Aromatic Residues and Neighboring Arg \(^{414}\) in the \((6R)-5,6,7,8\)-Tetrahydro-L-Biopterin Binding Site of Full-length Neuronal Nitric-oxide Synthase Are Crucial in Catalysis and Heme Reduction with NADPH* 

Nitric-oxide synthase (NOS) requires the cofactor, \((6R)-5,6,7,8\)-tetrahydrobiopterin (H4B), for catalytic activity. The crystal structures of NOSs indicate that H4B is surrounded by aromatic residues. We have mutated the conserved aromatic acids, Trp\(^{676}\), Trp\(^{678}\), Phe\(^{691}\), His\(^{692}\), and Tyr\(^{706}\), together with the neighboring Arg\(^{414}\) residue within the H4B binding region of full-length neuronal NOS. The W676L, W678L, and F691L mutants had no NO formation activity and had very low heme reduction rates (<0.02 min\(^{-1}\)) with NADPH. Thus, it appears that Trp\(^{676}\), Trp\(^{678}\), and Phe\(^{691}\) are important to retain the appropriate active site conformation for H4B/L-Arg binding and/or electron transfer to the heme from NADPH. The mutation of Tyr\(^{706}\) to Leu and Phe decreased the activity down to 13 and 29%, respectively, of that of the wild type together with a dramatically increased EC\(_{50}\) value for H4B (30–40-fold of wild type). The Tyr\(^{706}\) phenol group interacts with the heme propionate and Arg\(^{414}\) amine via hydrogen bonds. The mutation of Arg\(^{414}\) to Leu and Glu resulted in the total loss of NO formation activity and of the heme reduction with NADPH. Thus, hydrogen bond networks consisting of the heme carboxylate, Tyr\(^{706}\), and Arg\(^{414}\) are crucial in stabilizing the appropriate conformation(s) of the heme active site for H4B/L-Arg binding and/or efficient electron transfer to occur.

Nitrergic oxide has many diverse biological functions as an important signaling and cytotoxic molecule in the cardiovascular, nervous, and immune systems (Refs. 1–6 and references therein). NO is generated from L-Arg via formation of \((6R)-5,6,7,8\)-tetrahydrobiopterin (H4B), for catalytic activity. The intrinsic function of H4B in NOS catalysis is still unclear, although H4B binding affects the conformation of the heme domain (13–15, 17, 18), facilitates substrate binding (16), and also promotes subunit dimerization (9–12, 19, 20). From previous experimental observations, however, it is likely that the most important function of H4B would be participation in the reaction as a redox-active cofactor (14, 17, 20, 21, 25, 26). This paper is available on line at http://www.jbc.org

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1 The abbreviations used are: NOS, nitric oxide synthase; mNOS, neuronal NOS; iNOS, inducible NOS; eNOS, endothelial NOS; H4B, \((6R)-5,6,7,8\)-tetrahydro-L-biopterin; CaM, calmodulin; DTT, dithiothreitol.

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sequences among NOSs are shown in the bottom lines from the reductase domain to the heme active site of nNOS, both the NO formation activity and the electron transfer rate of various NOSs. Amino acid residues for rat nNOS (37), the eNOS backbone (28). Blue letters come from one subunit (A), and the residues indicated by black letters come from the another subunit (B) of NOS.

Mutagenesis—The cDNA for rat nNOS was kindly gifted from Dr. S. H. Snyder (Johns Hopkins University School of Medicine). Site-directed mutagenesis were performed with the polymerase chain reaction-based strategy using a kit from Takara Shuzo. CDNA fragments containing wild type and the desired mutations were cloned into NdeI and XhoI sites of a vector, pCWRi +, and transformed into Escherichia coli strain BL21(DE3) (32) in expressing most mutants and the wild type (32, 33). However, we used a yeast expression system (34–36) for obtaining the R414L mutant protein because of the instability of this mutant.

