Time-resolved Infrared Spectroscopy Reveals a Stable Ferric Heme-NO Intermediate in the Reaction of Paracoccus pantotrophus Cytochrome cd$_1$ Nitrite Reductase with Nitrite*

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Cytochrome cd$_1$ is a respiratory enzyme that catalyzes the physiological one-electron reduction of nitrite to nitric oxide. The enzyme is a dimer, each monomer containing one c-type cytochrome center and one active site cd$_1$ heme. We present stopped-flow Fourier transform infrared data showing the formation of a stable ferric heme cd$_1$-NO complex (formally d$_1$Fe(II)-NO$^+$) as a product of the reaction between fully reduced Paracoccus pantotrophus cytochrome cd$_1$, and nitrite, in the absence of excess reductant. The Fe$^{14}$NO ν(NO) stretching mode is observed at 1913 cm$^{-1}$ with the corresponding Fe$^{15}$NO band at 1876 cm$^{-1}$. This cd$_1$ heme-NO complex is still readily observed after 15 min. EPR and visible absorption spectroscopic data show that within 4 ms of the initiation of the reaction, nitrite is reduced at the cd$_1$ heme, and a cFe(III) d$_1$Fe(II)-NO complex is formed. Over the next 100 ms there is an electron redistribution within the enzyme to give a mixed species, 55% cFe(III) d$_1$Fe(II)-NO and 45% cFe(II) d$_1$Fe(II)-NO$^+$. No kinetically competent release of NO could be detected, indicating that at least one additional factor is required for product release by the enzyme. Implications for the mechanism of P. pantotrophus cytochrome cd$_1$ are discussed.

Reduction of nitrite (NO$_2^-$) to NO is the committed step in denitrification, the process within the biological nitrogen cycle by which nitrate (NO$_3^-$) is sequentially reduced to dinitrogen gas (1). The reaction is catalyzed by periplasmic dissimilatory nitrite reductases, of which two types are known (2, 3). The first type comprises the copper nitrite reductases. These are trimeric enzymes, each monomer containing one type I copper center that acts as an electron acceptor from donor proteins and a type II copper center as the active site (3). The other type of dissimilatory nitrite reductase is a heme enzyme, cytochrome cd$_1$. This enzyme is a dimer, each monomer containing one c-type cytochrome center that accepts electrons from soluble donor proteins (c-type cytochromes and cupredoxins) and one cd$_1$-heme (3–6). The cd$_1$-heme is the active site and is unique to this class of enzyme (7–9).

High resolution x-ray crystallographic structures have been obtained for cytochrome cd$_1$ from Paracoccus pantotrophus (formerly Thiosphaera pantotropha (10)) in two oxidation states (9, 11). When oxidized, the cd$_1$ heme has bis-histidinyl ligation with residues His-200 and Tyr-25 coordinated to the cd$_1$-heme. Remarkably, upon reduction Met-106 replaces His-17 as axial ligand to the cd$_1$ heme, and Tyr-25 is displaced from the cd$_1$-heme, leaving it penta-coordinate and able to bind substrate. Ligands to both hemes in the oxidized enzyme, His-17 and Tyr-25, are connected by a very short amino acid loop, and there is evidence that they bind the hemes cooperatively (12, 13). Distances are such that electron transfer across the dimer interface cannot occur on a catalytically relevant time scale (11, 14).

There has been considerable speculation about the role of these unprecedented structural changes in the enzyme during the physiological reduction of nitrite (9, 13, 15, 16). The product of this one-electron reaction is nitric oxide, which is known to have a high affinity for heme (particularly ferrous heme). It is generally believed that product NO dissociates from the ferric state of the cd$_1$ heme of nitrite reductase (for example see Refs. 2–4). A model has been proposed involving displacement of NO from the cd$_1$-heme by Tyr-25 re-binding to the heme during each catalytic cycle (11). Further, it has been postulated (9) that axial ligand switching at the cd$_1$ heme between His/Met and His/His coordination may provide a method of gating reduction potentials to prevent formation of an all ferrous enzyme with NO bound. In the case of Pseudomonas aeruginosa cd$_1$, such a species was formed in the presence of excess reductant and nitrite and showed no further activity (4).

