Very Early Reaction Intermediates Detected by Microsecond Time Scale Kinetics of Cytochrome cd₁-catalyzed Reduction of Nitrite*

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Paracoccus pantotrophus cytochrome cd₁ is a nitrite reductase found in the periplasm of many denitrifying bacteria. It catalyzes the reduction of nitrite to nitric oxide during the denitrification part of the biological nitrogen cycle. Previous studies of early millisecond intermediates in the nitrite reduction reaction have shown, by comparison with pH 7.0, that at the optimum pH, approximately pH 6, the earliest intermediates were lost in the dead time of the instrument. Access to early time points (~100 μs) through use of an ultra-rapid mixing device has identified a spectroscopically novel intermediate, assigned as the Michaelis complex, formed from reaction of fully reduced enzyme with nitrite. Spectroscopic observation of the subsequent transformation of this species has provided data that demand reappraisal of the general belief that the two subunits of the enzyme function independently.

Cytochrome cd₁ is a homodimeric enzyme found in the periplasm of denitrifying bacteria such as Paracoccus pantotrophus and Pseudomonas aeruginosa. It catalyzes the one electron reduction of nitrite to nitric oxide, which is the first committed step in the denitrification pathway of the biological nitrogen cycle (1, 2). There are two classes of enzyme that catalyze this reaction. The copper nitrite reductases, comprising the first category, contain an electron-accepting type I copper center (3). Cytochromes cd₁ belong to a second group of nitrite reductase (1, 2). These are heme-containing enzymes with one c heme and one d₁ heme per monomer. The c heme is the site of electron donation from external electron donor proteins; for the enzyme from P. pantotrophus, these have been shown to be pseudooxyazurin and cytochrome c₅₅₀ (4). The d₁ heme forms the active site of the enzyme. The 1.55-Å crystal structure of oxidized cytochrome cd₁ from P. pantotrophus reveals that in its oxidized as-isolated state, the c heme is axially ligated by histidines 69 and 17, and the d₁ heme binds histidine 200 and tyrosine 25 (5). The c heme is located in a predominantly α-helical domain of the enzyme, whereas the d₁ heme resides in a β-propeller structure. The tyrosine ligand to the d₁ heme is part of the N-terminal c heme domain and is connected to the c heme distal ligand, His-17, by a short polypeptide loop of just 8 amino acids. Upon reduction of the enzyme, His-17 is replaced by Met-106, and Tyr-25 dissociates leaving the active site pentacoordinate and able to bind substrate (5).

Our previous study of nitrite reduction by P. pantotrophus cytochrome cd₁ described the use of stopped flow methodology to study the kinetics of nitrite reduction by P. pantotrophus cytochrome cd₁ (6). At the earliest time point (2–3 ms) using a conventional stopped flow apparatus, at pH 6.0, a significant proportion of the enzyme had already undergone one turnover. At this time point three separate species were assigned at the d₁ heme, Fe(II)-NO, Fe(II)-NO⁺, and ferrous d₁ heme, with neither substrate nor NO bound (6). The latter, it was postulated, was an intermediate of the enzyme that had released product generated in the first turnover. The findings of this conventional stopped flow study indicated that a faster technique was required to observe intermediates of the first turnover.

A rapid freezing technique known as microsecond freeze-hyperquenching (MHQ)² has been developed in the de Vries laboratory (7, 8). This technique allows mixing and freezing of enzyme and substrate on a microsecond time scale, with an effective dead time of 75 μs (8), and thus enables previously undetectable intermediates to be observed. We report a completely novel species observed at the d₁ heme just 130 μs after mixing of the fully reduced enzyme with nitrite in the absence of excess reductant. The formation of other early intermediates was also observed within the first 800 μs of the reaction, and novel insight into their involvement in the catalytic cycle is presented; these intermediates provide evidence that the long standing assumption that the two monomers are independent needs reappraisal.