Preparation of Neuronal NOS—Full-length wild type and mutant nNOSs were purified using DEAE-TOYOPEARL 2.5'-ADP-Sepharose, and calmodulin-Sepharose column chromatographies as described previously (34–36). The purified and concentrated enzyme was dialyzed against 50 mM Tris-HCl (pH 7.5) buffer containing 5 μM H4B, 20 μM DTT, 0.1 mM EDTA, and 10% glycerol. For the preparation of H4B-deficient enzyme, E. coli cells expressing wild type and mutant enzymes were sonicated in the H4B-deficient buffer, and the enzymes were further purified in the H4B-deficient buffer. Because H4B was deficient in E. coli cells, the enzymes prepared without addition of exogeneous H4B should be H4B-free. All purified full-length nNOSs were more than 95% pure as judged by SDS-polyacrylamide gel electrophoresis stained with Coomassie Blue R-250. The concentration of nNOSs was determined optically from the [CO-reduced] – [reduced] difference spectrum using Δε444–607 nm = 55 μM−1 cm−1. This Δε value was estimated by the pyridine hemochromogen method (34–36) assuming that one heme is bound to one subunit of this enzyme.

**Fig. 1.** Amino acid sequence alignment of H4B binding domains of various NOSs. Amino acid residues for rat nNOS (37), human nNOS (38), bovine eNOS (39), human eNOS (40), mouse iNOS (41), human iNOS (42), and Drosophila NOS (43). The conserved sequences among NOSs are shown in the bottom lines. Each asterisk designates the amino acid residue mutated in this study.

**Fig. 2.** Hypothetical structure of the H4B binding domain of nNOS dimer interface created by placing the nNOS sequence on the eNOS backbone (28). Trp276, Trp278, Phe311, His602, and Tyr706 of NOS correspond with Trp411, Trp413, Phe462, His463, and Tyr477 of eNOS. The residues and heteromolecules indicated by black letters come from one subunit (A), and the residues indicated by blue letters come from the another subunit (B) of NOS.

stacking interactions influence the dimeric structure of the oxygenase domain of iNOS, the heme environment, and NO synthesis but are not essential for H4B binding and NO formation activity.

In the present study, we have mutated the conserved aromatic amino acids Trp276, Trp278, Phe311, His602, and Tyr706 of full-length nNOS and studied the effects on the spectroscopic characteristics, dimer formation, catalytic activity, and electron transfer rate from NADPH to the heme. We also generated mutants at Arg114, which forms hydrogen bonds between Tyr706 and Trp278. Mutation of these residues greatly reduced both the NO formation activity and the electron transfer rate from the reductase domain to the heme active site of nNOS, even if dimer dissociation by the mutations was not marked. We will discuss the role of these aromatic amino acids in catalysis and electron transfer in full-length nNOS.

**Experimental Procedures**

**Materials**—A polymerase chain reaction kit for site-directed mutagenesis was obtained from Takara Shuzo (Tokyo, Japan). H4B was purchased from Schircks Laboratories (Jona, Switzerland). 2',5'-ADP-Sepharose and CaM-Sepharose were products of Amersham Pharmacia Biotech. Other reagents were obtained from Sigma or Wako Pure Chemicals (Osaka, Japan).

**Mutagenesis**—The cDNA for rat nNOS was kindly gifted from Dr. S. H. Snyder (Johns Hopkins University School of Medicine). Site-directed mutagenesis were performed with the polymerase chain reaction-based strategy using a kit from Takara Shuzo. CDNA fragments containing wild type and the desired mutations were cloned into NdeI and XhoI sites of a vector, pCWRi +, and transformed into Escherichia coli strain BL21(DE3) (32) in expressing most mutants and the wild type (32, 33). However, we used a yeast expression system (34–36) for obtaining the R414L mutant protein because of the instability of this mutant.

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trophoresis stained with Coomassie Blue R-250. The concentration of nNOSs was determined optically from the [CO-reduced] – [reduced] difference spectrum using Δε444–607 nm = 55 μM−1 cm−1. This Δε value was estimated by the pyridine hemochromogen method (34–36) assuming that one heme is bound to one subunit of this enzyme.

