Cytochrome cd₁, Reductive Activation and Kinetic Analysis of a Multifunctional Respiratory Enzyme*

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Paracoccus pantotrophus cytochrome cd₁ is an enzyme of bacterial respiration, capable of using nitrite in vivo and also hydroxylamine and oxygen in vitro as electron acceptors. We present a comprehensive analysis of the steady state kinetic properties of the enzyme with each electron acceptor and three electron donors, pseudoazurin and cytochrome c₅₅₀, both physiological, and the non-physiological horse heart cytochrome c. At pH 5.8, optimal for nitrite reduction, the enzyme has a turnover number up to 121 s⁻¹ per d₁ heme, significantly higher than previously observed for any cytochrome cd₁. Pre-activation of the enzyme via reduction is necessary to establish full catalytic competence with any of the electron donor proteins. There is no significant kinetic distinction between the alternative physiological electron donors in any respect, providing support for the concept of pseudospecificity, in which proteins with substantially different tertiary structures can transfer electrons to the same acceptor. A low level hydroxylamine disproportionase activity that may be an intrinsic property of cytochromes c is also reported. Important implications for the enzymology of P. pantotrophus cytochrome cd₁ are discussed and proposals are made about the mechanism of reduction of nitrite, based on new observations placed in the context of recent rapid reaction studies.

Cytochrome cd₁ was first isolated from Pseudomonas aeruginosa as a soluble cytochrome oxidase, capable of catalyzing the four-electron reduction of O₂ to water (1). It was established quickly that this class of enzyme can also catalyze the one-electron reduction of nitrite (NO₂⁻) to nitric oxide (2). Later, Singh (3) demonstrated a third reaction, the two-electron reduction of hydroxylamine (NH₂OH) to ammonia. The principal physiological role of cytochromes cd₁ is now believed to be the reduction of nitrite. The enzyme is produced by bacteria that catalyze the respiratory process of denitification, the sequential reduction of nitrate via nitrite, nitric oxide, and nitrous oxide to dinitrogen gas, an essential component of the biogeochemical nitrogen cycle (4). However, there continue to be suggestions that the oxidase activity may have some physiological importance, for instance in the transition between anaerobiosis and aerobiosis (e.g. Ref. 5).

The cytochromes cd₁ from P. aeruginosa and Paracoccus pantotrophus (former species Paracoccus denitrificans GB17 and Thiosphaera pantotropha (6)) have now been characterized extensively. The enzymes are dimeric; each monomer contains one c-type cytochrome center, which accepts electrons from donor proteins, and one specialized d₁ heme that provides the catalytic site. *P. pantotrophus* cd₁ has recently been the subject of rapid reaction kinetic studies using each known substrate (7–9) and crystallographic studies on the reactions with nitrite and oxygen (5, 10). However, this array of data is not underpinned by extensive and reliable information on the steady state kinetic properties of the enzyme.

The descriptions of specific activity and analyses of steady state kinetics for cytochromes cd₁ are far from complete. Moreover, it is difficult to make comparisons between different publications because it is rare to find two studies that employed the same pH, temperature, and other conditions. Assays can be established using either proteins (e.g. azurin, cytochrome c) or small redox active molecules (e.g. ascorbate, tetramethylphenylenediamine, viologens, quinols) as an electron donor. Comparison of nitrite with the alternative substrates, oxygen or hydroxylamine, also requires that the same electron donor be used in each case. Finally, the vast majority of the available data has been recorded for *P. aeruginosa* cytochrome cd₁ (see Ref. 11 for discussion), which may not help in the analysis of the considerable body of pre-steady state data now available for the *P. pantotrophus* enzyme, because the enzymes from the two sources show considerable structural differences (12, 13).

Recently, the physiological electron donor proteins to *P. pantotrophus* cytochrome cd₁, pseudoazurin and cytochrome c₅₅₀, have, uniquely for a cd₁, been unambiguously identified (14). The concept of pseudospecificity has been invoked to explain how two such proteins with very different structures can transfer electrons to the same enzyme (15). This idea is based on the finding that both the donor proteins have a surface patch consisting of hydrophobic and positively charged residues, whereas the electron accepting cytochrome c domain of cd₁ has a complementary patch containing hydrophobic and negatively charged residues. It has been suggested (15) that only a transient and promiscuous interaction between the surface patches of cd₁ and the donor protein is required for electron transfer to occur. However, the relative quantitative properties of cytochrome c₅₅₀ and pseudoazurin in their steady state turnover

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1 The abbreviations used are: cd₁, cytochrome cd₁; MES, 2-[N-morpholino]ethanesulfonic acid; MOPS, 3-[N-morpholino]propanesulfonic acid.
interactions with \textit{P. pantotrophus} cytochrome \textit{cd} \textit{1} have not yet been systematically investigated. The non-physiological, but readily available, horse heart cytochrome \textit{c} can also serve as an electron donor \textit{in vitro}. Based on work with \textit{Pseudomonas stutzeri} cytochrome \textit{cd} \textit{1}, use of this cytochrome has been advocated as a general assay method in steady state kinetic studies (16). It is clear that, given three potential protein electron donors to \textit{P. pantotrophus} cytochrome \textit{cd} \textit{1} and three different electron acceptors, many kinetic constants can, in principle, be acquired. There is no reason to expect that, for example, a \( K_M \) value determined using nitrite as substrate will be independent of the electron donor used.

