Intramolecular Electron Transfer from *c* Heme to d_1 Heme in Bacterial Cytochrome cd_1 Nitrite Reductase Occurs over the Same Distances at Very Different Rates Depending on the Source of the Enzyme†

Kazuo Kobayashi,*,‡ Alrik Koppenhöfer,§ Stuart J. Ferguson,§ Nicholas J. Watmough,|| and Seiichi Tagawa‡

The Institute of Scientific and Industrial Research, Osaka University, Mihogaoka 8-1, Ibaraki, Osaka 567-0047, Japan, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K., and Centre for Metalloprotein Spectroscopy and Biology, School of Biological Science, University of East Anglia, Norwich NR4 7RU, U.K.

Received November 3, 2000; Revised Manuscript Received May 14, 2001

ABSTRACT: Intramolecular electron transfer over 12 Å from heme *c* to heme d_1 was investigated in cytochrome cd_1 nitrite reductase from *Pseudomonas aeruginosa*, following reduction of the *c* heme by pulse radiolysis. The rate constant for the transfer is relatively slow, $k = 3\ s^{-1}$. The present observations contrast with a corresponding rate of electron transfer, $1.4 \times 10^3\ s^{-1}$, measured for cytochrome cd_1 from *Paracoccus pantotrophus*, though the relative positions of the two heme groups are the same in both enzymes. The rate of intramolecular electron transfer within the enzyme from *P. aeruginosa* was accelerated 10^4-fold ($1.4 \times 10^4\ s^{-1}$) by the binding of cyanide to the d_1 heme. A coordination change at the d_1 heme upon its reduction is suggested to be a major factor in determining the slow rate of electron transfer in the *P. aeruginosa* enzyme in the absence of cyanide.

Cytochrome cd_1 is a bacterial nitrite reductase that has a respiratory function and is located in the periplasm. The enzyme catalyzes the reduction of nitrite to nitric oxide ($NO_2^- + e^- + 2H^+ \rightarrow NO + H_2O$). Each monomer of the homodimeric molecule contains two heme groups, a *c*-type cytochrome and a d_1 heme. The *c*-heme is the electron acceptor site in cytochrome cd_1 and mediates the electron transfer to the catalytic site heme d_1 of the enzyme. High-resolution X-ray crystal structures have recently been obtained for the oxidized cytochrome cd_1 molecules from *Paracoccus pantotrophus* (formerly *Thiosphaera pantotropha*) (*1*) and *Pseudomonas aeruginosa* (*2*). Remarkably, these structures show substantial differences. Whereas an essentially identical eight-bladed β-sheet propeller structure binds the d_1 heme in protein from both sources, the organization of the N-terminal cytochrome *c* domain differs. The latter domain of nitrite reductase from *P. aeruginosa* (NIR-Ps)[1] has a typical class I *c*-type cytochrome fold with axial His and Met ligands to the heme iron. In contrast, the *c* cytochrome center in nitrite reductase from *P. pantotrophus* (NIR-Par) is His/His coordinated, and the corresponding Met residue to that found as a heme ligand in NIR-Par is at the bottom of a loop some distance from the heme iron. A further difference occurs with respect to the ligation of the d_1 heme iron. In enzymes from both sources the proximal ligand is His; the distal ligand in NIR-Par is Tyr25, which is only eight residues further along the polypeptide chain than one of the His ligands to the *c*-type cytochrome center. In contrast, in the structure of NIR-Ps, the second d_1 heme ligand is a hydroxide which in turn is bonded to Tyr10, which is not structurally equivalent to Tyr25 in NIR-Par. However, despite the differences in both heme iron ligation and tertiary protein structure between the cytochrome cd_1 from the two sources, the relative positions of the hemes are the same, in terms of both edge to edge cofactor distance (11.4 Å for NIR-Ps, 11.0 Å for NIR-Par) and their relative orientation (60°) to each another.

