Crucial Role of Lys\(^{423}\) in the Electron Transfer of Neuronal Nitric-oxide Synthase\(^*\)

(Received for publication, April 13, 1999, and in revised form, June 1, 1999)

Tomomas Shimanuki, Hideaki Sato, Simon Daff, Ikuko Sagami, and Toru Shimizu\(^\dagger\)

From the Institute for Chemical Reaction Science, Tohoku University, Sendai 980-8577, Japan

Nitric-oxide synthase (NOS) is composed of an oxygenase domain having cytochrome P450-type heme active site and a reductase domain having FAD- and FMN-binding sites. To investigate the route of electron transfer from the reductase domain to the heme, we generated mutants at Lys\(^{423}\) in the heme proximal site of neuronal NOS and examined the catalytic activities, electron transfer rates, and NADPH oxidation rates. A K423E mutant showed no NO formation activity (\(<0.1\) nmol/min/nmol heme), in contrast with that (72 nmol/min/nmol heme) of the wild type enzyme. The electron transfer rate (0.01 min\(^{-1}\)) of the K423E on addition of excess NADPH was much slower than that (>10 min\(^{-1}\)) of the wild type enzyme. From the crystal structure of the oxygenase domain of endothelial NOS, Lys\(^{423}\) of neuronal NOS is likely to interact with Trp\(^{408}\) which lies in contact with the heme plane and with Cys\(^{415}\), the axial ligand. It is also exposed to solvent and lies in the region where the heme is closest to the protein surface. Thus, it seems likely that ionic interactions between Lys\(^{423}\) and the reductase domain may help to form a flavin to heme electron transfer pathway.

Nitric-oxide synthase (NOS)\(^1\) produces nitric oxide (NO) for a range of important biological functions (see Refs. 1–7 and references therein). NOS consists of an oxygenase domain with a thiol-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. For NOS to catalyze efficient monooxygenation reactions, the presence of effectors such as calmodulin (CaM) and tetrahydrobiopterin (H\(_4\)B) as well as the formation of the homodimer are prerequisite. It is conceivable that CaM plays an important role in arranging the protein structure for efficient electron transfer to occur from the reductase domain to the heme domain (8). H\(_4\)B is bound to a site distant from the L-Arg-binding site located on the heme distal side within the oxygenase domain, based on the x-ray crystal structure of the dimeric oxygenase domain of inducible NOS (iNOS) and endothelial NOS (eNOS) (9–11). Another structurally important factor, consisting of a cysteine-bound zinc center, was indicated by two groups (10, 11).

A recent report suggested an emerging role for H\(_4\)B, suggesting that it may deliver a second electron to the intermediate Fe(II)-O\(_2\)-L-Arg ternary complex for the activation of molecular oxygen during P450-type monooxygenation at the heme active site (12). Another role for H\(_4\)B recently proposed suggests that a non-heme iron-pterin complex is involved in the activation of molecular oxygen during the monooxygenation of L-Arg, functioning in a similar way to aromatic amino acid hydroxylase (13). The x-ray crystal structure of eNOS also suggests that a pterin radical is directly involved in the catalysis (10). Thus, the first step of NO synthesis, monooxygenation of L-Arg to N\(_2\)-hydroxy-L-Arg (NHA), is a matter of debate in regard to the role of H\(_4\)B. The mechanism of the second monooxygenation from the intermediate compound, NHA, to NO and L-citrulline has also been controversial, although it seems likely that the heme iron is directly involved in this process (15–19).

Whichever mechanism is followed, introduction of electrons into the oxygenase domain is necessary for the activation of molecular oxygen during catalysis (20–23). If H\(_4\)B is involved in the introduction of the second electron to the heme active site (12), the electron must initially reach H\(_4\)B via the heme distal side from NADPH per se or directly via the reductase domain. Likewise, electron transfer via the heme distal side would be possible if the non-heme iron-pterin complex is involved in the activation of molecular oxygen for the monooxygenation of L-Arg (13), or if a pterin radical is involved in the process (10). However, it seems likely that electrons pass directly to the heme iron for the second step, monooxygenation of NHA, since the heme iron seems certain to be the site for this process. Interestingly, a recent report proposed that intermolecular electron transfer from the adjacent reductase domain to the oxygenase domain occurs in the homodimer of iNOS (24). Previous work in this laboratory suggested that basic amino acids such as Lys and Arg on the proximal surface of microsomal P450s are important for the interaction between the reductase and P450 for efficient electron transfer to occur (25, 26). It is possible that similar interactions are required for electron transfer to occur in the homodimer of NOS, particularly in view of the structural rearrangement which probably occurs at the reductase domain-heme domain interface on CaM binding.

