Rapid Calmodulin-dependent Interdomain Electron Transfer in Neuronal Nitric-oxide Synthase Measured by Pulse Radiolysis*

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Electron transfer within rat neuronal nitric-oxide synthase (nNOS) was investigated by pulse radiolysis. Radiolytically generated 1-methyl-3-carbamoyl pyridinium (MCP) radical was found to react predominantly with the heme of the enzyme with a second-order rate constant for heme reduction of $3 \times 10^9$ M$^{-1}$ s$^{-1}$. In the calmodulin (CaM)-bound enzyme a subsequent first-order phase was observed which had a rate constant of $1.2 \times 10^8$ s$^{-1}$. In the absence of CaM, this phase was absent. Kinetic difference spectra for nNOS reduction indicated that the second phase consisted of heme reoxidation accompanied by formation of a neutral flavin semiquinone, suggesting that it is heme to flavin electron transfer. Experiments with the heme proximal surface mutant, K423E, had no second phase, confirming that the mutation blocks interdomain electron transfer. With the autoinhibitory loop deletion mutant, Δ40, the slow phase was observed even in the absence of CaM consistent with the role of the loop in impeding interdomain electron transfer. The rate of heme to FMN electron transfer observed in the wild-type enzyme is ~1000 times faster than the FMN to heme electron transfer rate predicted during catalysis from kinetic modeling, suggesting that the catalytic process is slowed by kinetic gating.

Nitric oxide (NO) is a key signaling molecule in a diverse array of cellular processes. These range from essential roles in signal transduction in the nervous system to blood pressure regulation and involvement in the immune response (1–7). It is synthesized in mammals by the nitric-oxide synthases (NOS). The NOS enzyme is composed of an oxygenase domain with a thiold-coordinated heme active site similar to that of cytochrome P450 (P450), and an electron transfer domain related to NADPH-cytochrome P450 reductase which binds FMN and FAD (8–12). Neuronal NOS (nNOS) and endothelial NOS (eNOS) require Ca$^{2+}$-promoted CaM binding for activation. CaM binds tightly an amphipathic a-helical region containing 12 basic and hydrophobic residues located in the linker region between the reductase and heme domains (13). Once CaM is bound, electrons provided by NADPH are transferred in a linear sequence across the domains, first entering the flavins and then passing to the heme iron (14). Upon reduction, NOS catalyzes an O$_2$-dependent conversion of l-arginine to NO and l-citrulline. Several studies have shown a role for CaM in controlling from flavin to heme interdomain electron transfer (15–21) and an intradomain electron transfer between flavins (22). However, these studies are based on the effect of CaM on NO synthesis, NADPH oxidation, cytochrome c reduction, and superoxide formation (14, 18–21). There has been no previous report of the effect of CaM on the individual intramolecular electron transfer steps.

To investigate how CaM regulates electron transfer between the cofactors of nNOS we mutated residues thought to lie in the interface between the domains (23, 24). Mutation of a moderately conserved basic amino acid residue (Lys$^{225}$) in the heme proximal site of nNOS to several neutral and acidic amino acids caused the loss of NO formation activity and a large decrease in the rate of heme reduction by NADPH via the reductase domain (23). On the other hand, deletion of a putative autoinhibitory loop within the FMN-binding domain (Δ40 and Δ42 mutants) caused nNOS to retain activity in the absence of Ca$^{2+}$/CaM (24). The Δ40 mutant was found to be able to pass electrons through from NADPH to the heme in the absence of CaM, whereas both Ca$^{2+}$ and CaM are required for this to occur in the wild enzyme.

Pulse radiolysis is a powerful tool for investigating electron transfer within proteins, often allowing an electron to be introduced rapidly and selectively into one redox center of an enzyme (25–30). This paper describes the pulse radiolysis of nNOS, utilizing different types of radical as the reductant. Differences in the electron transfer reactions occurring in the wild-type, K423 mutants, and Δ40 deletion mutant are reported. We present a detailed study of backward electron transfer from heme to FMN and the factors which control its rate.

EXPERIMENTAL PROCEDURES

Materials—Reagents were purchased from Sigma or Wako Pure Chemicals (Osaka, Japan), and were of the highest guaranteed grade and without purification.

