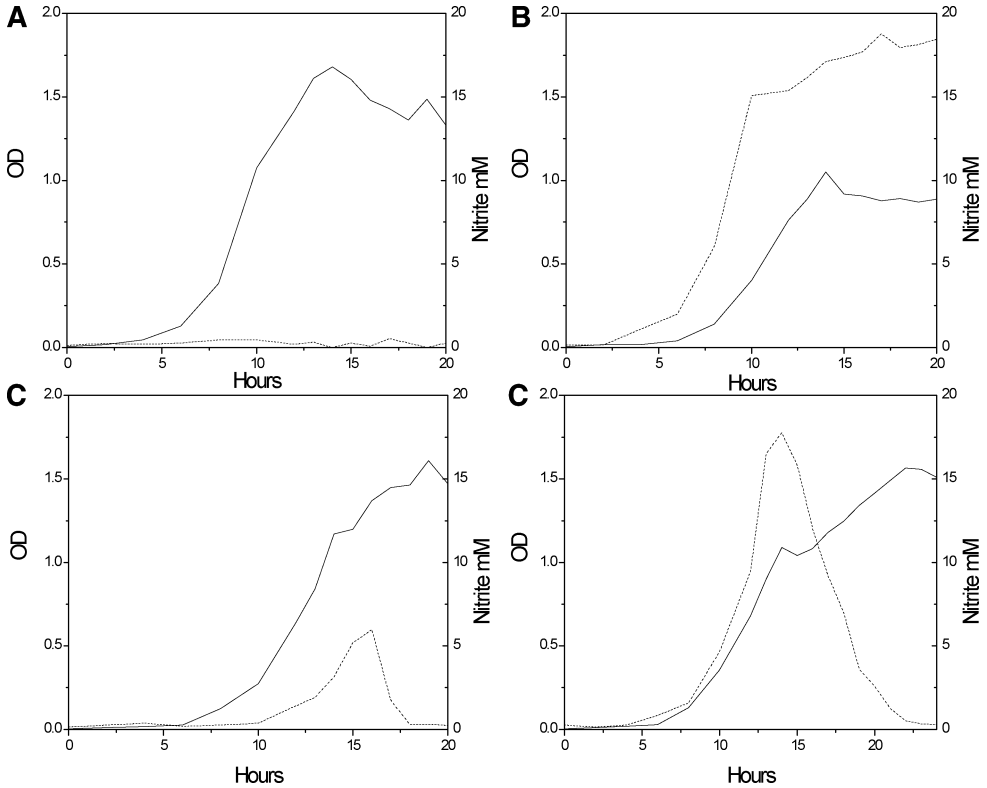


FIGURE 1: Nitrite concentrations (- - -) and optical densities (OD) (—) of growth trials of *P. pantotrophus* wild type and strain EG6202, with or without nitrite reductase expression vectors, under anaerobic conditions: (A) wild-type *P. pantotrophus*; (B) EG6202 (ΔnirS); (C) RZ005 (EG6202 + pRZ020); (D) RZ011 (EG6202 + pRZ203). All growth trials were grown in 1 L Duran bottles filled with minimal media and grown anaerobically.

teristic split α-band at 550 nm for the reduced protein, exactly as been reported before for wild-type semi-apoprotein (*33*). The absence of $d_1$ heme may have been a consequence of the lack of production of nitric oxide during anaerobic growth.

It was, therefore, necessary to try to introduce a source of nitric oxide into the media with a view to stimulation of $d_1$ heme production and its insertion into the semi-apo form of M106H cytochrome $cd_1$. To this end it was decided to express, from an NO-independent promoter, a copper-nitrite reductase, for which NO is the reaction product, in EG6202. The nitrite reductase from *A. faecalis* was chosen because of the high degree of homology between the physiological electron donors (pseudoazurins) to the nitrite reductases of both *A. faecalis* and *P. pantotrophus*. Figure 1D shows the nitrite concentrations and OD for a culture of EG6202 complemented with the *A. faecalis* nitrite reductase (NirK). The concentration of nitrite in the media reached 20 mM but then decreased to zero in contrast to EG6202 where a plateau level was reached. Reduction of nitrite by *A. faecalis* NirK should produce NO which might stimulate $d_1$ heme production. In addition to a decrease in nitrite, the OD of this strain, compared with its parent that lacked *nirK*, reached a value closer to that of wild-type *P. pantotrophus*. Coexpression of this nitrite reductase with M106H cytochrome $cd_1$ required that the *A. faecalis* nitrite reductase gene (*nirK*) was ligated into a different plasmid that was suitable for coexistence with the plasmid expressing M106H cytochrome $cd_1$ (see Methods). Upon coexpression of both forms of nitrite reductase, a holo form of M106H cytochrome $cd_1$ was expressed. Figure 2 shows the spectra of oxidized as isolated and dithionite-reduced M106H cytochrome $cd_1$; it is clear