**Gel Filtration**—To examine the dimer contents of the mutants, purified full-length NOSs were incubated with 1 mM L-Arg and 0.1 mM H4B overnight at 4 °C and analyzed on a Superose 6 HR10/30 size exclusion chromatography column (Amersham Pharmacia Biotech), equilibrated with 50 mM Tris-HCl (pH 7.5) buffer containing 0.2 mM NaCl, 0.1 mM EDTA, and 0.1 mM L-Arg, and connected to an fast protein liquid chromatography system (Amersham Pharmacia Biotech). The molecular masses of the protein peaks were estimated relative to the molecular mass of standard proteins: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), and albumin (67 kDa).

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 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 μM oxyhemoglobin, 0.1 mM NADPH, 5 μM each of FAD and FMN, 10 μg/ml CaM, 1 mM CaCl2, 100 units/ml aldolase, 10 units/ml superoxide dismutase, 5 μM H4B, 20 μM DTT, and 0.05–0.1 μM nNOS in the presence or absence of 0.1 mM L-Arg or N4'-hydroxy-L-Arg. Cytochrome c reductase activity was determined by monitoring the absorbance at 550 nm using an extinction coefficient of 21.1 mM−1 cm−1. The H2O2 generation rate was measured by the formation of ferric thiocyanate under similar conditions as described for the NO formation activity and NADPH oxidase activity but without catalase and superoxide dismutase.

**Optical Absorption Spectra**—Spectral experiments under aerobic conditions were carried out on a Shimadzu UV-2500 spectrophotometer maintained at 25 °C by a temperature controller. Anaerobic spectral experiments were conducted on a Shimadzu UV-160A spectrophotometer maintained at 15 °C in a glove box under a nitrogen atmosphere with an O2 concentration of less than 50 ppm. To ensure that the temperature of the solution was appropriate, the cell was incubated for 10 min prior to spectroscopic measurements. Titration experiments were repeated at least three times for each complex. Regression analyses were performed, and lines giving an optimum correlation coefficient were selected. Experimental errors were less than 10%.

**Crystal Structure**—The crystal structure coordinates of bovine eNOS...
Biopterin Binding Site of Nitric-oxide Synthase

RESULTS

Aromatic Mutants

Spectral Properties—The mutants were expressed as full-length enzymes in 1 liter of culture of E. coli cells yielding 100–400 nmol. Purification of the mutants in the absence of H4B resulted in unstable enzymes. Therefore, all buffers used for enzyme preparation except for H4B-deficient enzyme preparation contained 5 μM H4B. The resting Fe(III) form of the W678L mutant had a Soret peak at 438 nm ascribed to the low spin type heme as shown in Fig. 3. These results suggest that the substrate binding site was changed by the mutation in terms of the absorption spectral character. The Fe(II) heme complex of the W678L mutant was 0.1 mML -Arg, Fe(II) complexes (sorption spectral change in the presence of 5 μM H4B, respectively. When L-Arg is used as the substrate, the NO formation rates of the W676L, W678L, and F691L mutants were less than 0.1 nmol/min/nmol heme. The rate of the W678H mutant was 17 nmol/min/nmol heme, which is 35% that of the wild type. The H692L mutant had a catalytic activity comparable with that of the wild type enzyme. The mutation of Tyr706 to Leu and Phe decreased the rate of NO formation down to 13 and 29% that of the wild type, respectively. When N^2-hydroxy-L-Arg, the reaction intermediate, was used as the substrate, the W676L, W678L, and F691L mutants again did not show any detectable NO formation activity. The other mutants had slightly higher NO formation rates with N^2-hydroxy-L-Arg compared with that with L-Arg, similar to that observed for the wild type enzyme. Thus, the mutations of aromatic residues in the dimer interface had similar effects on the NO formation with either L-Arg or N^2-hydroxy-L-Arg.

