Pulse Radiolysis Studies on Cytochrome cd_1 Nitrite Reductase from *Thiosphaera pantotropha*: Evidence for a Fast Intramolecular Electron Transfer from *c*-Heme to d_1-Heme†

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ABSTRACT: Electron transfer within cytochrome cd_1 from *Thiosphaera pantotropha* was investigated by the technique of pulse radiolysis. The reduction of the heme centers in this nitrite reductase occurred in two phases as judged from kinetic difference spectra. In the faster phase, radiolytically generated *N*-methylnicotinamide (NMA) radicals selectively reduced the *c*-heme of the enzyme. From the absorbance increase at 420 nm, a characteristic of formation of the ferrous *c*-heme, the second-order rate constant for this electron transfer process was estimated to be 3.8×10^9 M^{-1} s^{-1} at pH 7.0. In the slower phase, a decrease of absorption around 420 and 550 nm, corresponding to a reoxidation of the *c*-heme, was accompanied by an increase of absorption around 460 and 640 nm, characteristic of formation of the reduced d_1-heme. This indicated that an intramolecular electron transfer from the *c*-heme to the d_1-heme occurred. The first-order rate constant of this process was calculated to be 1.4×10^3 s^{-1} at pH 7.0 and was independent of the enzyme concentration. In the presence of nitrite the interheme electron transfer rate was not affected, but on a time scale of seconds a new species associated with the d_1-heme, having an absorption maximum at 640 nm, was detected and is proposed to reflect ligand binding to this heme. These results suggest the role of the *c*-heme as the electron acceptor site in cytochrome cd_1 and in mediating the electron transfer to the catalytic site of the enzyme. Moreover, the fast interheme electron transfer rate argues against this process being the rate determining step in catalysis.

The reduction of nitrate to nitrogen gas *via* nitrite, nitric oxide, and nitrous oxide is the biological process known as denitrification. The molecular mechanisms underlying these four important reactions are not well characterized. However, the recent description of high-resolution crystal structures for the two types of nitrite reductase participating in denitrification, the copper enzyme (Adman *et al.,* 1995; Godden *et al.,* 1991), and the cytochrome cd_1 enzyme (Fülöp *et al.,* 1995) provide frameworks for understanding the reduction of nitrite to nitric oxide at the molecular level.

The structure of cytochrome cd_1 showed that each monomer of the homodimeric enzyme contains within separate domains two redox centers, one a *c*-type heme and the other the d_1-heme that is unique to this type of enzyme. The *c*-type cytochrome domain is generally regarded as the entry point for electrons arriving from the respiratory chain *via* molecules like cytochrome c_{550} or pseudoazurin, while the Fe-atom of the d_1-heme is the site of catalytic reduction of nitrite to nitric oxide. The distance between these two redox centers within one monomeric unit is 11 Å if the heme−heme edge distance is considered and no more than 21 Å if the Fe−Fe distance is taken. Such a distance between the hemes is expected to be compatible with an interheme electron transfer rate on a submillisecond time scale (Moser & Dutton, 1996), yet in a stopped-flow study on the kinetics of oxidation of reduced cytochrome cd_1 from *Pseudomonas aeruginosa* a rate of only 1 s^{-1} was determined (Silvestrini *et al.,* 1990). Although the crystal structure of cytochrome cd_1 is that of the protein from *Thiosphaera pantotropha*, it is improbable that its structure will differ significantly from that of the enzyme from *P. aeruginosa*. Further investigation of the interheme electron transfer is clearly required.

A powerful approach for investigating electron transfer within proteins is that of pulse radiolysis through which an electron can often be introduced rapidly and selectively into one redox center of an enzyme (Kobayashi *et al.,* 1989, 1993; Kyritsis *et al.,* 1993; Suzuki *et al.,* 1994). The present paper describes application of this method to the cytochrome cd_1 nitrite reductase of *T. pantotropha* for which previously there has been no information available on rates of internal electron transfer. Here, we present observations of fast intramolecular electron transfer from *c*-heme to d_1-heme within the enzyme.

MATERIALS AND METHODS

Cytochrome cd_1 nitrite reductase was purified from *T. pantotropha* by the method of Moir *et al.,* (1993) with one modification. As the third chromatographic step, a FPLC Mono-Q ion exchange column (Pharmacia) was introduced which resulted in electrophoretically and spectroscopically pure enzyme ($A_{406}/A_{280} > 1.3$).