The conformation of cytochromes cd_1 from both sources depends on the redox state of the protein, and major changes in the structural organization were found to occur upon reduction. The most dramatic occur for NIR-Par where the His17 ligand to the Fe of the *c*-type cytochrome center is replaced by Met106 and the Tyr25 is displaced from the d_1 heme iron with the result that this iron becomes five coordinate (*3*). In the case of NIR-Ps, the conformational changes are less extensive but nevertheless cause loss of the hydroxide ion that is a ligand to the d_1 heme of the oxidized enzyme (*4*, *5*). In both cases, very recently, it has been proposed that a loop of the *c* heme domain (56−62 in NIR-Ps, 99−162 in NIR-Par) moves following the reduction of the d_1 heme rather than as a consequence of the reduction of the *c* heme (*5*).

Several studies have shown that the rate of electron transfer between the *c* and d_1 hemes in NIR-Ps is of the order of 1 s^{-1} (*6*−*8*). This is a strikingly low rate in the context of the

† This work was supported by Grant-in Aid 08249104 for scientific research on the priority area molecular biometallics, Grant-in Aid 12147205 from the Japanese Ministry of Education, Science and Culture (K.K.), the European Community (Grants ERB FMB 1CT95 0066 and BioTECH BI104 CT96-0281), the Sasakawa Fund of Oxford University, the Biotechnology and Biological Research Council of the U.K. (Grant B11988) (S.J.F.), and a Career Development Award from the Wellcome Trust (042103/2/94/2) to N.J.W.

* To whom correspondence should be addressed: fax, +81-6-6876-3287; e-mail, kobayasi@sanken.osaka-u.ac.jp.

‡ Osaka University.

§ University of Oxford.

|| University of East Anglia.

[1] Abbreviations: NIR-Ps, nitrite reductase from *Pseudomonas aeruginosa*; NIR-Par, nitrite reductase from *Paracoccus pantotrophus*; NMA, *N*-methylnicotinamide.

10.1021/bi002534i CCC: $20.00
Published on Web 06/23/2001

heme to heme separation of only 11 Å. It is important to note that the interheme electron-transfer rate within NIR-Ps is accelerated to ca. 100 s^{-1} when oxygen is bound at the d_1 heme (*9*). In the case of NIR-Par, on the other hand, this rate has been determined under two sets of conditions. Pulse radiolysis experiments showed that the *c* to d_1 electron-transfer rate within NIR-Par was 1.4×10^3 s^{-1} (*10*). A different approach showed that reoxidation of ascorbate-reduced enzyme by oxygen resulted in electron transfer from the *c* to the d_1 heme at a rate of over 100 s^{-1} (*11*). Thus to date the very slow (1 s^{-1}) electron-transfer rate identified in NIR-Ps has not been observed for NIR-Par. The difference in structure between the oxidized forms of NIR-Ps and NIR-Par, together with the much larger structural change that occurs upon reduction of the latter, means that the interheme electron transport rates may differ in an instructive manner between the two proteins. A very significant difficulty in comparing the rates obtained hitherto for cytochrome cd_1 from the two sources is that the experiments have been done under completely different conditions, as discussed before (*10*).

The pulse radiolysis approach (*10*, *12*−*15*) has several features that are distinct from those of other methods used to study electron transfer within proteins. First, it permits the extremely rapid donation of a single electron to a metal center in an enzyme; in the case of NIR-Par this was previously shown to be specific for the *c*-type cytochrome center (*10*). The rate of reduction was such that the absence of any conformational change could be assumed. Second, the method does not require any substrates of the enzyme; thus complications arising from kinetic constraints imposed by chemical events at, for example, the d_1 heme center or site of docking of an electron donor protein, will not occur. Indeed, these considerations mean that extrapolation cannot automatically be made from the results obtained with the pulse radiolysis technique applied to NIR-Par to those obtained using different approaches on NIR-Ps. Thus the understanding of the electron-transfer process between the two types of heme in cytochrome cd_1, and the consequences for this process of the difference in structure, requires the same conditions to be applied to both enzymes. This paper presents the first such study, describing the results of studying electron transfer within NIR-Ps using the pulse radiolyis technique previously applied to the NIR-Par enzyme (*10*).

MATERIALS AND METHODS

NIR-Ps was purified by the method of Parr et al. (*16*).

Pulse radiolysis experiments were performed with an electron linear accelerator at the Institute of Scientific and Industrial Research, Osaka University (*10*, *12*−*15*). The pulse width and energy were 8 ns and 27 MeV, respectively. The concentration of *N*-methylnicotinamide (NMA) 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} (*17*). The reactions were carried out at 20 °C.

