Pseudoazurin Dramatically Enhances the Reaction Profile of Nitrite Reduction by Paracoccus pantotrophus Cytochrome cd₁ and Facilitates Release of Product Nitric Oxide*

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Cytochrome cd₁ is a respiratory nitrite reductase found in the periplasm of denitrifying bacteria. When fully reduced Paracoccus pantotrophus cytochrome cd₁ is mixed with nitrite in a stopped-flow apparatus in the absence of excess reductant, a kinetically stable complex of enzyme and product forms, assigned as a mixture of cFe(II) d₁Fe(II)-NO⁷ and cFe(III) d₁Fe(II)-NO (cd₁-X). However, in order for the enzyme to achieve steady-state turnover, product (NO) release must occur. In this work, we have investigated the effect of a physiological electron donor to cytochrome cd₁, the copper protein pseudoazurin, on the mechanism of nitrite reduction by the enzyme. Our data clearly show that initially oxidized pseudoazurin causes rapid further turnover by the enzyme to give a final product that we assign as all-ferric cytochrome cd₁ with nitrite bound to the d₁ heme (i.e. from which NO had dissociated). Pseudoazurin catalyzed this effect even when present at only one-tenth the stoichiometry of cytochrome cd₁. In contrast, redox-inert zinc pseudoazurin did not affect cd₁-X, indicating a crucial role for electron movement between monomers or individual enzyme dimers rather than simply a protein-protein interaction. Furthermore, formation of cd₁-X was, remarkably, accelerated by the presence of pseudoazurin, such that it occurred at a rate consistent with cd₁-X being an intermediate in the catalytic cycle. It is clear that cytochrome cd₁ functions significantly differently in the presence of its two substrates, nitrite and electron donor protein, than in the presence of nitrite alone.

Cytochrome cd₁ is a nitrite reductase found in the periplasm of many denitrifying bacteria, including Paracoccus pantotrophus and Pseudomonas aeruginosa (1, 2). The enzyme is a homodimer; each monomer contains one c-type heme center and one d₁ heme cofactor (3). The c heme is located in the N-terminal region of the enzyme, which is predominantly α-helical (with a fold similar to that of mitochondrial cytochrome c). As in the vast majority of c-type cytochromes (4, 5), the heme (Fe-protoporphyrin IX) is covalently bound to the polypeptide via two thioether bonds that form between the heme vinyl groups and cysteine thiols that occur in a Cys-Xaa-Cys-His motif. In cytochromes cd₁, the c heme is the site of electron acceptance from the external donor proteins (6, 7), which are, in P. pantotrophus, the cupredoxin pseudoazurin and cytochrome c₅₅₀ (8). The d₁ heme is a dioxoisobacteriochlorin (a four-electron reduced porphyrin relative to “normal” b heme and with other variant features) (9) and is unique to this class of enzyme. It is noncovalently bound to the polypeptide chain and is at the active site (10); the d₁ heme binding domain has an eight-bladed β-propeller structure. Nitrite (NO₂⁻) binds via its nitrogen atom to the iron of the d₁ heme, where it is reduced by one electron to form nitric oxide (10); the other reaction product is water, with the two required protons being provided by two highly conserved histidine residues in the d₁ heme pocket (11). In addition to reduction of nitrite to NO, cytochrome cd₁ is capable of catalyzing the two-electron reduction of hydroxylamine to ammonia and the four-electron reduction of oxygen to water. The enzyme was long thought to be an oxidase; however, it is now widely accepted that its physiological role is the reduction of nitrite to nitric oxide during denitrification (12–15).

The crystal structure of oxidized P. pantotrophus cytochrome cd₁ “as isolated” revealed that the c heme is axially ligated by histidines 69 and 17 and that the d₁ heme is coordinated by histidine 200 and tyrosine 25 (3). The distal tyrosine ligand to the d₁ heme is provided by the c heme domain of the protein and is connected to the c heme distal ligand, His-17, by a short loop. Upon reduction of the enzyme, a remarkable switch in heme coordinations occurs. His-17 is replaced by Met-106 as a ligand to the c heme, resulting in His/Met coordination, and Tyr-25 dissociates from the d₁ heme, leaving the iron pentacoordinate and able to bind substrate (10). The oxidized “as isolated” state of the enzyme is catalytically inert, but the enzyme can be activated by reduction (12, 16). When reduced enzyme is reoxidized, the oxidized form generated (which retains His/Met c heme coordination) is catalytically active in the presence of electron donor and substrate but, in their absence, reverts slowly (over a few min) to the inactive “as isolated” conformation (which has His/His-coordinated c heme) (16, 17). There is no evidence for c heme ligand switching occurring during the catalytic cycle of the enzyme.