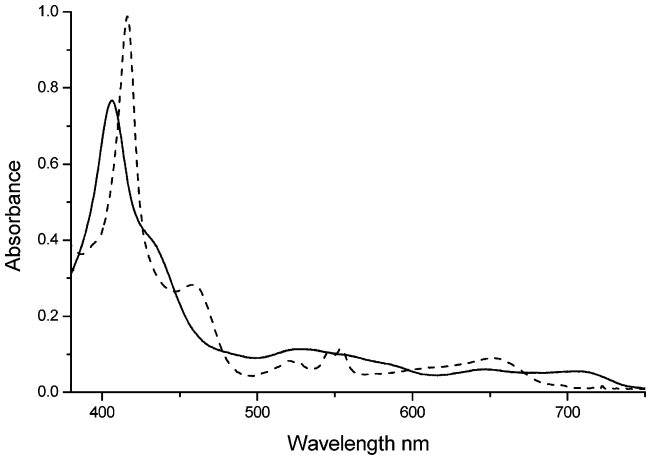


FIGURE 2: Visible absorption spectra of oxidized as isolated (solid line) and reduced (dashed line) *P. pantotrophus* M106H cytochrome $cd_1$. The reduced sample was prepared by addition of a few grains of dithionite. All samples were in 50 mM potassium phosphate buffer, pH 7.0. Spectra were recorded at 25 °C. The protein concentration was ~3 $\mu$M.

that the provision of the copper-nitrite reductase (NirK) restored $d_1$ heme production in *P. pantotrophus* EG6202 by catalyzing the formation of NO from nitrite, a reaction that could not be catalyzed by the M106H enzyme.

As expected, the potentiometric titration of the M106H variant cytochrome $cd_1$ differed greatly from that of the wild-type protein. Köppenhofer et al. (*19*) showed the titration of the wild-type protein to be a hysteretic process with both hemes titrating together in both the reductive and oxidative directions, albeit at different potentials for each titration (+60 mV and +210 mV, respectively, $n = 1.4$). However, the

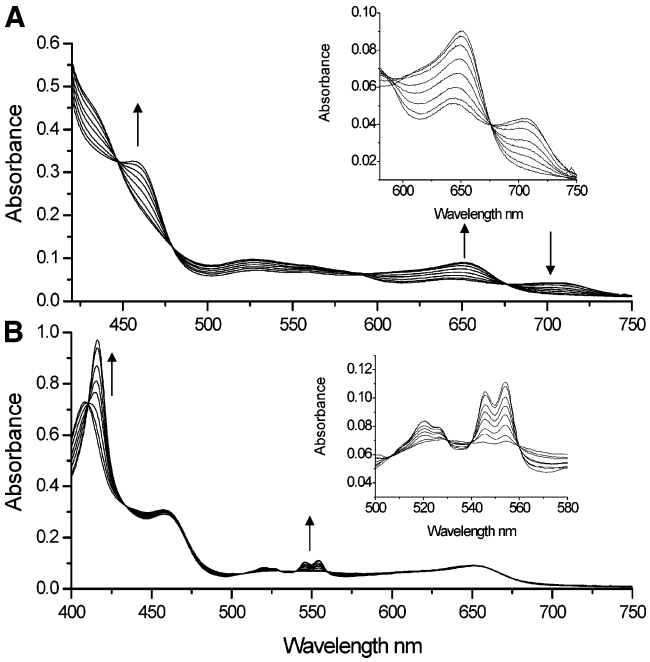


FIGURE 3: Reductive potentiometric titration of *P. pantotrophus* M106H cytochrome $cd_1$ conducted over the range +350 to −350 mV. (A) Spectra recorded over the range +350 to +100 mV showing the reduction of the $d_1$ heme. (B) Spectra recorded over the range +100 to −200 mV showing the reduction of the *c* heme. The arrows indicate the direction of the absorbance change during the course of the titration with dithionite. The sample was ~2.5 M in 100 mM potassium phosphate buffer, pH 7.0 at 25 °C.