In the present study, we mutated a moderately conserved basic amino acid, Lys\(^{423}\) of neuronal NOS (nNOS), to several neutral and acidic amino acids and studied the mutation effect on the catalytic activity and electron transfer rate from NADPH to the heme. Note that the 423 position is conserved as Lys for both nNOS and eNOS, while it is Asn for iNOS (Fig. 1). A K423E mutant had no NO formation activity with either L-Arg or NHA as substrate. This mutant also showed a very low electron transfer rate from the reductase domain to the heme iron under both aerobic and anaerobic conditions. Also, the
heme of the K423E mutant proved difficult to reduce by sodium dithionite. Thus, we suggest that Lys\(^{423}\) is involved in catalysis, perhaps in regulating the rate of electron transfer from the reductase domain to the heme active site of nNOS.

**EXPERIMENTAL PROCEDURES**

**Materials—**H\(_4\)B was purchased from Schircks Laboratories (Jona, Switzerland). Other reagents, which were from Wako Pure Chemicals (Osaka, Japan), were of the highest guaranteed grade and were used without further purification.

Preparation of Neuronal NOS—Rat nNOS cDNA was kindly gifted by Dr. S. H. Snyder (Johns Hopkins University School of Medicine). nNOS was expressed in *Saccharomyces cerevisiae* using the acid phosphatase promoter previously used for the expression of cytochrome P450 1A2 (25–27). The oligonucleotide primers for the mutations of Lys\(^{423}\) to Glu, Met, Leu, and Asn were 5′-CCAGTGTCGACGCGATCAAG-3′, 5′-CACTGTCGTCGTCGTCAGGT-3′, 5′-CACTGTCGTCGTCGTCAGGT-3′, and 5′-ATGTCGTCGTCGTCAGGT-3′, respectively. The polymerase chain reaction-based mutagenesis were performed using oligonucleotide-directed dual amber long and accurate PCR kits (Takara Shuzo, Kyoto, Japan).

Purification of wild-type and mutant nNOS enzymes were carried out using 2.5′-ADP-Sepharose and calmodulin-Sepharose column chromatographies as described previously (28, 29). 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. The reductase activity was determined by monitoring the absorbance at 340 nm, using an extinction coefficient of 6.22 mM\(^{-1}\) cm\(^{-1}\). The NADPH oxidation rate was determined spectrophotometrically as an absorbance decrease at 340 nm, using an extinction coefficient of 6.22 mM\(^{-1}\) cm\(^{-1}\). Unless otherwise indicated, assays were carried out at 25 °C in 50 mM Tris-HCl (pH 7.5) buffer containing 10 mM oxyhemoglobin, 0.1 mM NADPH, 5 \(\mu\)M each of L-H\(_4\)B, 0.1 mM DTT, and 0.05–0.1 \(\mu\)M nNOS in the presence or absence of 0.5 mM l-Arg or NHA. Cytochrome c reductase activity was determined by monitoring the absorbance at 550 nm using an extinction coefficient of 21 mM\(^{-1}\) cm\(^{-1}\). The reductase activities of the Lys\(^{423}\) mutants were essentially the same as those of the wild type. The \(\mathrm{H}_2\mathrm{O}_2\) generation rate was measured by the formation of ferric thiocyanate under similar conditions as described previously (1). The NADPH oxidation rate was determined optically from the [CO-reduced] → [reduced] difference spectrum using \(\Delta A_{444–467}\) = 55 mM\(^{-1}\) cm\(^{-1}\). This \(\Delta A\) value was estimated by the pyridine hemochromogen method (28) assuming that one heme is bound to one subunit of this enzyme.