There remain many mechanistic uncertainties for cytochrome cd₁, prominent among which is the mode of release of nitric oxide from the d₁ heme. Full, catalytically competent dis-
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association of NO has so far not been observed from the P. pantotrophus enzyme other than in steady-state turnover experiments. George et al. (18) demonstrated that fully reduced cytochrome cd₁, in the absence of excess reductant forms a stable complex with an optical absorbance maximum at 632 nm when mixed in a stopped-flow apparatus with 5 mM potassium nitrite (key spectroscopic data in this paper are summarized in Table 1). The complex was assigned as a mixture of $\text{cFe(II)} \cdot \text{Fe(II)}$-NO$^+$ and $\text{cFe(III)} \cdot \text{Fe(II)}$-NO, and was shown to persist over many h in the dark, although in intense light it decayed with a rate constant of about 0.2 s$^{-1}$. Throughout this work, this complex will be referred to, for simplicity, as $cd₁$-X.$^3$

Steady-state turnover of fully reduced P. pantotrophus cytochrome cd₁ can be achieved in the presence of the reduced electron donors cytochrome $c_{550}$, pseudoazurin, and the non-physiological horse heart cytochrome c; thus, product release must occur, and, by implication, the presence of electron donor must contribute to effecting product release (12). This may be through a protein–protein interaction, or it may be as a result of the electron donation itself. To test these possibilities, we have mixed fully reduced cytochrome cd₁, plus potassium nitrite, in the absence of excess reductant, with fully oxidized pseudoazurin (in the absence of excess oxidant) and also with pseudoazurin from which the normal copper had been extracted and replaced with zinc. The results of these experiments are presented here, and implications for the physiological mechanism of nitric oxide dissociation from cytochrome cd₁ are discussed.

**EXPERIMENTAL PROCEDURES**

**Preparation of Cytochrome cd₁—P. pantotrophus** was grown in anaerobic conditions at 37 °C. Cytochrome cd₁ was purified from the periplasm of the cells according to the method of Moir et al. (19), as modified by Koppenhöfer et al. (20). The purity of the enzyme was determined by the $R_s$ value ($A_{400}/A_{280}$), and all cytochrome cd₁ used in this work had an $R_s > 1.25$. The concentration of the enzyme was determined at 406 nm for the oxidized enzyme and 418 nm for the reduced, using the respective extinction coefficients of 142.5 mm$^{-1}$ cm$^{-1}$ (21) and 161.5 mm$^{-1}$ cm$^{-1}$ (20). These extinction coefficients refer to the concentration of the enzyme monomer; throughout this work, the enzyme concentration will be given as monomer concentration. Fully reduced cytochrome cd₁, which has oxidase activity (20), was produced by reduction with sodium dithionite in an anaerobic glove box ($<2$ ppm of O$_2$; Faircrest Ltd.); the excess reductant was removed by passing the enzyme down a desalting column packed with P6-DG resin (Bio-Rad) and equilibrated with 50 mM potassium phosphate buffer.

**Preparation of Pseudoazurin**—Pseudoazurin was purified according to the method of Moir et al. (19) from the total soluble cell extract of Escherichia coli XL-1 Blue transformed with the plasmid pJR2 (22). Cells were grown aerobically for 16 h on 2× TY medium containing 2 mM CuSO$_4$ and 100 μg ml$^{-1}$ ampicillin. To ensure that the pure pseudoazurin was fully oxidized for use in kinetics experiments, a small excess of potassium ferricyanide was added. The excess oxidant was separated from the protein using a desalting column packed with P6-DG resin (Bio-Rad).

**Preparation of Zinc Pseudoazurin**—Apopseudoazurin was prepared essentially according to the method previously described (23). The apopseudoazurin was washed by passing down a desalting column packed with P6-DG resin and equilibrated in water. Buffering apopseudoazurin in 50 mM potassium phosphate solution was found to cause protein precipitation. The apopseudoazurin was incubated with 20 mM ZnCl$_2$ for 1 h at room temperature and then washed with water as above. The concentration of zinc pseudoazurin, which is colorless, was estimated from its predicted extinction coefficient at 280 nm calculated using the program ProtParam (available on the World Wide Web).