Samples for pulse radiolysis were prepared as follows. Solutions of the enzyme contained 2 mM NMA and 0.1 M *tert*-butyl alcohol (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 or 1 cm.

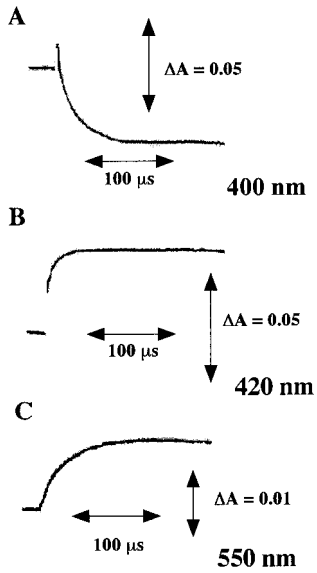

FIGURE 1: Absorption changes on the microsecond time scale after pulse radiolysis of cytochrome cd_1 nitrite reductase from *P. aeruginosa* measured at 400 nm (A), 420 nm (B), and 550 nm (C). Samples contained 70 μM enzyme in a 0.3 cm optical pathway cell for spectra A and B and a 1 cm path for spectrum C, respectively. All samples contained 2 mM NMA and 0.1 M *tert*-butyl alcohol in 10 mM phosphate buffer (pH 7.0).

The concentration of the oxidized NIR-Ps was determined by using millimolar extinction coefficients of 282 mM^{-1} cm^{-1} at 410 nm (*18*). Optical absorption spectra were measured with a Hitachi U-3000 or a Perkin-Elmer Lambda 2 spectrophotometer.

RESULTS

The pulse radiolysis experiment involves the almost instantaneous generation of NMA radicals which in turn can reduce a redox center within a protein. Our previous report showed that the NMA radical gave a rapid and specific reduction of the *c*-type heme center of NIR-Par (*10*). A similar result was also obtained with NIR-Ps, as shown in Figure 1. This is apparent from the decrease in absorbance at 400 nm and increases in absorbance at 420 and 550 nm, characteristic of *c* heme reduction, and the lack of absorbance increases around 460 nm (data not shown), characteristic of d_1 heme reduction, observations that are diagnostic of reduction of the *c*, but not of the d_1, heme iron centers on the microsecond time scale. The initial transient increase in absorbance at 400 and 420 nm indicated the formation of NMA radicals. A second-order rate constant of 2×10^9 M^{-1} s^{-1} could be calculated for the reduction of the enzyme by NMA radicals, a comparable value to that found previously with NIR-Par (3.8×10^9 M^{-1} s^{-1}). However, subsequent events were quite different for NIR-Ps (*10*). Whereas the electron passed essentially quantitatively to the d_1 heme on the millisecond time scale in NIR-Par, the transfer between the hemes within NIR-Ps took place more slowly, as shown in Figure 2. Following the initial very rapid reduction of the enzyme at the *c*-type heme by NMA radicals, there was a much slower change in absorbance at 400 and 420 nm and an increase at 460 nm, corresponding to the oxidation of the *c* and the reduction of the d_1 heme centers, respectively. Only after 5 s had the electron-transfer process been completed.

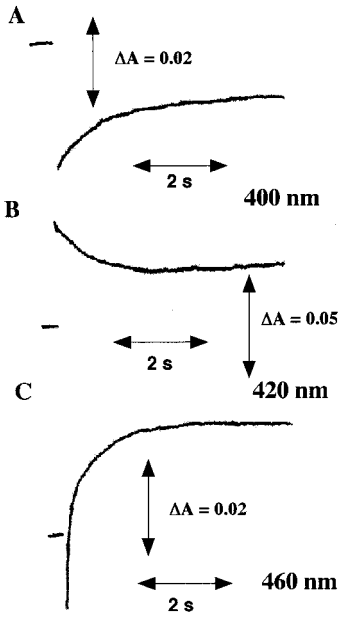

FIGURE 2: Absorption changes on the seconds time scale after pulse radiolysis of cytochrome cd_1 nitrite reductase from *P. aeruginosa* measured at 400 nm (A), 420 nm (B), and 460 nm (C). Samples contained 70 μM enzyme in a 0.3 cm path. All samples contained 2 mM NMA and 0.1 M *tert*-butyl alcohol in 10 mM phosphate buffer (pH 7.0).