**Enzyme Assay—**The rate of NO formation was determined from the NO-mediated conversion of oxyhemoglobin to methemoglobin, monitored at 401 nm using a methemoglobin minus oxyhemoglobin extinction coefficient of 49 mM\(^{-1}\) cm\(^{-1}\) (1). The NADPH oxidation rate was determined spectrophotometrically as an absorbance decrease at 340 nm, using an extinction coefficient of 6.22 mM\(^{-1}\) cm\(^{-1}\). Unless otherwise indicated, assays were carried out at 25 °C in 50 mM Tris-HCl (pH 7.5) buffer containing 10 \(\mu\)M oxyhemoglobin, 0.1 mM NADPH, 5 \(\mu\)M each of FAD and FMN, 10 \(\mu\)g/ml CaM, 1 mM CaCl\(_2\), 100 units/ml superoxide dismutase, 5 \(\mu\)g/ml BSA, 5 \(\mu\)M DTT, and 0.05–0.1 \(\mu\)M nNOS in the presence or absence of 0.5 mM l-Arg or NHA. Cytochrome c reductase activity was determined by monitoring the absorbance at 550 nm using an extinction coefficient of 21 mM\(^{-1}\) cm\(^{-1}\). The reductase activities of the Lys\(^{423}\) mutants were essentially the same as those of the wild type. The \(\mathrm{H}_2\mathrm{O}_2\) generation rate was measured by the formation of ferric thiocyanate under similar conditions as described previously (1).

**RESULTS**

We generated K423E, K423M, K423L, and K423N mutants. The Soret spectral band of the resting Fe(III) form of the all mutants appeared to consist of a mixture of the high spin and low spin complexes as observed for the wild type enzyme (A and B in Fig. 2). The Soret bands of the mutants were moved to 395 nm and became narrower on addition of l-Arg, similar to the wild type enzyme, suggesting that the l-Arg-binding site was not altered by the mutation of the proximal site of nNOS and that the spin state change still occurred as normal. The lower part of Fig. 2 shows difference absorption spectra of the Fe(II)-CO complexes of the wild type and K423E mutant purified from the supernatant of the yeast crushed homogenized solution. No absorption band around 420 nm ascribed the denatured complex, P420, was observed for the K423E mutant as with the wild type enzyme (28, 29). The K423M, K423L, and K423N mutants also generated in the present study all had similar spectra.

Table I summarizes various kinetic parameters associated with the catalysis of this enzyme, when l-Arg is used as a substrate. The NO formation rate of K423E was less than 0.1 nmol/min/nmol heme, in contrast with that of the wild type enzyme (72 nmol/min/nmol heme). The K423M and K423L mutants had relatively low NO formation activities, 18 and 25 nmol/min/nmol heme, respectively. In contrast, K423N had a similar catalytic activity (78 nmol/min/nmol heme) to the wild type enzyme. We also examined the NO formation rate of the Lys\(^{423}\) mutants using NHA, the reaction intermediate, as the substrate (Table II). When NHA was used, the NO formation activity of the K423E mutant was 3 nmol/min/nmol of heme.
The solution composed of nNOS (0.5 mM), 50 mM Tris-HCl (pH 7.5), 10 μg/ml CaM, 1 mM CaCl₂, 5 μM FAD, 5 μM FMN, 5 μM H₂B, and 5 μM DTT. 

**TABLE II**

Kinetic parameters (nmol/min/nmol of heme) of the Lys423 mutants of nNOS in the presence of NHA

| Enzymes | NO formation<sup>a</sup> | NADPH<sup>b</sup> oxidation |
|---------|--------------------------|-----------------------------|
| Wild type | 72                        | 139                         |
| K423E   | 3                         | 84                          |
| K423M   | 52                        | 185                         |
| K423L   | 59                        | 146                         |
| K423N   | 78                        | 163                         |

<sup>a</sup>Solutions consisted of 0.5 mM H₂O₂, 0.05–0.1 μM nNOS, 50 mM Tris-HCl (pH 7.5), 10 μg/ml CaM, 1 mM CaCl₂, 5 μM FAD, 5 μM FMN, 100 units/ml catalase, 10 units/ml superoxide dismutase, 5 μM H₂B, and 5 μM DTT.