M106H cytochrome $cd_1$ titration did not display these features. In an initial reductive titration each heme had a different potential, the $d_1$ heme at +250 mV ($n$ = 1.4), whereas the *c* heme had a potential of −30 mV ($n$ = 1.0) (all midpoint potentials quoted are versus SHE). Figure 3 clearly shows these separate titration profiles. The peaks above 600 nm are those arising due to the $d_1$ heme; in the early titration points between +350 and +100 mV it is these peaks that change significantly with reduction (Figure 3A). However, as seen in Figure 3B, between +100 and −200 mV these peaks are unchanged, and only signal changes associated with the reduction of the *c* heme were observed (416 and ~555 nm). Consequently, in a midpoint between these two titrations there existed a state of *P. pantotrophus* M106H cytochrome $cd_1$ in which the $d_1$ heme was fully reduced and the *c* heme was oxidized.

In the subsequent oxidative titration (Figure 4) the reduction potentials varied slightly (relative to the initial reductive titration) at both heme centers, and this change was also reflected in the visible absorption spectrum. Upon oxidation of the *c* heme the midpoint potential was −60 mV ($n$ = 1), a figure slightly lower than that during the original reductive direction (Figure 5). The $d_1$ heme had a lowered midpoint potential in the oxidative direction at +175 mV ($n$ = 1). However, upon further cycles of reduction and oxidation the midpoint potentials of the *c* and $d_1$ hemes remained at ~−60 and ~+175 mV, respectively (Figure 5). In addition to the change in redox potential, a change in the visible absorption spectrum in the region associated with the $d_1$ heme was observed. As shown in Figure 2 the as isolated oxidized form of the M106H variant has an absorption spectrum identical to the oxidized wild-type protein and specifically has two peaks above 600 nm. These peaks at 640 and 700 nm are

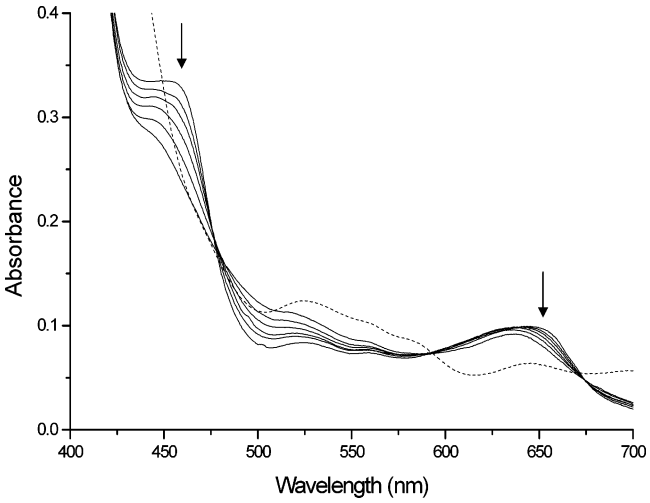


FIGURE 4: Oxidative potentiometric titration of *P. pantotrophus* M106H cytochrome $cd_1$ conducted over the range +100 to +350 mV. The arrows indicate the direction of the absorbance change during the course of the titration with potassium ferricyanide. The final spectrum is that of the fully oxidized redox-cycled protein which is distinctly different from the as isolated M106H cytochrome $cd_1$ (---) shown. The sample was ~2.5 $\mu$M in 100 mM potassium phosphate buffer, pH 7.0 at 25 °C.

