A novel, kinetically stable, catalytically active, all-ferric, nitrite-bound complex of *Paracoccus pantotrophus* cytochrome cd_1

James W. A. ALLEN*, Christopher W. HIGHAM*, Richard S. ZAJICEK*, Nicholas J. WATMOUGH† and Stuart J. FERGUSON*[1]

*Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K., and †School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.

The oxidized form of *Paracoccus pantotrophus* cytochrome cd_1 nitrite reductase, as isolated, has bis-histidinyl co-ordination of the *c* haem and His/Tyr co-ordination of the d_1 haem. On reduction, the haem co-ordinations change to His/Met and His/vacant respectively. If the latter form of the enzyme is reoxidized, a conformer is generated in which the ferric *c* haem is His/Met co-ordinated; this can revert to the 'as isolated' state of the enzyme over approx. 20 min at room temperature. However, addition of nitrite to the enzyme after a cycle of reduction and reoxidation produces a kinetically stable, all-ferric complex with nitrite bound to the d_1 haem and His/Met co-ordination of the *c* haem. This complex is catalytically active with the physiological electron donor protein pseudoazurin. The effective dissociation constant for nitrite is 2 mM. Evidence is presented that d_1 haem is optimized to bind nitrite, as opposed to other anions that are commonly good ligands to ferric haem. The all-ferric nitrite bound state of the enzyme could not be generated stoichiometrically by mixing nitrite with the 'as isolated' conformer of cytochrome cd_1 without redox cycling.

Key words: d_1 haem, enzyme activation, ligand switching, nitrite reductase.

INTRODUCTION

Paracoccus pantotrophus cytochrome cd_1 catalyses the one-electron reduction of nitrite (NO_2^-) to nitric oxide and water as part of the respiratory process of denitrification, during which nitrate (NO_3^-) is reduced stepwise to dinitrogen gas (N_2) [1–3]. The enzyme is a dimer, each monomer contains one *c* haem centre, which accepts electrons from partner donor proteins, and one specialized d_1 haem at the active site. X-ray crystallographic studies have shown that the *c* haem of the oxidized enzyme, as isolated, has bis-histidinyl co-ordination, whereas the d_1 haem is co-ordinated by a histidine residue and a tyrosine residue [4]. The tyrosine (Tyr^{25}) is connected to one of the histidine ligands of the *c* haem (His^{17}) by a polypeptide loop of only seven residues; on reduction, these two closely linked residues dissociate from the haems to which they were co-ordinated [5,6]. At the *c* haem, a methionine sulphur (from Met^{106}) then binds the iron atom to give His/Met co-ordination, whereas the d_1 haem becomes five co-ordinate, providing a binding site for substrate at the active site. Solution spectroscopic studies on the oxidized enzyme were consistent with the ligation of the haems assigned by the crystallographers; in particular, His/His co-ordination of the *c* haem was demonstrated unequivocally by near-infrared magnetic CD spectroscopy [7].

Studies using physiological electron donor proteins to *P. pantotrophus* cytochrome cd_1 with various electron acceptors have shown that the 'as isolated' conformer of the enzyme, with bis-histidinyl co-ordination of the *c* haem, is far from catalytically competent [8–10]. Rather, we have identified an oxidized conformer of the enzyme, in which the *c* haem has His/Met co-ordination, as a catalytically active species [8,9]. This activated form of the enzyme reverted to the 'as isolated' state over 20 min at room temperature following reoxidation of the enzyme with the non-physiological oxidant hydroxylamine. It remains unclear how the enzyme in the 'as isolated' state might be activated *in vivo*, but we have speculated that if *P. pantotrophus* cytochrome cd_1 is synthesized in the active state in the cell it may never reach the catalytically incompetent state [9,11]. A consequential prediction is that there must be at least one mechanism by which reversion to the 'as isolated' state of the enzyme is prevented. This could in part be due to the enzyme being predominantly reduced during steady-state turnover. However, *P. pantotrophus* cytochrome cd_1 exhibits a very strong tendency to return to the 'as isolated' conformer when oxidized (see e.g. [12]). Nevertheless, in experiments in which fully reduced cytochrome cd_1 was reacted with its physiological substrate nitrite in the absence of excess reductant, the final product, although not characterized, was tentatively assigned to be all-ferric cytochrome cd_1 with nitrite bound to the d_1 haem [13].