A further complication arises from a highly unusual property of \textit{P. pantotrophus} cytochrome \textit{cd} \textit{1}. In the oxidized, "as isolated," form of the enzyme, the \( c \) heme iron has his-bistidinyl coordination, whereas the \( d_1 \) heme has histidine and tyrosine as axial ligands (12, 17). Upon reduction, the coordination changes at both hemes, to His/Met at the \( c \)-type center and His/vacant (penta-coordinate) at the \( d_1 \) heme (10). The function of this ligand switching is unclear, but if the reduced protein is reoxidized using hydroxyamine or oxygen, reversion to the oxidized "as isolated" conformation occurs on a time scale of minutes (5, 9). We have recently demonstrated that the enzyme can exist in an oxidized \( c \)-heme His/Met coordinated form and that it is this conformer that accepts electrons from one physiological electron donor, pseudoazurin (9, 18).

In this paper, we present a comprehensive analysis of the steady state kinetic parameters of \textit{P. pantotrophus} cytochrome \textit{cd} \textit{1} using both its physiological electron donors and horse heart cytochrome \textit{c}, in combination with three alternate electron acceptors. In each case, catalysis is studied commencing with the enzyme chemically reduced (\textit{i.e.} initially \( c \) heme His/Met).

**EXPERIMENTAL PROCEDURES**

\textit{P. pantotrophus} cytochrome \textit{cd} \textit{1} was purified according to the method of Moir \textit{et al}. (19) from the periplasm of cells grown anaerobically with nitrate as terminal electron acceptor. Semiaphore cytochrome \textit{cd} \textit{1} (which contains the \( c \) heme but no \( d_1 \) heme) was prepared from holoprotein according to the method described by Kobayashi \textit{et al}. (20). \textit{P. pantotrophus} pseudoazurin was purified as described (19) from the total soluble extract of \textit{Escherichia coli} XL1-Blue transformed with the plasmid pJIR2 (21). Cells were grown aerobically on \( 2 \times \) TY medium supplemented with 2 mM CuSO\(_4\) and 100 \( \mu \)g ml\(^{-1}\) ampicillin, at 37 °C. Horse heart cytochrome \textit{c} was purchased from Sigma and used as supplied. \textit{P. denitrificans} cytochrome \textit{c} \textit{550} was overproduced in \textit{E. coli} strain 7123 using a similar method to that described by Gordon \textit{et al}. (22). Cells were co-transformed with plasmids pKPD1 containing the gene for cytochrome \textit{c} \textit{550} (23) and pEC86 containing the genes for the cytochrome \textit{c} maturation system (24). Cultures were grown aerobically on LB medium, supplemented with 100 \( \mu \)M ml\(^{-1}\) ampicillin and 35 \( \mu \)g ml\(^{-1}\) chloramphenicol, for at least 24 h.

Cytochrome \textit{c} \textit{550} was purified using fast protein liquid chromatography equipment (Amersham Biosciences) at 4 °C. Initially, the cells were resuspended in isotonic buffer, 100 mM Tris-HCl, 100 mM NaCl, pH 8.0; they were disrupted by the addition of 1 mg ml\(^{-1}\) polymixin B sulfate (Melford) and incubation for 20 min at 37 °C. A periplasmic fraction was recovered by centrifugation at 39,000 \( \times \) g for 30 min. The soluble material so obtained was diluted 3-fold, applied to a fast-flow DEAE-Sepharose anion exchange column pre-equilibrated in 50 mM Tris-HCl, pH 8.0, and eluted using a 0–300 mM NaCl gradient in the same buffer. Red fractions were recovered, diluted with the same buffer, and applied to a fast-flow Q-Sepharose column; the protein was eluted using a 0–500 mM NaCl gradient, also in 50 mM Tris-HCl, pH 8.0. Cytochrome-containing fractions were concentrated to a total volume of 2 ml, applied to a G-50 (Sigma) size exclusion column (2.6 × 90 cm), and eluted at 14 ml h\(^{-1}\). Those fractions judged pure by SDS-PAGE were retained. A yield of ~4 mg of pure cytochrome \textit{c} \textit{550} per liter of culture was obtained. Note that \textit{P. denitrificans} cytochrome \textit{c} \textit{550} is 85.9% identical to cytochrome \textit{c} \textit{550} from \textit{P. pantotrophus} (25), the species from which cytochrome \textit{cd} \textit{1} was isolated for this work; the major differences are at the functionally unimportant \( C \) terminus.