Preparation of nNOS—Rat nNOS cDNA was a kind gift from Dr. S. H. Snyder (Johns Hopkins University School of Medicine). The expression plasmid pCWO$^1$ was a kind gift from Dr. M. R. Waterman (Vanderbilt University School of Medicine). nNOS was expressed in Escherichia coli cells using pCWO$^1$ expression plasmid as previously mentioned (31). The expression plasmids for full-length of nNOS of wild types and the mutants at Lys$^{225}$ (23) and the Δ40 mutant (24) of nNOS were prepared as described previously. The oxygenase domain of the wild-type enzyme (Wildox) was prepared as previously mentioned (32).
Purification of wild-type and mutant nNOS enzymes were carried out using 2',5'-ADP-Sepharose and calmodulin-Sepharose column chromatographies as described previously (33, 34). Purification of Wildox was carried out using an Ni-NTA-agarose column, as described previously (32).

For all enzymes, purified nNOS was more than 95% pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis stained with Coomassie Blue R-250 and Western blot analysis. The purified and concentrated enzyme was dialyzed against 50 mM Tris-HCl (pH 7.5) buffer containing 4 mM H4B, 0.1 mM dithiothreitol, 0.1 mM EDTA, and 10% glycerol. The concentration of nNOS was determined optically from the [CO-reduced] forms of FMN of nNOS, and 570 nm, respectively. Second-order rate constant values were calculated by plotting log ΔA versus time for absorbance changes. Reduction of nNOS and Wildox were monitored at 500 nm, 600 nm, and 660 nm by sodium dodecyl sulfate-polyacrylamide gel electrophoresis stained with Coomassie Blue R-250 and Western blot analysis. The purified and concentrated enzyme was dialyzed against 50 mM Tris-HCl (pH 7.5) buffer containing 4 mM H4B, 0.1 mM dithiothreitol, 0.1 mM EDTA, and 10% glycerol.

The concentration of nNOS was determined optically from the [CO-reduced] forms of FMN of nNOS, and 570 nm, respectively. This ΔΔ value was estimated by the pyridine hemochromogen method (33) assuming that one heme is bound to one subunit of the enzyme. The enzyme activity was measured by the rate of NO formation, as described previously (33, 34).

**Pulse Radiolysis—**Samples of nNOS and Wildox for pulse radiolysis were prepared as follows. Before use, nNOS and Wildox were passed through a Sephadex G-25 column equilibrated with 10 mM potassium phosphate buffer (pH 7.4). In the case of Wildox, the equilibration buffer contained 0.2 M KCl. Solutions of nNOS contained 2 mM MCP and 0.1 mM tert-butyl alcohol (for scavenging OH radicals) in 10 mM phosphate buffer (pH 7.4) and were deoxygenated in sealed cells by repeated evacuation and flushing with argon. 0.1 mM tert-Butyl alcohol had no effect on the enzyme activity or the optical absorption spectrum of NOS.

The samples in the presence of CO were formed as follows. Solutions containing phosphate buffer, 2 mM MCP, and 0.1 mM tert-butyl alcohol were bubbled with CO gas for 10 min. Concentrated solutions of nNOS and Wildox were added to the solution and deoxygenated in sealed cells by evacuation and flushing with argon.

Pulse radiolysis experiments were performed with an electron linear accelerator at the Institute of Scientific and Industrial Research, Osaka University (25–30). The pulse width and energy were 8 ns and 27 MeV, respectively. The sample was placed in a quartz cell with an optical path length of 1 or 0.3 cm. The temperature of the sample was maintained at 20 °C. The light source for the spectrophotometer was a 150 W halogen lamp or a 200 W Xe 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. For each measurement, a fresh sample was used, even though the process did not cause any damage to the sample as judged by its visible absorption spectrum. The concentration of MCP radical generated by pulse radiolysis was estimated from the absorbance at 420 nm, using a molar extinction coefficient of 3390 M⁻¹ cm⁻¹ (35). This concentration was adjusted by varying the dose of electron beams.