<sup>b</sup>Solutions consisting of 0.5 mM l-Arg, 0.05–0.1 μM nNOS, 50 mM Tris-HCl (pH 7.5), 10 μg/ml CaM, 1 mM CaCl₂, 5 μM FAD, 5 μM FMN, 5 μM H₂B, and 5 μM DTT.

whereas those of the other mutants were between 52 and 78 nmol/min/nmol of heme, which is comparable to that of the wild type enzyme (72 nmol/min/nmol of heme). These relatively high activities could be caused by the shuttling reaction with H₂O₂, a by-product of O₂ reduction on catalytic uncoupling (14–18, 20, 21). However, this is unlikely in the presence of catalase, therefore, it may indicate that the second step of the reaction (monooxygenation of NHA) is less dependent on the supply of electrons to the heme iron than the first step (monooxygenation of l-Arg). This is consistent with the fact that monooxygenation of NHA requires only 1 electron equivalent per NO generated, whereas NO generation from l-Arg requires 3 electron equivalents.

Since the low activity of the K423E mutant appeared to be associated with the heme reduction rate and/or the electron transfer rate from NADPH via FAD and FMN in the reductase domain to the heme iron, the rate of the heme reduction on addition of excess NADPH was examined under both aerobic and anaerobic conditions. Fig. 3 shows the Soret absorption spectral changes of the wild type and K423E mutant in the presence of CO on addition of NADPH under anaerobic conditions. The 0.5 μM wild type nNOS quickly (in less than 0.5 min) showed a peak at around 445 nm on addition of 0.1 mM NADPH, while the K423E mutant showed only a small peak at around 445 nm even after 40 min incubation under the same conditions. A similar trend was seen under aerobic conditions. Table I summarizes the rate of heme reduction in the presence of NADPH for the other Lys423 mutants. All the mutants generated in this study showed significantly slower rates of heme reduction than the wild type enzyme.

It was also interesting to note that the intensity of the wild-type Fe(II)-CO complex caused by adding excess NADPH under anaerobic conditions reached up to about 50% of that caused by adding sodium dithionite. This intensity with excess NADPH never increased to the same intensity as with sodium dithionite even in the presence of l-Arg and H₂B. The Lys423 mutants behaved similarly. This phenomenon may be caused by the slow disproportionation of electrons required for full reduction by NADPH, and/or the fact that the reduction potential of NADPH is not as negative as dithionite so that it is a less effective reducing agent even after equilibration. Under anaerobic conditions in the presence of NHA, the Soret intensity of the wild type Fe(II)-CO with NADPH was 60–70% of that with sodium dithionite. These relatively high NADPH oxidation rates, whereas both the wild type and the K423L and K423N mutants, which have large NO formation activities, also had relatively low NADPH oxidation rates. In the presence of NHA, the NADPH oxidation rates of the K423M, K423L, and K423N mutants were comparable to that of the wild type enzyme, whereas that of the K423E mutant was lower (Table II). The electrons from NADPH may be used...
Electron Transfer of Nitric-oxide Synthase

DISCUSSION

The absorption spectrum of the Fe(II)-CO complex of P450 or P450-type heme proteins reflects the integrity of the heme active site structure in that the presence of a 420-nm peak indicates enzyme denaturation. Since all the Lys423 mutants we generated had normal Fe(II)-CO spectra, with no band around 420 nm, the Lys423 residue is unlikely to be directly involved in the binding of heme to the active site. If Lys423 were to directly contact the heme plane and/or the heme propionate, heme binding to the apoprotein would be expected to be destabilized if these interactions were disrupted by the mutations.

As can be seen in the amino acid sequence comparison (Fig. 1), Lys423 is not well conserved throughout the NOS isoforms. Lys423 of nNOS corresponds with Asn202 of iNOS. The K423N mutant of nNOS we generated in this study had similar catalytic activity, changes in salt concentration should influence the enzyme’s kinetic parameters (31). Fig. 5 shows the effect of KCl on the NO formation activities of the wild type enzyme and Lys423 mutants. The NO formation activity of the wild type enzyme increased by 1.8-fold when the KCl concentration was increased up to 200 mM. However, the activity of K423N did not essentially change even on adding up to 500 mM KCl. The activities of K423M and K423L decreased by half on addition of 500 mM KCl.