Activity of cytochrome \textit{cd} \textit{1} was assayed spectrophotometrically by monitoring the change in absorbance of electron donor proteins at wavelengths characteristic of their redox state. Oxidized pseudoazurin absorbs in the visible region with a maximum at 590 nm, whereas reduced pseudoazurin is colorless. The reduced \( c \)-type cytochromes both exhibit a sharp absorbance band centered on 550 nm, whereas the oxidized proteins have a broader spectrum with much weaker absorbance at that wavelength. Thus, changes in the intensity of these transitions are a direct probe of the oxidation state of the donor protein and hence the rate of change of that state as electrons are donated to cytochrome \textit{cd} \textit{1} during steady state turnover. The concentration of \textit{P. pantotrophus} pseudoazurin was determined for the oxidized protein using the extinction coefficient 1360 m\(^{-1}\) cm\(^{-1}\) at 590 nm (19); this value was also taken as the difference extinction coefficient (reduced minus oxidized). The concentrations of \textit{P. denitrificans} cytochrome \textit{c} \textit{550} and horse heart cytochrome \textit{c} were determined from the extinction coefficients 30 (26) and 29.5 m\(^{-1}\) cm\(^{-1}\) (16) at 550 nm for the reduced proteins, respectively. Difference extinction coefficients (reduced minus oxidized) of 21 (26) and 18.5 m\(^{-1}\) cm\(^{-1}\) (27) at 550 nm, respectively, were used in activity assays. The concentration of \textit{P. pantotrophus} cytochrome \textit{cd} \textit{1} was determined from the absorbance of the oxidized protein at 406 nm (\( e = 285 \) m\(^{-1}\) cm\(^{-1}\) (20)) or of the reduced protein at 418 nm (\( e = 323 \) m\(^{-1}\) cm\(^{-1}\) (7, 26)).

All assays were conducted at 25 °C in gas tight cuvettes with path lengths of 1 or 0.2 cm for pseudoazurin or cytochrome \textit{c} as electron donor, respectively. Unless otherwise stated, all experiments were conducted in 50 mM potassium phosphate buffer at pH 7.0. In experiments to examine the pH dependence of reactions, a mixed buffer system containing acetate, MOPS, MES, and Tris, each at 12 mM, was used. Reaction was initiated by addition via a Hamilton syringe of cytochrome \textit{cd} \textit{1} to a cuvette containing substrate and electron donor. Potassium nitrite and hydroxylamine hydrochloride solutions were made from the chemicals as supplied by Sigma. Oxygen solutions were prepared by flushing buffer with pure oxygen (British Oxygen Company) for 30 min to produce a saturated solution (1.2 mM (28)), which was then diluted with argon-sparged buffer to give the desired oxygen concentration. All solutions (except those containing oxygen as assay substrate) were thoroughly deoxygenated by extended sparging with humidified argon. In addition, in assays where oxygen was not substrate, 0.6% (w/v) \( \beta \)-p-glucose, 0.5 units ml\(^{-1}\) glucose oxidase (Sigma) and 200 units ml\(^{-1}\) catalase (Sigma) could be added to the system to scrub any remaining traces of oxygen. Unless stated, pseudoazurin, cytochrome \textit{c}, and cytochrome \textit{c} \textit{550} were used at a concentration of 200 \( \mu \)M; cytochrome \textit{cd} \textit{1} was used at convenient concentrations in the range 15–300 nm.

Where oxidized cytochrome \textit{cd} \textit{1} was assayed, it was used as purified. Stock solutions of reduced \textit{cd} \textit{1} were prepared by care-
ful addition of a 1 mL 0.1 mol/L dithionite solution to thoroughly deoxygenized protein solution. 10–70 mL of this reduced enzyme was added to a 0.4–0.8 mL assay solution, such that essentially no excess dithionite would be present; the lack of dithionite was confirmed by the absence of an apparent initial lag phase in the reoxidation of the electron donor and by monitoring the dithionite absorption peak at 315 nm. Throughout the period during which measurements were taken, the stock solution of cd1 was repeatedly examined spectrophotometrically to confirm that it remained fully reduced. Solutions of reduced electron donor proteins were prepared by addition of excess ascorbate. The mixtures were then passed down a PD10 desalting column (Amersham Biosciences), which had been pre-equilibrated with deoxygenated buffer. The first 80% of the protein to be eluted was retained for assays and was stored on ice in a degassed vessel until required. Reduced pseudoazurin was identified by its absorbance at 280 nm.

Kinetic data from steady state assays were analyzed using the curve fitting program TableCurve 2D (Jandel Scientific). In all cases, the initial rate of reaction was considered. (When electron donor proteins become reoxidized, the oxidized material may, in principle, be inhibitory of reaction between cytochrome cd1 and the remaining reduced electron donor. Following reduction of nitrite in vitro, accumulation of product nitric oxide is inhibitory (29, 30).) The experimental data recorded during the first 30 s of reaction were fitted to a single exponential function from which the initial rate was calculated. These values were then subjected to Hanes analysis (plots fitted using MS Excel) to produce kinetic constants.