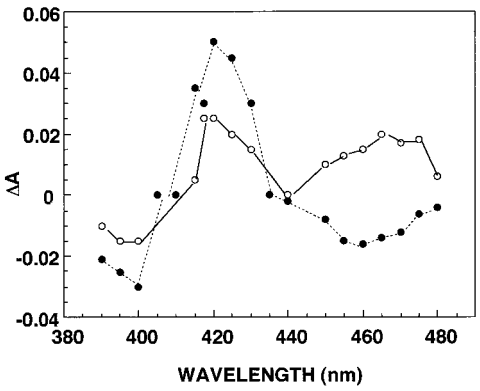

FIGURE 3: Kinetic difference spectra at 100 μs (●) and 5 s (○) after the pulse radiolysis of cytochrome cd_1 nitrite reductase from *P. aeruginosa*. The experimental conditions were as described in Figure 1.

The calculated first-order rate constant for electron transfer from the *c* to d_1 heme was 3 s^{-1}. The intramolecular nature of the process was established by observing that this rate constant was independent of the enzyme concentration in the range from 30 to 240 μM (data not shown). The slow rate for NIR-Ps (3 s^{-1}) is consistent with that obtained by the stopped-flow method (0.2−1 s^{-1}) (*6*−*8*) under certain completely different conditions.

Figure 3 shows the kinetic difference spectra in the Soret region at 100 μs and 5 s after pulse radiolysis of NIR-Ps. The spectrum at 100 μs, which has an absorption maximum at 420 nm, is consistent with the reduction of *c* heme. At 5 s after the pulse, an absorption increase around 460 nm appeared together with a decrease in absorbance at 420 nm. However, the absorbance at 420 nm (or 400 nm) did not return to its initial value, and about 50% of the initial absorption change remained after 5 s. Thus, only approximately half of the electrons originally delivered to the

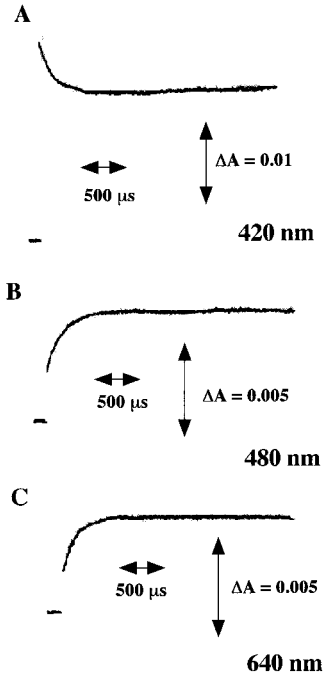

FIGURE 4: Absorption changes after pulse radiolysis of cytochrome cd_1 nitrite reductase from *P. aeruginosa* measured at 420 nm (A), 480 nm (B), and 640 nm (C) in the presence of KCN. Samples contained 12.5 μM enzyme, 2 mM NMA, 2 mM KCN, and 0.1 M *tert*-butyl alcohol in 10 mM phosphate buffer (pH 7.0).

c-type cytochrome center were passed on after 5 s to the d_1-type heme. This can be explained by an equilibrium constant close to unity for electron transfer between *c* and d_1 heme. This is consistent with the reports (*19*, *20*) of a relatively small difference between the redox potentials of hemes *c* and d_1.

Reduction of the d_1 heme of NIR-Ps is known to correlate with change in coordination of the Fe atom (*21*) which might be connected with a slow electron-transfer rate. It was of interest to study effects of the binding of cyanide to the d_1 heme on the intramolecular electron transfer within the NIR-Ps enzyme. Electron transfer should occur without any rearrangement at the two heme centers, because cyanide binds both ferrous and ferric heme d_1 (*22*, *23*). As in the case without cyanide, the *c* heme was reduced first, and subsequently, interheme electron transfer occurred. The rate constant for the reduction of *c* heme by the NMA radical was not affected by binding of cyanide to d_1 heme (data not shown). The initial absorbance increase at 420 nm subsequently reversed (Figure 4A), indicating the reoxidation of heme *c*. Concomitantly, the absorption at 480 and 640 nm, characteristic of a ferrous−CN d_1 heme, increased with a half-time of 50 μs (Figure 4B,C). Figure 5 shows the kinetic difference spectra in the Soret region at 100 and 500 μs after the pulse. The initial spectrum of the *c* heme was not affected by binding of cyanide to the d_1 heme. Formation of ferrous−CN d_1 heme was supported by the kinetic difference spectrum, with a characteristic broad absorption from 460 to 500 nm, obtained at 500 μs after the pulse (Figure 5). The rate constant for this process (1.4×10^4 s^{-1}) was independent, within experimental error, of both the enzyme and cyanide (1−4 mM) concentrations. Therefore, this process is due to the intramolecular