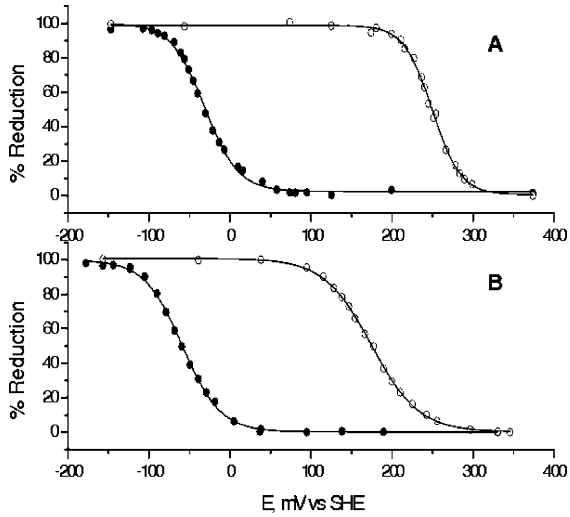


FIGURE 5: Plot of absorbance (650 and 416 nm) versus electrode potential during both reductive (A) and oxidative (B) potentiometric titrations of *P. pantotrophus* M106H cytochrome $cd_1$. The absorbance at 650 nm (○) is diagnostic of the $d_1$ heme. At this wavelength for $d_1$ heme the initial reductive data $E'_{red}$ = +250 mV and $n$ = 1.4, and for the oxidative data $E'_{ox}$ = +175 mV and $n$ = 1. The absorbance at 416 nm (●) is diagnostic of the *c* heme. At this wavelength for *c* heme the initial reductive data $E'_{red}$ = ±30 mV and $n$ = 1, but for the oxidative and subsequent reductive data $E'_{ox}$ = −60 mV and $n$ = 1.

due to a high/low-spin thermal equilibrium caused by the binding of Tyr25 to the $d_1$ heme (*34*). Upon redox cycling of this initial form of the protein, a new oxidized spectrum was seen (Figure 4); this has a peak at 640 nm, but not at 700 nm, implying the loss of Tyr25 binding to the $d_1$ heme. The latter observation suggests that the original structural state of the oxidized enzyme is not regained after an initial reduction. In contrast, if sufficient dithionite was added to the original oxidized enzyme to reduce only the $d_1$ heme, then a subsequent oxidative titration gave a midpoint potential of ~+250 mV and showed a return to the original visible absorption spectrum. This suggests that a putative

structural rearrangement occurred only after the reduction of the *c*-type heme center. It is important to note that when the redox-cycled form of M106H variant oxidized cytochrome $cd_1$ was left for >24 h at 4 °C, a small signal at 700 nm was observed, implying a very slow reversion to the original "non-redox-cycled" protein.

Previously, Steensma et al. (*35*) have shown that it is possible to express a truncated cytochrome $cd_1$ which contains only the domain binding of the *c* heme (*18*). In contrast to holocytochrome $cd_1$, this *c*-domain protein has His/Met (M106) ligation at the *c* heme in both the oxidized and reduced forms, as shown by NMR spectroscopy (*35*). A redox titration of this protein gave a midpoint potential of +250 mV (as expected for a His/Met coordinated heme) (*18*). In the present work, a M106H variant of the *c*-domain was subsequently made and expressed, using the method of Steensma et al. (2001), and the reduction potential of the *c* heme was investigated. The midpoint potential of this variant protein was −55 mV, a value that is assumed not to be affected by any heme ligand switches and thus provides a reference point for the more complex redox titration observed for the holo M106H protein. This result is in excellent agreement with the midpoint potential of the *c* heme of holo M106H protein in the oxidative and second and subsequent reductive titrations; presumably each heme group in both variant proteins is binding the same ligand, namely, His106, as well as the histidine of the CXXCH motif. The possibility of five-coordinate or otherwise high-spin heme was ruled out by the creation of a *c*-domain M106A variant which exhibited the characteristics of a high-spin heme. The midpoint potential of this variant was −200 mV, and the spectrum of the oxidized protein showed a broad feature at 650 nm, characteristic of a high-spin ferric heme with histidine as one axial ligand and either nothing or water/hydroxide bound in the other axial position.

Previous experiments performed on Y25S variant *P. pantotrophus* cytochrome $cd_1$, which has His/Met ligands to both the oxidized and reduced *c* heme in solution, demonstrated that reduced pseudoazurin was able to reduce the protein (*31*). In contrast, we found that pseudoazurin failed to reduce M106H cytochrome $cd_1$. The reason for this is the lower reduction potential of a bishistidinyl coordinated *c* heme in comparison to a His/Met coordinated *c* heme. This result highlights the sensitivity of the physiological electron donor to the change in reduction potential of the *c* heme in cytochrome $cd_1$.