RESULTS AND DISCUSSION

Kinetic Parameters for Three Electron Acceptors—Steady state kinetic parameters were determined for each of the electron acceptors, nitrite (the physiological substrate), oxygen, and hydroxylamine, for P. pantotrophus cytochrome cd1 using both of its physiological electron donors, pseudoazurin and cytochrome c550, as well as horse heart cytochrome c (Tables I and II). The concentration of electron donor was kept constant, and the electron acceptor concentration was varied in these assays. The numerical values are from Hanes analyses of the experimental data. Reaction rates were monitored by recording the change as a function of time in the intensity of absorbance bands of the electron donor proteins. The data in Table I were obtained when the cytochrome cd1 was introduced to the reaction mixture in its oxidized form as purified ("as isolated"), with bis-histidyl coordination of the c heme (12, 17). The data in Table II were obtained when the cytochrome cd1 was initially fully reduced (hence with His/Met coordination at the c heme) (10, 31). Fig. 1 shows examples of the Hanes plots used to generate the parameters in Tables I and II.

Activation of P. pantotrophus Cytochrome cd1—Our data confirm the need for activation of P. pantotrophus cytochrome cd1 before it can efficiently perform catalysis. The oxidized enzyme (as isolated) has a very low level of catalytic activity (compare Table I with Table II). A similar phenomenon was observed previously with pseudoazurin as electron donor and either nitrite or hydroxylamine as acceptor (9). The present data shows the same to be true irrespective of the electron donor used with any of the electron acceptors. Furthermore, when oxidized "as isolated" cytochrome cd1 was mixed with either reduced pseudoazurin (9) or cytochrome c550 (data not shown) in a stopped-flow apparatus, in the absence of an electron acceptor, no electron transfer reaction was observed. The oxidized His/His coordinated c heme of "as isolated" P. pantotrophus cytochrome cd1 is thus far from fully competent to accept electrons from its physiological protein electron donors. Its coordination must be switched to His/Met, in which case catalytic competence is established (see also Refs. 9 and 18). Given that in vivo experiments show that pseudoazurin and cytochrome c550 are the only electron donors that allow viable cell growth by Paracoccus on nitrite (14), there is now compelling evidence that cytochrome cd1 must either be synthesized in its active conformation in the cell or that there is an as yet unidentified activation factor in the bacterial periplasm.

Current electron transfer theories suggest that thermodynamically unfavorable steps in electron transport chains are permissible as long as the overall chain is favorable (32). Due to the very unusual redox properties of P. pantotrophus cytochrome cd1, the reduction potentials of the individual heme centers cannot be determined precisely (33), but the apparent midpoint potential of the His/His coordinated c heme of cd1 is +60 mV or lower at room temperature. Nevertheless, it might be expected that electron donor proteins such as pseudoazurin (E0’ = 230 mV) would transfer electrons to the His/His c heme of cytochrome cd1, even though the reduction potential of the latter is lower than that of the former, because subsequent electron transfer from the c heme to the d1 heme and then to an electron accepting substrate means that the overall electron transfer is thermodynamically favorable (the standard reduction potential for NO2−/NO is +375 mV). A good example of this type of behavior is the heme chain of Rhodopsseudomonas viridis, where the cytochrome c2 (E0’ approximately +280 mV) reduces a heme of the R. viridis reaction center (E0’ approximately −50 mV); the potential of the terminal electron-accepting center is approximately +500 mV so the intermediate endergonic step can occur (32). The fact that the tyrosine ligand to d1 heme in the "as isolated" form of P. pantotrophus cytochrome cd1 blocks nitrite binding to the heme iron may be responsible for our failure to observe reduction of that enzyme conformation (and hence full catalytic activity) in the presence of reduced electron donor proteins. If the midpoint reduction potential of His/Tyr-coordinated d1 heme is below +230 mV, there will be no thermodynamic driving force...
for electron transfer from pseudooxazurin. His/His coordination of the c heme is very closely coupled to His/Tyr coordination of the d1 heme (9, 33, 34). Therefore, a cooperative switch in the coordination of both hemes of the “as isolated” enzyme, by which the c heme becomes His/Met-ligated but more significantly the d1 heme becomes accessible to nitrite, may be what is required to allow electron donation from partner proteins and hence establish catalytic competence. In other words, it might be that the observation that the c heme is His/Met-coordinated in catalytically active conformers of P. pantotrophus cytochrome cd1 (9) is important only because the d1 heme cannot be ligated by residue Tyr-25 in those conformers. This matter warrants further investigation to reconcile the experimental observations with theory.