**Characterization of Zinc Pseudoazurin**—Zinc pseudoazurin, like reduced copper pseudoazurin, is colorless; therefore, it was not possible to verify the presence of zinc holopseudoazurin by any characteristic absorbance in the visible region of the spectrum. However, a comparison of the UV region of the spectrum with that of copper holopseudoazurin indicated the same absorbance pattern (data not shown). MALDI-TOF mass spectrometry indicated that the zinc pseudoazurin was of the expected molecular weight and had not been degraded during the preparation process, although the metal dissociates from the polypeptide in this experiment. The addition of potassium ferricyanide, in an attempt to oxidize any residual copper bound to the protein, did not cause any increase in absorbance at 590 nm, indicating that the process of making the zinc pseudoazurin had not merely yielded reduced copper pseudoazurin. The addition of copper to the zinc pseudoazurin, followed by oxidation with potassium ferricyanide, did not result in any increase in absorbance at 590 nm, indicating that apopseudoazurin was not present; apopseudoazurin takes up copper to reform holopseudoazurin, which absorbs maximally at 590 nm in the oxidized state (19).

The circular dichroism spectrum of the zinc pseudoazurin was compared with that of copper pseudoazurin (data not shown). The spectrum of copper pseudoazurin was as previously published (23), and that of the zinc pseudoazurin was very similar, although the spectrum of the latter possibly indicated the presence of a minor amount of unfolded protein, which is likely to have been produced during the process of extracting the copper. The zinc pseudoazurin was also denatured with 4 M urea; the unfolded form gave a completely different CD spectrum showing that, although there may have been a small percentage of misfolded protein present in the zinc pseudoazurin stock, the majority was not unfolded.

**Stopped-flow UV-visible Spectroscopy**—Stopped-flow experiments were performed on a Hi-Tech SF-61 DX2 double mixing stopped-flow spectrophotometer interfaced with a CU-61 control unit (Hi-Tech Scientific). The path length of the optical cell was 10 mm, and the dead-time of the instrument was ~2 ms. The stopped-flow unit was housed entirely within an anaerobic glove box maintained at <2 ppm O$_2$. Unless otherwise stated, all solutions of enzyme and substrate were prepared in 50 mM potassium phosphate buffer, pH 7.0, and experiments were carried out at 25 °C. Both single and multimixing experiments.

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$^3$ The abbreviations used are: $cd₁$-X, the stable complex of cytochrome cd₁ and NO previously assigned as a mixture of $\text{cFe(II)} \cdot \text{Fe(II)}$-NO$^+$ and $\text{cFe(III)} \cdot \text{Fe(II)}$-NO.
were carried out, and details of these are given under "Results" and in each of the figure legends. For multimixing experiments, enzyme and substrates were prepared at 4 times the final concentration required, and in single mixing experiments, they were prepared at double the final required concentration.

**EPR Spectroscopy**—EPR spectra were recorded using conditions similar to those previously described (18) on a Bruker ELECSYS 500 spectrometer with an ER049X SuperX microwave bridge and shq cavity, fitted with an Oxford Instruments ER-900 liquid helium-cooled cryostat. Integrations of EPR signals were performed according to the method of Aasa and Vännigård (24).

**RESULTS**

**Pseudoazurin Promotes Decay of cd₁-X**—A stopped-flow multimixing experiment was carried out in which fully reduced *P. pantotrophus* cytochrome cd₁, mixed with 4 mM potassium nitrite to form cd₁-X, which is an approximately equimolar mixture of cdFe(III) dFe(II)-NO and cdFe(II) dFe(II)-NO⁺ (18). This mixture was allowed to age for ~82 ms, the time scale on which George et al. (18) reported full formation of cd₁-X, and was then mixed with oxidized pseudoazurin. When a stoichiometric ratio of initially oxidized pseudoazurin to cd₁ heme was used, the disappearance of cd₁-X was lost in the dead time of the instrument (~2 ms), and, therefore, the experiment was repeated using pseudoazurin at one-tenth the stoichiometry of enzyme monomer. Diode array spectra were collected on a logarithmic time base for 2 s after mixing with pseudoazurin (Fig. 1).