In this paper, we report the first rapid reaction study of the reduction of nitrite by P. pantotrophus cytochrome cd$_1$. We have employed the novel approach of stopped-flow Fourier transform infrared (SF-FTIR) spectroscopy (17, 18), which is a method ideally suited to detecting transient heme-NO intermediates that are difficult to observe by other techniques. We have combined this with visible absorption stopped-flow and freeze-quench EPR methodologies. We show that the following reaction between reduced enzyme and nitrite in the absence of excess reductant, a cd$_1$-heme Fe(II)-NO$^+$ complex forms rapidly. There follows a redistribution of electrons between the heme centers of the enzyme over 100 ms, resulting in formation of a mixture of d$_1$Fe(II)-NO$^+$ and d$_1$Fe(II)-NO complexes. This state of the enzyme is stable on a time scale of minutes. Implications for the mechanism of the enzyme are discussed.

**EXPERIMENTAL PROCEDURES**

**Preparation of Enzyme Samples**—Cytochrome cd$_1$ was purified from the periplasm of P. pantotrophus, grown under denitrifying conditions,
according to the method of Moir et al. (19), as modified by Koppenhöfer et al. (20). All enzyme used in this work had an $R_{\text{A}_{280}/A_{260}}$ ratio of at least 1.32. The concentration of cytochrome $c_d$ was determined using the extinction coefficients 285 $\text{mm}^{-1} \text{cm}^{-1}$ at 406 nm, for the oxidized dimer (21) and 325 $\text{mm}^{-1} \text{cm}^{-1}$ at 415 nm (20, 22) for the reduced enzyme. All enzyme and substrate solutions used in this study were made in 50 mM potassium phosphate buffer at pH 7.0, unless otherwise stated.

Fully reduced enzyme, in the absence of excess reductant, was prepared in an anaerobic cabinet ($<5$ ppm O$_2$). Enzyme at approximately double the final desired concentration was reduced with an excess of sodium dithionite or with electrochemically reduced methyl viologen. The mixture was passed down a desalting column containing P6-DG resin (Bio-Rad). Dithionite reduced eluent was tested for the absence of excess reductant using papers pre-soaked in methyl viologen, which turn blue on contact with dithionite. When viologen was used as reductant, the dark green reduced enzyme was observed to clearly separate from the blue reducing agent on the desalting column. Enzyme thus prepared was immediately transferred to a stopped-flow machine or gas tight vessel. The UV-visible absorption spectrum of the reduced enzyme was identical using each reductant, and the visible region of the spectrum was as reported by Koppenhöfer et al. (20), who used ascorbate and hexamethylenurathenium(III) chloride to reduce the enzyme.

**Stopped-Flow FTIR Spectroscopy**—SF-FTIR experiments were performed using an adapted Bruker IFS 66/S spectrometer (Bruker UK Ltd., Coventry, UK) fitted with a liquid nitrogen cooled mercury cadmium telluride detector. The stopped-flow circuit and cell are home-built and essentially those described by Wharton and co-workers (23).

The drive system and stopped-flow cell are entirely housed within an anaerobic dry glove box (<2 ppm O$_2$) (Belle Technology, Portesham, Dorset, UK) as detailed elsewhere (18). The cell had pathlength of approximately 50 $\mu$m. For these experiments the instrument collected data across a range of 200 $\text{cm}^{-1}$. Typically, for an infrared resolution of 4 $\text{cm}^{-1}$, the time resolution was 25 ms per spectrum, and the dead time before detection was 18 ms. Exact sample conditions are given in the figure legends.

**UV-Visible Stopped-Flow Spectroscopy**—Stopped-flow kinetic experiments were performed using a Hi-Tech SF-61 DX2 double mixing stopped-flow spectrometer interfaced with a CU-61 control unit (Hi-Tech Scientific). The drive syringes and optical cell were entirely housed within an anaerobic dry glove box (Faircrest Ltd) (<10 ppm O$_2$). Both the glove box atmosphere and drive system were thermostatted to give a sample temperature of 25 °C. The optical flow cell had a pathlength of 10 mm. The dead time before detection was ~2 ms. This work used the instrument in both single wavelength photomultiplier and diode array detection modes. In diode array mode the maximum time resolution was 2.25 ms/spectrum. Data were analyzed using the KinetAsyst 2.2 software package (Hi-Tech Scientific).