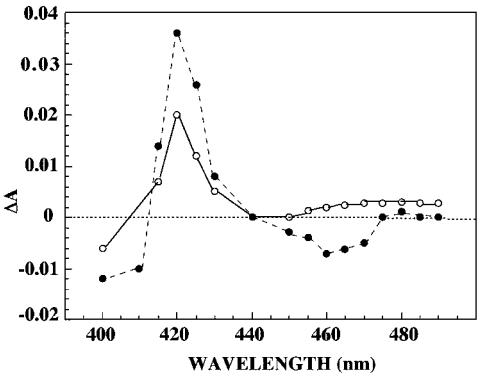

FIGURE 5: Kinetic difference spectra at 100 μs (●) and 500 μs (○) after the pulse radiolysis of cytochrome cd_1 nitrite reductase from *P. aeruginosa*. The experimental conditions were as described in Figure 4.

electron transfer from *c* heme to CN-bound d_1 heme, as follows:

$$c^{2+}d_1{}^{3+}-\mathrm{CN} \rightarrow c^{3+}d_1{}^{2+}-\mathrm{CN}$$

The absorbance at 420 nm did not return to its initial value (Figure 4A), just as observed without cyanide. This suggests that the redox potential of the d_1 heme may not be greatly affected by the binding of CN^-, rather than hydroxide, to the d_1 heme, at least under the conditions of the pulse radiolysis experiment. However, an alternative explanation stems from the previously described effects of ligand binding to the d_1 heme on the potential of the *c* heme (*24*). Thus, coincidentally, it may be that the difference in reduction potentials between the two hemes changes little upon introduction of cyanide at the d_1 heme. This is consistent with the results reported (*20*), where the difference in reduction potential between the two hemes (−25 mV) does not change upon cyanide binding. We note that there are variations among the published values of reduction potentials for the two hemes in NIR-Ps cytochrome cd_1 (*19*, *24*). However, although precedent with some other heme proteins might suggest that an anionic ligand such as hydroxide or cyanide can lower the redox potential to ca. −200 mV (*25*), there is no evidence that this is the case for cytochrome cd_1 (*24*). The factors determining the redox potential of the unusual d_1 heme center, with various ligands bound, have not been studied systematically, but we note that at least for the *P. pantotrophus* enzyme the ferrous−cyanide state is unusually stable (*26*), a factor that would contribute to a more positive potential for the cyano form of d_1 heme than usually found for hemes with cyanide bound.