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The semi-apo form of cytochrome cd_1, containing only the *c*-type heme, was prepared as described by Hill and Wharton (1978) with some modifications. A solution of the purified enzyme in 50 mM phosphate buffer (pH 7.0) was added to acid−acetone with constant stirring. The mixture was incubated at 37 °C for 20 min, which caused precipitation of the protein and extraction of the d_1-heme into the acidic acetone phase. The red precipitate was collected by centrifugation at 3800*g* for 2 min. The semi-apoenzyme was washed twice with ice-cold acetone, and then resuspended in 50 mM phosphate buffer (pH 7.0) at 4 °C. The solution was dialyzed against the same buffer overnight.

Pulse radiolysis experiments were performed with an electron linear accelerator at the Institute of Scientific and Industrial Research, Osaka University (Kobayashi *et al.*, 1989, 1993; Suzuki *et al.*, 1996). The pulse width and energy were 8 ns and 27 MeV, respectively. The light source was a 150 W halogen lamp or a 1 kW xenon lamp. After passing through an optical path, the transmitted light intensities were analyzed and monitored by a fast spectrophotometric system composed of a Nikon monochromator, an R-928 photomultiplier, and a Unisoku data analyzing system. The concentration of *N*-methylnicotinamide (NMA)[1] radicals generated by pulse radiolysis was determined by absorbance change at 420 nm using a millimolar extinction coefficient of 3.2 mM^{-1} cm^{-1} (Hill & Anderson, 1991). This concentration could be adjusted by varying the dose of the electron beams.

Samples for pulse radiolysis were prepared as follows. Solutions of nitrite reductase contained 2 mM NMA and 0.1 M *tert*-butylalcohol (for scavenging OH·radicals) in 10 mM phosphate buffer (pH 7.0) and were deoxygenated in sealed cells by repeated evacuation and flushing with argon. The quartz cells had an optical path length of 0.3 cm or 1 cm. No effect of 0.1 M *tert*-butylalcohol on the optical absorption spectrum of the enzyme was seen. For each pulse, a new sample was used, even though pulse radiolysis did not cause any damage to the sample as judged by its visible absorption spectrum. Each data point was averaged over three to six pulses.

The concentrations of the oxidized holoenzyme and semi-apoenzyme were determined by using millimolar extinction coefficients of 285 mM^{-1} cm^{-1} at 406 nm and 220 mM^{-1} cm^{-1} at 420 nm, respectively. These values were obtained by correlating the visible spectrum with the Ala, Arg, His, Lys, and Tyr content of hydrolyzed enzyme for which the amino acid sequence is known (Baker *et al.*, 1997). Optical absorption spectra were measured with a Hitachi U-3000 or a Perkin-Elmer Lambda 2 spectrophotometer.

RESULTS

The visible difference spectrum (Figure 1) of cytochrome cd_1 from *T. pantotropha* is complex owing to the presence of two types of hemes. Assignment of the parts of the spectrum originating from the *c* and d_1-centers was possible by reference to the spectrum of the semi-apoenzyme (Figure 1), which contains only the *c*-type cytochrome. Thus, in the reduced form of the enzyme, the d_1-heme absorbs mainly between 450 and 470 nm plus between 580 and 730 nm; this is distinct from the principal absorption bands of the *c*-type cytochrome. However, in the oxidized state the d_1-heme absorbs also in the regions of the *c*-heme. Knowledge of the assignment of the spectrum in Figure 1 permits a clear interpretation of the pulse radiolysis experiments described below.

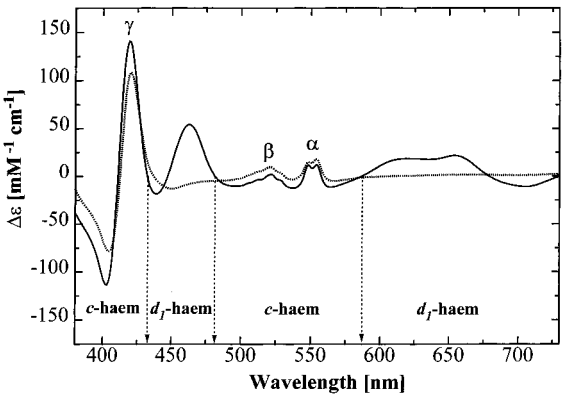

FIGURE 1: The visible difference spectrum of *T. pantotropha* cytochrome cd_1 (—) obtained by subtracting the spectrum of the fully oxidized form from the spectrum of the fully dithionite-reduced form. The difference spectrum of the semiapoenzyme (- - -) showing absorbance maxima only of the *c*-heme. In the reduced state, the d_1-heme absorbs mainly between 430 and 500 nm and between 600 and 700 nm, but in the oxidized state it also absorbs in the region of the *c*-heme.