It is noteworthy that in all cases our assays involving “as isolated” cd1 did show an activity ~5% of that when chemically pre-reduced enzyme was assayed (Table I cf. Table II). It is possible that this fraction of the enzyme is in a different conformation from the remainder. This would not have been detected by x-ray crystallography, the technique used to determine the structure of cytochrome cd1 (12), but in a solution study of the enzyme using magnetic circular dichroism spectroscopy, Cheesman et al. (17) noted “A weak feature in the (P. pantotrophus cd1) near-infrared magnetic circular dichroism spectrum at 1900 nm suggests that a small population of heme c has methionine coordination.” Oxidized enzyme with c heme His/Met coordination is fully catalytically active (9); it will not have His/Tyr coordination of the d1 heme (see above). However, if there is an equilibrium between an oxidized c heme His/His conformer of the enzyme and an oxidized c heme His/Met conformer, then when the latter becomes reduced by an electron donor the equilibrium will tend to produce more of the oxidized His/Met species from the His/His. If this happens, the catalytic activity of the assay sample should progressively increase because the His/Met state is the catalytically competent form. We did not observe this increase in rate with time, although it is very possible that the rate of formation of the oxidized c heme His/Met enzyme conformer from the oxidized His/His state is slower than the time scale of our assays (cf. Refs. 9 and 33). A further consideration is that if the 5% activity is due to an initially oxidized population of the enzyme with His/Met coordination of the c heme that is otherwise identical to the pre-reduced enzyme (100% activity), one might expect the observed KM for a given reaction to be the same when either initially oxidized or pre-reduced cd1 is assayed. KM is an intrinsic property of an enzyme and should not depend on the concentration of active cd1 present. However, the values of KM obtained for reduction of nitrite using pseudooxazurin as electron donor did differ (initially oxidized, 19 μM; initially pre-reduced, 71 μM), implying that the reaction was not the same in the two cases, although the KM values obtained using the two cytochromes c were similar in each assay (Tables I and II).

### Table II

| Nitrite  | Oxygen | Hydroxylamine |
|---------|--------|---------------|
| KM      | kcat  | kc          |
| a       | s⁻¹   | s⁻¹         |
| b       | M⁻¹   | s⁻¹         |
| Pseudooxazurin | 71 (6.6) | 144 (13.6) | 2.0 |
| Cytochrome cd1 | 12 (2.4) | 74 (12.5) | 6.2 |
| Horse heart cytochrome c | 11 (1.4) | 41 (4.9) | 3.7 |

**Notes:**
- Table II includes kinetic parameters for electron acceptors with pre-reduced cytochrome cd1.
- Data are an average of multiple experiments; S.D. values are shown in parentheses.

**References:** 9, 33, 34.

**Fig. 1:** Examples of Hanes plots used to determine steady state kinetic parameters for reductive reactions catalyzed by P. pantotrophus cytochrome cd1. These reactions were conducted at pH 7.0 and 25°C in 50 mM potassium phosphate buffer. The vertical axis shows substrate concentration divided by the rate of change of absorbance of the electron donor protein at the wavelength used to monitor its oxidation state (see text). A, 200 μM horse heart cytochrome c as electron donor in the presence of 32 nM pre-reduced cytochrome cd1, and various concentrations of oxygen; KM = 164 μM and kcat = 3.2 s⁻¹. B, 200 μM P. pantotrophus pseudooxazurin, 19 nM pre-reduced cytochrome cd1, and various concentrations of nitrite; KM = 66 μM and kcat = 162 s⁻¹. C, 200 μM P. denitrificans cytochrome cd1, 24 nM initially oxidized (as isolated) cytochrome cd1, and various concentrations of nitrite; KM = 7.2 μM and kcat = 1.8 s⁻¹. These numerical values were obtained from linear fits to the respective data sets.
Oxidized, “as isolated” *P. pantotrophus* cytochrome cd$_1$ can also be kinetically “activated” by pre-exposure to nitrite. The results in Table I report assays where reaction was initiated by the addition of cd$_1$ to a mixture of reduced electron donor and substrate. However, if the cd$_1$ was mixed with nitrite for 5 min before being mixed with electron donor, the initial rate increased by a factor of 4.5 where cytochrome cd$_{550}$ was reductant. Only the initial rate was affected (i.e., the sample of cd$_1$ not pre-exposed to nitrite “caught up” with the pre-exposed enzyme). Absorption spectra show that when “as isolated” cd$_1$ is mixed with nitrite, there are subtle changes in those bands that can be assigned to the d$_1$ heme (data not shown), but these changes are not consistent with changes in heme ligation in a large portion of the enzyme sample. These observations may represent an ability of nitrite to loosely coordinate in the d$_1$ heme pocket without being bound to the heme itself. They may also reflect nitrite binding to the d$_1$ heme in the state of the enzyme discussed above that is responsible for 5% of the maximum activity without pre-activation.

**Analysis of the Steady State Kinetic Data**—It is difficult to compare directly steady state kinetic data for cytochromes cd$_1$ from different publications because of the wide range of electron donors and reaction conditions that have been used. However, the pre-reduced form of the enzyme from *P. pantotrophus* appears to be appreciably more catalytically effective when using its physiological substrate (Table II) than has been observed for this or any other cytochrome cd$_1$ to date. With pseudoazurin as electron donor and nitrite as acceptor, the dimeric turnover number at pH 7.0 (i.e., for two active sites per dimer) was 144 s$^{-1}$ and with cd$_{550}$ it was 74 s$^{-1}$ (Table II). Typical values for dimeric *P. aeruginosa* cytochrome cd$_1$ are of the order of 8 s$^{-1}$ (e.g., Refs. 29 and 35). Reduction of oxygen by *P. aeruginosa* cd$_1$, with proteinaceous electron donors occurs at 0.6–6.4 s$^{-1}$ (reviewed in Ref. 36), highly comparable with our values for the *P. pantotrophus* enzyme of 3–6 s$^{-1}$ (Table II). A more extensive review of previous steady state analyses for cytochromes cd$_1$ can be found in Ref. 11.