It was evident from the above titrations that it was possible to reduce the $d_1$ heme leaving the *c* heme oxidized. This gave us the unprecedented opportunity to create a one electron reduced form of *P. pantotrophus* cytochrome $cd_1$ with the electron clearly residing on the $d_1$ heme. In addition, this allowed the study of a one-electron turnover reaction upon addition of nitrite. Therefore, oxidized *P. pantotrophus* M106H cytochrome $cd_1$ was reduced with dithionite to produce a one electron reduced complex (at ~120 mV), and nitrite was then added to a final concentration of 1 mM. Figure 6 shows the initial spectrum and that of the complex 30 s after adding nitrite. This spectrum remained unchanged after further 5 and 30 min intervals. The major change in the spectrum after the addition of nitrite was a shift in the $d_1$ heme peak at ~640 nm (in the one electron reduced complex) to 631 nm (after the addition of nitrite). When the final spectra were compared to previously observed spectra, it is evident that the peak at 631 nm is that of oxidized cytochrome $cd_1$ with NO bound at the $d_1$ heme (*15*; K. Sam and S. J. Ferguson, unpublished results). Previously, FTIR spectroscopy was used to identify this species of ferric $d_1$ heme with NO bound (*15*). The spectrum of oxidized Y25S variant cytochrome $cd_1$ with $NO_2^-$ bound at the $d_1$ heme has a maximum at 636 nm (R. Zajicek, unpublished results). It is clear that a one-electron reduction of $NO_2^-$ has occurred at the $d_1$ heme to produce NO. An unlikely alternative interpretation was that this spectrum of the protein actually represented ferrous $d_1$ heme with $NO_2^-$ bound as an enzyme−substrate complex. In order to rule out this possibility, excess $Fe(CN)_6^{3-}$ was added to the putative $d_1(Fe^{3+})$−NO complex. The spectrum did not show any change in the $d_1$ heme region. Thus there was no steady- state formation of $d_1(Fe^{2+})-NO_2^-$, unless its spectrum is identical to $d_1(Fe^{3+})$−NO. A $d_1(Fe^{2+})$−NO species could also be ruled out as this has an absorption centered close to 640 nm (*15*). It is also apparent that the excess $NO_2^-$ in the cuvette is unable to displace the NO from the $d_1$ heme, even at a concentration of 1 mM. This result implies that an additional factor(s) is/are required for the dissociation of NO from the $d_1$ heme of oxidized cytochrome $cd_1$ (this factor is likely to be the addition of reductant and/or the presence of a physiological electron donor, i.e., cytochrome $c_{550}$ or pseudoazurin).

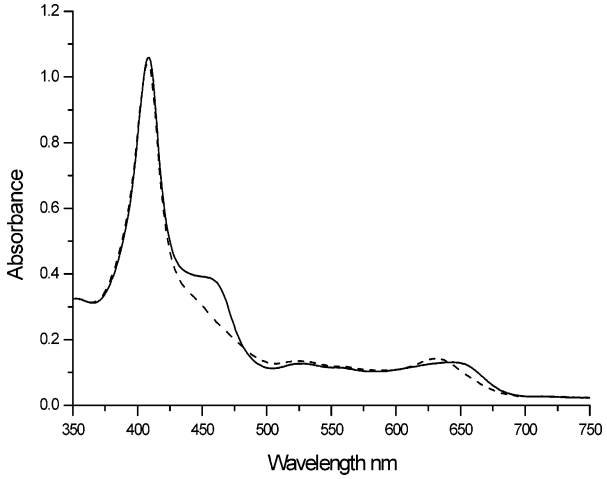


FIGURE 6: One electron reduced *P. pantotrophus* M106H cytochrome $cd_1$ before and after addition of 1 mM nitrite. The protein was carefully reduced with dithionite until a potential of ~+120 mV was reached therefore reducing only the $d_1$ heme (—). Sodium nitrite was then added to a final concentration of 1 mM and a spectrum recorded after 30 s (---).The sample was ~3.5 $\mu$M in 100 mM potassium phosphate buffer, pH 7.0 at 25 °C.