Since hydrated electrons (e_{aq}^-) failed to reduce the hemes in cytochrome cd_1, *N*-methylnicotinamide (NMA) was used as a mediator. All the e_{aq}^-, generated by pulse radiolysis, reacted with the NMA present to give NMA radicals at an approximate concentration of 20 μM. The formation of these radicals is accompanied by an increase in absorbance at both 405 and 430 nm.

The radicals reacted very rapidly with the nitrite reductase, resulting in selective reduction of the *c*-type heme. An increase in absorbance at 420 nm and a decrease at 405 nm reflected this reduction (Figure 2). The initial transient increase in absorbance at 405 nm thus indicated the formation of NMA radicals; the corresponding absorbance change at 420 nm was not resolved. Subsequently, the initial changes in absorption reversed, indicating reoxidation of the *c*-heme, while absorption increases at 460 and 640 nm, which are characteristic of the reduction of the d_1-heme, were observed in the kinetic difference spectra, Figure 2. Variation in pH between 5.5 and 8 had little effect on the two phases of reduction.

After pulse radiolysis of the semi-apoenzyme, a similar but not identical (see later), absorption increase at 420 nm was observed; however, no subsequent absorption changes were seen (data not shown). This indicates that NMA radicals reduced the *c*-heme of both the holoenzyme and the semi-apoenzyme.

Figure 3 shows the kinetic difference spectra of the Soret region (A) and the α-β-region (B) at 100 μs and 2 ms after pulse radiolysis of the holoenzyme. The spectrum, corresponding to the faster phase, which has absorption maxima at 410 nm (Soret-band), 520 nm (β-band), and around 550 nm (α-band), is consistent with the reduction of *c*-heme. The kinetic difference spectrum for the slower phase has absorption maxima around 460 and 640 nm; these are characteristic for the reduced d_1-heme of cytochrome cd_1. From these findings, it can be concluded that the *c*-heme was reduced initially and, subsequently, the reoxidation of the *c*-heme and the reduction of the d_1-heme were observed.

[1] Abbreviations: e_{aq}^-, hydrated electron; NMA, *N*-methylnicotinamide.

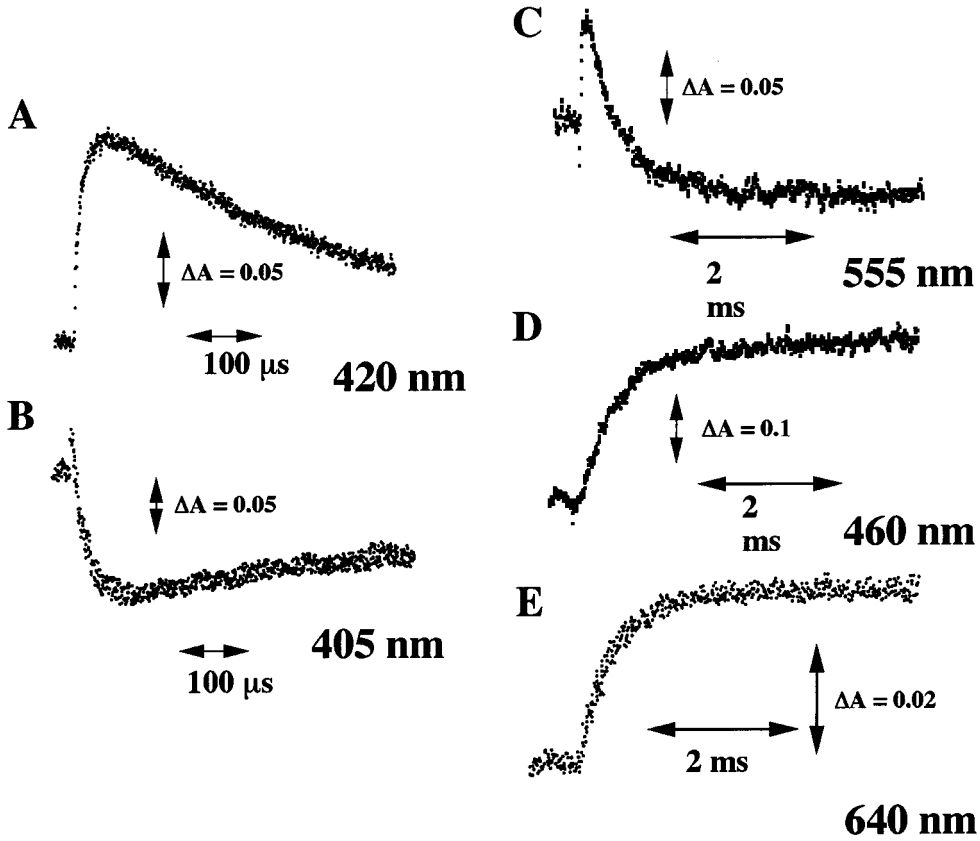

FIGURE 2: Absorption changes after pulse radiolysis of cytochrome cd_1 nitrite reductase measured at 420 nm (A), 405 nm (B), 555 nm (C), 460 nm (D), and 640 nm (E). Samples for spectra A and B contained 70 μM in a 0.3 cm optical path. Samples for spectra C, D, and E contained 28 μM enzyme in a 1 cm optical path. All samples contained 2 mM NMA and 0.1 M *tert*-butylalcohol in 10 mM phosphate buffer (pH 7.0).