EXPERIMENTAL PROCEDURES

Production of Cytochrome cd₁.—P. pantotrophus was grown under anaerobic conditions at 37 °C. Cytochrome cd₁ was purified from the periplasm of the cells according to the method of Moir et al. (9) as modified by Koppenhöfer et al. (10). The purity of the enzyme was determined by the Rₚ value (A₄₀₅/A₂₈₀), and all cytochrome cd₁ used in this work had an Rₚ of >1.25. The

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‡ The abbreviations used are: MHQ, microsecond freeze-hyperquenching; FTIR, Fourier-transform infrared.

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concentration of the enzyme was determined at 406 nm for the oxidized enzyme and 418 nm for the reduced, with the respective extinction coefficients of 142.5 mM$^{-1}$ cm$^{-1}$ and 161.5 mM$^{-1}$ cm$^{-1}$ (10). These extinction coefficients refer to the concentration of the enzyme monomer. Throughout this work the monomeric concentration will be reported.

Anaerobic Preparation of the Enzyme for Rapid Kinetic Experiments—The purified enzyme was transferred to an anaerobic glove box (Coy Laboratory Products Inc.) that was maintained at less than 2 ppm O$_2$. Cytochrome $cd_1$ was reduced with a small excess of sodium dithionite and then passed down a desalting column packed with P6-DG resin (Bio-Rad) and equilibrated with 50 mM potassium phosphate of the desired pH. All buffers were sparged overnight in the anaerobic glove box to remove oxygen. The enzyme was loaded into a gas-tight syringe that had been presoaked in sodium dithionite and washed to remove traces of excess reductant. The absence of excess dithionite was confirmed by testing buffer expelled from the syringe with methyl viologen, which turns blue on contact with dithionite. 10 mM potassium nitrite was made up in 50 mM potassium phosphate buffer of the desired pH and loaded into a second gas-tight syringe that had undergone the same process as the enzyme-containing syringe to remove traces of oxygen. This was important because cytochrome $cd_1$ also functions as an oxidase (10).

MHQ—MHQ measurements were performed as described previously (7) and as modified by Ref. 24. Optical measurements were performed on an SLM-Aminco DW2000 scanning spectrophotometer, which was adapted for low temperature measurements of spectra of the frozen powders obtained from the MHQ experiments; the entire MHQ design and set up of the spectrophotometer are described in Ref. 7.

RESULTS

Fully reduced cytochrome $cd_1$ was mixed with potassium nitrite in the absence of excess reductant in the MHQ apparatus. Because cytochrome $cd_1$ also functions as an oxidase (10), great care was taken to ensure the equipment was sufficiently anaerobic to prevent re-oxidation of the enzyme, from which excess reductant had been removed, before mixing. To test that this requirement had been met, the enzyme was mixed with anaerobic phosphate buffer in the absence of excess reductant, and the optical spectrum produced was compared with the spectrum recorded when the enzyme was mixed with phosphate buffer in the presence of excess reductant. The position of the c heme Soret band (418 nm), which is characteristic of reduced c heme in this enzyme, and the $d_1$ heme spectral features at 460 and 653 nm both indicated that the enzyme remained fully reduced in the apparatus, even in the absence of excess reductant (Fig. 1 and Fig. 3 ($t = 0$ spectra)).

Fully reduced cytochrome $cd_1$ was mixed with potassium nitrite in the MHQ apparatus at pH 7.0, and the reaction was quenched at various time points between 130 μs and 11 ms after mixing to build up a profile of the early reaction interme-
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Conventionally, the percentage oxidation of P. pantotrophus cytochrome cd$_1$ is judged by the relative intensity of the c heme $\alpha$-band. However, this requires that spectra are first normalized according to protein concentration. Using the MHQ technique, it was not possible to determine the concentration of the enzyme in the final sample from which the optical spectra were produced because an unknown and slightly variable amount of cold reacted powder was mixed with cold isopentane. The percentage oxidation of the c heme of the enzyme can therefore only be judged by the position of the Soret band. The Soret band of P. pantotrophus cytochrome cd$_1$ shifts from 410 to 418 nm between its fully oxidized (His/Met coordinated) and fully reduced forms. Assuming a linear relationship between oxidation state and Soret position, the extent of c heme oxidation was estimated from the percentage Soret shift; however, the values obtained are inevitably approximate.