The first time point after mixing with pseudoazurin, at which a peak at 632 nm is clearly visible and a significant proportion of the c heme is still reduced, as judged by the characteristic split α-band absorbance around 550 nm, is shown in boldface type. These spectral features indicate the presence of cd₁-X (18) (Table 1). At later time points after mixing with pseudoazurin at one-tenth the stoichiometry of enzyme monomer, the peak at 632 nm attenuated and was red-shifted to 641 nm, with oxidation occurring at the c heme. The final species was almost completely oxidized at the c heme and had a cd₁ heme absorbance maximum at 641 nm (Fig. 1; Table 1). In other experiments (e.g. Fig. 2), the final product was fully oxidized at the c heme, and the presence of the residual absorbance around 550 nm in Fig. 1 is probably explained by the presence of a small amount of reduced semiapocytochrome cd₁ (i.e. lacking the cd₁ heme) (13). The most likely assignment of the final product of the reaction between pseudoazurin and cd₁-X is all-ferric cytochrome cd₁ with nitrite bound (i.e. cdFe(III) dFe(III)-NO₂⁻), as was suggested by George et al. for the product of photocatalysis of cd₁-X (18). If so, the enzyme has been oxidized relative to cd₁-X, and NO has been released. The final products of photocatalyzed and pseudoazurin-dependent NO dissociation from cytochrome cd₁ have essentially identical absorption spectra (Fig. 2); this was the same whether the pseudoazurin concentration was stoichiometric with cd₁ heme or at one-tenth the stoichiometry. Another possible assignment for the species obtained after reaction of initially oxidized pseudoazurin with cd₁-X would be the all-ferric enzyme with NO bound at the cd₁ heme. However, this is unlikely, since the spectrum does not vary with pH in the manner that we have observed for the all-ferric NO-bound form of cd₁.

**TABLE 1**

**Absorption maxima for P. pantotrophus cytochrome cd₁ and its complexes referred to in this work**

| Protein or protein complex | Absorption maxima | Reference/Source |
|----------------------------|-------------------|------------------|
| i. Oxidized “as isolated” cytochrome cd₁ | cd₁ heme, 458 nm (shoulder), 640 nm, 702 nm; c heme, 406 nm | Refs. 19 and 35 |
| ii. Fully reduced cytochrome cd₁ | cd₁ heme, 654 nm; c heme, 418 nm; split α-band at 549 and 554 nm | Refs. 19 and 35 |
| iii. 6 s after mixing fully reduced cytochrome cd₁ with nitrite (“first observed intermediate”) | cd₁ heme, 630 and 660 nm; c heme, some residual α-band absorbance around 550 nm | Ref. 18 |
| iv. cd₁-X (an approximately equimolar mixture of cdFe(III) dFe(II)-NO, and cdFe(II) dFe(II)-NO⁺) | cd₁ heme, 632 nm; c heme, some residual α-band absorbance around 550 nm | Ref. 18 |
| v. Photolyzed cd₁-X (final product) | d₀ heme, 642 nm (pH 7.0), 639 nm (pH 6.0); c heme, no α-band absorbance | Ref. 18, this work |
| vi. All ferric Y25S cytochrome cd₁, with NO₂⁻ bound | d₀ heme, 641 nm (pH 7.0), 639 nm (pH 6.0); c heme, no α-band absorbance | Ref. 36 |
| vii. Pseudoazurin (final product) | 642 nm (pH 7.0), 639 nm (pH 6.0) | This work |
| viii. All ferric Y25S cytochrome cd₁, with NO bound | 640 nm (pH 7.0), 632 nm (pH 6.0) | This work |
| ix. Reduced wild type cytochrome cd₁, reoxidized by hydroxylamine, NO₂⁻ added | 643 nm | Ref. 25 |
cytochrome cd₁ (Table 1). The final product of the reaction between pseudoazurin and cd₁-X at pH 7.0 had a visible absorption peak at 641 nm, and when the same experiment was repeated at pH 6.0, the peak was at 639 nm. Model spectra for both nitrite and NO bound to fully oxidized cytochrome cd₁ were obtained using the Y25S variant of P. pantotrophus cytochrome cd₁, which, unlike the “as isolated” oxidized wild type enzyme, is fully competent for exogenous ligand binding (25–27). The d₁ heme peak in the spectrum of nitrite bound to all-ferric Y25S enzyme varied from 639 to 642 nm as the pH shifted from 6.0 to 7.0 (Table 1). However, in the spectrum of NO bound to all-ferric Y25S cytochrome cd₁, the d₁ heme peak varied between 632 nm at pH 6.0 and 640 nm at pH 7.0. The possibility of the final species produced by the reaction between pseudoazurin and cd₁-X being ferrous d₁ heme with NO bound was ruled out using EPR spectroscopy. A signal corresponding to d₁Fe(II)-NO (28) was observed in the spectrum of a sample of cd₁-X reacted with pseudoazurin, but quantitation showed that it represented only ~1% of the total d₁ heme that was present.