In order to understand how mutations influence the monomer-dimer equilibrium, we examined low-temperature SDS-polyacrylamide gel electrophoresis and gel filtration column chromatography. Essentially no change in the equilibrium was found when the mutant enzymes were compared with the wild type (not shown).

The crystal structure of the oxygenase domain of bovine eNOS indicates that the amino nitrogen of the axial ligand, Cys415 (Cys414 of human eNOS, Ref. 11), interacts with several proximal site amino acids, including Gly188, Arg189, and Trp180 via ionic or hydrogen bonds (10). The proximal structure of nNOS is likely to be similar to that of eNOS, in which case the axial ligand, Cys415, will interact with Trp409 of nNOS. The crystal structure of eNOS also indicates that Lys194, which corresponds with Lys423 of nNOS, probably interacts with Trp180, which corresponds with Trp409 of nNOS, via a hydrogen bond over about 2.4 Å distance (with RasMac 2.6-ucb1.0 software) (Fig. 6). In the present study, the replacement of Lys423...
with acidic or neutral residues (Glu, Met, or Leu) resulted in a clear decrease in the NO formation activity observed. Therefore, these results suggest that ionic interactions or hydrogen bonding between Lys423 and Trp409 in the oxygenase domain or adjacent residues in the reductase domain are tightly associated with NO formation and electron transfer from the reductase domain. Thus, the disruption of these ionic interactions by mutation of Lys423 markedly reduced the NO formation activity and the rate of electron transfer from NADPH to the heme iron. The K423N mutant retained NO formation activity, confirming its compatibility with this position indicated by the sequence of iNOS. Presumably, Asn is able to form similar contacts to Lys, recreating a viable electron transfer route from the reductase domain to the heme. In the crystal structure of the eNOS oxygenase domain, the heme is clearly displaced to one side of the protein. The region in which the heme lies closest to the solvent exposed surface of the domain includes the Lys194 residue (Lys423 in nNOS), which crystallizes in direct contact with several water molecules. In fact, the heme is only 6 Å from this residue and only 5 Å from the solvent exposed surface. In view of this, it seems likely that Lys423 may form direct contact with the reductase domain, acting as the start of an electron transfer pathway also including the aromatic residue Trp409 which would bridge the gap between the heme and Lys423. As a matter of fact, the molecular surface near the FMN-binding site of NADPH-cytochrome P450 reductase has several acidic residues which are conserved in nNOS (Fig. 7) (32, 33). It is conceivable that these acidic amino acids of the reductase domain interact with basic amino acids of the oxygenase domain for effective electron transfer (Fig. 7).

Ionic interactions in the heme vicinity may also be important to control the redox potential of the heme iron maintaining an appropriate driving force for electrons to transfer to the heme iron (34). It was difficult, however, to obtain redox potentials for the mutant enzymes because the absorption bands of FAD and FMN hamper the monitoring of the heme absorption accompanying the redox change. However, the reduction rate of the K423E ferric heme by sodium dithionite appeared to be lower than that of the wild type enzyme under the same conditions. Thus, it is possible that the lowered NO formation activity and electron transfer rate of the K423E mutant is also associated with a change in the redox potential of the heme iron. However, dithionite may reduce the heme directly via an alternative pathway. As such, the slow reduction of the heme in the K423E mutant could be simply a consequence of charge repulsion between dithionite ion and the glutamic acid side chain.

Increasing the KCl concentration increases the rate of cytochrome c reduction by NADPH-cytochrome c reductase (35). Also, P450 monooxygenase activity increases with increasing KCl concentration when the P450:NADPH-P450 reductase molar ratio is 10 (36). Thus, it is not uncommon for protein-protein interactions to be stabilized at high concentrations of KCl and intermolecular electron transfer rates increased. The NO formation rate of the wild type enzyme increased with the KCl concentration (Fig. 5), similar to the results obtained elsewhere (31). Following the Lys423 mutations, this tendency was mark-