Kinetic data were analyzed by plotting log ΔA versus time for absorbance changes. Reduction of nNOS and Wildox were monitored at 500 nm, an isosbestic point between the oxidized and the semiquinone forms of FMN of nNOS, and 570 nm, respectively. Second-order rate constants were determined under the condition, where ~1 μM MCP radical was generated in a solution containing 10-80 μM enzyme. Reduction of nNOS flavin was monitored at 580 nm, which is an isosbestic point between the oxidized and the reduced forms of heme iron of nNOS. Optical absorption spectra were measured with a Hitachi U-3000 or a Shimadzu UV-2500 spectrophotometer.

**RESULTS**

**Reactions of 1-Methyl-3-carbamoyl Pyridinium Radical with the Wild-type nNOS and the Isolated Oxygenase Domain of the Wild-type Enzyme (Wildox)—**Pulse radiolysis experiments involve the almost instantaneous generation of the MCP radical which in turn can reduce redox center(s) within a protein. The initial transient increase in absorbance indicated the formation of the MCP radical with an absorption maximum at 420 nm (data not shown). In the absence of the enzyme, the lifetime of the MCP radical is longer than 6 ms (36), whereas the lifetime of the MCP radical in the presence of 70 μM nNOS was 444 ns (Fig. 1A). This indicates that MCP radical with nNOS determined by the decrease in absorbance at 500 nm. The monoexponential loss in absorbance corresponds to reduction of nNOS by MCP radical. B, concentration dependence of apparent rate constants for the reaction of MCP radical with nNOS determined by the decrease in absorbance at 500 nm. Samples contained 12 μM enzyme in a 1-cm optical path. All samples contained 2 mM MCP and 0.1 mM tert-butyl alcohol in 10 mM phosphate buffer (pH 7.4) in the presence and absence of 500 μM L-Arg. In the presence of 70 μM nNOS, the MCP radical decayed within 100 μs (data not shown). The absorbance at 490 and 600 nm decreased and the absorbance at 460 nm increased with the decay of the MCP radical (Fig. 2). In the presence of CaM, a subsequent slower absorbance change was observed in the time range of milliseconds (Fig. 2B). The absorbance at 490 nm consisted of a faster and a slower decrease. The initial decrease of absorbance at 600 nm and the increase at 460 nm subsequently reversed. However, no second phase was observed in the absence of CaM (Fig. 2A). Fig. 3 shows the difference spectra monitored at 200 μs and 10 ms after the pulse. The spectrum at the end of the faster phase, which has a broad absorption decrease around 500 nm, is similar to that obtained in the absence of CaM. The slower phase had an absorbance decrease at 460 nm and an increase around 580 nm, characteristic of blue flavin semiquinone formation. This suggests that the slow phase arises from the electron reduction of the flavin of the enzyme.

Since the visible difference spectrum of nNOS is complex owing to the presence of absorption of heme iron, FAD, and FMN, a similar experiment was performed using the isolated oxygenase domain of the wild type enzyme (Wildox). After pulse radiolysis of Wildox, the absorbance at 520 and 570 nm decreased and increased, respectively, with the decay of the MCP radical (Fig. 4A). However, no subsequent absorption changes were observed, in contrast to the experiments on the holoenzyme in the presence of CaM. The kinetic difference spectrum at 500 μs after pulse radiolysis is essentially identical to the static difference spectrum of the reduced minus oxidized form of Wildox (Fig. 4B) and, furthermore, is similar to the kinetic difference spectrum observed in the fast phase of the holoenzyme experiments (Fig. 3A). This indicates that MCP radicals exclusively reduced the heme iron of both the holoenzyme and Wildox. The rate constant for the reduction of the heme iron of Wildox was calculated from the absorbance change at 570 nm to be 4.2 × 10⁶ M⁻¹ s⁻¹, which is comparable to that of the heme reduction in holo-nNOS. Addition of 500 μM...
L-arginine increased the second order rate constants for the reduction to $7.2 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$.