Upon close inspection of the data shown in Fig. 1, it is apparent that there are two distinct kinetic phases of the reaction between cd₁-X and initially oxidized pseudoazurin at one-tenth the stoichiometry of the cd₁ monomer. In the region above 600 nm, where only the d₁ heme absorbs (21), the phases can be clearly separated from one another. Up to 100 ms after mixing cd₁-X with pseudoazurin, the first phase was dominant, and an isosbestic point was apparent at 642 nm. From 100 ms onward, the second phase dominated the absorbance change with an isosbestic point at 635 nm. The first 100 ms of the absorbance change at 635 nm (Fig. 3A) fitted to a single exponential with k_obs = 24 s⁻¹. The rate constant of the second phase of the reaction was determined by plotting the absorbance change at 642 nm, the wavelength at which there is no change during the first phase of the reaction, against time (Fig. 3B). After an initial lag of ~100 ms, the change at 642 nm fitted to a single exponential with k_obs = 5 s⁻¹. Oxidation of the c heme was measured by subtraction of the change in absorbance at 539 nm from the change in absorbance at 549 nm (Fig. 3C). This enables determination of the absorbance change at the c heme by substantially eliminating the d₁ heme contribution and is necessary because there is no wavelength at which the c heme absorbs without some contri-
Pseudoazurin Facilitates NO Release from Cytochrome cd$_1$

The current work has shown that pseudoazurin causes product release from cytochrome cd$_1$. Of at least equal importance is whether it prevents formation of the stable complex, cd$_1$-X, between the enzyme and its product NO. At pH 7.0, cd$_1$-X forms slowly in relation to the rate of steady-state turnover of nitrite by cytochrome cd$_1$ (18). The latter occurs at 72 s$^{-1}$ per monomer (12), indicating that the length of the catalytic cycle is ≈14 ms at pH 7.0, whereas cd$_1$-X forms with $k_{obs} = 40$ s$^{-1}$ at this pH. To determine whether this stable complex of enzyme and product is formed in the presence of pseudoazurin, fully reduced cytochrome cd$_1$ was mixed in the stopped-flow apparatus with a combination of 4 mM potassium nitrite and either one-tenth stoichiometric (Fig. 4) or stoichiometric (Fig. 5) initially oxidized pseudoazurin.

As can be seen from the spectra in Figs. 4 and 5, the concentration of pseudoazurin has a significant effect on the reaction. In the presence of pseudoazurin at one-tenth the stoichiometry of enzyme monomer, cytochrome cd$_1$ first reacts with nitrite to form a species with an absorbance maximum at 632 nm, the absorbance maximum of cd$_1$-X, which then decays in a biphasic process to give the final product described earlier with a maximum at ≈641 nm and fully oxidized c heme (Table 1). Formation and subsequent decay of the 632 nm peak are shown separately in Fig. 4, A and B. In the presence of a 1:1 ratio of
Pseudoazurin to enzyme monomer, separate formation and decay of the 632 nm peak is not observed, and there appears to be a smooth transition toward the final product (Fig. 5); the difference between this experiment and the reaction in the absence of pseudoazurin (Fig. 5, inset) is striking. Particularly noteworthy is that in the absence of pseudoazurin, the first observed intermediate of reaction between cytochrome cd₁ and nitrite in the stopped-flow apparatus has significant absorbance at 660 nm (Table 1); the absorbance at 660 nm then diminishes as cd₁-X forms (Figs. 4 and 5). When fully reduced cytochrome cd₁ was mixed in the stopped-flow apparatus with pseudoazurin at one-tenth the stoichiometry of enzyme monomer and nitrite, the first time point showed significant absorbance at 660 nm, which decreased concomitantly with an increase in absorbance at 632 nm, as is seen in the absence of pseudoazurin. When the same experiment was conducted in the presence of stoichiometric pseudoazurin, the initial observed time point of the reaction showed much less absorbance at 660 nm than with substoichiometric pseudoazurin (Fig. 5). It appears that, remarkably, pseudoazurin, in a concentration-dependent manner, accelerates formation of intermediates in the reaction between cytochrome cd₁ and nitrite and promotes the release of product from the enzyme.