**EPR Spectroscopy**—EPR spectra were recorded on a X-band Bruker 200D spectrometer (Bruker UK Ltd., Coventry, UK) interfaced to an ESP 1600 computer and fitted with an Oxford Instruments ESR600 liquid helium cooled flow cryostat (Oxford Instruments Ltd., Abingdon, UK). Samples were prepared by mixing enzyme and substrate and freezing in liquid isopentane (~140 °C) on a millisecond time scale, using the freeze quench apparatus previously described (24). Instrument settings are given in the figure legends.

**RESULTS**

The aim of this study of the kinetics of reduction of nitrite by cytochrome $c_d$ was to characterize the formation of the nitric oxide product as well as other intermediates of the catalytic cycle and possibly to detect a product release step. The experiments were performed using a Hi-Tech SF-61 DX2 double mixing stopped-flow machine or gas tight vessel. The UV-visible absorption spectrum of the reduced enzyme was identical using each reductant, and the visible region of the spectrum was as reported by Koppenhöfer et al. (20), who used ascorbate and hexamethylenurathenium(III) chloride to reduce the enzyme.

Thus concludes the nature of $c_d$ and shows that this peak arises from bound NO. The energy of this peak is consistent with the $\gamma$(NO) fundamental stretching mode of NO bound to ferric heme (25, 26). (The observed complex is in fact more accurately described as a heme-Fe(II)-NO$^-$ species.) Repeating the experiment using H$_2$O buffers did not cause any significant shift in this band, suggesting that any hydrogen bonding interactions to the bound NO must arise from nonexchangeable protons.

Wang and Averrill (27) reported similar spectra when NO was mixed with oxidized cytochrome $c_d$ from Pseudomonas stutzeri. They observed peaks at 1910 $\text{cm}^{-1}$ (14NO) and 1874 $\text{cm}^{-1}$ (15NO), with half-height widths of 7 $\text{cm}^{-1}$. In contrast to their experiment, the species we detected was unequivocally the product of enzymatic reduction of substrate nitrite. We could not produce a species where NO bound to oxidized, “isolated” $P$. pantotrophus $c_d$ despite extensive attempts to do so. Presumably the Tyr-25 ligand to the $d_1$ heme cannot be displaced under such conditions. The energy of the peak we observed by infrared is also in close agreement with those recorded for model compounds of $d_1$ heme, with ferric iron coordinated by NO and N-methylimidazole (25).

We were able to measure the rate of formation of the peak observed by SF-FTIR; the experiment was conducted as above, in H$_2$O buffer at pH 7.0, using Na$_2$NO$_2$ as substrate. Spectra were recorded approximately every 25 ms for 5 s. The intensity of the base-line corrected peak observed at 1913 $\text{cm}^{-1}$ is shown in Fig. 2, as a function of time (longer time points are taken from the experiment described in Fig. 1; see figure legends). The superimposed curve in Fig. 2 is a calculated single exponential with a rate constant of 38 s$^{-1}$. It is apparent that the Fe(II)-NO$^-$ species we detect substantially formed within 100 ms; the peak was still intense 15 min after reaction.

In this paper we use $d_1$Fe(II)-NO$^-$ to describe the ferric $d_1$ heme-NO$^-$ complex we observe by SF-FTIR rather than the isoelectronic species $d_1$Fe(III)-NO. In general, when NO binds to a metal in a high oxidation state, the NO ligand can donate charge to the metal, resulting in a complex with appreciable NO$^-$ character. This typically results in a linear metal-N-O geometry and an elevated $\gamma$(NO) stretching frequency, implying that the NO bond order has increased. The $\gamma$(NO) of 1913 $\text{cm}^{-1}$ we observe is higher than that of free NO (1876 $\text{cm}^{-1}$) and substantially higher than those of ferrous heme-NO complexes (1690–1590 $\text{cm}^{-1}$ (26)). This suggests that the notation $d_1$Fe(II)-NO$^-$ we use here is more correct than $d_1$Fe(III)-NO when describing the ferric $d_1$ heme-NO complex.
reported (20) for reduced starting material had an identical spectrum to that previously apparent following mixing of reduced enzyme with nitrite. The conditions very similar to those used for the FTIR measurements.