For further data analysis, we compared the sum of the two transient spectra, corresponding to the faster and the slower phases (Figure 4) with the static difference spectrum of the fully reduced minus the fully oxidized forms of the holoenzyme (Figure 1). The similarity indicates that electron transfer from the *c*-heme to the d_1-heme occurs nearly quantitatively. It is noteworthy that the difference spectra in the Soret region differ in their maxima. The transient spectrum (Figure 4A) has an absorption maximum at 412 nm and an isosbestic point at 408 nm, whereas the steady state spectrum has an absorption maximum at 420 nm and an isosbestic point at 412 nm. However, the latter spectrum is similar to the kinetic difference spectrum obtained at 200 μs by pulse radiolysis of the semi-apoenzyme in Figure 5.

The reduction of *c*-heme obeyed pseudo-first-order kinetics and its rate constant increased with the concentration of the enzyme. (For these experiments, the concentration of NMA radicals was lowered to approximately 1 μM and the enzyme concentration was varied between 10 and 30 μM.) This indicates that the reduction of the *c*-heme is a consequence of a bimolecular reaction of NMA radicals with *c*-heme of the enzyme. The second-order rate constant of the reaction was calculated to be 3.8×10^9 M^{-1} s^{-1}. On the other hand, the increase of the absorption at 460 and 640 nm obeyed first-order kinetics. These rate constants of 1.4×10^3 s^{-1} were independent, within experimental error, of the enzyme concentration, over an 8-fold range. Therefore, the slower process is due to the intramolecular electron transfer from *c*-heme to d_1-heme in the enzyme.

It was of particular interest to study the spectroscopic changes and rate constants for the reduction of nitrite reductase in the presence of nitrite. As in the case without any substrate, the *c*-heme was initially reduced and, subsequently, interheme electron transfer was observed. The initial spectra of the *c*-heme and d_1-heme obtained after the pulse and the rate constants of the interheme electron transfer were not affected by the presence of nitrite in the range 100 μM to 1 mM. Following the spectral changes due to the reduction of the d_1-heme, however, an increase of absorption at 620 nm and a decrease at 460 nm were observed with a half-time of 100 ms in the presence of nitrite. In the absence of nitrite, the spectrum of the d_1-heme changed very slowly toward that corresponding to the initial oxidized d_1-heme.

Figure 7 shows the kinetic difference spectra at 2 ms and 2 s after pulse radiolysis of cytochrome cd_1 in the presence of 500 μM $NaNO_2$; clearly, only the d_1-heme difference spectrum was affected by the presence of nitrite. The spectroscopic changes at 2 ms account for the reduction of the d_1-heme and are identical to those in the absence of nitrite. This species generating the 2 s spectrum may be a NO-bound form of the oxidized d_1-heme. The species responsible in the presence of nitrite for the increase of absorption in the range 580−640 nm was stable, and persistent for at least 30 s after the pulse radiolysis.

DISCUSSION

The introduction of an electron from the NMA radical into the *c*-heme, also believed to be the electron entry point from physiological donors (Williams *et al.*, 1997), of cytochrome cd_1 nitrite reductase is followed by a relatively rapid electron transfer to the d_1-heme. The rate is in the same range as for the copper-containing nitrite reductase (Suzuki *et al.*, 1994), in which two copper centers are ca. 12.5 Å apart (Godden

