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)

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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$^{409}$ which lies in contact with the heme plane and with Cys$^{412}$, 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 intramolecular 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 Lys423 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—H4B 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 αCD phoshatase promoter previously used for the expression of cytochrome P450 IA2 (25–27). The oligonucleotide primers for the mutations of Lys423 to Glu, Met, Leu, and Asn were 5′-CCAGTGGTCGAGCTGAC-GG-3′, 5′-CACTGGTCATGTCAGCGGT-3′, 5′-CACTGGTCGCTGCGGT-3′, and 5′-AGTGGTCAGGTCAAGGTG-3′, respectively. The polymerase chain reaction-based mutageneses 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 and by Western blot analysis. The purified and concentrated 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. 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 (FeII)-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.

**RESULTS**

We generated K423E, K423M, K423L, and K423N mutants. The Soret spectral band of the resting Fe(III) form of all the 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).

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**Fig. 1.** Amino acid sequences at the proximal site of nNOS (1–7). ϒ designates the amino acid residue mutated in this study.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Mutation</th>
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<tbody>
<tr>
<td>Lys423</td>
<td>Glu</td>
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<tr>
<td>Lys423</td>
<td>Met</td>
</tr>
<tr>
<td>Lys423</td>
<td>Leu</td>
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<td>Lys423</td>
<td>Asn</td>
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**Fig. 2.** Optical absorption spectra (upper) of Fe(III) complexes in the absence (red) and presence (green, 1 min; blue, 6 min after addition) of 0.5 mM L-Arg for the wild type (A) and the K423E mutant (B). Difference absorption spectra (lower) of Fe(II)-CO complexes of the wild type (C) and K423E mutant (D).

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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 Lys423 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,
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 shunt reaction with H$_2$O$_2$, a by-product of O$_2$ 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 formation, 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 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 H$_2$O$_2$. 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 for the wild type (not shown). For the Lys423 mutants, no essential difference between l-Arg and NHA solutions was observed. The heme reduction rate with NADPH in the presence of NHA was merely 10–20% higher than in the presence of l-Arg for the wild type. Similar increase of the reduction rate was observed for the Lys423 mutants.

In order to understand the coupling of electron transfer to NO formation, we obtained the NADPH oxidation rate during catalysis (Table I). The rates of NADPH oxidation of the wild type and the mutants compare well with the corresponding NO formation rates. Namely, both the wild type and the K423L and K423N mutants, which have large NO formation activities, showed relatively high NADPH oxidation rates, whereas both the K423E and K423M mutants, which have low 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
to generate \( \text{H}_2\text{O}_2 \) to a certain extent, which may then be used for propagating NHA monoxygenation via the shunt reaction. It should be also noted as a possibility that a significant amount of reducing equivalents leaked to form superoxide anion radicals.

During P450-type catalysis, \( \text{H}_2\text{O}_2 \) is formed when O-O bond cleavage is not coupled well to electron transfer (20–23). The \( \text{H}_2\text{O}_2 \) formation rates of the K423E and K423M mutants, which exhibit low catalytic activities with L-Arg, were higher than those of the wild type enzyme and the K423N and K423L mutants (Table I). The reciprocal relationships between catalytic activity and \( \text{H}_2\text{O}_2 \) formation rates for the wild type and Lys423 mutants were also observed in the P450 system.

It is difficult to obtain the redox potential of the heme iron of the holoenzyme since FAD and FMN absorption bands overlap with the heme absorption band (30). However, we estimated the relative reduction ability of the heme iron of the mutant enzymes on addition of sodium dithionite. Fig. 4 shows how the heme of the wild type and the K423E mutant were reduced by addition of the same amount of sodium dithionite under anaerobic conditions. The 0.3 \( \mu \text{M} \) wild type heme was almost completely reduced in 3 min in the presence of CO by adding 40 \( \mu \text{M} \) sodium dithionite, while only 15% of the K423E mutant heme was reduced even after 20 min following the same procedure. Thus, it appears that the heme iron of the K423E is difficult to reduce by both sodium dithionite and NADPH.

If ionic interactions are important in the formation of protein-protein complexes, determining protein conformation and 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).

**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 Asn423 of iNOS. The K423N mutant of nNOS we generated in this study had similar catalytic activity to the wild type enzyme and its other kinetic parameters were comparable. Thus, there appears to be no significance in the lack of conservation of this residue in nNOS and iNOS.

The crystal structure of the oxygenase domain of bovine eNOS indicates that the amino nitrogen of the axial ligand, Cys184 (Cys186 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 Lys414, which corresponds with Lys423 of nNOS, probably interacts with Trp410 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 alanine decreased both the catalytic activity and H2O2 formation rates for the wild type and Lys423 mutants. The NO formation activity of the wild type and Lys423 mutants. The NO formation activity of the wild type and Lys423 mutants. The NO formation activity of the wild type and Lys423 mutants. The NO formation activity of the wild type and Lys423 mutants.
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 Lys$^{423}$ and Trp$^{409}$ 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 Lys$^{423}$ 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 Lys$^{194}$ residue (Lys$^{423}$ 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 Lys$^{423}$ may form direct contact with the reductase domain, acting as the start of an electron transfer pathway also including the aromatic residue Trp$^{409}$ which would bridge the gap between the heme and Lys$^{423}$. 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 Lys$^{423}$ mutations, this tendency was mark-

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**FIG. 6.** Hypothetical model of the active site of nNOS oxygenase domain created by placing the nNOS sequence on the eNOS backbone (10). Trp$^{409}$, Cys$^{415}$, and Lys$^{423}$ of nNOS correspond with Trp$^{180}$, Cys$^{186}$, and Lys$^{194}$ of eNOS, respectively.

**FIG. 7.** Hypothetical surfaces of interaction of NOS oxygenase domain (left) and FMN-binding domain (right). The oxygenase domain is based on the structure odimeric 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 Lys$^{423}$ 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.
edly changed. The salt (KCl) effect probably implies a possible direct solvent exposure of this area including Lys423. Therefore, 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 NOS 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 interdomain action 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 1-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 strict 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 only able to supply electrons in pairs, limiting the equilibration process severely. When reduced, the electrons will be shared only able to supply electrons in pairs, limiting the equilibration to aid full reduction, which would be a slow process. NADPH is that electron transfer between reductase domains is necessary because NO formation would be negligible. It is also possible conceivable that catalytically generated NO quickly binds to the reduced heme. However, this is unlikely under anaerobic conditions including Lys423. Therefore, 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 NOS 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 interdomain action appears to be involved in the case of nNOS.

In summary, it was found that Lys423 is very much involved in the catalytic generation of NO and in electron transfer from NADPH to the heme iron. Based on the NADPH oxidation rate and the heme reduction rate of the Lys423 mutants, it appears that Lys423 forms a key contact in the electron transfer route from the FMN of the reductase domain to the heme of the oxygenase domain via the proximal site.

References

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doi: 10.1074/jbc.274.38.26956

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