The $K_M$ values reported here for reduction of nitrite where cytochrome cd$_{550}$ was electron donor (−12 μM) are generally consistent with other work. For *P. aeruginosa* cd$_1$, Cutruzzola *et al.* (29) quote a value of 6 μM in combination with azurin; for (initially oxidized) *P. denitrificans* cd$_1$, Timkovitch *et al.* (37) reported 6 μM with cytochrome cd$_{550}$ as electron donor. However, we did observe the somewhat higher value of 71 μM in our assays using pseudoazurin (Table II). The values of $K_M$ we observed for oxygen (−160 μM with each electron donor) are significantly higher than values reported for *P. aeruginosa* cd$_1$: 2 μM (29) or 1.6–49 μM (36). Using *P. denitrificans* cd$_1$, Timkovitch *et al.* (37) reported a $K_M$ for oxygen of $80$ μM with cytochrome cd$_{550}$ as electron donor. Little data exist on reduction of hydroxylamine by cytochromes cd$_1$; however, our values for *P. pantotrophus* cd$_1$ (Table II) of $K_M$ (3.0–6.4 s$^{-1}$) and $K_M$ (0.4–2.5 mm) compare reasonably with those of Singh (3) for *P. aeruginosa* cd$_1$ (0.7 s$^{-1}$ (cytochrome cd$_{551}$ as electron donor) and 0.6 mm, respectively.

We have derived specificity constants ($k_i$), defined as $k_i/\bar{K}_M$, from our steady state kinetic data (Tables I and II). For a multifunctional enzyme such as cytochrome cd$_1$, this value gives a measure of the preference for one substrate over another; the higher the value of $k_i$, the more preferred a substrate is, so $k_i$(substrate)#$k_i$(substrate) is a direct indication of the specificity of enzyme toward substrate x relative to substrate y. Consideration of the specificity constants for the activated enzyme (Table II) shows that nitrite is very clearly the preferred substrate for *P. pantotrophus* cytochrome cd$_1$. When pseudoazurin was electron donor, $k_i$(nitrite)/$k_i$(oxygen) = 50 and $k_i$(ni-

| Pseudoazurin | Cytochrome cd$_{550}$ |
|--------------|-----------------------|
| **Optimum pH** | **Max $k_{cat}$** | **Optimum pH** | **Max $k_{cat}$** |
| Nitrite      | 5.8 242  | 5.7 135 |
| Oxygen       | 6.3 182  | 6.2 6  |
| Hydroxylamine| 5.1–5.4  | 14      |

Pseudoazurin cytochrome cd$_{550}$ is 30% $k_i$(nitrite)/$k_i$(hydroxylamine) = 670. For cytochrome cd$_{550}$, $k_i$(nitrite)/$k_i$(oxygen) = 310 and $k_i$(nitrite)/$k_i$(hydroxylamine) = 1240.

**Comparison of the Alternative Electron Donors to cd$_1$**—We have studied the pH dependence of the reactions catalyzed by pre-reduced *P. pantotrophus* cytochrome cd$_1$ (Table III; Fig. 2) using saturating concentrations of each electron acceptor (c/f the $K_M$ values in Table II) in combination with pseudoazurin and cytochrome cd$_{550}$. The data are plotted relative to the activity at pH 7.0 (Fig. 2). Each reaction showed significant pH dependence, but the results using cytochrome cd$_{550}$ and pseudoazurin were essentially identical, implying that there is no functional difference in their mode of interaction with cytochrome cd$_1$. For most of the reactions, the maximum rates were approximately constant in a plateau region of −0.5 pH units around the optimum (Fig. 2). The maximum value of $k_{cat}$ was observed for reduction of nitrite by the enzyme dimer (422 s$^{-1}$ at pH 5.8 with pseudoazurin as electron donor) sets a lower limit of 121 s$^{-1}$ on both the intramonomeric c to d$_1$ heme and intermolecular pseudoazurin to cd$_1$ electron transfer rates during steady state turnover, because only the c heme can accept electrons from donor proteins (15, 30, 35).