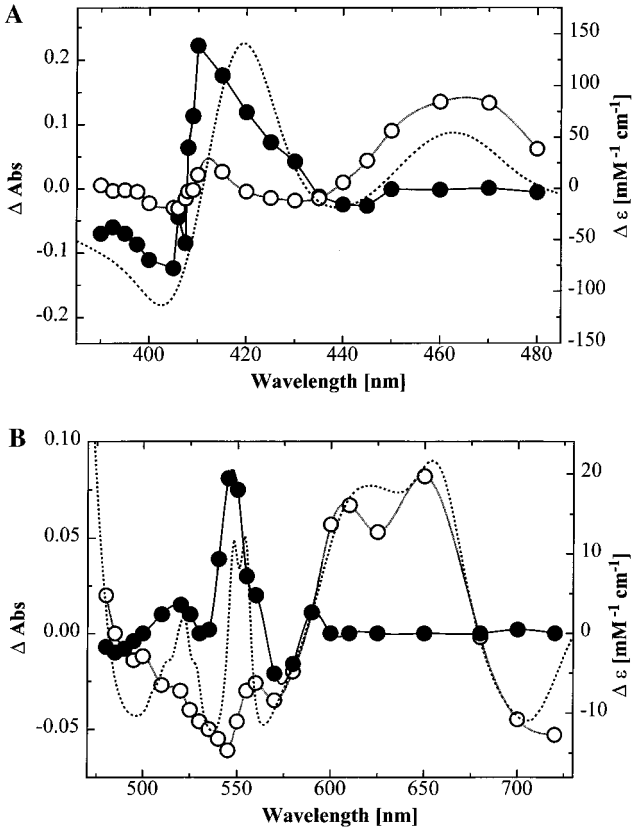

FIGURE 3: Comparison of the kinetic difference spectra from (A) 380 to 480 nm and (B) 480 to 730 nm at 100 μs (●) and 2 ms (○) after pulse radiolysis of cytochrome cd_1 with the visible reduced minus oxidized difference spectrum (···). Note that the left hand ordinate is in units of Δ absorbance corresponding to the kinetic difference spectrum, while the right-hand ordinate is in units of extinction coefficients corresponding to the reduced minus oxidized spectrum as shown in Figure 1.

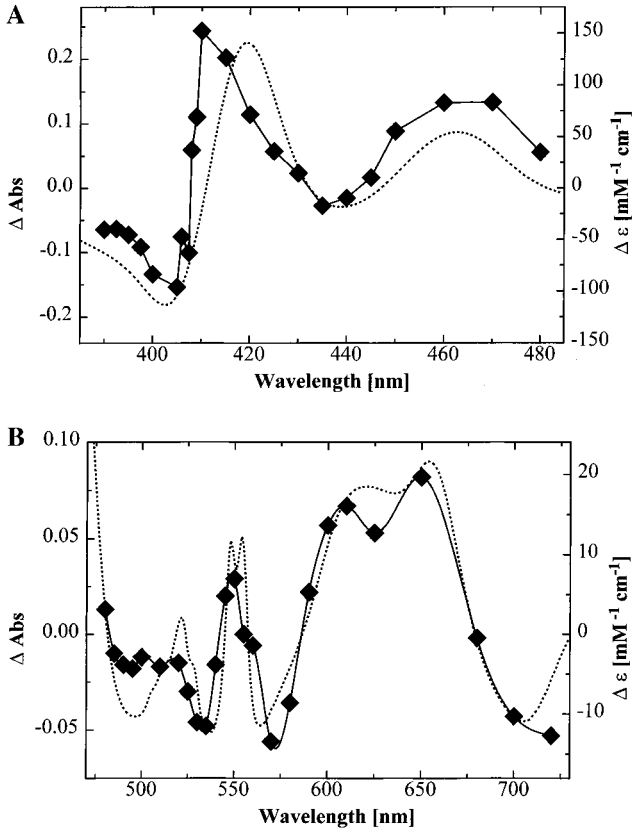

FIGURE 4: Comparison of the sum (◆) of the two kinetic difference spectra in the Soret region (A) and the α-β-region (B), obtained at 100 μs and 2 ms after pulse radiolysis, with the reduced minus oxidized difference spectrum (···) of cytochrome cd_1. Note that the left-hand ordinate is in units of Δabsorbance corresponding to the kinetic difference spectrum, while the right-hand ordinate is in units of extinction coefficients corresponding to the reduced minus oxidized spectrum as shown in Figure 1.

et al., 1991). The first-order rate constant for the interheme electron transfer in cytochrome cd_1 is approximately 10^3 times greater than that previously obtained for the cytochrome cd_1 from *P. aeruginosa* (Parr *et al.,* 1977; Silvestrini *et al.,* 1990). However, it is important to realize that rates were obtained for the *P. aeruginosa* enzyme under different conditions from those used in the present work in which the enzyme was initially fully oxidized and received only one electron, very rapidly, following pulse radiolysis. Thus the electron transfer event was

$$c^{2+}\, d_1{}^{3+} \rightarrow c^{3+} d_1{}^{2+}$$

In contrast, the work of Silvestrini *et al.,* (1990) involved observing the consequence of adding nitrite to the fully prereduced enzyme. Spectroscopic evidence (EPR) was presented (Silvestrini *et al.,* 1990) that nitric oxide was formed at the d_1-heme and that the internal electron transfer within the protein was

$$c^{2+} d_1{}^{3+}\text{-NO} \rightarrow c^{3+} d_1{}^{2+}\text{-NO}$$

On the other hand, Parr *et al.,* (1977) measured the electron transfer rate between hemes under the conditions where reduced azurin had first generated the c^{2+} state of the cytochrome cd_1 with neither nitrite nor NO present as potential ligands for the d_1 heme. The similarity between the electron transfer rates measured for the *P. aeruginosa* enzyme under these two sets of conditions is not necessarily