The final species seen after 11 ms at pH 7.0 still contains clear contributions from the 630 and 660 nm species that are also observed in the conventional time stopped flow experiments at this time point. It has been shown previously from later time points (6, 15) that this species decays over a period of 100 ms to produce a spectrum containing a single peak in the d$_1$ heme region with maximum absorbance at 630 nm. Stopped flow FTIR shows that the peak corresponding to d$_1$ Fe(II)-NO$^+$ forms at the same rate as the optical species at 630 nm, and therefore, as previously explained, the 630 nm absorbing species is assigned as containing d$_1$ Fe(II)-NO$^+$ (15).

The mixing of fully reduced cytochrome cd$_1$ (in the absence of excess reductant) with potassium nitrite was repeated at pH 6.0 because our recent study of the pH dependence of the nitrite reduction reaction on a slower time scale showed that at pH 6.0 the final reaction product was different and formed approximately three times faster compared with pH 7.0. The data in the current ultra-rapid reaction study show that at pH 6.0 a similar pattern of early intermediates to those at pH 7.0 was observed (Fig. 3). However, their lifetimes differed significantly from those at pH 7.0. As with the reaction at pH 7.0, a 620-nm peak was observed in the optical spectrum of the fastest time point (130 $\mu$s), which as before is assigned as the Michaelis complex of c Fe(II)-d$_1$ Fe(II)-NO$^+$.

At pH 6.0, oxidation of the c heme was similar to pH 7.0 with the majority of this oxidation occurring between 780 $\mu$s and 1.31 ms (Fig. 2). The most notable difference between pH 6.0 and 7.0 is that at pH 6.0 the species with a peak at 660 nm decayed on a much faster time scale (compare Fig. 1 and Fig. 3). At both pH 6.0 and pH 7.0, a small amount of absorbance at 660 nm was evident in the spectrum at 780 $\mu$s. By 1.31 ms in the pH 6.0 reaction, which is the time point at which the greatest level of c heme oxidation was observed, clear resolution of a peak at 630 nm, which was assigned as d$_1$ Fe(II)-NO$^+$, and an increase in intensity of the 660 nm peak occurred. Unlike at pH 7.0, where both the 660 and 630 nm absorbance peaks remained for longer than 11 ms after mixing of enzyme and substrate, at pH 6.0, the 660 nm peak decayed much faster and had substantially disappeared by 2.2 ms, leaving a species generating a single 630 nm peak by 11 ms.

The c heme remained essentially reduced for the first 410 $\mu$s of the reaction and then underwent oxidation between 410 $\mu$s and 1.31 ms. It then continued to oxidize further but at a much slower rate until the final time point at 11 ms. This second slower phase of oxidation was interpreted as a balance of continued oxidation in conjunction with the back reduction of the c heme shown by George et al. (15). After 11 ms, the c heme had reached ~55% oxidation (Fig. 1), which is again in good agreement with the conventional stopped flow studies of the nitrite reduction reaction at this pH (6, 15).
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FIGURE 2. Percentage oxidation of the c heme at pH 6.0 (---) and pH 7.0 (-----) at specific time points (indicated in Figs. 1 and 3) during the reaction of P. pantotrophus cytochrome cd₁ with potassium nitrite, which was in -10-fold excess to enzyme monomer. Enzyme and substrate were mixed in the MHQ apparatus at 25 °C, and the reaction was quenched at various time points. The percentage oxidation of the c heme was measured as the % shift between 418 nm (fully reduced His/Met coordinated) and 410 nm (fully oxidized His/Met coordinated).