The assumed reaction mechanism of cytochrome cd_1 involves nitrite binding to ferrous [Fe(II)] haem, where it can be reduced to nitric oxide by the haem iron. However, this hypothesis is not proven; anionic nitrite may bind to ferric d_1 haem before the d_1 haem is reduced by electron transfer from the *c* haem. This would be analogous to the mechanism proposed for the copper-containing nitrite reductases, which also catalyse formation of nitric oxide. For these enzymes, it has been suggested that nitrite binds to the oxidized type II Cu active site, increasing the reduction potential of the latter to allow reduction by an electron from a nearby type I Cu centre; this electron is then used to reduce nitrite to nitric oxide [14]. In general, anions have a much higher affinity for ferric, rather than ferrous haem. Thus a comparison of nitrite binding to ferrous and ferric d_1 haem is necessary. In the present study, we characterize and assess the importance of an all-ferric nitrite-bound form of *P. pantotrophus* cytochrome cd_1.

EXPERIMENTAL

P. pantotrophus cytochrome cd_1 was purified by the method of Moir et al. [15], from the periplasms of cells grown anaerobically with nitrate as terminal electron acceptor. *P. pantotrophus* pseudoazurin was purified as described previously [15], from the total soluble extract of *Escherichia coli* XL1-Blue transformed with the plasmid pJR2 [16]. The concentration of *P. pantotrophus*

[1] To whom correspondence should be addressed (e-mail stuart.ferguson@bioch.ox.ac.uk).

cytochrome cd_1 was determined from the absorbance of the oxidized protein at 406 nm ($\epsilon = 285000\ M^{-1} \cdot cm^{-1}$ [17]). The concentration of *P. pantotrophus* pseudoazurin was determined for the oxidized protein using $\epsilon = 1360\ M^{-1} \cdot cm^{-1}$ at 590 nm [15]; this value was also taken as the difference in the molar absorption coefficient (oxidized minus reduced). All enzyme and substrate solutions used in this study were made in 50 mM potassium phosphate buffer at pH 7.0, unless otherwise stated. All solutions were thoroughly sparged with argon before use. Potassium nitrite, sodium nitrite, hydroxylamine hydrochloride, disodium dithionite, sodium nitrate (SigmaUltra), sodium sulphate, sodium acetate, sodium thiocyanate, 4-chloropyridine, sodium formate, sodium azide and imidazole were purchased from Sigma. Potassium ferricyanide and potassium dichromate were purchased from Fisons plc (Loughborough, Leics., U.K.).

For experiments in which the excess reductant was removed from cytochrome cd_1 before reaction, procedures were conducted in an anaerobic cabinet (Belle Technology) (< 2 ppm O_2). Cytochrome cd_1 was reduced and the reductant removed as described by George et al. [13] and Allen et al. [8], using a desalting column containing P6-DG resin (Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K.). Catalytic activity of cytochrome cd_1 was assayed with pseudoazurin as electron donor, using procedures described previously [10]. EPR spectra were recorded on an X-band ER 200D spectrometer (Bruker Spectrospin) interfaced to an ESP 1600 computer and fitted with a liquid-helium flow-cryostat (ESR-9, Oxford Instruments, Oxford, U.K.).

To determine equilibrium-binding parameters, nitrite was added to aliquots of cytochrome cd_1 that had been reduced with dithionite and reoxidized with 10 mM hydroxylamine (nitrite was added to the appropriate concentration 10 s after the addition of hydroxylamine). The solutions were allowed to equilibrate for 1.5–2 h before analysis by absorption spectroscopy. Results were analysed at 434 and 640 nm, wavelengths characteristic of the d_1 haem [17]. Spectra corresponding to maximum and minimum binding of nitrite (representing the two end points of the titration) were obtained after addition of 250 and 0 mM nitrite respectively after reoxidation with hydroxylamine. Hence at each titration point it was possible to determine the concentrations of free and bound nitrite.