DISCUSSION

The present results clearly show that electron transfer within NIR-Ps (3 s^{-1}) contrasts with the corresponding rate of the electron transfer of 1.4×10^3 s^{-1} in NIR-Par (*10*), even though the relative positions of two heme groups are the same in both enzymes. A possible interpretation of the present data is that the difference in redox potential between the *c* and d_1 centers is a factor in determining the rate of electron transfer. It is evident that under the conditions of the pulse radiolysis experiments the redox potentials of the *c*- and d_1-type hemes must be approximately equal for NIR-Ps; in contrast, they must differ by at least approximately 100 mV in NIR-Par in which electron transfer from *c* to d_1 is stoichiometric (*10*). Therefore, the difference in redox potential is suggested to be one factor in determining the different rates. However, the 10^3 times difference in the rate cannot be explained simply by the redox potential. It is noted that the rate in NIR-Par (1.4×10^3 s^{-1}) is comparable to the intramolecular electron transfer from type I Cu to type II Cu in copper-containing nitrite reductase (1.4×10^3 s^{-1}) (*12*) and from copper A to heme *a* in bovine cytochrome oxidase (1.8×10^4 s^{-1}) (*13*). The separation distance and the difference in the redox potential of these redox centers are 12.5 Å (*27*) and 20 mV (*28*) for Cu−NIR and 19 Å (*29*) and 0−35 mV (*30*, *31*) for cytochrome oxidase, respectively. Rather, it can be argued that the much slower rate in NIR-Ps is due to a particular character of the enzyme. From an experiment with zinc protoporphyrin IX-reconstituted NIR-Ps (*32*) the internal electron-transfer rate constant (7.0×10^5 s^{-1}), even after correction for the driving force, is still much higher (over 100-fold) than those determined by the present study and stopped flow (*7*−*9*). Therefore, it is likely that the very slow rate observed in the present paper is due to a reorganization step involving a coordination change concomitant with electron transfer from the *c* to d_1 heme. The reduction of d_1 heme causes loss of the hydroxide ion from the d_1 heme iron of NIR-Ps (*4*, *5*). Thus, the d_1 heme might need to lose the bound hydroxide in order to accept an electron from the *c* heme; an associated conformational change could determine the rate of electron transfer. This proposal is supported by the finding that CN^- bound to the ferric form of d_1 heme increased by about 10^4-fold the electron-transfer rate from the *c* heme, which is explained by cyanide remaining bound to resultant ferrous d_1 heme. Though the binding of cyanide to the d_1 heme may affect the redox potential of the heme, the 10^4 times difference in the rate constant for the intramolecular transfer cannot be explained simply by the redox potential. Furthermore, recently it has been shown by X-ray crystallography that a form of the NIR-Ps with the *c* heme reduced and the d_1 heme oxidized retains the hydroxide ligation to the d_1 heme (*5*). This observation contradicts a previous hypothesis (*4*) that reduction of the *c* heme triggered hydroxide loss from the oxidized d_1 heme and strongly suggests that reduction of the d_1 heme is correlated with dissociation of the hydroxide. This supports our interpretation of the slow electron-transfer rate from *c* to d_1 heme in native NIR-Ps. In the case of NIR-Par, on the other hand, the d_1 heme can accept an electron from the *c* heme without prior displacement of Tyr25 from the d_1 heme (*10*). The difference between the two enzymes would be due to the coordination structures His/Tyr25 for NIR-Par and His/OH^- for NIR-Ps. We note that it is not possible to study the electron transfer between the *c* and d_1 hemes with cyanide present for the NIR-Par because cyanide will not bind to the oxidized state of the enzyme (*26*).

Recently, a study of the intermolecular electron-transfer kinetics between *c*-type cytochromes and cytochrome cd_1 from *Pseudomonas nautica* has been reported (*33*). It was estimated that the first-order rate constant for the intramolecular transfer of electrons from the *c*-type to the d_1 heme center within the cytochrome cd_1 was a minimum of 33 s^{-1} at a pH value of 6.3 but declined to approximately 1 s^{-1} at pH 8. It was concluded that at pH 8 this is a rate-limiting step, whereas at pH 6.3 the intermolecular transfer to

cytochrome cd_1 from a *c*-type donor is rate limiting. Thus, it was argued (*33*) that, at pH 6.3, the intramolecular electron-transfer rate might be as high as the 1400 s^{-1} obtained in a previous pulse radiolysis study of NIR-Par (*10*). The enzyme from *P. nautica* appears to be closely related to that of NIR-Ps because the N-terminal sequences, not generally well conserved between different cytochromes cd_1, from the two proteins are very similar (*34*) and overall they share 70% sequence homology (A. S. Pereira, I. Cabrito, J. J. G. Moura, and I. Moura, personal communication). Thus it is very probable that the rate for intramolecular electron transfer of 3 s^{-1} obtained in the present work for NIR-Ps will also apply to the *P. nautica* cytochrome cd_1. Unless there is a large increase in the intramolecular electron rate on going from pH 7 to pH 6.3, the pulse radiolysis data presented here argue against the intermolecular electron-transfer reaction being the rate-determining step at pH 6.3. Alternatively, one must consider the possibility that the binding of a *c*-type cytochrome to cytochrome cd_1 of *P. nautica* alters the intramolecular electron-transfer rate; the pulse radiolysis approach measures the intrinsic rate of electron transfer in the isolated protein. Whereas these issues will require further investigation in the future, it is notable that the redox potentials of the *c* and d_1 hemes in the *P. nautica* enzyme are reported as being relatively pH independent, with the d_1 being slightly less positive than the *c* (*34*). Thus a large change in electron-transfer rate with pH is not predicted, while the similarities of the potentials of the two centers suggest that, as with the *P. aeruginosa* enzyme, single electron reduction following pulse radiolysis should result in an approximately equal distribution of electrons between the two centers after the reaction.