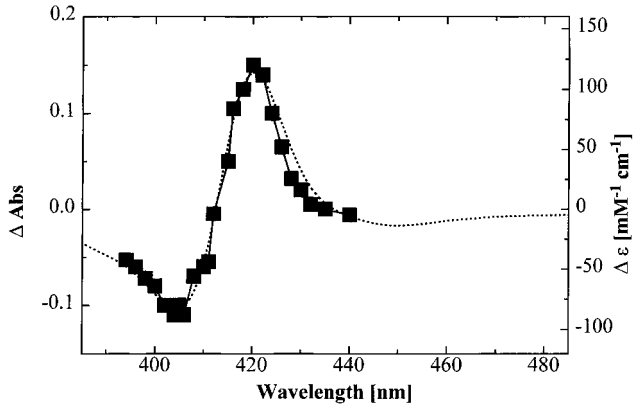

FIGURE 5: Comparison of the kinetic difference spectrum (■) for semiapoenzyme of cytochrome cd_1 at 200 μs after pulse radiolysis with the fully reduced minus oxidized visible difference spectrum (···) of the semiapoenzyme. The sample contained 28 μM semiapoenzyme, 2 mM NMA, and 0.1 M *tert*-butyl alcohol in 10 mM phosphate buffer (pH 7.0). Note that the left hand ordinate is in units of Δabsorbance corresponding to the kinetic difference spectrum, while the right-hand ordinate is in units of extinction coefficients corresponding to the reduced minus oxidized spectrum as shown in Figure 1.

expected and illustrates the complex factors that may influence electron transfer within cytochrome cd_1. The presence of NO bound to the Fe^{3+} form of d_1-heme might have been expected to increase very considerably the driving force for electron transfer from the *c*-heme. This is because the NO bound form of Fe^{2+} d_1-heme is expected to be much more stable than the Fe^{3+} form compared with Fe^{2+} d_1 heme

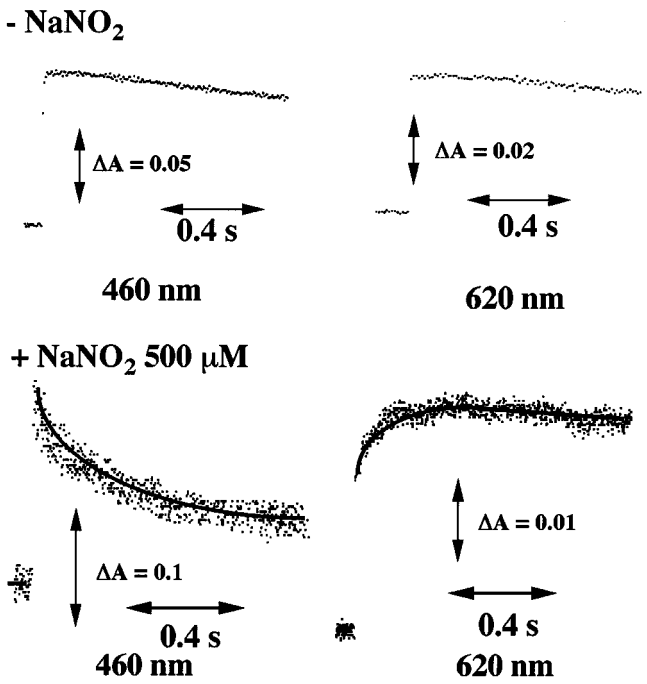

FIGURE 6: Absorption changes at two wavelengths after pulse radiolysis of cytochrome cd_1 in the absence and presence of $NaNO_2$. The sample contained 23.4 μM enzyme, 2 mM NMA, and 0.1 M *tert*-butylalcohol in 10 mM phosphate buffer (pH 7.0), with 500 μM $NaNO_2$ as appropriate.