RESULTS

P. pantotrophus cytochrome cd_1 was reduced with disodium dithionite and fully reoxidized with the non-physiological electron acceptor hydroxylamine. This produces a form of the enzyme in which the *c* haem is ferric but His/Met co-ordinated and the d_1 haem is six co-ordinate low spin with an unidentified distal ligand [8]. On addition of potassium nitrite to this state of the enzyme, a species with novel EPR (Figure 1) and absorption (Figure 2) spectra was generated. Essentially, the same spectra were obtained if potassium ferricyanide was used as oxidant before the addition of nitrite. Absorption spectra of such mixtures did not change over 2 h following initial equilibration; thus the complex they represent is kinetically stable. These observations are in contrast with the case in which the enzyme was reoxidized by hydroxylamine without subsequent addition of nitrite, when reversion to the 'as isolated' conformation occurred with a rate constant of approx. 0.2 min^{-1} [8].

The X-band EPR spectrum of the *P. pantotrophus* cytochrome cd_1 species generated by the addition of nitrite to hydroxylamine-reoxidized enzyme had major signals at $g = 2.94$, 2.32 and 1.39 (Figure 1). It has been shown previously that this rhombic trio is characteristic of the histidine/methionine co-ordination of the *c* haem of this enzyme [9]. The spectra also contained minor

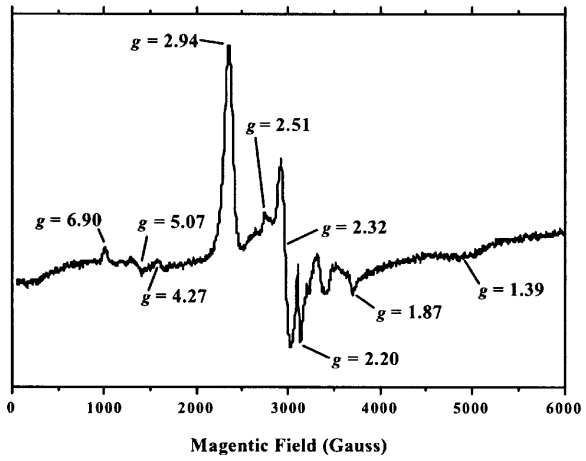

Figure 1 EPR spectrum of all-ferric *P. pantotrophus* cytochrome cd_1 with nitrite bound to the d_1 haem

Cytochrome cd_1 was reduced with dithionite and the excess dithionite was removed using a desalting column. The enzyme was reoxidized using 1 mM hydroxylamine, then 10 mM potassium nitrite was added 15 s after oxidation; the final cytochrome cd_1 concentration was approx. 100 μM. The mixture was incubated for 1 h to allow equilibration before the sample was quenched in liquid nitrogen. All reaction steps were performed in an anaerobic cabinet. The spectrum was recorded at 10 K with 2 mW microwave power, signal gain of 1.12×10^5, modulation amplitude of 10 G and microwave frequency of 9.658 GHz.

signals at $g = 2.51$, 2.20 and 1.87. These are characteristic of d_1 haem with His/Tyr co-ordination, as observed for the 'as isolated' conformer of the enzyme [7]. They arise from a small fraction of the enzyme that reverted to its 'as isolated' state before equilibrium between ferric enzyme and nitrite was reached. Notably, no other significant signals from the d_1 haem were observed in the EPR spectrum of the oxidized enzyme–nitrite complex (see the Discussion section). The spectra do, however, contain other minor features that require comment. The signal at $g = 4.27$ arises from adventitious bound iron. The signals at $g = 5.07$ and 6.90 arise from a trace of high-spin d_1 haem

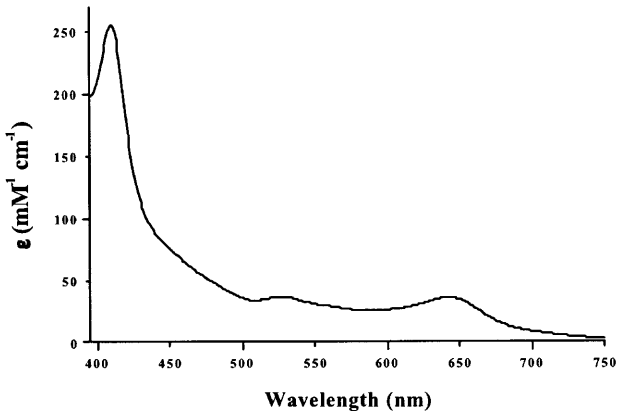

Figure 2 Absorption spectrum of all-ferric *P. pantotrophus* cytochrome cd_1 with nitrite bound to the d_1 haem

Cytochrome cd_1 in 50 mM potassium phosphate buffer (pH 7.0) at 25 °C was reduced with dithionite, the enzyme was reoxidized using 10 mM hydroxylamine, and 50 mM potassium nitrite was added 10 s after oxidation. The mixture was incubated for 2 h before the spectrum was recorded.