The reaction of fully reduced cytochrome cd₁ with potassium nitrite and pseudoazurin at one-tenth the stoichiometry of enzyme monomer was analyzed kinetically. The first phase was fitted at 635 nm, the wavelength at which no absorbance change occurs during the subsequent phases, and showed that formation of cd₁-X occurred with a rate constant of 77 s⁻¹ (Fig. 6A). This is faster than the rate of formation of cd₁-X in the absence of pseudoazurin, which occurs with a rate constant of 40 s⁻¹ at pH 7.0 (18) (this work). The decay of cd₁-X was fitted to a double exponential at 650 nm, the wavelength at which no absorbance change occurs during the first phase of the reaction (Fig. 6B). The observed rate constants were 17 s⁻¹ and 3 s⁻¹, comparable with those observed for the reaction between this concentration of pseudoazurin and preformed cd₁-X (above), with respective amplitudes of 0.02 and 0.01. Changes in oxidation state at the c heme were measured by plotting the absorbance at 549 nm minus 539 nm to eliminate the contribution from the d₁ heme. The c heme appeared to rereduce rapidly (see Ref. 18) and then subsequently showed biphasic oxidation (Fig. 6C), the rate constants of which were 14 and 3 s⁻¹, with the same relative amplitudes as attributed to the two phases occurring at the d₁ heme. This strong agreement between both the rate constants and amplitudes of the phases occurring at the c and d₁ hemes indicates that the same biphasic process is being observed at both heme centers. The phases of the reaction of fully reduced cytochrome cd₁ with stoichiometric initially oxidized pseudoazurin and 4 mM potassium nitrite could not easily be kinetically resolved. However, it is clear (Fig. 5) that cd₁-X does not accumulate; at this concentration of pseudoazurin, either it does not form, or its initial rate of decay exceeds its rate of formation. The latter is consistent with the observation above that the reaction between preformed cd₁-X and stoichiometric oxidized pseudoazurin was lost in the dead time of the stopped-flow apparatus.

The Effect of Zinc-substituted Pseudoazurin—The data presented thus far have shown that initially oxidized pseudoazurin is capable of effecting dissociation of the product, nitric oxide, from cd₁-X; the enzyme also becomes fully oxidized. Further-
more, at sufficient concentration, initially oxidized pseudoazurin prevents accumulation of cd₁-X during the reaction of fully reduced cytochrome cd₁ with potassium nitrite. These effects may be due to electron transfer processes dependent on pseudoazurin or simply because of protein-protein interaction between pseudoazurin and cytochrome cd₁. To test whether or not electron transfer is required, the above experiments were repeated using redox-inactive zinc-substituted pseudoazurin (23) in place of normal pseudoazurin, which contains copper. The production of zinc pseudoazurin and its characterization is documented under ”Experimental Procedures.”

Initially, multimixing experiments were carried out in which fully reduced cytochrome cd₁ in the absence of excess reductant was mixed first with 5 mM potassium nitrite in the stopped-flow apparatus, aged for ~70 ms, and then mixed rapidly with zinc pseudoazurin at one-tenth the stoichiometry of enzyme monomer. Over the course of 60 s, cd₁-X was seen to decay; however, the rate of decay was comparable with the rate of photocatalyzed dissociation, indicating that the zinc pseudoazurin had little or no effect on dissociation of cd₁-X. It was also necessary to determine whether zinc pseudoazurin affects formation of cd₁-X. Fully reduced cytochrome cd₁, in the absence of excess reductant, was mixed with a combination of one-tenth stoichiometric zinc pseudoazurin to enzyme monomer and potassium nitrite. Following mixing, formation of cd₁-X occurred with the same rate constants as in the absence of pseudoazurin, and cd₁-X did not decay above the rate of photocatalyzed dissociation. Thus, zinc pseudoazurin had no detectable effect on the reaction.