The oxidation/reduction behavior of NIR-Par and NIR-Ps presents many unresolved issues which relate to the observations made with the pulse radiolysis technique. With the NIR-Par a quasi-equilibrium redox titration has shown that the *c* and d_1 hemes are reduced with high cooperativity which is associated with ligand switching at both hemes (*35*); it is not possible to obtain enzyme with just one type of heme center reduced under titration conditions. Under the pulse radiolysis condition the NIR-Par enzyme is supplied with only one electron per *c* and d_1 hemes (*10*). The migration of that electron from the *c* to the d_1 heme may well occur without a preceding ligand switch at either the *c* heme, which would remain His/His throughout, or the d_1 heme. There is not such evidence for cooperativity among the two types of heme group in NIR-Ps, but the oxidation/reduction behavior depends on the conditions. Thus in the crystal (*5*) an electron on the *c* heme shows little tendency to migrate to the d_1 heme, but under our solution conditions using pulse radiolysis such migration does occur on the seconds time scale. Perhaps one electron per polypeptide chain can slowly drive the dissociation of the hydroxide from the d_1 heme in solution whereas in the crystal both hemes need to be reduced. On the other hand, even in solution there are variations in the reports of the oxidation/reduction behavior of the enzyme (*19*, *23*); resolution of these issues is beyond the scope of the present work.

Finally, the results presented here show how the rate of electron transfer can be regulated by the environment of the donor and acceptor groups. Such conformational changes, without movement of the donor and acceptor groups, could offer a mechanism of control of electron transfer. Although there are differences between the enzymes from *P. pantotrophus* and *P. aeruginosa*, in each case a relatively slow controlled electron transfer from the *c* to the d_1 heme may be important to avoid the buildup of a dead-end inhibitory ferrous d_1 heme NO complex.

ACKNOWLEDGMENT

We thank the members of the Radiation Laboratory in the Institute of Scientific and Industrial Research, Osaka University, for their assistance in operating the accelerator.