In addition to determining kinetic parameters for the various electron acceptors of *P. pantotrophus* cytochrome cd$_1$, we have used the same methodology to assess Michaelis constants for the electron donors. Nitrite was selected as substrate, at a concentration of 1 mM. For cytochrome cd$_{550}$ we observed a $K_M$ of 13 μM with “as isolated” cytochrome cd$_1$, and 40 μM when pre-reduced cytochrome cd$_1$ was assayed. For horse heart cytochrome c, the values were 74 and 83 μM, respectively. Thus the data presented in Tables I and II were obtained using electron donor concentrations significantly above $K_M$. We have not determined a $K_M$ for pseudoazurin, as our methodology would make accurate measurement difficult. The maximum reduced minus oxidized difference extinction coefficient for pseudoazurin is 1.36 mm$^{-1}$ cm$^{-1}$ (19); thus complete reoxidation of a 10 μM solution in a 1-cm path length cuvette results in a maximum absorbance change of only 0.013 absorbance units. However, we consider the initial rate, the useful change will be even smaller. Therefore, we cannot report a value for $K_M$ with significant confidence. Using a method based on chemical assays of nitrite consumption, Koutny *et al.* (38) reported a $K_M$ of 9.7 μM for pseudoazurin as reductant for *P. denitrificans* cytochrome cd$_1$. Their value is comparable with the value we report for cytochrome cd$_{550}$ with *P. pantotrophus* cd$_1$.

No substantial difference in the kinetic efficacy of cyto-

$^2$Thus, in an assay of mixed substrates, nitrite and oxygen would be reduced at equal rates if the concentration of oxygen exceeded that of nitrite by a factor of 50.

$^4$*P. denitrificans* cytochrome cd$_1$ is 97% identical to the enzyme from *P. pantotrophus* and is considered to be identical in structure and functionality (47).
of Martinkus et al. (39) who reported that P. denitrificans “azurin” was not an effective electron donor to cytochrome cd1. In their assays, they detected a very low level oxidase activity for cytochrome cd1 with “azurin” as donor (1.3 μM “azurin” oxidized (μM cd1)$^{-1}$ s$^{-1}$, at pH 6.0). The blue copper protein they purified and assayed was later reclassified as pseudoazurin (40), as used in the present study. The assay described by Martinkus et al. (39) was conducted by reducing the pseudoazurin with a small excess of dithionite, shaking it in air to remove the excess reductant and then mixing it with cytochrome cd1. Thus, the cd1 would have been in the inert “as isolated” conformation described in the present work (Table I cf. Table II) and hence apparently inactive. Indeed, using an analogous assay we were unable to determine kinetic parameters for cd1, reducing oxygen with pseudoazurin as electron donor (Table I). However, as we have shown (Table II), if the cd1 is activated, e.g. by pre-reduction, pseudoazurin is an effective electron donor.

Cytochromes c Show a Low Level Hydroxylamine Disproportionation Activity—Hydroxylamine (NH$_2$OH) is a substrate that can be reduced to ammonia and water by cytochrome cd1 (Ref. 3; Table II). In addition to NH$_2$OH, cytochrome cd1 will also catalyze reduction of substituted hydroxylamines, including both N-methylhydroxylamine (CH$_3$NH$_2$OH) and O-methyl hydroxylamine (NH$_2$OCH$_3$), with comparable rates (data not shown). Furthermore, both horse heart cytochrome c and P. denitrificans cd550 are themselves capable of catalyzing the reduction of hydroxylamine (judged by the fact that the cytochromes became reoxidized even in the absence of cytochrome cd1). For horse heart cytochrome c, the $k_{cat}$ for this activity was $5 \times 10^{-4}$ s$^{-1}$, with a $K_M$ of 40 mM. Additionally, hydroxylamine will directly reduce oxidized horse heart cytochrome c or P. denitrificans cytochrome cd550. For the former, the $K_M$ was 55 mM and $k_{cat}$ was $7.5 \times 10^{-4}$ s$^{-1}$. Thus, the c-type cytochromes we used for assays can inefficiently catalyze the disproportionation of hydroxylamine. This low level activity may be an intrinsic property of some c-type cytochromes, analogous to the peroxidase activity of heme (41).

Given this previously unreported ability of c-type cytochromes to reduce hydroxylamine, we became concerned that the values of $k_{cat}$ given for cytochrome cd1 in Tables I and II reflect catalysis with this substrate taking place only, or substantially, at the c heme center of the enzyme, rather than at the d$_1$ heme. To test this hypothesis, we prepared a sample of semi-apo cytochrome cd1, enzyme from which the d$_1$ heme had been chemically extracted and that contained only the c heme. Reduced semi-apoenzyme was assayed with 200 μM horse heart cytochrome c and 200 mM hydroxylamine, using identical methodology to assays of the holoenzyme (Table II). On addition of the semi-apoenzyme to the reaction mixture (to a final concentration of 20 nM, an enzyme concentration similar to those used in assays described in Table II), no measurable increase in the rate of reaction was observed relative to the background reaction between hydroxylamine and horse heart cytochrome c. Thus, in common with the oxidase and nitrite reductase activities (5, 10, 42, 43), the active site for the hydroxylamine reductase activity of cytochrome cd1 is the d$_1$ heme.