DISCUSSION

This work exemplifies the novel insight into enzyme mechanisms, in this case cytochrome cd₁, which is available through the use of the recently developed MHQ apparatus. The novel species observed just 130 μs after mixing of enzyme and substrate, with an optical d₁ heme signature at 620 nm, is argued to be the Michaelis complex of fully reduced enzyme with nitrite bound at the d₁ heme, cFe(II)-d₁Fe(II)-NO₃⁻. A peak at 620 nm has not previously been observed during the catalytic cycle of cytochrome cd₁, and indeed there are very few references to d₁ heme complexes with absorbance maxima below 630 nm. The closest reported d₁ heme absorbance maximum to 620 nm is that of the d₁ heme pyridine hemochrome, d₁Fe(II)-bis-pyr, the absorbance maximum of which has been reported at 617 nm (16) and 620 nm (17). Also of note is that ferrous, cyanide-bound cytochrome cd₁ has an absorbance maximum at 628 nm (13, 18). Both the pyridine hemochrome and the ferrous CN⁻ bound species are examples of ferrous d₁ heme with strong field ligands. Nitrite is a strong field π-acceptor ligand, and the fact that these two complexes of ferrous d₁ heme, with strong field axial ligands, result in observed absorbance maxima below 630 nm supports the proposal that the 620-nm peak seen in this study arises from nitrite bound to ferrous d₁ heme, the Michaelis complex. The time scale on which the 620-nm peak is present (between 130 and 780 μs after mixing of enzyme and substrate) is also consistent with it being the Michaelis complex, given the length of the catalytic cycle, which is in the order of 14 ms at pH 7.0 and 8 ms at pH 6.0, as determined by Richter et al. (14). This also makes it unlikely that the first molecule of product NO is formed within the first 130 μs. The c heme apparently remains fully reduced at the first time point of 130 μs; thus no intramolecular electron transfer has occurred by this stage. Therefore, the only possibilities, other than the Michaelis complex, for the species absorbing at 620 nm, are ferric d₁ heme with either NO or nitrite bound; however, the above discussion of kinetic parameters argues against these possibilities. As reported in previous work (19), model spectra were obtained for the oxidized Y25S cytochrome cd₁ with nitrite and NO bound; it was not possible to use the wild type enzyme for these experiments because tyrosine 25 blocks the active site preventing exogenous ligand coordination to the oxidized enzyme. The Y25S enzyme is competent to exogenous ligand binding in its oxidized as-isolated state, and therefore circumvents this problem. No peak between 550 and 630 nm was seen for the Y25S enzyme with either nitrite or NO bound, further indicating that the 620 nm peak does not arise from either of these ligands bound to ferric d₁ heme. Recent work has invoked the possibility of electron donation from an amino acid side chain during nitrite reduction by cytochrome cd₁ (20), and therefore formation of cFe(II)-d₁Fe(II)-NO was also addressed. However, when the 130-μs sample was subjected to EPR, no signal arising from Fe(II)-NO was observed. The only logical assignment of the 620 nm peak, therefore, remains the Michaelis complex. At pH 7.0, following observation of the proposed Michaelis complex at 130 μs, the 620 nm absorbance maximum is seen to red shift and flatten. This is concurrent with another species being formed at the d₁ heme, the absorbance of which is not sufficiently separated from 620 nm to distinguish formation of a separate peak. There is considerable evidence for non-equivalence between monomers of P. pantotrophus cytochrome cd₁ (13, 21, 22), but the general assumption has been that the monomers are kinetically independent. However, it is possible that the residual absorbance at 620 nm, in conjunction with increased absorbance at longer wavelengths, reflects nitrite reduction occurring at monomer 1, whereas the Michaelis complex remains at monomer 2. By 1.31 ms, significant absorbance has become apparent at 660 nm, and the absorbance between 620 and 630 nm remains broad and flat although it is further shifted toward 630 nm. By 2.2 ms, the previously apparent absorbance at 620 nm appears to have significantly diminished, with the emergence of a much sharper peak at 630 nm and