Arg410 near the Heme Proximal Ligand of Neuronal Nitric Oxide Synthase Is Critical for Both Substrate Recognition and Electron Transfer

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Mutations at a well-conserved Arg410 located near the heme proximal axial ligand in the oxygenase domain of neuronal NOS (nNOS) abolished both substrate recognition and electron transfer to the heme, suggesting that this residue plays an important role in the architecture of the substrate recognition site and the electron transfer process.

Nitric oxide (NO) is generated by nitric oxide synthase (NOS), and has multiple vital functions in physiology and pathology.[1–6] To date, three NOS isoforms have been cloned from a variety of sources.[1–6] Interestingly, although the modes of expression and post-translational control of the NOS isoforms are distinct, their primary sequences and protein structures are similar. All NOS enzymes are homodimers, with each subunit comprising an N-terminal oxygenase domain that binds iron protoporphyrin IX (heme) and (6*R*)-5,6,7,8-tetrahydro-L-biopterin (H4B) as prosthetic groups, and a C-terminal reductase domain that binds FAD, FMN, and NADPH. Two stepwise monooxidation reactions on the substrate, L-Arg, via an intermediate, N^{ω}-hydroxy-L-Arg (NHA), occur at the heme distal side using molecular oxygen with the aid of electrons transferred through the reductase domain from NADPH. Calmodulin (CaM) binding to a site between the two domains is essential to facilitate electron transfer within the reductase domain and between the two domains. H4B is additionally important for electron transfer to activate molecular oxygen via cleavage of the O–O bond.

Although the essential roles of CaM and H4B in NOS catalysis are different from those of P450 enzymes, the mechanism of interdomain electron transfer from the reductase domain to the heme is possibly similar to that of intermolecular electron transfer between P450 and NADPH-P450 reductase. In P450 (and perhaps NOS) appropriate interaction(s) between surface anionic side chain(s) of Glu or Asp of the reductase domain and surface cationic side chain(s) of Arg or Lys of the oxygenase domain are essential for efficient electron transfer to initiate catalysis.[7] In fact, a previous study by our group on neuronal NOS (nNOS)[8] revealed that conserved Lys423 located on the surface of the oxygenase domain is important for catalysis associated with electron transfer to the heme from the reductase domain. The amino acid sequences (Figure 1) and structures of the oxygenase domains of NOS enzymes disclose a well-conserved Arg410 near the surface of the heme proximal side.[9–11] In view of these findings, it is important to determine the role of Arg410 in catalysis and electron transfer.

In the present study, we generated Ala, Gln, Glu, Leu, and Lys mutants of Arg410, and examined NO formation and electron transfer activities. Surprisingly, Arg410 mutants lost substrate recognition properties and heme reduction by NADPH.

The Soret spectral bands of the resting Fe(III) complexes of all mutants consist of a mixture of low- and high-spin species, as shown previously.[12–14] The Fe(III) complex of wild-type NOS in the resting state has a broad Soret absorption band around 400 nm, which readily shifts to 395 nm, following the addition of L-Arg.[1–6,12–14] In contrast to wild-type NOS, the Soret bands of the Fe(III) complexes of the Arg410Ala, Arg410Gln, Arg410Glu, and Arg410Lys mutants did not display a spin shift upon addition of excess L-Arg, while a small change was observed in the position of the Soret band of the Fe(III) Arg410Leu mutant (Table 1). The results suggest that the substrate binding pocket is preserved in the Arg410Leu mutant. In contrast, for the other Arg410 mutants, substrate recognition capability was defective in terms of the substrate-induced Soret spectral change.[15] The heme coordination structures of all Arg410 mutants generated in the present study with Cys415 as an axial ligand were well preserved, since essentially no denatured forms (represented by a 420-nm absorption peak) were generated, as observed in the absorption spectra of the Fe(II)–CO complexes around 450 nm.

Most of the Arg410 mutants, except Arg410Leu, lost NO formation activity (Table 1). The NO formation rate of the Arg410Leu mutant with L-Arg substrate was half that of the wild-type, whereas that with NHA as a substrate was 83% that of the wild-type rate. These catalysis data are consistent with spectral findings. Specifically, optical absorption spectral changes due to substrate binding were not observed for the Ala, Gln, Glu, and Lys mutants, but were evident for the Leu mutant. Therefore, the significant decrease in catalytic activity of most Arg410 mutants is caused by the loss of substrate recognition, and not defects in the electron-transferring capability.[15]

To determine the specific role of Arg410 in electron transfer, heme reduction was examined by adding NADPH in the presence of CO.[12–14] The increase in absorption at 444 nm of the Fe(II)–CO complex of Arg410 mutants following the addition of NADPH (indicative of heme reduction) was monitored under anaerobic conditions. Ala, Gln, Glu, and Lys mutants did not exhibit heme reduction, whereas only a half of heme of the Leu mutant was reduced by NADPH compared with the wild-type enzyme ($2.0\,min^{-1}$). The data suggest that Arg410 plays an important role in intersubunit electron transfer between the two domains and/or intramolecular electron transfer within the oxygenase domain.