[7] and are always observed (in variable amounts) in spectra of the 'as isolated' form of *P. pantotrophus* cytochrome cd_1.

The absorption spectrum of the nitrite-bound cd_1 complex had prominent maxima at 410 and 643 nm (Figure 2). The former band, which arises from the *c* haem, is at a wavelength indicative of His/Met co-ordination in this enzyme [8,18]. Indeed, the spectrum of the complex formed in the presence of 50 mM nitrite can be used to estimate an $\epsilon = 255\ mM^{-1} \cdot cm^{-1}$ at 410 nm for the ferric *c* haem His/Met conformer of *P. pantotrophus* cytochrome cd_1, based on $\epsilon = 285\ mM^{-1} \cdot cm^{-1}$ at 406 nm [17] for the unreacted 'as isolated' (*c* haem His/His co-ordinated) conformer of the enzyme. The band at 643 nm is assigned to arise from nitrite bound to ferric d_1 haem. The low-spin absorption band from ferric His/Tyr co-ordinated d_1 haem in this enzyme is broad, but observed at approx. 644 nm [7]. However, complexes between the ferrous d_1 haem of *P. pantotrophus* cytochrome cd_1 and cyanide [12] or nitric oxide (J. W. A. Allen and S. J. George, unpublished work) produce absorption maxima at 628 and 634–636 nm (broad peak) respectively, considerably blue-shifted from the value we observe. Thus the position of the absorption peak assigned to d_1Fe(III)-NO_2^- in the present study suggests that the d_1 haem is ferric, despite the lack of EPR signals from that haem. This conclusion is reinforced by the observation that the absorption spectra do not change over 10 min in the region of the 643 nm absorption band on addition of the strong oxidants potassium ferricyanide or potassium dichromate, to final concentrations of 1 mM with 3 μM cytochrome cd_1–nitrite complex.

The all-ferric nitrite-bound complex of *P. pantotrophus* cytochrome cd_1 is fully catalytically active. Complex prepared by mixing nitrite with hydroxylamine-reoxidized enzyme was assayed using 250 μM *P. pantotrophus* pseudoazurin as electron donor and 10 mM nitrite ($\gg K_M$ [10]) as acceptor, at pH 7.0. The k_{cat} was measured to be $106\ s^{-1}$ for the enzyme dimer (results not shown). This compares with $5\ s^{-1}$ for the oxidized 'as isolated' form of the enzyme and $144\ s^{-1}$ for the initially fully reduced (and hence activated) enzyme, all assayed under similar conditions [10]. In the cd_1–nitrite complex assayed, an estimated 25 % of the enzyme had reverted to the inert 'as isolated' conformation, judged by the intensity of the 700 nm absorption band, which is characteristic of His/Tyr co-ordinated d_1 haem [7]. Thus for enzyme samples of which approx. 75 % were in the all-ferric nitrite-complexed state, we observed 73 % of the predicted catalytic activity [10] of 100 % activated enzyme. These results are consistent with previous studies in which it has been shown that when the *c* haem is His/Met co-ordinated, the enzyme is catalytically active [8,10].