## DISCUSSION

The results in this work demonstrate the ability of one or more electron donors of *P. pantotrophus* to transfer electrons to the Cu-NIR of *A. faecalis*, at a physiologically significant rate, so that it can catalyze reduction of all nitrite present in the media. It is reasonable to assume that pseudoazurin is the major donor due to the homology described above; however, cytochrome $c_{550}$ could be involved instead of, or as well as, pseudoazurin in keeping with the theory of pseudospecific docking (*36*). Before the present work, a Cu-Nir from *Pseudomonas aureofaciens* had been used to enable nitrite reduction in a Δ*nirS* strain of *P. stutzeri* (*37*). This

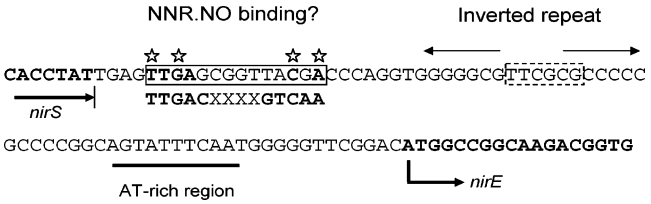


FIGURE 7: Nucleotide sequence of the *P. pantotrophus* genome highlighting the 74 bp intergenic region between *nirS* and *nirE* and the putative NNR binding motif. The region where an NNR−NO complex may bind is boxed with the NNR box consensus sequence below. The essential nucleotides of this box are highlighted with stars. The dashed box highlights the conserved sequence seen adjacent to other NNR induced promoters (see text). Other features of this region include an inverted repeat and an AT-rich region.

observation along with those described here can now be used to expand the theory of pseudospecific docking to include large structural and cofactor variation in the electron acceptor as well as the electron donor.

The finding in the present work that the synthesis of $d_1$ heme in *P. pantotrophus* is dependent on nitric oxide can be understood by inspection of the intergenic region upstream of *nirE* (Figure 7). There is a partially palindromic sequence that is related to the sequence recognized by NNR, the only known NO-dependent transcriptional regulator in *Paracoccus* species. The most important nucleotides (*37*) of an NNR consensus sequence are present (Figure 7). The presence of a "partial" rather than "full" NNR binding site might be explained in the context that a lower amount of mRNA for the $d_1$ heme biogenesis genes is needed compared with that required for the structural genes for nitrite and nitric oxide reductases. Previously, various nucleotides of an FNR binding consensus sequence, which are remarkably similar to those for NNR binding, have been altered to study the consequences for transcription of an FNR-dependent gene (*38*). Such mutations reduce the efficiency of FNR, to which NNR is closely related, but do not prevent its partial activity. Taken together, all of these considerations render very likely a nitric oxide dependent regulation of the $d_1$ biogenesis genes that involves NNR.

In addition to the partial NNR box, there is an upstream TTCGCG sequence (or a sequence very similar) that has also been seen in the same position in the promoter regions of *nirS* and *norC*. It is unlikely that the presence of this sequence adjacent to three different genes is coincidental. Although there is yet no established role for the TTCGCG sequence (*39*), its presence also supports NNR-dependent regulation of the $d_1$ biogenesis genes.

The change in the proximal ligand to the *c* heme in M106H cytochrome $cd_1$ has disrupted the highly cooperative nature of heme reduction/oxidation seen in *P. pantotrophus* wild-type cytochrome $cd_1$, and it is clear that in making this mutant we have successfully separated the individual midpoint potentials of both heme cofactors. The cooperativity in the wild-type protein has been attributed to a kinetically gated conformationally dependent process (*19*) which must be lost in the M106H protein. Nevertheless, the M106H variant cytochrome $cd_1$ redox titration has a complexity, not least the difference in visible absorption spectra and redox properties between the starting and redox-cycled forms of the oxidized enzyme. Due to the similarity in the $d_1$ heme region for the oxidized "as prepared" absorption spectra of both the M106H variant protein and the wild type, the former can be assumed to have His17/His69 coordination at the *c* heme and His200/Tyr25 coordination at the $d_1$ heme. Whereas the wild-type $d_1$ heme has a cooperative midpoint titration potential of +60 mV for both hemes, the M106H variant $d_1$ heme now has a potential of +250 mV that titrates independently of the *c* heme. The values for the reduction of the *c* hemes in the two proteins, however, are in closer agreement. In the as isolated forms of both the wild-type and M106H proteins both are thought to bind histidine as the distal ligand to the oxidized *c* heme. Therefore, *c* hemes of both the wild-type and M106H enzyme have a lower midpoint reductive potential than the $d_1$ heme. However, a stark contrast is seen during the oxidative titration. The wild-type *c* heme has a midpoint potential of +250 mV, whereas the M106H *c* heme potential remains at a negative value (−60 mV). This variation in potentials can partially be explained by the difference in *c* heme ligand. In the wild-type protein the ligand switch has occurred upon reduction, replacing His17 with Met106. However, in the M106H variant such a change in ligand type cannot happen, and a histidine residue is likely to remain as the sixth ligand to the *c* heme. It is well documented that the average midpoint redox potential of a His/Met coordinated *c* heme is commonly ∼200 mV in excess of a bishistidinyl coordinated *c* heme because methionine is more effective than histidine at stabilizing the reduced *c* heme iron (*19*, *40*). This is highlighted by the observation that, in this work, reduced pseudoazurin could not reduce the bishistidinyl M106H variant protein; however, it was able to reduce a Y25S variant with His/Met *c* heme coordination due to the higher midpoint potential of a His/Met *c* heme center (*31*).