**DISCUSSION**

The principal observations in this work are summarized in Scheme 1. As previously reported (18), when fully reduced cytochrome cd₁, in the absence of excess reductant, is mixed with excess nitrite in a stopped-flow apparatus, an initial rapid phase is observed in which the heme becomes largely oxidized and the cd₁ heme forms a complex with two absorbance peaks, one at ~660 nm and one at ~630 nm (key spectroscopic data are summarized in Table 1). There then follows a second phase in which the 660 nm peak decays with concomitant increase in absorbance at 632 nm, and the cd₁ heme is partially reduced, to yield a species that is ~50% reduced at the cd₁ heme and has a cd₁ heme absorbance maximum at 632 nm. This second phase occurs with a rate constant of 40 s⁻¹ at pH 7.0. The resulting product was assigned by George et al. (18) as a mixture of cd₁-X (Scheme 1). Oxidation of the cd₁ heme was also a biphasic process with isosbestic points, enabling the rates of these phases to be determined (rate constants of 24 and 5 s⁻¹). The decomposition of preformed cd₁-X in the presence of one-tenth stoichiometric pseudoazurin to enzyme monomer was biphasic (Figs. 1 and 3). The two processes occurring at the cd₁ heme can be clearly separated by isosbestic points, enabling the rates of these phases to be determined (rate constants of 24 and 5 s⁻¹). Oxidation of the cd₁ heme was also a biphasic process with rate constants of 10 and 4 s⁻¹.
exchange between the c heme of cytochrome cd₁ and pseudoazurin. Such electron transfer would be consistent with the relative reduction potentials (19, 30).

The observation that pseudoazurin with zinc substituted in place of the usual copper ion does not increase the rate of dissociation of cd₁-X above the background rate of photolysis implies that electron transfer is essential for pseudoazurin-dependent NO release from cytochrome cd₁. All analytical techniques used suggested that the zinc pseudoazurin was essentially fully folded. Even if a small amount of unfolded protein had been present, the fact that copper pseudoazurin increases the rate of NO dissociation ~100-fold above the rate of photolysis when present in just a one-tenth stoichiometric ratio to enzyme monomer suggests that if a protein–protein interaction alone were the driving force behind NO dissociation from cytochrome cd₁, zinc pseudoazurin should have enabled NO dissociation even if a small percentage was not correctly folded.

Initially oxidized copper pseudoazurin is able to promote apparently full decomposition of cd₁-X when present in a 10-fold substoichiometric ratio to enzyme monomer. Therefore, the effect of pseudoazurin on the enzyme is catalytic. The observation that zinc pseudoazurin has little or no effect on dissociation of NO from cd₁-X indicates a crucial role for the presence of the redox-active metal, copper, in pseudoazurin to bring about product dissociation. These results imply that it is not the addition of electrons to the system per se that is required for dissociation of NO from cd₁-X but more precisely electron movement. The introduction of pseudoazurin to preformed cd₁-X must enable shuttling of electrons between monomers or individual enzyme dimers, which results in further turnover, oxidation of the cd₁, and apparently release of the reaction product, NO, from the cd₁ heme. From the available data, it also remains possible, if intuitively unlikely, that copper pseudoazurin (but not zinc pseudoazurin) plays a direct role in releasing NO from the cd₁ heme of cytochrome cd₁ (e.g. by a ligand exchange between the heme and copper centers).

As shown in Fig. 2, the spectrum of the product of photocatalyzed decomposition of cd₁-X is essentially identical to the spectrum following pseudoazurin–dependent decomposition, indicating that the products are likely to be the same. This was the case whether pseudoazurin was stoichiometric with d₁ heme or present at one-tenth of the stoichiometry. The final product of pseudoazurin–dependent decomposition of cd₁-X is most likely to be the all-ferric enzyme with nitrite bound (Table 1 and Scheme 1). This assignment is based first on good agreement of the pH dependence of the optical absorbance maxima of the dissociated product of cd₁-X with that of the all-ferric nitrite-bound Y255 cytochrome cd₁. Further evidence is provided by Allen et al. (25), who reported the spectrum of reduced wild type P. pantotrophus cytochrome cd₁, reoxidized with hydroxylamine and with nitrite added; the d₁ heme absorbance maximum of the enzyme was at 643 nm, which is also very similar to that of the final product resulting from pseudoazurin-catalyzed dissociation of cd₁-X. d₁(Fe(II)-NO) has its absorption maximum at 632 nm (18); thus, the other plausible assignment of the cd₁-X dissociation product is all-ferric enzyme with NO bound at the d₁ heme as d₁(Fe(III)-NO). However, as previously discussed, this explanation is not favored, because the pH dependence of absorbance maxima of the cd₁-X-dissociated spectrum was very different from that of all-ferric Y255 cytochrome cd₁ with NO bound. Furthermore, there is considerable evidence that formation of d₁(Fe(II)-NO)⁺, rather than the isoelectronic state d₁(Fe(III)-NO), is favored in cytochromes cd₁ (18, 31). Fe(III)-NO, Fe(II)-NO⁺, and Fe(III)-NO²⁻ are all EPR-silent complexes of d₁ heme, and therefore, none of these states can be unequivocally demonstrated (or eliminated) using this technique. However, d₁(Fe(II)-NO) was ruled out as the final product of pseudoazurin-catalyzed cd₁-X dissociation, because integration of the d₁(Fe(II)-NO) EPR signal from a sample of cd₁-X treated with pseudoazurin demonstrated that it constituted an insignificant portion of the total enzyme (~1%).