A similar experiment was performed in the presence of CO, making it possible to follow heme reduction by measuring the build-up of the Fe$^{2+}$-CO complex at 450 nm. Following the spectral changes of the heme reduction of the enzyme, an increase of absorbance at 450 nm was seen in the time range of milliseconds (Fig. 5A). The kinetic difference spectrum at 10 ms after the pulse shows that CO-bound ferrous nNOS was formed. A similar CO binding process was seen in Wildox (data not shown). The rates of the reduction in both the holoenzyme and Wildox were not affected in the presence of CO at all. From these findings, it can be concluded that the heme irons of the holoenzyme and Wildox were initially reduced in the presence or absence of CaM. No subsequent changes were seen in the absence of CaM. However, in the presence of CaM, subsequent events were quite different for the mutants. In the K423M mutant, a slow absorbance decrease from 460 to 490 nm and an increase at 600 nm were observed, as the case of the wild enzyme (Fig. 6A). On the other hand, in the K423E mutant, the slow process was not observed (Fig. 6B). Fig. 7 shows the difference spectra at 200 ms and

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**Fig. 2.** Absorption changes after pulse radiolysis of the wild-type nNOS in the absence (A) and presence (B) of CaM measured at 460, 490, 580, and 600 nm. Samples contained 70 nM enzyme in a 1-cm optical path. All samples contained 2 mM MCP and 0.1 M tert-butyl alcohol in 10 mM phosphate buffer (pH 7.4). Samples for B contained 10 mg/ml CaM and 1 mM CaCl$_2$.

**Fig. 3.** Kinetic difference spectra of pulse radiolysis of wild-type nNOS in the absence (A) and presence (B) of CaM monitored at 200 ms (filled circle) and 10 ms (open circle) after pulse radiolysis. Experimental conditions were the same as described in the legend to Fig. 2.
10 ms after the pulse of the K423M and K423E mutants. The difference spectra for the fast and slow phases in the K423M mutant are essentially the same as those obtained after pulse radiolysis of the CaM-bound wild-type enzyme, although the amplitude of the slow phase was smaller. The rate constant for the backward electron transfer in the K423M mutant was also found to be slower than for the wild type enzyme at 5.1 × 10^2 s⁻¹. These results indicate that the interdomain (heme to FMN) electron transfer does not occur in the K423E mutant even in the presence of CaM.

Reaction of 1-Methyl-3-carbamoyl Pyridinium Radical with the Δ40 Deletion Mutant—The inducible form of NOS (iNOS) differs from the constitutive NOS isoforms (nNOS and eNOS) in the fact that it is insensitive to changes in Ca²⁺ concentration (39). One of the main structural differences between iNOS and the constitutive NOSs is that the latter contain an additional 40–50-amino acid insert in the middle of a conserved region of the FMN-binding domains. The role of the insert in controlling Ca²⁺/CaM sensitivity was confirmed by nNOS loop deletion mutants (Δ40, Δ42) which retained their activity in the absence of Ca²⁺/CaM (24). Therefore, the interdomain electron transfer in the deletion mutant must also occur in the absence of CaM. As in the case of the wild-type enzyme, reduction of the Δ40 deletion mutant with MCP radical proceeded via two phases (Fig. 8). The initial absorbance increase at 460 nm and the decrease at 600 nm subsequently reversed. The absorption change at 490 nm consists of a faster and a slower decrease. It is noted that these changes were largely unaffected by CaM. The kinetic difference spectra of the faster and the slower phases obtained with the deletion mutant are similar to those obtained after pulse radiolysis of the CaM-bound wild-type enzyme, however, the Δ40 spectra were not affected by the presence of CaM (Fig. 9). The rate constant of the interdomain electron transfer was calculated to be 8.7 × 10^2 s⁻¹, and independent of CaM binding. However, the difference spectra of Fig. 9 indicate other changes in addition to the reduction of the heme iron in the fast phase. The absorbance from 460 to 490 nm decreases initially, significantly more than with the wild-type enzyme. This suggests that some flavin reduction is also taking place in the fast phase.
that NADP/H interacts with one of the flavins of the enzyme. In the CaM-free enzyme, on the other hand, the absorbance at 460 nm increased with a decrease in absorbance at 600 nm (Fig. 10A). The kinetic difference spectrum, which has an absorption minimum around 490 nm, is quite different from that of the CaM-bound form, but is similar to the spectrum of the initial reduction product on reaction with the MCP radical. This indicates that NADP/H directly reacts with heme iron of the CaM-free enzyme. Similar results were obtained with the K423M and K423E mutants (Fig. 11).