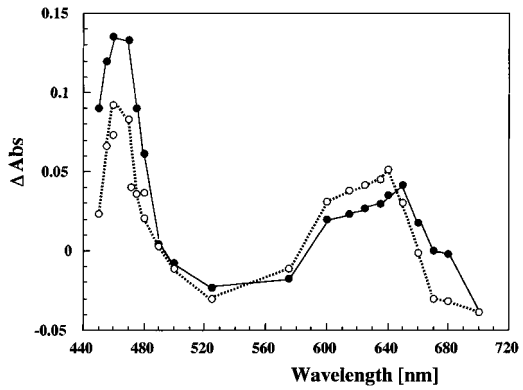

FIGURE 7: Kinetic difference spectra at 2 ms (●) and 2 s (○) after the pulse radiolysis of cytochrome cd_1 in the presence of 500 μM $NaNO_2$. The experimental conditions were the same as described in Figure 6.

relative to the Fe^{3+} state in the absence of the NO ligand. The failure of NO binding to accelerate the rate of electron transfer between the two hemes might be explained by changes in the redox potential of the *c*-type cytochrome center caused by binding of azurin to cytochrome cd_1 and/or the binding of NO to the d_1-heme, which would be analogous to the reported effects of CO binding to the d_1-heme (Silvestrini *et al.*, 1982). Alternatively, the presence of NO at the d_1-heme may cause such a large increase in the driving force that the Marcus inversion region is reached with the result that the rate of electron transfer to the d_1-heme is coincidentally similar to that in the absence of NO. Some support for this possibility may be taken from the finding of Greenwood *et al.*, (1978) that the presence of oxygen at the d_1-heme, which might reasonably be expected to increase the driving force for electron transfer from *c* to d_1 but to a lesser extent than binding of NO, results in a rate for this process of over 100 s^{-1}. However, whatever the basis for the electron transfer rates in the enzyme from *P. aeruginosa*, it nevertheless remains the case that they have all been measured under different conditions than used in the present work.

Cytochromes cd_1 from *T. pantotropha* and *Paracoccus* denitrificans are almost identical (Baker *et al.*, 1997, Fülöp *et al.*, 1995). A k_{cat} of 4 s^{-1} has been reported for the enzyme from *P. denitrificans* (Timkovich *et al.*, 1982). The much faster rate (>1000 s^{-1}) of interheme electron transfer measured here may thus mean that either this is not the rate-determining step or this rate becomes much slower when ligands are bound to the d_1-heme during catalysis (*cf.* previous paragraph).

Recently, the structure of the reduced form of cytochrome cd_1 has been determined by X-ray diffraction (Williams *et al.*, 1997). In the fully reduced enzyme, there are important ligand changes for both heme groups. A methionine has replaced one of histidines of the *c*-type cytochrome while one of the axial ligands to the d_1-heme iron, Tyr-25, has dissociated to leave a vacant coordination site. Thus, the electron transfer events monitored by the two techniques, pulse radiolysis and stopped-flow (Parr *et al.*, 1977, Silvestrini *et al.*, 1990), may take place from different initial structures of the enzyme, assuming that the high rate of reduction of *c*-type cytochrome by the NMA radical is so fast that no protein conformational change would be possible. Thus, we envisage that the electron transfer in the present work has occurred from a transiently formed reduced His/His state of the *c*-type cytochrome center while in the various studies of the *P. aeruginosa* enzyme the transfer is from a His/Met coordination, either because reduction of the enzyme has been followed by sufficient time to permit ligand switching to a His/Met form or because the *P. aeruginosa* enzyme never enters a His/His coordinated state. In the case of the *T. pantotropha* enzyme, it is possible, on the basis of precedent with other cytochromes, that the His/His ligation gives a lower redox potential than the His/Met state. Thus, in the present work there could be a larger driving force for electron transfer from *c* to d_1-heme and, thus higher rate than, in circumstances where the electron transfer is from the reduced His/Met coordinated *c*-cytochrome center. We expect that this coordination would be present in the *T. pantotropha* enzyme if it were subject to the same experiments are those done by Silvestrini *et al.*, (1990) with the *P. aeruginosa* enzyme. Although the structures of the oxidized and reduced states of the enzyme indicate that the distance separating the two hemes is similar in each form (Williams *et al.*,, 1997), it is possible that other relative factors in the two oxidation states, for example, reorganization energy, can account for the difference in rate. Clearly, it will be important to carry out similar stopped-flow experiments with *T. pantotropha* enzyme to those performed with the protein isolated from *P. aeruginosa*.