Visible absorption stopped-flow spectrophotometry, under conditions shown in Fig. 2 might have been so fast as to escape detection in the experiment shown in Fig. 2a. Insight into this issue was sought using visible absorption stopped-flow spectrophotometry, under conditions very similar to those used for the FTIR measurements.

Using visible spectrophotometry, three phases of reaction were apparent following mixing of reduced enzyme with nitrite. The starting material had an identical spectrum to that previously reported (20) for reduced P. pantotrophus cytochrome cd₁ (Fig. 3a). After 4 ms both the d₁ heme and c heme contributions had changed (Fig. 3b). In particular, the d₁ heme peak and shoulder at 652 nm had been replaced by a broader less intense feature centered at about 642 nm, whereas the d₁ Soret region was bleached. Figs. 3b and 4a show that 4 ms after reaction, the c-type cytochrome center was largely oxidized. The rate constant for this oxidation is estimated to be at least 500 s⁻¹. Simulation shows that the c-type center was oxidized by 87% during this phase, which largely occurred within the dead time of the stopped-flow apparatus. The most straightforward interpretation of these spectral changes is that after 4 ms a reduced form of nitrite, presumably NO, was coordinated to the d₁ heme.

The second phase of the reaction was apparent approximately 5 ms after mixing enzyme with substrate, as the c heme started to become re-reduced (Fig. 4a). This was evident from the doublet around 550 nm in the a-band region of the spectrum, which is characteristic of reduced c heme in cytochromes cd₁ (15). By 100 ms a stable state was reached (Fig. 3c), in which 45% of the c-type center (in the bulk sample) was ferrous. Re-reduction of the c-type center correlated with the appearance of a new visible absorption band at 632 nm, which we assign to the d₁ heme center. This developed with a concomitant reduction in intensity at 670 nm.

Example time courses illustrating the first two phases (from 2 to 250 ms after reaction) are presented in Fig. 4. These were recorded at 629 nm (d₁-heme) and from the c heme a-band doublet around 550 nm. Indicated are the starting intensities from the unreacted ferrous enzyme (○). Note that the c heme time course is a difference time course designed to eliminate background contributions from the d₁-heme (see the figure legend). Between 10 and 250 ms both curves can be fitted to the same single exponential process with a monophasic rate constant of 38 s⁻¹ (broken lines in Fig. 4). This rate curve also correlates well with the appearance of the d₁Fe(II)-NO⁺ species observed by SF-FTIR (Fig. 2). These kinetic data strongly imply that the same (or coupled) processes were being observed by visible spectroscopy at both the c and d₁ hemes and by SF-FTIR. Varying the concentration of nitrite in the reaction mixture between 200 and 5 mM for 15 µM enzyme did not significantly change these time courses.

The third phase of the reaction was slow, taking many hours to complete in the dark. It was also photosensitive. It was, for example, photocatalyzed by the high light intensity incident on the sample in the diode array apparatus, which caused the reaction to complete within a few minutes. This phase in-

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involved the loss of the new band at 632 nm and essentially complete oxidation of the c heme. The spectrum of the final product of the reaction (Fig. 3d) was not that of the “ferric enzyme” as isolated. The high spin d₄ heme α-band at 702 nm was absent; this feature is characteristic of Tyr-25 ligation to the heme (28). Therefore, we postulate that the final reaction product was an all ferric form of the enzyme with nitrite bound to the d₄ heme. The position of the Soret band was similar to that in intermediates observed in recent studies that were proposed to have His/Met coordinated ferric c heme (13, 20). When near stoichiometric heme-d₄:nitrite ratios were used, the reaction did not show this third phase; this strongly suggests that it arises from further turnover by the enzyme.

We have also studied the reaction of stoichiometrically reduced cd₁ with nitrite using EPR spectroscopy. As expected, all-ferrous nitrite reductase did not exhibit any EPR signal (data not shown); in general it is ferric heme that exhibits intense EPR signals, because it is a non-integer spin paramagnet. The X-band EPR spectrum of a sample of reduced nitrite reductase freeze-quenched 4 ms after the addition of excess nitrite showed three prominent features (Fig. 5). It was dominated by a sharp band at g = 2.01, which can be assigned to a ferrous heme-NO species (29). In the case of heme-NO complexes it is the ferrous heme that is EPR active, with the ferric heme EPR silent, as the unpaired electron on NO delocalizes onto the metal. The other significant signals in the spectrum were observed at g = 2.93 and 2.32. By analogy with the P. pantotrophus cytochrome cd₁-oxygen and hydroxylamine complexes, we assign these to oxidized heme-c with His-Met coordination (13, 20).