**DISCUSSION**

The data presented here provide new insight into electron transfer within nNOS. The backward electron transfer from the heme to the FMN of nNOS is essentially an artificial process, which occurs only under specific conditions. However, electron transfers such as this one are freely reversible and the factors controlling them are identical in both directions. Biological electron transfers are governed by the type of cofactor involved, the distance between them, the nature of the intervening medium, the temperature, and the driving force as defined by the appropriate reduction potentials. In reversing the direction of an electron transfer, the reversal of the driving force is the only factor changing. Consequently, the analysis of the dependence of electron transfer rate constants on structural factors is equally applicable to the reverse direction. However, it should be noted that the rate of an electron transfer event may also be limited by slow changes occurring to any of the above factors, i.e. the process may be kinetically “gated.” In some cases this may compromise the free reversibility of an electron transfer event.

Pulse radiolysis of MCP radical with wild-type nNOS in the presence and absence of CaM caused rapid heme reduction, with intramolecular electron transfer to FMN occurring as a
slow phase observed only in the presence of CaM. This is confirmed by the experimental results of the oxygenase domain of the wild enzyme, with which the MCP radical reacts in a similar way. Confirmation of the link between the slow phase and intramolecular electron transfer was obtained by studying nNOS mutants known to have modified FMN to heme electron transfer properties. The results also provide further insight into the effects of the mutations on electron transfer.

In the heme proximal mutant K423E, FMN to heme electron transfer does not occur even in the presence of CaM (23). Here we show that backward electron transfer (heme to FMN) does not occur either. This is consistent with the role of Lys423 in forming an important electrostatic contact between the heme domain and the reductase domain. In the Δ40 mutant, in which the forward electron transfer occurs even in the absence of CaM (24), the same was observed for the backward electron transfer. The fact that CaM binding controls backward (heme to FMN) electron transfer in nNOS consistent with the theory that CaM’s mechanism of action is to control the distance between the cofactors via induction of a large-scale structural rearrangement rather than a shift in the cofactor reduction potentials. The results obtained with both mutants corroborate this theory. In Table I the rate constants for interdomain backward electron transfer obtained for the wild-type and mutant enzymes are compared. It is clear that CaM binding is a prerequisite for electron transfer to occur in the wild-type enzyme. However, the difference in NADPH oxidation rates and NO synthase activity (23, 24) among the various enzyme forms cannot be explained solely by the difference in the rates of the interdomain electron transfer.

We previously reported the midpoint reduction potentials for the flavin cofactors in the reductase domain of nNOS (40). The values clearly show that the backward electron transfer from the heme to FMN is thermodynamically favorable, since the redox couple of the nNOS heme (−257 mV) (35) is operative in the reduction of FMN (−30 mV for FMNH/FMN) (40). It is an essentially quantitative electron transfer because the redox potentials of the two cofactors differ by −230 mV. In general, pulse radiolysis precedes electron equilibration between two redox sites by reversible electron transfer, the observed rate constant is the sum of the forward and backward rates (25, 27). However, with such a large redox potential difference, this step is essentially unidirectional, for a 230 mV driving force the rate of forward electron transfer is 50 times faster than the rate of reverse electron transfer, i.e. for $k_{obs} = 1200 \text{ s}^{-1}$, the heme to FMN and FMN to heme rates are 1175 s$^{-1}$ and 25 s$^{-1}$, respectively. This partly explains why the FMN semiquinone is air-stable and can only be accessed by the oxyferrous form of the heme in the catalytic cycle (41, 42). Nevertheless, these electron transfer rate constants are extremely fast and appear to be at odds with predictions of 1–10 s$^{-1}$ for FMN to heme during catalysis (43). The FMN to heme electron transfer occurring during catalysis takes place from the FMN hydroquinone to the substrate-bound heme, driven by a 30 mV redox potential difference. Assuming a linear relationship between the free energy change and the logarithm of the rate constant (i.e. $\Delta G \approx \lambda$, reorganization energy), this corresponds to a rate constant of 220 s$^{-1}$. If the FMN hydroquinone to heme electron transfer rate is 2 orders of magnitude greater than that expected from modeling the catalytic cycle (43), it is likely that conformational gating limits the delivery of electrons to the

FIG. 9. Kinetic difference spectra of pulse radiolysis of the nNOS Δ40 deletion mutant in the absence (A) and presence (B) of CaM at 200 µs (filled circle) and 10 ms (open circle) after pulse radiolysis. Experimental conditions were the same as described in the legend to Fig. 6.