![Fig. 6. Hypothetical model of the active site of nNOS oxygenase domain created by placing the nNOS sequence on the eNOS backbone (10). Trp409, Cys415, and Lys423 of nNOS correspond with Trp180, Cys186, and Lys194 of eNOS, respectively.](image)

![Fig. 7. Hypothetical surfaces of interaction of NOS oxygenase domain (left) and FMN-binding domain (right). The oxygenase domain is based on the structure dimeric eNOS (10). On one subunit all basic and acidic atoms are in blue and red, respectively. The other subunit is colored yellow. The heme is in magenta. The residue Lys423 is indicated. The FMN-binding domain is based on the structure of the corresponding domain of NADPH-cytochrome P450 reductase (32, 33). All surface residues in the proximity of the FMN are colored as above according to their corresponding residues in nNOS. The FMN is in yellow.](image)
Electron Transfer of Nitric-oxide Synthase

Electron Transfer of Nitric-oxide Synthase

1. Feelisch, M., and Stamler, J. S. (eds) (1996) Methods in Nitric Oxide Research, John Wiley & Sons, Chichester
2. Ignarro, L., and Murad, F. (eds) (1996) Nitric Oxide, Biochemistry, Molecular Biology, and Therapeutic Implications, Academic Press, San Diego
3. Lancaster, J., Jr. (ed) (1996) Nitric Oxide, Principles and Actions, Academic Press, San Diego
4. Maines, M. D. (ed) (1996) Nitric Oxide Synthase, Characterization and Functional Analysis, Academic Press, San Diego
5. Moncada, S., Stamler, J., Gross, S., and Higgs, E. A. (eds) (1996) The Biology of Nitric Oxide, Part 5, Portland Press, London
6. Stuehr, D. J. (1997) Annu. Rev. Pharmacol. Toxicol. 37, 339–359
7. Masters, B. S. S. (1994) Annu. Rev. Nutr. 14, 131–145
8. Matsuzaki, A., Stuehr, D. J., Olson, J. S., Clark, P., and Ikeda-Saito, M. (1994) J. Biol. Chem. 269, 20335–20339
9. Crane, B. R., Arvai, A. S., Ghosh, D. K., Wu, C., Getzoff, E. D., Stuehr, D. J., and Tainer, J. A. (1998) Science 279, 2121–2125
10. Raman, C. S., Li, H., Martasek, P., Keal, V., Masters, B. S. S., and Poulos, T. L. (1998) Cell 95, 927–937
11. Fischmann, T. O., Hruza, A., Niu, X. D., Posssetta, J. D., Lunn, C. A., Dolphin, E., Prongay, A. J., Reichert, P., Lundell, D. J., Narula S. K., and Weber, P. C. (1999) Nature Struct. Biol. 6, 233–242
12. Bec, N., Gorren, A. C. F., Voelker, C., Mayer, B., and Lange, R. (1998) J. Biol. Chem. 273, 13562–13568
13. Perry, J. M., Stuehr, D. J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11101–11106
14. Groves, J. T., and Han, Y.-Z. (1995) in Cytochrome P450, Structure, Mechanism, and Biochemistry (Ortiz de Montellano, P. R., ed) 2nd Ed., pp. 3–48, Plenum Publishing Corp., New York
15. Korth, H.-G., Sustman, R., Thater, C., Butler, A. R., and Ingold, K. U. (1994) J. Biol. Chem. 269, 17776–17779
16. Pufahl, R. A., Wishnok, J. S., and Martella, M. A. (1995) Biochemistry 34, 1930–1941
17. Claque, M. J., Wishnok, J. S., and Martella, M. A. (1997) Biochemistry 36, 14465–14473
18. Rusche, K. M., Spiering, M. E., and Martella, M. A. (1996) Biochemistry 37, 15053–15052
19. Abu-Soud, H. M., Presta, A., Mayer, B., and Stuehr, D. J. (1997) Biochemistry 36, 10811–10816
20. Johnson, E. F., and Waterman, M. R. (1996) Methods Enzymol. 272, 1–430
21. Ortiz de Montellano, P. R. (ed) (1995) Cytochrome P-450, Structure, Mechanism and Biochemistry, 2nd Ed., Plenum Press, New York
22. Porter, T. D., and Coon, M. J. (1991) J. Biol. Chem. 266, 13469–13472
23. Guengerich, F. P. (1991) J. Biol. Chem. 266, 10019–10022
24. Siddhanta, U., Presta, A., Fan, B., Wolan, D., Lunn, C. A., and Stuehr, D. J. (1998) J. Biol. Chem. 273, 18950–18958
25. Shimizu, T., Tateishi, T., Hatano, M., and Fujii-Kuriyama, Y. (1991) J. Biol. Chem. 266, 3372–3375
26. Mayuzumi, H., Sambongi, C., Hiraya, K., Shimizu, T., Tateishi, T., and Hatano, M. (1993) Biochemistry 32, 5622–5628
27. Nakano, R., Sato, H., Watanabe, A., Ito, O., and Shimizu, T. (1996) J. Biol. Chem. 271, 8570–8574
28. Sagami, I., and Shimizu, T. (1998) J. Biol. Chem. 273, 2105–2108
29. Sato, H., Sagami, I., Daff, S., and Shimizu, T. (1998) Biochem. Biophys. Res. Commun. 253, 845–849
30. Presta, A., Weher-Main, A. M., Stankovich, M. T., and Stuehr, D. J. (1998) J. Am. Chem. Soc. 120, 9460–9465
31. Nishimura, J. S., Narayanasami, R., Miller, R. T., Roman, L. J., Panda, S., and Masters, B. S. S. (1999) J. Biol. Chem. 274, 5399–5406
32. Sevrioukova, I. F., Li, H., Zhang, H., Petersen, J. A., and Poulos, T. L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1863–1868
33. Wang, M., Roberts, D. L., Paschke, R., She, T. M., Masters, B. S. S., and Kim, J.-J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8411–8416
34. Raag, R., and Poulos, T. L. (1989) Biochemistry 28, 917–922
35. Shen, A., Christensen, M. J., and Kasper, C. B. (1991) J. Biol. Chem. 266, 19976–19980
36. Voznesensky, A. I., and Schenkman, J. B. (1992) J. Biol. Chem. 267, 1060–1067
37. Martasek, P., Miller, R. T., Liu, Q., Roman, L. J., Salerno, J. C., Migita, C. T., Raman, C. S., Gross, S. S., Ikeda-Saito, M., and Masters, B. S. S. (1998) J. Biol. Chem. 273, 3469–34705