These EPR signals were also apparent in samples frozen at longer times. However their intensities fell rapidly, having approximately halved by 110 ms. Fig. 6 is a plot of the intensities of the ferrous heme-NO and heme-c EPR signals superimposed on a portion of the optical stopped-flow time courses recorded for the 632 and 550 nm absorption features, respectively. There is a clear correspondence between the growth of the 632 nm band and the decay of the ferrous heme-NO EPR signal. Similarly, the intensity of the ferric heme-c EPR band can be plotted over the changes in the 550 nm doublet. In samples frozen in liquid nitrogen after reacting for longer times (between 2 and 11 min), the intensity of the ferrous heme-NO band attenuated further, whereas the ferric heme-c spectrum was seen to increase slowly (data not shown), correlating with the slow third phase of the reaction observed by absorption spectroscopy. We note that the bis-His form of ferric heme-c in native enzyme exhibits an EPR signal with g = 3.05 (28). At no time did we see any indication that the heme-c was reverting to bis-His coordination.⁴

**DISCUSSION**

This work provides much new insight into the mechanism of P. pantotrophus cytochrome cd₁. Scheme 1 summarizes the mechanism suggested by these data.

The primary observation of this work is the first unequivocal spectroscopic detection of a ferric heme-NO (d₄Fe(II)-NO⁻) complex as a reaction product of nitrite reduction by cytochrome cd₁. This result arose from use of the novel technique of SF-FTIR. Evidence for formation of such a species is strong, including a ¹⁵NO versus ¹⁴NO isotopic shift of the expected

⁴ In preparation for this study we recorded an EPR spectrum of an isolated P. pantotrophus cd₁. The form of the spectrum was essentially as reported by Cheesman et al. (28); however, the signal at g = 2.00 was absent, and we were able to clearly resolve the gₓ component of the c heme spectrum at g = 1.4, which Cheesman et al. did not observe.
value and excellent agreement in peak positions with model compounds (25) and a complex of oxidized P. stutzeri cd1 with NO (27). Previously, the existence as a reaction intermediate of the d1Fe(II)-NO complex (often referred to as d1Fe(III)-NO) has been inferred from nucleophilic trapping experiments with 15O-labeled water or hydroxylamine (30, 31) or from the lack of an EPR signal following quenching of the reaction between reduced enzyme and nitrite (d1Fe(II)-NO) is EPR silent but d1Fe(II)-NO is active (4). In this work we have made kinetic measurements by the complementary techniques of absorption, EPR, and FTIR spectroscopies. These data are in excellent agreement. Thus, SF-FTIR is shown to be a valuable addition to the techniques used to study the interaction of metalloproteins with substrates and other ligands.

The mechanism of formation of the d1Fe(II)-NO+ species is complicated. The optical data presented here show that a reaction occurs within 4 ms of mixing reduced P. pantotrophus cytochrome cd1 with nitrite (Fig. 3b and Scheme 1). EPR confirms that at this time the enzyme has ferrous d1 heme with NO bound (i.e. d1Fe(II)-NO) and oxidized c heme (Fig. 5). The extent of the oxidation of the c heme (and stoichiometric formation of ferrous d1 heme-NO) varies with enzyme sample up to a maximum of ~90%. The conclusion we draw is that on addition of nitrite to reduced enzyme, the substrate binds to the d1 heme and is rapidly reduced by that heme. This is followed by transfer of the remaining electron on the monomer from the c heme to the heme d1-NO complex, with a rate constant of at least 500 s⁻¹.