Unusual uncoupling of electron transfer with catalysis was additionally observed in the Arg410 mutants. In general, in the absence of substrate, the NADPH oxidation rate (80 nmol/min/nmol heme) by wild-type NOS is high because of uncoupling, whereas in the presence of the substrate, the rate is decreased (49 nmol/min/nmol heme) as a result of efficient cou-

pling of electron transfer with catalysis. In contrast, NADPH oxidation rates for the Arg410Ala and Arg410Leu mutants (89 and 79 nmol/min/nmole heme, respectively) in the presence of substrate were higher than those (49 nmol/min/nmol heme for both mutants) in the absence of substrate.[16] Thus, our results confirm that Arg410 plays an important role in electron transfer.

In summary, data from the present investigation strongly suggest that the well-conserved Arg410 near the heme proximal axial ligand, Cys415, is critical in the architecture of the heme distal side and electron transfer to the heme. The indirect effect of proximal mutations on substrate recognition at the heme distal side is a novel finding in this study.

	410	415	423
Rat eNOS	VFGAKQAWRNAPRCVGRIQWGKL		
Rat iNOS	IFATKMAWRNAPRCIGRIQWSNL		
Human nNOS	IYGAKHAWRNASRCVGRIQWSKL		
Rat nNOS	IYGAKHAWRNASRCVGRIQWSKL		

Figure 1. Amino acid sequences of NOSs. Note that C415 is the proximal axial ligand to the heme.

Table 1. Catalytic activities of Arg410 mutants (nmol/min/nmol heme)

Enzyme	NO formation rate		Substrate-induced Spin change
	+Arg	NHA	
wild-type	30 ± 9	24 ± 3	+
Arg410Ala	<2	<2	−
Arg410Gln	<2	<2	−
Arg410Glu	<2	<2	−
Arg410Leu	15 ± 1	20 ± 0	±
Arg410Lys	<2	<2	−

Rat nNOS cDNA was a kind gift from Dr. S. H. Snyder (Johns Hopkins University School of Medicine). For site-directed mutagenesis, polymerase chain reaction-based procedures were performed using oligonucleotide-directed dual amber long and accurate PCR kits (Takara Shuzo, Kyoto, Japan). Wild-type and mutant nNOS enzymes were purified using 2′,5′-ADP-Sepharose and calmodulin-Sepharose column chromatography, as described previously.[12–14] In all cases, purified nNOS was more than 95% homogenous, as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by staining with Coomassie Blue R-250, and Western blot analysis. The concentrations of nNOS enzymes were calculated optically from the [CO-reduced] − [reduced] difference spectrum using $\Delta\varepsilon_{444-467\,nm} = 55\,mM^{-1}\,cm^{-1}$.[12,13] 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 $49\,mM^{-1}\,cm^{-1}$.[12–14] Assays were performed at 25 °C in 50 mM Tris-HCl (pH 7.5) buffer containing 10 mM oxyhemoglobin, 0.1 mM NADPH, 5 μM FAD and FMN each, 10 mg/mL CaM, 1 mM $CaCl_2$, 100 units/ml catalase, 10 units/mL superoxide dismutase, 5 mM H4B, 5 mM dithiothreitol (DTT), and 0.05–0.1 mM nNOS in the presence or absence of 0.5 mM L-Arg or NHA.

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15 It appears that functions of the Arg410Leu mutant regarding substrate recognition and NO formation activity are similar to those of the wild-type enzyme. At this moment, it is not clear why these two enzymes have similar functional character. Perhaps, hydrophobic character and length of the side chain of Leu on the molecular surface are suitable for appropriate protein–protein interaction in the dimeric enzyme and thus the Leu mutation may not have largely altered the substrate recognition site. In contrast, those factors of other inactive mutants are different and may have cuased improper folding in the same site.
16 Higher NADPH oxidation rates of the Arg410Ala and Arg410Leu mutants reflect uncoupling of catalysis and electron transfer. Although electron leakage should exit, still certain portions of electrons from the reductase domain are used for catalysis of the Arg410Leu mutant.

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