Reduced cytochrome cd_1 has a high affinity for nitrite binding to the d_1 haem (approx. $K_d = 1\ \mu M$) [19]. For comparison, we assessed the dissociation constant for nitrite binding to ferric *P. pantotrophus* cytochrome cd_1. Dithionite-reduced enzyme was reoxidized with hydroxylamine. After 10 s (time to allow complete oxidation), nitrite was added. Samples were allowed to equilibrate and absorption spectra were recorded. At low nitrite concentrations, the enzyme had the spectrum of the 'as isolated' conformer; at high concentrations the spectrum described above (Figure 2) was observed. Using a binding titration (Figure 3A) and a Hill plot of the data (Figure 3B), an effective dissociation constant $K_d = 2 \pm 0.5$ mM was obtained for nitrite. An isosbestic point at 675 nm showed that the titration was a two-state system, i.e. the d_1 haem had His/Tyr or His/NO_2^- co-ordination only. The Hill coefficient was 0.94 ± 0.07 and a Scatchard plot of the data (results not shown) was slightly concave, indicating some slight negative cooperativity or inequivalence of binding sites within the enzyme dimer. This is consistent with the array of crystallographic data available for this enzyme, in which the two

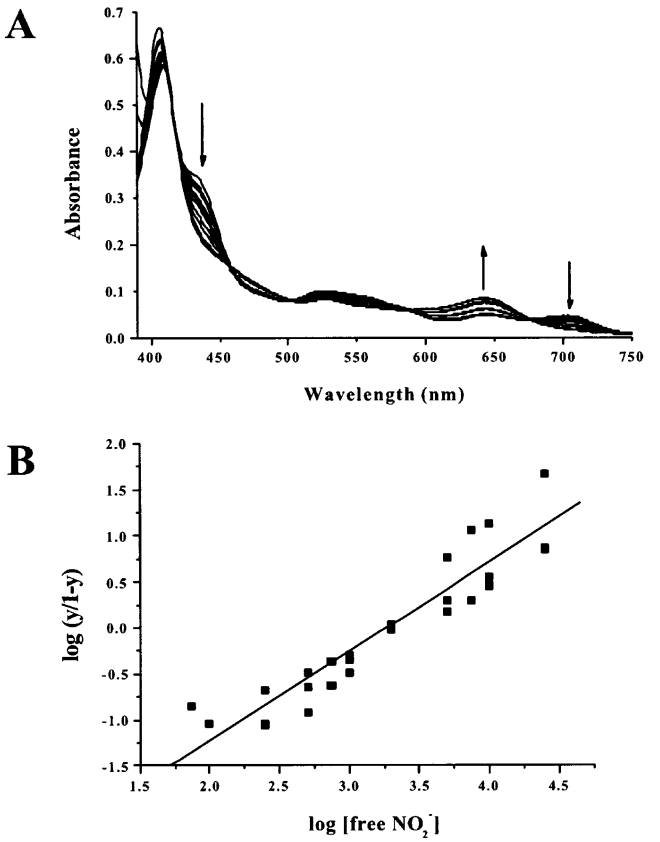

Figure 3 Nitrite binding curve and Hill plot for all-ferric *P. pantotrophus* cytochrome cd_1 with nitrite bound to the d_1 haem

Cytochrome cd_1 was reduced with dithionite; the enzyme was reoxidized using 10 mM hydroxylamine, and various concentrations of potassium nitrite were added 10 s after oxidation. The mixtures were incubated for 1.5–2 h before the spectra were recorded. All samples were in 50 mM potassium phosphate buffer (pH 7.0) at 25 °C. (**A**) Absorption spectra with various concentrations of nitrite. Arrows indicate the direction of change with increasing nitrite concentration. (**B**) Hill plot. Results were analysed using values obtained at 434 and 640 nm, wavelengths characteristic of the state of the d_1 haem [17]. Log[$y/(1-y)$] represents the fractional occupancy of nitrite bound to the d_1 haem. From the linear fit to this plot and others, the dissociation constant for nitrite from ferric cd_1 was 2 ± 0.5 mM and the Hill coefficient was 0.94 ± 0.07.

monomers within the dimer are always inequivalent; e.g. the structure of reduced cd_1 with cyanide binding shows differences between the monomers in the immediate ligand environment [12].

To understand the significance of the all-ferric, nitrite-bound cytochrome cd_1 complex, we determined whether the effect is specific to nitrite. For example, it is known that cyanide binds strongly to ferrous cd_1 but if the enzyme is reoxidized it substantially reverts to its 'as isolated' conformation and the cyanide is displaced [12]. In the presence of 25 mM acetate, sulphate, 4-chloropyridine, formate, imidazole and thiocyanate, reduced cd_1 reverted fully to the 'as isolated' conformation following hydroxylamine reoxidation (as was observed with hydroxylamine alone [8]). However, 25 mM azide, and to a much lesser extent 25 mM nitrate, were able to maintain a fraction ($\ll 100$ %) of the oxidized cd_1 in a conformation other than that of the 'as isolated' enzyme. Thus nitrite does not uniquely prevent reduced cytochrome cd_1 returning to the 'as isolated' conformation on oxidation, but it is the optimal ligand for that purpose.