Implications for the Physiology and Reaction Mechanism of Cytochrome cd1—A notable feature of our data is that it illustrates further differences between the cytochromes cd1 from P. pantotrophus and P. aeruginosa. It was already known that the axial ligation of the hemes in the two enzymes “as isolated” differs (12, 13) and that their apparent mechanisms of reduction of nitrite (8, 29, 30) and rates of intramonomeric electron transfer (8, 20, 29, 30, 44) are far from the same. We now report differences in steady state kinetic properties, for instance a $k_{cat}$
of ~90 s⁻¹ monomer⁻¹ for reduction of nitrite by the activated *P. pantotrophus* enzyme at pH 6.2 (Fig. 2) compared with ~8 s⁻¹ (29) for the *P. aeruginosa* protein at the same pH. Similarly, we observed a $K_m$ for oxygen of 160 μM for *P. pantotrophus* cd₁ in marked contrast to the values as low as 2 μM reported for *P. aeruginosa* cd₁ (29, 36). Some of these variances might arise from differences in experimental conditions, but the trend seems to suggest true differences in the functionality of the enzymes, such that there may be two distinct classes of cytochrome cd₁-type nitrite reductase. This could represent evolutionary refinement or different needs of the organisms. There may be an as yet unrecognized need for “activation” of the *P. aeruginosa* enzyme as for *P. pantotrophus* cd₁ (9; this work) that would eliminate some of the differences.

The steady state kinetic characterization of *P. pantotrophus* cytochrome cd₁ in this paper provides further insight into the mechanism of reduction of nitrite by the enzyme. We recently reported (8) a pre-steady state investigation of this reaction. Fully reduced enzyme in the absence of excess reductant was mixed with nitrite in stopped-flow apparatus at pH 7.0 and 25 °C. After 5 ms, a cFe(III) dFe(II)-NO species was detected, formed with a rate constant of at least 500 s⁻¹. Because the d₁ heme is the site of nitrite binding and reduction, then by inference a cFe(II) dFe(III)-NO intermediate must have formed transiently during this time. Between 5 and 100 ms after reaction, there was electrode distribution within the enzyme to form an approximately equimolar mixture of cFe(II) dFe(III)-NO and cFe(III) dFe(II)-NO (8). Release of nitrite oxide from the enzyme was not detected on a catalytically relevant time scale, being observed to occur over hours.

The present work reveals that at pH 7.0, *P. pantotrophus* cytochrome cd₁ can reduce nitrite during steady state turnover at a rate of at least 72 s⁻¹ monomer⁻¹ (Table II); thus the catalytic cycle must be completed in (at most) 14 ms. This suggests that the species formed between 5 and 100 ms after reaction, observed by stopped-flow methods, may not be a relevant catalytic intermediate or at least may represent a “catalytically relaxed” state. Moreover, it implies that the species detectable after 5 ms, cFe(III) dFe(II)-NO, is likely to be significant. The principal difference between the experiments reported by George et al. (8) and those in this paper is the presence of excess equivalents of electron donor protein in the present work. Thus, it may be inferred that interaction between the cd₁ cFe(III) dFe(II)-NO complex and a further molecule of reducing protein facilitates release of product NO between 5 and 14 ms into the catalytic cycle. If such interaction both caused NO dissociation and further reduction of the cd₁, then all-ferrous cd₁ with penta-coordinate d₁ heme, the initial species of the catalytic cycle, would be regenerated. This concurs with the comments of Sjogren and Hajdu (31) based on the observations of George et al. (8) and crystallographic studies, “it is possible that the enzyme requires a constant supply of both electrons and substrate for reduction of nitrite to occur at physiological rates, possibly using a metastable “active form” which is dominantly present during steady state turnover.” Their results, and ours, are consistent with the idea that electron donor, enzyme, and electron acceptor must all be present for facile detachment of product NO.

The mechanism of NO dissociation itself is not clear, although given that cd₁ is a highly conformationally flexible molecule (e.g. Ref. 31), some important structural change on electron donor-acceptor complexation might be involved. In the two available crystal structures of reduced *P. pantotrophus* cytochrome cd₁, the position of the cytochrome c binding domain (which is also the site of interaction with partner electron donor proteins) differs by 20 Å, and the angle of rotation in one structure relative to the other differs by 60° (10, 31). Thus there exists at least the possibility of mechanistically significant domain movement in the enzyme. Sjogren and Hajdu (31) also highlight the little considered distal side of the d₁ heme, where the highly conserved loop from His-200 (the distal ligand to the heme iron) to Tyr-197 undergoes substantial movement during redox cycling. This could affect pathways for protons and products, as well as influencing other conformational changes important for catalysis. Additional or alternate factors may include monomer-monomer interaction within the enzyme dimer (the enzyme is always a non-crystallographic dimer, which hints at cooperativity) (5, 10, 45) and changes in protonation state of the conserved histidine residues on the distal side of the d₁ heme pocket (29, 46). However, it must be reiterated (8) that no rapid reaction study has yet detected catalytically competent NO release from cytochrome cd₁, and thus dissociation of product NO is not simply spontaneous but requires some specific factor that has not yet been tested experimentally in stopped-flow conditions.

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Cytochrome cd$_1$, Reductive Activation and Kinetic Analysis of a Multifunctional Respiratory Enzyme

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