An important issue is whether the above assumption that the rapid arrival of an electron at the *c*-heme of cytochrome cd_1 did not trigger the histidine to methionine ligand switch. The kinetic spectrum, obtained after pulse radiolysis, of the *c*-type cytochrome is not identical, especially in the Soret region where there are distinct maxima are 412 and 420 nm, to that of the static reduced enzyme (Figure 4A). This suggests that the reduction of *c*-type cytochrome center after the pulse is not accompanied by an axial ligand switch. It follows that this switch may be driven by the reduction of the d_1-heme and/or the arrival of a second electron at *c*-heme. The reduction of the d_1-heme would thus displace the Tyr-25 from the ferrous heme, and this change may trigger the

axial ligand switch of the c-type cytochrome domain. X-ray diffraction analysis of the enzyme (Fülöp *et al.*, 1995) shows that the main chain of the α-helical *c*-type cytochrome domain makes an excursion into the d_1-domain, providing Tyr-25 of the *c*-domain as one of the axial ligands to the iron of the d_1-heme, though the two types of heme are located in separate domains.

In the semi-apoenzyme, Tyr-25 cannot be anchored to the d_1-heme, and it is not known whether the oxidized state of this form of the enzyme has His/His or His/Met coordination of the *c*-type cytochrome center. However, the close correspondence between the Soret region spectrum of the semi-apoenzyme within 200 μs of pulse radiolysis and the corresponding spectra of the fully reduced holo or semi-apoenzyme suggests that, in each case, a reduced His/Met coordination *c*-type cytochrome is observed. Because of the rate of reduction of this redox center following pulse radiolysis, these similarities in spectra suggest His/Met coordination in the oxidized state of semi-apoenzyme.

Previous reports showed that the electron equilibrates between type-I Cu and type-II Cu in copper-containing nitrite reductase (Suzuki *et al.*, 1994; Suzuki *et al.*, 1997) and between copper A and the *a*-heme in bovine cytochrome oxidase (Kobayashi *et al.*, 1989) following reductions of the type-I copper of nitrite reductase or copper *A* of cytochrome oxidase by pulse radiolysis. These results reflect the relatively small difference between the redox potentials of the pairs of sites in the two enzymes. In the present experiments, on the other hand, following the reduction of the *c*-type cytochrome center on the 100 μs time scale, there was an essentially quantitative transfer of the electron to d_1-heme. This requires that, at least in the structural state of the enzyme studied, the redox potential of two heme groups must differ by approximately 100 mV. This is a bigger difference than has been reported, 7 mV, hitherto for cytochrome cd_1 from other sources (Schichman & Gray, 1981). Unfortunately, attempts to determine the redox potential of the *c* and d_1 centers in cytochrome cd_1 of *T. pantotropha* by equilibrium redox potentiometry have been thwarted by nonreversible behavior. This implies the existence of interactions between the *c*-heme and d_1-heme in the enzyme.

The results presented here provide the following important implications for the catalytic mechanism of the enzyme. The spectrum of the d_1-heme and the intramolecular electron transfer from *c*-heme to d_1-heme observed after the pulse were not affected by the presence of nitrite. This result suggests that nitrite binds to the vacant coordination site of the reduced d_1-heme after dissociation of the Tyr-25, as observed by Williams *et al.*, (1997). Subsequent reduction of nitrite to nitric oxide and concomitant oxidation of the d_1-heme would give Fe^{3+}-NO, which could account for the spectrum, Figure 7, obtained at 2 s after the pulse in the presence of nitrite. Addition of either nitric oxide or nitrite to oxidized cytochrome cd_1 does not generate the spectrum reported here. This may be because, whereas in the present experiments the d_1-hemes have been reduced with concomitant opening of its binding site (Williams *et al.*,, 1997), the binding of nitric oxide or nitrite to oxidized cytochrome cd_1 is prevented by the tyrosine ligand to d_1-heme (Fülöp *et al.*, 1995).

The process of the reduction of nitrite to nitric oxide by the d_1-heme would correspond to the slower process of spectroscopic changes due to d_1-heme observed in Figure 6; this is supported by the rate constants of this process being independent of the concentrations of the enzyme and nitrite. From stopped-flow analysis of the reaction between the reduced cytochrome cd_1 from *P. aeruginosa* and nitrite, on the other hand, the reduction of nitrite to nitric oxide by d_1-heme is very fast, being lost within the dead time of a rapid mixing apparatus (Silvestrini *et al.*, 1990). This difference between the two types of experiments could be due to the oxidation state of the *c*-heme. In the catalytic process, the subsequent reduction step of d_1-heme with an electron from *c*-heme is an important process. In the present experiment, however, we can follow the events at d_1-heme only after one-electron reduction of the enzyme. Unfortunately, the methology of pulse radiolysis does not allow one to add a second electron to the enzyme from NMA radicals (only a small cross section of a sample is analyzed and diffusion would occur between the first and second pulses); otherwise, it would have been of great interest to have monitored the time dependence of the *c*-heme and d_1-heme spectra following addition of a second electron.

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