One must also consider the interaction between the ferric 'as isolated' state of *P. pantotrophus* cytochrome cd_1 (*c* haem His/His; d_1 haem His/Tyr) and nitrite. In all the experiments described above, cd_1 interacted with nitrite when the enzyme was reduced-reoxidized, before reversion to the 'as isolated' state. We have recently reported that the 'as isolated' state of the enzyme is kinetically activated, slightly, by pre-exposure to nitrite, i.e. cd_1 exposed to nitrite before reductant initially turned over 4-fold faster than cd_1 exposed to reductant before nitrite (although the absolute rate was still low compared with enzyme activated by complete pre-reduction) [10]. Whether this implies that a small fraction (approx. 10 %) can reach the same activated all-ferric nitrite-bound state as described elsewhere in the present study is difficult to determine. Spectroscopic analyses of 'as isolated' enzyme plus nitrite are dominated by the features seen in the absence of nitrite. However, they allow us to conclude that there is a very significant kinetic barrier to nitrite displacing Tyr^{25} as a ligand to the d_1 haem.

DISCUSSION

Previous studies have shown that when reduced *P. pantotrophus* cytochrome cd_1 is reoxidized with hydroxylamine, an activated oxidized state of the enzyme forms but then reverts to the catalytically inert 'as isolated' conformation [8,9]. It is shown in the present study that in the presence of sufficient nitrite the enzyme will not return to the 'as isolated' conformation. Instead, an all-ferric state of the enzyme forms, in which nitrite is bound to the d_1 haem and the *c* haem maintains His/Met co-ordination. This cd_1 complex is catalytically active with the physiological electron donor protein, pseudoazurin, as would be predicted from the His/Met ligation of its *c* haem [8–10]. In the absence of an electron donor, the complex is kinetically very stable, and thus for the first time allows us to access an oxidized state of *P. pantotrophus* cytochrome cd_1 with His/Met co-ordination of its *c* haem that is not a transient species. The effective dissociation constant for nitrite from the ferric d_1 haem of cd_1 is approx. 2 mM. Therefore during conditions of growth on low nitrite concentrations, all-ferric cytochrome cd_1 may not form part of the catalytic cycle because the enzyme could revert to the catalytically inert 'as isolated' state. This view is fully consistent with the previously proposed reaction mechanism (e.g. [19,20]), in which nitrite binds to ferrous, rather than ferric, d_1 haem. The dissociation constant for nitrite binding to ferrous d_1 haem is estimated to be approx. 1 μM [19] and the binding reaction is kinetically facile [21]. Nonetheless, it is important to note that there is no formal proof that the normal catalytic cycle involves binding of nitrite to the reduced d_1 haem. Binding of nitrite to the oxidized d_1 haem followed by rapid reduction of the haem–nitrite complex (by intramolecular electron transfer from the *c* haem) would trap bound substrate at the active site, enabling reduction and dehydration of NO_2^- to NO.

It is not immediately clear why binding of nitrite to oxidized d_1 haem causes disappearance of the d_1 haem EPR spectrum (Figure 1). The most obvious explanation would be that the haem is in fact reduced ($s = 0$) and hence EPR silent, but several lines of evidence establish that this cannot be the case. (i) We generated the nitrite-bound complex by completely reoxidizing cytochrome cd_1 with an excess of hydroxylamine, an effective electron-accepting substrate [8,22], and then adding nitrite. Hydroxylamine ($E^{\circ\prime} = +900$ mV [23]) is a two-electron oxidant; thus in polypeptides, where the *c* haem is reoxidized, the d_1 haem is also reoxidized [8] in experiments in which nitrite is added subsequently. The *c* haem clearly is oxidized in our nitrite-bound complex, judging both from its strong EPR signals ($g = 2.94$, 2.33 and 1.40) and the Söret-band wavelength in the absorption spectrum (410 nm). (ii) The wavelength of the absorption band we assign to arise from the d_1 haem–NO_2^- complex (643 nm) is much more consistent with low-spin ferric–ligand complexes for *P. pantotrophus* cytochrome cd_1 (642–644 nm) [7,8] than with low-spin ferrous complexes {e.g. d_1Fe(II)–CN^- (628 nm) [12]; d_1Fe(II)–NO (broad peak, 634–636 nm); J. W. A. Allen and S. J. George, unpublished work}. (iii) On addition of large excesses of strong oxidants (ferricyanide or dichromate) to our putative ferric d_1 haem–nitrite complex, the absorption spectra did not change, implying that the haem was indeed ferric, unless the reduction potential of a putative d_1Fe(II)–ligand complex is extremely positive.