By changing the ionic interactions of Lys423 with other amino acids, it appears that electron transfer from NADPH to the heme iron is involved in catalyzing NO formation and in electron transfer from NADPH to the heme. Based on the NADPH oxidation rate in the catalytic generation of NO and in electron transfer from NADPH to the heme iron, it seems clear that the ionic interaction of Lys423 with other amino acids is important in catalyzing NO formation and in facilitating electron transfer from NADPH to the heme. Such a change in ionic strength dependence is characteristic of intermolecular electron transfer, but in the case of NO this would involve interdomain or intersubunit electron transfer. However, if the domains involved retain a degree of relative conformational mobility, the same principles would apply. Intermolecular electron transfer across the interfacial surface between the oxygenase and adjacent reductase of the microsomal P450 system was found to involve the conserved Lys/Arg residues of the proximal surface which interact with Asp/Glu residues of the NADPH-P450 reductase surface (25, 32). A similar interaction appears to be involved in the case of nNOS. The optical absorption intensity of the Fe(II)-CO complex caused by NADPH reduction (Fig. 3) was only about 50% of that induced by sodium dithionite in the presence of l-Arg. Similar observations are reported for wild-type iNOS (24) and a C331A mutant of nNOS (37). The intensity never reached the same level with sodium dithionite even in the presence of a large excess of NADPH under anaerobic conditions. It is conceivable that catalytically generated NO quickly binds to the reduced heme and hampers further binding of CO to the heme. However, this is unlikely under anaerobic conditions because NO formation would be negligible. It is also possible that electron transfer between reductase domains is necessary to aid full reduction, which would be a slow process. NADPH is a large excess of NADPH under strict anaerobic conditions. It is possible that electron transfer between reductase domains is necessary to aid full reduction, which would be a slow process. NADPH is only able to supply electrons in pairs, limiting the equilibration process severely.