A very informative result presented here is that obtained from mixing fully reduced cytochrome cd₁ with oxidized pseudoazurin and nitrite in the stopped-flow apparatus. In the presence of stoichiometric pseudoazurin, formation of cd₁-X occurred at an accelerated rate relative to the absence of pseudoazurin. Subsequent decay of cd₁-X occurred quickly enough to prevent observation of the discrete increase in absorbance at 632 nm that was seen with substoichiometric pseudoazurin and in the absence of pseudoazurin (Figs. 4 and 5) (although the data are also consistent with cd₁-X being bypassed altogether at this concentration of pseudoazurin) (Scheme 1). The rate of cd₁-X formation observed in the presence of even substoichiometric pseudoazurin was ~80 s⁻¹, putting it on a time scale that is relevant to the rate of steady-state turnover (the kcat of P. pantotrophus cytochrome cd₁ is 72 s⁻¹ at pH 7.0 (12)). It is thus our current view that species identified following the addition of nitrite to fully reduced cytochrome cd₁ in the absence of electron donor proteins are relevant to the catalytic cycle, but pseudoazurin increases at least some of the rate constants for the formation and decay of these species.

The state of cytochrome cd₁ that normally releases NO is still the subject of much debate. It has been axiomatic that NO is released from oxidized d₁ heme, because “normal” hemes (e.g. b heme in hemoglobin) have a much greater affinity for NO when reduced. However, recent work has shown, unexpectedly, that NO can be released from the ferrous d₁ heme of P. aeruginosa cytochrome cd₁, in the presence of excess ascorbate, at a rate greater than kcat for the enzyme from that source (32, 33). In principle, given two hemes on each monomer of the enzyme, there are five possible overall oxidation states of the cd₁ dimer; these are the all-ferrous form (four-electron reduced), three-, two-, and one-electron reduced dimer, and the fully oxidized (all-ferric) state. In the partially reduced forms, multiple permutations of oxidation state are possible for the c and d₁ hemes (e.g. in the two-electron reduced dimer, the electrons could be
distributed between the four hemes in six distinct ways, since the monomers are not absolutely equivalent (10)). It is clear that at least some of the partially reduced states of cytochrome cd₁ do not spontaneously release NO, irrespective of the oxidation state of the d₁ heme (18). This conclusion is reinforced by the current work in which the ability of initially oxidized pseudoazurin to shuttle electrons between cd₁ monomers (or dimers) is apparently an essential part of its role in catalyzing NO release from the enzyme. Our assignment of the final product of reaction between pseudoazurin and cd₁-X as all-ferric enzyme with nitrite bound implies that fully oxidized cytochrome cd₁ is an effective NO-releasing state. It is conceivable that, for example, cytochrome cd₁ monomers work optimally when cycling between one-electron reduced and fully oxidized states; in the present experiments, the addition of oxidized pseudoazurin might have provided a sink for surplus electrons that was absent in earlier studies, allowing some or all of the cytochrome cd₁ molecules (depending on the pseudoazurin concentration) to maintain their favored oxidation state(s). It will be interesting to learn whether P. pantotrophus cytochrome cd₁ can, like its counterpart from P. aeruginosa (32, 33), also release NO from the fully reduced enzyme at a catalytically competent rate.

The present work provides significant new insight into the mechanism of nitrite reduction by P. pantotrophus cytochrome cd₁ and shows that pseudoazurin enables what was previously thought to be a dead end complex of the enzyme (cd₁-X) to rapidly undergo further turnover and apparently release the final product NO. Remarkably, the presence of pseudoazurin also accelerates formation of intermediates in reduction of nitrite by the enzyme. These effects are facilitated by a combination of electron transfer via the copper of pseudoazurin and protein-protein interaction between pseudoazurin and cytochrome cd₁. It is very clear that cytochrome cd₁ functions significantly differently in the presence of its two substrates, nitrite and electron donor protein, than in the presence of nitrite alone.

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