Ferric d_1 haem with nitric oxide bound is EPR silent (the $s = \frac{1}{2}$ iron couples with the unpaired electron on NO), but it is not easy to rationalize how this would be produced under the conditions we have used. Since we add nitrite to ferric (hydroxylamine- or ferricyanide-reoxidized) d_1 haem, an autoreduction would be required to produce d_1Fe(III)–NO (isoelectronic with, and often described as, d_1Fe(II)–NO^+ [13,24]). EPR excludes the presence of d_1Fe(II)–NO in our samples, and thus such an autoreduction would need to be a very specific one-electron process. Furthermore, there is spectroscopic evidence to suggest that our reaction conditions do not produce NO bound to ferric d_1 haem. For *Pseudomonas aeruginosa* cytochrome cd_1, the absorption spectrum of d_1Fe(II)–NO^+ has its maximum at 634 nm [25,26]; for *P. pantotrophus* cd_1 a peak was observed at 635 nm when oxidized 'as isolated' enzyme was flushed with gaseous NO [27]. For *P. stutzeri* cd_1, the analogous visible absorption peak was observed at 637 nm and the d_1Fe(II)–NO^+ complex was also characterized by Fourier-transform infrared spectroscopy [24]. Therefore the d_1 haem Fe(II)–NO^+ absorption band of cytochrome cd_1 is clearly blue-shifted compared with the 643 nm peak consistently observed for our putative *P. pantotrophus* cytochrome cd_1 d_1Fe(III)–NO_2^- complex.

There are, however, several precedents for the EPR phenomenon we have observed. Muhoberac and Wharton [28] mixed sodium nitrite with oxidized cytochrome cd_1 from *Ps. aeruginosa*; in this enzyme the *c* haem and d_1 haem co-ordinations in the oxidized (as isolated) enzyme are different from those in *P. pantotrophus* cd_1 [29,30]. In low-temperature EPR spectra they observed pronounced signals only from the *c* haem ($g = 2.95$, 2.30 and 1.42) but they did not characterize the enzyme–nitrite complex further. Walsh et al. [31] showed that the d_1 haem–azide complex of *Ps. aeruginosa* cytochrome cd_1, like the complex described in the present study, gave no prominent d_1 haem signals, but they proved, using magnetic CD, that the haem was low-spin ferric (e.g. not autoreduced). Day et al. [32] studied the nitrite-bound complex of the oxidized sirohaem-type nitrite reductase. Sirohaem and d_1 haem are both isobacteriochlorins (4-electron reduced compared with protoporphyrin IX); the two haems share the same unusual electronic ground state [7]. Similar to the d_1 haem-nitrite complex reported in this paper and the d_1 haem–azide complex reported by Walsh et al. [31], the sirohaem–nitrite complex of Day et al. [32] was EPR silent. The latter was shown to be low-spin ferric using magnetization experiments. A definitive explanation for these EPR-silent ferric isobacteriochlorins is outside the scope of the present study. However, we conclude that the lack of an EPR signal from the d_1 haem of our samples (Figure 1), although unusual, is not evidence against nitrite binding to ferric cytochrome cd_1. On the contrary, consistent with all the available spectroscopic data including at least three literature precedents, we argue that we have indeed formed a ferric His/NO_2^- co-ordinated d_1 haem. It

will be a major experimental and theoretical challenge in future studies to explain why this species is EPR silent. One possibility is extreme heterogeneity in g values (g strain) resulting in an EPR signal broadened beyond detection; this was the explanation proposed by Day et al. [32] for their observations with the sirohaem–nitrite complex (see above). Note, however, that in the sirohaem-containing nitrite reductase, the haem centre is coupled to a [4Fe–4S] cluster through a shared (bridging) cysteine thiolate ligand [33], which may mean that the reason for the EPR silence of the nitrite-bound ferric haem in that enzyme is different from that for the similar phenomenon reported here for cytochrome cd_1. Alternatively, the observed EPR silence of our complex may be due to, for example, relaxation effects in the EPR experiment.