FIG. 10. Absorption changes after pulse radiolysis of wild-type nNOS in the absence (A) and presence (B) of CaM. C, kinetic difference spectra of pulse radiolysis of wild-type nNOS in the absence (filled circle) and presence (open circle) of CaM monitored at 10 ms after pulse radiolysis. Samples contained 70 µM enzyme in a 1-cm optical path. All samples contained 2 mM NADP$^+$ and 0.1 M tert-butyl alcohol in 10 mM phosphate buffer (pH 7.4). Other conditions were same as described in the legend to Fig. 1.
cytochrome P450BM-3, on the other hand, the 7-methyl group of the FMN is 18.4 Å away from the heme iron (48).

We have shown in this study that the heme, FAD, and FMN of nNOS can be effectively reduced with different reducing species directly and via different centers. The heme iron is the preferred site of entry of an electron into the enzyme when the MCP radical is used. However, the rate constant for reaction of the MCP radical with the heme iron of nNOS \((3 \times 10^8 \text{ M}^{-1} \text{s}^{-1})\) is 1 order of magnitude smaller than those of Cu\(_A\) of bovine cytochrome oxidase \((1.5 \times 10^9 \text{ M}^{-1} \text{s}^{-1})\) (25) and heme c of cytochrome cd\(_1\) nitrite reductase \((3.8 \times 10^8 \text{ M}^{-1} \text{s}^{-1})\) (28), but is larger than those found previously with the iron-sulfur center of xanthine dehydrogenase \((6.5 \times 10^7 \text{ M}^{-1} \text{s}^{-1})\) (26) and heme b of E. coli cytochrome bo \((10^7 \text{ M}^{-1} \text{s}^{-1})\) (30). The electron entry points in the former proteins are located near the surface of the protein molecule, whereas the redox sites in the latter proteins are buried in a hydrophobic pocket, according to the x-ray crystal structures (49, 50). In nNOS, however, there is a large access channel for substrate and cofactor that allows access for solvent to both the active site and the heme (51). The mechanism of one-electron transfer from MCP radical to the heme iron of the oxygenase domain can be explained by similar considerations, since the heme iron of Wildox was reduced by the MCP radical at a comparable rate to that of the holoenzyme. The rate constant for heme reduction appeared to be largely unaffected by the addition of l-arginine. This suggests that the reduction rate depends on the steric accessibility of the heme to solvent, not the redox potential. It also appears that the MCP radical can access the heme iron of nNOS more easily, than the FAD and FMN in the reductase domain. No direct reduction of the FMN by the radicals could be detected, suggesting that the access of FMN is restricted relative to that of the heme. The molecular surface near the FMN-binding site of NADPH-cytochrome P450 reductase is conserved in NOS (48, 52). The acidic amino acids of the reductase are expected to interact with basic amino acids on the surface of the oxygenase domain. The present data suggest that the oxygenase domain masks the reduction site of FMN completely.

On the other hand, NADP\(^+\) transferred an electron to one of the flavins of the CaM-bound enzyme directly. Although we could not identify which flavin (FAD or FMN) was reduced by NADP\(^+\), it is likely that, because NADPH binds adjacent to FAD in NOS, it is reduced by NADP\(^+\) initially. The intramolecular electron transfer between the flavins may occur very rapidly, since the two redox centers are probably only 4 Å apart, based on the x-ray crystal structure of NADPH-cytochrome P450 reductase (52). The difference between the interaction of the MCP and NADP\(^+\) radicals with nNOS lies mainly in the fact that NADP\(^+\) will preferentially bind to the NADPH dehydrogenase active site located at the FAD. There are no significant differences in the redox properties of these reductants to otherwise explain their respective selectivity for heme and flavin (53). CaM binding does cause a change in the environment of FAD in nNOS, as indicated by the CaM dependence of FAD reduction by NADPH (20), this is consistent with our observations.

In conclusion, CaM activates nNOS at two points in the electron transfer sequence: electron transfer into the flavins and interdomain electron transfer between FMN and the heme. Our results indicate that FMN to heme electron transfer is rapid and must be controlled by conformational gating during catalysis.

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