further increase in the absorbance at 660 nm. In previous work we have tentatively assigned the species with 660 nm absorbance as a high spin ferrous d₁ heme, which is either pen-tacoordinate or which has a 6th weak field ligand such as a water (6). The reason for the uncertainty of this assignment was because it is hard to justify how the d₁ heme, in the presence of a large excess of nitrite, could have anything bound other than this anion. However, in light of these new insights into much earlier time points in the reaction, several steps in the nitrite reduction reaction may, in fact, be gated by events occurring at the other cytochrome cd₁ monomer. The evidence for this is as follows: as already discussed, the earliest time point is assigned as the Michaelis complex of fully reduced enzyme with nitrite bound at the d₁ heme. Later time points show that this absorbance shifts to longer wavelengths, but no clear decrease in absorbance at 620 nm is observed until formation of the 660 nm species, at which time, clear absorbance at 630 nm is observed with a marked decrease in absorbance at 620 nm. A peak at 630 nm, later on in the reaction between cytochrome cd₁ and nitrite, has been shown to correspond to ferric d₁ heme with NO bound, formally d₁ Fe(II)-NO⁺. An interpretation of the observed absorbance changes at the d₁ heme is that reduction of nitrite to NO at monomer 2 cannot occur until product has
dissociated from monomer 1. Hence, residual absorbance at 620 nm, arising from the Michaelis complex at monomer 2, is observed until product dissociation occurs at monomer 1. If the assignment of the 660 nm species as ferrous pentacoordinate $d_1$ heme is accurate, then the product dissociated state of monomer 1 is evident from the 660 nm absorbance. As soon as the 660 nm absorbance becomes distinguishable in the spectral time course, the absorbance at 620 nm decreases markedly, and a 630 nm peak is apparent. This is consistent with the Michaelis complex at monomer 2 being converted to ferric $d_1$ heme with NO bound, as monomer 1 releases NO. As has been shown from later points in the time course of nitrite reduction by this enzyme (6, 15), the subsequent disappearance of the 660 nm peak occurs concomitantly with an increase in intensity at 630 nm. Again, this is consistent with the requirement for product formation at monomer 2 before rebinding of substrate is possible at monomer 1. It has been suggested in our previous work that at early time points in the reaction, when the 660 nm peak is observed at its maximum intensity, essentially no FTIR signal for $d_1$ Fe(II)-NO$^+$ is present (6). However, as is explained by George et al. (15), the FTIR signal is indicative of Fe(II)-NO$^+$ and not Fe(III)-NO. Fe(II)-NO$^+$ is predicted to adopt a linear geometry, whereas Fe(III)-NO is bent. It is therefore possible that in the absence of a driving force to effect product release, formation of Fe(II)-NO$^+$ at monomer 1 may force monomer 2 to adopt a different geometry, if non-equivalence of the monomers is a strict requirement. This proposal requires a reconsideration of the cytochrome $cd_1$ mechanism. Some evidence for non-equivalence between the monomers of cytochrome $cd_1$ exists. Of particular significance to the current work is that Williams et al. (22) observed nitrite at monomer A and NO at monomer B when crystals of cytochrome $cd_1$, which had been pre-reduced with sodium dithionite, were soaked in nitrite. In each crystal structure, monomer B appeared to have progressed further through the catalytic cycle than monomer A. Cooperativity between monomers has also been observed in cytochrome $cd_1$ from Pseudomonas stutzeri in which internal electron transfer is shown to be allosterically gated (23). No structure is available for the P. stutzeri enzyme; however, the N-terminal region of sequence shows significant difference to the $P$. pantotrophus enzyme. Our suggestion that catalytic events occurring at each monomer may be gated by the requirement for non-equivalence is supported by the previously described non-equivalence between monomers of $P$. pantotrophus cytochrome $cd_1$. Further support for this view comes from the evidence for cooperativity between monomers within cytochromes $cd_1$ from other sources.