REFERENCES

1. Fülöp, V., Moir, J. W. B., Ferguson, S. J., and Hajdu, J. (1995) *Cell 81*, 369−377.
2. Nurrizo, D., Silvestrini, M.-A., Mathieu, M., Cutruzzolá, F., Bourgeois, D., Fülöp, V., Hajdu, J., Brunori, M., Tegoni, M., and Cambillau, C. (1997) *Structure 5*, 1157−1171.
3. Williams, P. A., Fülöp, V., Garman, E. F., Saunders, N. F. W., Ferguson, S. J., and Hajdu, J. (1997) *Nature 389*, 406−412.
4. Nurizzo, D., Cutruzzola, F., Arese, M., Bourgeois, D., Brunori, M., Cambillau, C., and Tegoni, M. (1998) *Biochemistry 37*, 13987−13996.
5. Nurizzo, D., Cutruzzola, F., Arese, M., Bourgeois, D., Brunori, M., Cambillau, C., and Tegoni, M. (1999) *J. Biol. Chem. 274*, 14997−15004.
6. Parr, S. R., Barber, D., Greenwood, C., and Brunori, M. (1977) *Biochem. J. 167*, 447−455.
7. Silvestrini, M. C., Tordi, M. G., Musci, G., and Brunori M. (1990) *J. Biol. Chem. 265*, 11783−11787.
8. Schichman, S. A., Merer, T. E., and Gray, H. B. (1996) *Inorg. Chim. Acta 243*, 25−31.
9. Greenwood, C., Barber, D., Parr, S. R., Antonini, E., Brunori, M., and Colosimo, A. (1978) *Biochem. J. 173*, 11−17.
10. Kobayashi, K., Koppenhöfer, A., Ferguson, S. J., and Tagawa, S. (1997) *Biochemistry 36*, 13611−13616.
11. Koppenhöfer, A., Little, R. H., Lowe, D. J., Ferguson, S. J., and Watmough, N. J. (2000) *Biochemistry 39*, 4028−4036.
12. Suzuki, S., Kohzuma, T., Deligeer, Yamaguchi, K., Nakamura, N., Kobayashi, K., and Tagawa, S. (1994) *J. Am. Chem. Soc. 116*, 11145−11146.
13. Kobayashi, K., Une, H., and Hayashi, K. (1989) *J. Biol. Chem. 264*, 7976−7980.
14. Kobayashi, K., Tagawa, S., and Mogi, T. (1999) *Biochemistry 37*, 5913−5917.
15. Kobayashi, K., Miki, M., Okamoto, K., and Nishino, T. (1993) *J. Biol. Chem. 268*, 24642−24646.
16. Parr, S. R., Barber, D., Greenwood, C., and Brunori, M. (1977) *Biochem. J. 167*, 447−455.
17. Hill, R., and Anderson, R. F. (1991) *J. Biol. Chem. 266*, 5608−5615.
18. Silvestrini, M. C., Colosimo, A., Brunori, M., Walsh, T. A., Barber, D., and Greenwood, C. (1979) *Biochem. J. 183*, 701−709.
19. Schichmann, S. A., and Gray, H. B. (1981) *J. Am. Chem. Soc. 103*, 7794−7795.
20. Carson, S. D., Ching, Y. C., Wells, C. A., Wharton, D. C., and Ondrias, M. R. (1986) *Biochemistry 25*, 787−790.
21. Walsh, T. A., Johnson, M. K., Greenwood, C., Barber, D., Springall, J. P., and Thomson, A. J. (1979) *Biochem. J. 177*, 29−39.
22. Barber, D., Parr, S. R., and Greenwood, C. (1978) *Biochem. J. 175*, 239−249.
23. Sutherland, J., Greenwood, C., Perterson, J., and Thomson, A. J. (1986) *Biochem. J. 233*, 893−898.
24. Silvestrini, M. C., Falcinelli, S., Ciabatti, I., Cutruzzola, F., and Brunori, M. (1994) *Biochimie 76*, 787−790.
25. Varadarajan, R., Zewert, T. E., Gray, H. B., and Boxer, S. G. (1989) Science *243*, 69−72.

26. Jafferji, A., Allen, J. A., Ferguson, S. J., and Fülöp, V. (2000) *J. Biol. Chem. 275*, 25089−25094.
27. Godden, J. W., Turley, S., Teller, D. C., Adman, E. T., Liu, M. Y., Payne, W. J., and LeGall, J. (1991) *Science 253*, 438−442.
28. Suzuki, S., Deligeer, Yamaguchi, K., Kataoka, K., Kobayashi, K., Tagawa, S., Kohzuma, T., Shidara, S., and Iwasaki, H. (1997) *J. Inorg. Biol. Chem. 2*, 265−274.
29. Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Sinzawa-Itoh, K., Nakashima, R., and Yoshikawa, S. (1995) *Science 269*, 438−442.
30. Tiesjema, R. H., Muijsers, A. O., and Van Gelder, B. F. (1973) *Biochim. Biophys. Acta 162*, 327−337.
31. Wilson, D. F., Erecinska, M., and Owen, C. S. (1976) *Biochim. Biophys. Acta 175*, 160−172.
32. Bellelli, A., Brzezinski, P., Arese, M., Cutruzzola, F., Silvestrini, M. C., and Brunori, M. (1996) *Biochem. J. 319*, 407−410.
33. Lopes, H., Besson, S., Moura, I., and Moura, J. J. (2001) *J. Biol. Inorg. Chem. 6*, 55−62.
34. Besson, S., Carneiro, C., Moura, J. J. G., Moura, I., and Fauque, G. (1995) *Anaerobe 1*, 219−226.
35. Koppenhöfer, A., Turner, K. L., Allen, J. W., Chapman, S. K., and Ferguson, S. J. (2000) *Biochemistry 39*, 4243−4249.

BI002534I