These observations contrast with those of Silvestrini et al. (4), who concluded that reduced P. aeruginosa cd1 reacted rapidly with nitrite (in less than 2 ms) but to produce a ferric iron-NO complex at the d1 heme. This was followed by relatively slow electron transfer (k = 1 s⁻¹) from the c to the d1 heme to produce, presumably, the same d1 heme complex we observe after 4 ms. Although the origin of these differences is unclear, they may arise from variations in sequence and structure between the Paracoccus and Pseudomonas enzymes that result in substantially different electron transfer kinetics. It should also be noted that Silvestrini et al. performed their experiments at pH 8.0, whereas ours were conducted at pH 7.0. The optimum pH for P. aeruginosa cd1 in steady-state turnover is 6.5 (35); thus Silvestrini et al. may have been at sufficiently alkaline pH to change the character of the reaction.

The majority of literature reports for P. aeruginosa cd1 suggest that internal electron transfer (between the hemes within a monomer of the enzyme) occurs at ~1 s⁻¹, either in the absence of substrate or in the presence of nitrite (4, 36, 37). A pulse radiolysis experiment using P. pantotrophus cd1 hinted that faster internal electron transfer would be possible in that enzyme (21), but our data demonstrate it conclusively during the physiological reaction (k of at least 500 s⁻¹).

Between 4 and 100 ms after the reaction of P. pantotrophus cd1 with nitrite, optical and EPR data show that the c heme is re-reduced and the d1 heme center changes as a consequence of changes in oxidation and/or ligation state (Figs. 3, b and c, and 4 and Scheme 1). FTIR plays a key role in showing that ferric d1 heme-NO (formally d1Fe(II)-NO⁺) is formed. We suggest that the band observed in the absorption spectrum at 632 nm is this species, because its development is in excellent kinetic agreement with both the disappearance of the Fe(II)-NO EPR signal and the formation of the Fe(II)-NO⁺ signal seen by FTIR.

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5 This is consistent with the known tendency of the d1 heme to dissociate from the 97% identical enzyme from Paracoccus denitrificans (32). Enzyme lacking the d1 heme is catalytically inactive toward nitrite (33, 34).
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(Higs. 2, 4, and 6). Hence, despite the c heme having just reduced the $d_1$ heme, the $d_1$ heme now reduces the c heme until it is 45% ferrous (in the solution as a whole). From the EPR spectra there is no evidence that the His/His coordinated c heme seen in the ferrie “as stated” enzyme is formed in any quantity during the reaction. The observable components of the c heme EPR spectrum are the signals at $g = 2.93$ and 2.32, similar to those recently assigned to His/Met coordinated c heme by Koppenhöfer et al. (20) and Allen et al. (13). Kinetic data show that nitrite plays no part in this phase because the rate is independent of substrate concentration.

This remarkable oscillation of redox states of the heme centers requires that we comment on its possible origin. Reduction of nitrite (NO$_2^-$) by $cd_1$ involves two protons, believed to be provided by two conserved histidine residues, that extract an oxygen atom from the nitrite to produce water and NO (11). Nitrite is a bent molecule, and it is the nitrogen atom at the apex that is bound to the heme iron (9). Thus reduction of nitrite by ferrous $d_1$ heme will form nitric oxide with oxidized Fe-N-O geometry, bound to ferric heme. Typically, the preferred orientation of ferric heme-NO is linear (i.e. Fe(II)-NO$^-$), whereas the geometry of Fe(II)-NO is bent (38, 39). So if, for instance, adoption of a linear geometry is sterically hindered, it may be energetically favorable for the c heme to reduce the $d_1$ heme-NO complex to form Fe(II)-NO. The crystal structure of P. pantotrophus cd1 with NO bound (9) indicates that residues His-388, His-345, and Tyr-25 are all within hydrogen bonding distance of the product NO and could cause such steric hindrance. In that structure the Fe-N-O bond angle was 131°. We speculate that the $d_1$ Fe(II)-NO complex we detect after 4 ms is bent and formed essentially as described above. The second phase of the reaction may be reorientation of bound NO, with half of the $d_1$Fe(II)-NO complexes adopting linear geometry and re-reducing the corresponding c-hemes to produce the approximately equimolar mixture of cFe(II) $d_1$Fe(II)-NO$^+$ and cFe(II) $d_1$Fe(II)-NO that we observe after 100 ms.