When ferricyanide was used to reoxidize *P. pantotrophus* cytochrome cd_1 before addition of nitrite to form the all-ferric–NO_2^- complex, a higher proportion of the enzyme was observed to reach the 'as isolated' conformation than when hydroxylamine was used. This was apparent from the relative intensities of the 700 nm band in the absorption spectrum and the $g = 2.51$, 2.20 and 1.87 trio in the EPR spectrum, both characteristic of His/Tyr co-ordinated d_1 haem in this enzyme [7]. These results imply that reversion by the enzyme is quicker with ferricyanide as the oxidant and, therefore, that the rate of reversion is controlled by the ligand bound to haem d_1, rather than by the change from His/Met to His/His co-ordination at the c haem. Ammonia, or possibly hydroxylamine itself, will be bound to the d_1 haem following reoxidation of cd_1 by hydroxylamine [8]. In contrast, if the reduced, five-co-ordinate d_1 haem [5] is oxidized by ferricyanide there is no bound sixth ligand, at least in the initial stages of the reaction.

The results presented in this study show neatly that the d_1 haem, and its environment in cytochrome cd_1, is optimized to bind nitrite, relative to other anions. Only nitrite, and to a lesser extent azide, were able to significantly prevent reversion of *P. pantotrophus* cytochrome cd_1 to its 'as isolated' conformation when they were added to the enzyme immediately after hydroxylamine reoxidation. Sulphate, formate (a structural analogue of nitrite) and thiocyanate had essentially no effect; nitrate and cyanide [12] were able to maintain only a very small fraction of the enzyme in a conformation different from that of the 'as isolated' enzyme. The distal side of the d_1 haem binding pocket contains two highly conserved histidine residues (His^{345} and His^{388} in *P. pantotrophus* cd_1) [4]; in X-ray crystallography, these have been observed to hydrogen bond with one of the oxygen atoms of nitrite-bound d_1 haem, and hence to stabilize the interaction [5]; thus they seem to be a likely factor in providing specificity towards nitrite binding. Mutagenesis experiments on *Ps. aeruginosa* cytochrome cd_1 have demonstrated that one of the equivalent histidine residues (His^{369}) is essential for the stability of the ferrous d_1 haem–nitrite Michaelis complex [19]. There may also be an important contribution to nitrite-binding specificity from the specialized d_1 haem macrocycle, even when the haem iron is oxidized.

In summary, we have produced and characterized a novel state of *P. pantotrophus* cytochrome cd_1, in which the c haem of the fully oxidized enzyme is His/Met co-ordinated and the d_1 haem is His/NO_2^- co-ordinated. This complex is both kinetically stable and catalytically active and is thus distinct from the 'as isolated' state of the enzyme, which is catalytically almost inactive [10] and must be activated by a switch in the co-ordination of the haems [8]. The availability of the enzyme complex described in the present study will be valuable for further investigations of this complicated respiratory enzyme, for instance in rapid reaction studies where it is mixed with the reductant. Such experiments will provide further insight into the reaction mechanism of cytochrome cd_1, the details of which have proved elusive for more than 40 years.

This work was supported by a grant (B11988) from the U.K. Biotechnology and Biological Sciences Research Council (BBSRC) to S.J.F. and N.J.W. J.W.A.A. is the W. R. Miller Junior Research Fellow, St. Edmund Hall, Oxford. R.S.Z. is grateful to the BBRSC for a studentship. We thank Dr M. R. Cheesman for extensive helpful discussions and J. Thornton for technical assistance.

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Received 20 May 2002/24 June 2002; accepted 26 June 2002
Published as BJ Immediate Publication 26 June 2002, DOI 10.1042/BJ20020795