A mechanism can be proposed for the structural changes taking place in the M106H variant protein during redox cycling that explains the redox behavior. In the as isolated oxidized M106H protein the heme ligands are the same as the wild-type protein (*c*-His17/His69 and $d_1$-Tyr25/His200). It should be recalled that the visible absorption spectrum of the $d_1$ heme in the oxidized M106H variant retains the characteristics seen in the wild-type enzyme that are diagnostic of Tyr25 ligation. During the initial reductive titration the $d_1$ heme would reversibly undergo reduction at +250 mV because His17 would be bound to the *c* heme with Tyr25 still held within the $d_1$ heme pocket. It is important to note that for the wild-type protein most experiments have shown that when His17 is bound at the *c* heme, then Tyr25 is bound to the $d_1$ heme in the absence of nitrite or nitric oxide (*4*, *34*). Upon reduction, at −30 mV, of the *c* heme in the M106H enzyme His17 would be replaced by His106, whereupon significant conformational change should result in Tyr25 leaving the $d_1$ heme pocket. Upon oxidation, His106 would remain bound to the *c* heme and result in an alteration to the midpoint potential of the *c* heme, shifting it to −60 mV. This interpretation is supported by the redox potential measured for the *c*-domain protein carrying the M106H mutation; this single domain species has H106 as ligand to both oxidized and reduced *c* heme. The value was −55 mV, nearly identical to that we observe for the M106H cytochrome $cd_1$ following the initial reduction. When the $d_1$ heme is fully reoxidized in the titration of M106H cytochrome $cd_1$, His106 rather than His17 would be bound to the *c* heme, and thus there would be no driving force encouraging Tyr25

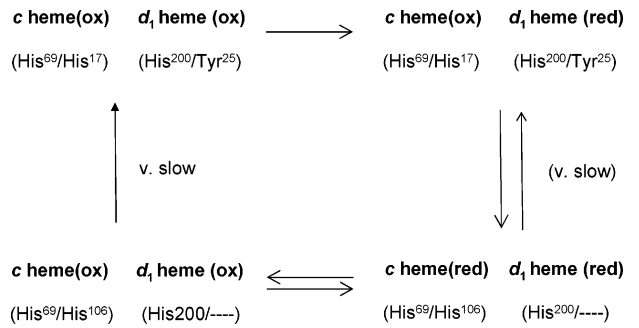


FIGURE 8: Proposed reaction scheme of the *P. pantotrophus* cytochrome $cd_1$ M106H variant protein. This scheme represents the changes that occur at each heme center of the protein following the initial reduction with dithionite followed by oxidation and redox cycling.

to bind. Therefore, the reduction potential of the $d_1$ heme is changed to +175 mV because Tyr25 is no longer bound, and the heme has either water/hydroxide or a vacancy at the proximal position. This is the first time the midpoint potential of *P. pantotrophus* cytochrome $cd_1$ $d_1$ heme lacking the Tyr25 ligand has been recorded. This value is expected to be relevant to the function of the $d_1$ heme during steady-state catalytic turnover, conditions under which no Tyr25 binding is believed to occur. Further redox cycling does not change the midpoint potentials significantly because neither His17 nor Tyr25 rebinds to the *c* heme or the $d_1$ heme, respectively, on this time scale. However, over a longer nonphysiological time scale they appear to do so.