Whether this oscillation of redox states is a physiologically relevant process also deserves comment. It is conceivable that were our experiments conducted in the presence of excess reductant we would not see formation of the $d_1$ Fe(II)-NO$^+$ species that we observe by infrared and correlate with the 632 nm visible absorption band. However, we propose that from a kinetic viewpoint this species is a genuine catalytic intermediate because it is formed at the rate of 38 s$^{-1}$ and the $k_{cat}$ of the enzyme, $-8$ s$^{-1}$, is much slower. In support of this view, we note that in a stopped-flow experiment where reduced enzyme was mixed with nitrite in the presence of excess ascorbate, formation of the 632 nm absorption band was clearly apparent. It is interesting that the turnover rate of the enzyme is so slow when the product NO, albeit bound to $d_1$ heme, forms at a rate of at least 500 s$^{-1}$. Thus product formation is not rate determining, and the catalytic cycle is kinetically controlled by a much slower process. It might be that in vivo conditions a much faster turnover rate would be observed. We note that, in vitro, copper nitrite reductases react with a $k_{cat}$ of the order of 50 s$^{-1}$ with no organic cofactor (3), whereas the much slower cytochrome $cd_1$ requires the biosynthetically complex heme $d_1$ (15).

The observation that the $d_1$Fe(II)-NO$^+$ complex is essentially stable at long times is startling. Almost all published mechanisms for reduction of nitrite by cytochrome $cd_1$ rely on this state spontaneously dissociating to allow the NO to leave. Our results suggest that the very slow third phase of the reaction (Scheme 1) arises from loss of the bound NO, followed by the binding of an additional nitrite leading to a further turnover and full oxidation of the enzyme. This process is dependent on the availability of sufficient excess substrate in the system and may well involve intermolecular electron transfer. The acceleration of this phase by intense light probably arises from photodissociation of the bound NO. In any case, this reactivity is far too slow to be physiologically relevant (P. pantotrophus does not require intense light for growth). This raises the crucial question of how the NO is released from the enzyme; no rapid reaction study, including this one, has detected kinetically competent NO release from cytochrome $cd_1$.

Although the experiments reported here do not reveal the mechanism of NO release, we have made several relevant observations. When fully reduced enzyme, in the absence of excess reductant, is mixed with nitrite it undergoes only one turnover when there are enough electrons on the monomer for two. Contrary to expectations, “ferric $d_1$-NO” does not spontaneously dissociate. Furthermore, we see absolutely no evidence of Tyr-25 rebinding to the $d_1$ heme during each catalytic cycle to displace the product. This was the mechanism proposed by Fülop et al. (11) based on the crystal structure, but if it were valid we would expect to see evidence both by absorption and EPR spectroscopies. Clearly something that is absent from our experiments must happen to complete the catalytic cycle.

Re-reduction of the enzyme by an external electron donor must cause product dissociation because it is possible to observe steady-state turnover. However, our data imply that an electron donor to $cd_1$ would have to reduce the electron-accepting ferric c heme, whereas the $d_1$ heme is ferrous with NO bound. Fully reducing this state of the enzyme yields the all-ferrous form of the enzyme previously described as an inactive “dead-end complex” (4). It is interesting that after 100 ms we have approximately 50:50 enzyme, with (in the bulk sample) half the enzyme having an oxidized heme $d_1$-NO accompanied by a reduced heme c, while half has reduced heme $d_1$-NO with an oxidized heme c. It may be that this distribution is not a simple equilibrium but arises from a conformational interaction across this dimeric protein, one monomer of each dimer having one electron distribution while its partner has the other. If so, it is possible that a relationship within the dimer controls product release. For instance, it may be that the three electron reduced dimer is the NO releasing state, whereas the all ferrous dimer is the “dead-end complex”. We note that in experiments where crystals of this enzyme soaked in nitrite and dithionite, the same ligation was never observed at both $d_1$ hemes in the dimer. In each case, nitrite was bound to the $d_1$ heme in one monomer, with NO or an unresolved ligand bound in the other (9). Thus, there is crystallographic evidence that during reduction of nitrite, the two monomers may not function completely independently.

In summary, we have shown that P. pantotrophus cytochrome $cd_1$ nitrite reductase readily forms a stable, oxidized heme $d_1$-NO complex as a product of the reaction between reduced enzyme and nitrite. We have made use of complementary techniques including stopped-flow FTIR, EPR, and absorption spectroscopy to examine product formation and to determine a plausible mechanism of the catalytic cycle until the point of NO release.

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