Analysis of the oxidation state of the $c$ heme during the early phases of nitrite reduction also provides new mechanistic insight. At pH 7.0, the $c$ heme remains essentially fully reduced until 410 µs (Figs. 1 and 2). Between 410 and 780 µs, the $c$ heme substantially oxidizes, and a small amount of absorbance becomes visible at 660 nm. By 1.31 ms, the $c$ heme has reached about 50% oxidation, and the two separate peaks are visible at
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![Scheme 1](image)

**SCHEME 1.** The sequence of events deduced to be occurring at the two redox centers of cytochrome cd₁, within the first ~2 ms (depending somewhat on pH) following the binding of nitrite to the fully reduced enzyme. As discussed in the text, the exact timing of these events on the two monomers is thought to differ, but there is currently insufficient information to assign on a scheme the relative rates of the transitions on each subunit. The wavelength maxima of the assigned species are given, and the species enclosed in a box is an implied intermediate. The dashed arrow indicates the movement of an electron from the c to the d₁ heme; it is currently unclear whether this event occurs before or after the release of NO from the enzyme, i.e. whether NO is released from the ferric or ferrous d₁ heme.

630 and 660 nm (Fig. 1), corresponding to previously discussed species at the d₁ heme. It appears that formation of the 660 nm absorbing species may occur shortly after oxidation of the c heme, meaning that electron movement from the c to the d₁ heme may enable product release from this monomer. Until recently, it was assumed that NO dissociated from the ferric heme, which is generally regarded as having a lower affinity than ferrous heme for this ligand (24, 25). However, recent work has shown that NO can dissociate from ferrous d₁ heme on a time scale that is relevant to the catalytic cycle of the enzyme from *P. aeruginosa* (26, 27). The latter study showed that, in the presence of hemoglobin which is an NO scavenger, or the competitors CN⁻ or NO₂⁻, NO can dissociate sufficiently rapidly from the ferrous d₁ heme for the Fe(II)-NO state of the enzyme to be considered a possible intermediate on the catalytic pathway. The authors discuss the modifications of the d₁ heme in relation to standard b heme, and they suggest that these modifications may, in addition to increasing the affinity of the ferric heme for anions, decrease the affinity of the ferrous heme for the reaction product NO. NO dissociation from ferrous d₁ heme is not inconsistent with the present work.

At pH 6.0, the pattern of intermediate formation is essentially similar to that at pH 7.0. As for pH 7.0, there is an initial d₁ heme absorbance maximum at 620 nm 130 μs after mixing of enzyme and substrate, at which point the c heme is still fully reduced. There then follows shifting of the 620-nm peak toward longer wavelengths and eventually formation of the absorbance maximum at 660 nm, which occurs with rapid oxidation of the c heme. The main difference between the spectra at pH 6.0 and 7.0 is that at pH 6.0 the absorbance at 660 nm is significantly shorter lived. It has already decayed substantially by 2.2 s, the time point at which it is observed at maximum intensity at pH 7.0, and is completely lost by 11 ms at pH 6.0. Another major difference between the reactions at pH 6.0 and 7.0, as previously observed (6), is that the c heme oxidation in the final product ultimately reached is far more complete at pH 6.0 than at pH 7.0. According to the data presented here, the c heme reaches about 75% oxidation at pH 6. However, this value appears to be closer to 85% when determined by the more precise method previously described (6). In our previous study of the pH dependence of nitrite reduction by cytochrome cd₁, the faster decay of the 660 nm peak at pH 6.0 was attributed to the requirement for protonation of NO₃⁻ by histidines 345 and 388, which would be predicted to be significantly less protonated at pH 7.0 compared with pH 6.0. An aspect of the generally accepted mechanism for cytochrome cd₁ is that a double protonation of nitrite is followed by loss of water and retention of nitric oxide bound to the ferric form of the d₁ heme (or the isoelectronic Fe(II)-NO⁺ state) (28, 29). Considering the significant dependence on pH of the rate of decay of the species that absorbs at 660 nm, the rate-limiting step for this part of the reaction may be the proton-dependent dehydration of nitrite at the active site.

This work adds considerably to our recent studies of nitrite reduction by cytochrome cd₁; Scheme 1 presents a current view of the sequence of events within one monomer. The most significant development is the observation, for the first time, of the Michaelis complex of the fully reduced enzyme with nitrite bound at the d₁ heme. This has never been reported previously, and its observation in the current work sets a lower limit on the rate of formation of the first product of nitrite reduction at the d₁ heme. In addition, the progression of the reaction on the microsecond/low millisecond time scale directs attention to the interplay between the two monomers, an aspect of the enzyme that has previously attracted little attention.

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