The proposed ligand switches which accompany the oxidation/reduction behavior of the M106H variant protein are shown in Figure 8. It remains to propose an explanation as to why the cooperativity between hemes seen in the titration of the wild-type protein is lost from the M106H variant. The reductive titration of the wild-type protein implies that the $d_1$ heme cannot be reduced until the *c* heme is also reduced and, presumably, the His17 to Met106 switch has occurred. This change would release the tyrosine from the $d_1$ heme, permitting its reduction. A driving force for this reductive step would be the relative affinity of the ferrous *c* heme for the methionine ligand. In the case of the M106H protein we propose that the $d_1$ heme can be reduced without the *c* heme ligand exchange leading to dissociation of Tyr25 from the $d_1$ heme. Thus we envisage that reduction of the *c* heme at −30 mV leads to completion of a conformational change that results in complete dissociation of Tyr25 from the $d_1$ heme pocket and switching of His17 to His106 at the *c* heme. The driving force for this reaction is less than that for the His17 to Met106 ligand switch in the wild-type.

A puzzling aspect of the present work is why only the initial reductive titration of the $d_1$ heme showed sign of cooperativity ($n = 1.4$) whereas the *c* heme did not ($n = 1$). This level of cooperativity is the same as that recorded for the wild-type enzyme during both reductive and oxidative titrations at both heme centers. It is clear that this cooperativity is in some way linked to Tyr25 ligation to the $d_1$ heme because upon irreversible dissociation from the $d_1$ heme in the M106H variant protein there is no longer any cooperativity. We suggest that this is a reflection of the fact that any dissociation of Tyr25 cannot occur on one subunit independently.

The potentiometric titration of the M106H variant cytochrome $cd_1$ shows that it is possible to reduce the M106H variant cytochrome $cd_1$ by one electron per monomer. Spectroscopically, it is easy to see that this electron resides on the $d_1$ heme whereas the *c* heme is oxidized. Addition of nitrite to M106H *c*(ox) $d_1$(red) protein resulted in the appearance of an absorption band that was readily interpreted in terms of formation of a $d_1$(ox)−NO complex. A corollary of this interpretation is that whereas the reduction of nitrite to nitric oxide can occur at the $d_1$ heme with an oxidized *c* heme, the affinity of the oxidized $d_1$ heme for nitric oxide is sufficiently high that it remains bound under the conditions of the present experiment. The concentration of $d_1$ heme active sites was ~8 $\mu$M, and so one can conclude that the affinity of the oxidized $d_1$ heme for NO must have been less than of the order of micromolar under these conditions. Previous experiments involving the one-electron reduction of *P. pantotrophus* cytochrome $cd_1$ have relied upon pulse radiolysis (*41*). This technique was able to introduce an electron specifically to the *c* heme at high speed. This electron would then, at a slower rate, transfer to the $d_1$ heme. When nitrite was added in this type of experiment, no difference in intramolecular heme transfer was seen; however, once the $d_1$ heme was reduced, a new species formed, at a slower rate, with a spectrum similar to the one reported above of oxidized $d_1$ heme with NO bound. However, the inevitably lower spectral resolution of the pulse radiolysis experiment makes confirmation of the exact spectral wavelengths very difficult. Further experiments have involved the addition of nitrite to fully reduced cytochrome $cd_1$, which resulted in a complex with not only NO bound to the $d_1$ heme but also an extra electron residing on a mixture of either the *c* or $d_1$ heme (*15*). The present work has then given us the opportunity to study the one-electron reduction of nitrite to nitric oxide and the dissociation behavior of NO from the fully oxidized enzyme. Previously, it has been shown that a fully reduced form of cytochrome $cd_1$ with NO bound is an extremely stable complex, often known as the "dead-end" complex (*14*, *15*). Conversely, in the experiments detailed above it has been shown that NO will also not readily dissociate from the $d_1$ heme of the oxidized M106H protein.

Finally, the methodology introduced here for production of mutant forms of *P. pantotrophus* cytochrome $cd_1$ will be important for the elucidation of the mechanism of the enzyme through the generation of other variant proteins.

## ACKNOWLEDGMENT

We thank Dr. James Allen for valuable discussion and Dr. M. E. Murphy for kindly providing pNIR501.

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BI0604983