To elucidate how the mutations in the H4B binding site couple electron transfer from NADPH to NO formation activity, we determined the NADPH oxidation rate during catalysis (Table I). The NADPH oxidation rate of the wild type corresponds well with the NO formation rate, indicating close coupling of NADPH consumption with NO formation. However, all the mutants had relatively high NADPH oxidation rates compared with NO formation rates. In these mutants, NO synthesis was not coupled well with NADPH consumption, and the electrons from NADPH appear to be used to generate H2O2 or H2O. In fact, for most of the mutants except for the Y706L and Y706F mutants, the H2O2 formation rates were larger than the rate of the wild type (Table I). This uncoupling of the electron transfer was more obvious for the W676L, W678L, and F691L mutants, where NO formation activities were undetectable. It is interesting to note that the NADPH oxidation rates with the F691L and H692L mutants were higher than those observed for the wild type enzyme. The cytochrome c reductase activity of each mutant was essentially the same as that of the wild type enzyme (data not shown).

| Mutants       | NO formation | NADPH oxidation | H2O2 formation |
|---------------|--------------|-----------------|----------------|
|               | + L-Arg      | + NHA           | + L-Arg        | + NHA          |
| Wild type     | 48           | 51              | 81             | 80             | 13             |
| W676L         | <0.1         | <0.1            | 19             | 24             | 27             |
| W678L         | <0.1         | <0.1            | 46             | 53             | 38             |
| W678H         | 17           | 22              | 93             | 79             | 52             |
| F691L         | <0.1         | <0.1            | 113            | 139            | 83             |
| H692L         | 39           | 46              | 118            | 137            | 39             |
| Y706L         | 6            | 9               | 36             | 43             | 17             |
| Y706F         | 14           | 16              | 38             | 48             | 7              |

a Determined at 25 °C as described under “Experimental Procedures.”

b Determined at 25 °C under similar conditions for α without catalase and superoxide dismutase.

c Determined at 25 °C.

d Determined at 25 °C.

e Determined at 25 °C.

Heme Reduction with NADPH—To understand the electron transfer from the reductase domain to the heme active site, we examined the heme reduction rate with NADPH under anaerobic conditions. Table II summarizes the rate of heme reduction with NADPH for all the mutants generated in this study. Fig. 4 shows the Soret absorption spectral changes of the W678L and W678H mutants in the presence of CO caused by addition of NADPH, upon which time-dependent Fe(II)-CO complex formation was monitored. The heme of the W678L

Fig. 3. Optical absorption spectra of Fe(III) complexes in the absence (black) and presence (green, 10 min after addition) of 0.1 μM L-Arg, Fe(II) complexes (blue) and Fe(II)-CO complexes (red) for the full-length W678L (A) and the W678H (B) mutants. The solutions contain 5 μM H4B.

Experimental errors were less than 10%.
TABLE II
Dimer formation, heme reduction rates, and H4B dependence of the full-length nNOS mutants

| Mutants   | Dimer formation (per dimer) | Relative activity (per dimer) | Heme reduction with NADPH | H4B dependence (EC50) |
|-----------|-----------------------------|------------------------------|---------------------------|-----------------------|
| Wild type | 94                          | 100                          | 1.0                       | 0.5                   |
| W676L     | 28                          | <0.1                         | <0.01                     | ND                    |
| W678L     | 15                          | <0.1                         | 0.02                      | ND                    |
| W678H     | 73                          | 46                           | 1.0                       | 9                     |
| F691L     | 43                          | <0.1                         | <0.01                     | ND                    |
| H692L     | 75                          | 100                          | 0.45                      | 12                    |
| Y706L     | 55                          | 21                           | 0.75                      | 15                    |
| Y706F     | 65                          | 42                           | 1.3                       | 20                    |

- Estimated by gel filtration chromatography after overnight incubation with 0.1 mM l-Arg and 5 mM H4B.
- NO formation activity from l-Arg relative to that of the wild type per dimer. Values were estimated from the data in Table I and the dimer formation fractions described in this Table.
- Solutions consisting of 0.1 µM full-length nNOS, 0.1 mM l-Arg, 0.1 mM NADPH, 1 mM Tris-HCl (pH 7.5), 10 µg/ml CaM, 1 mM CaCl2, 5 µM H4B, and 20 µM DTT. Experiments were repeated at least three times, and the average values are shown. Experimental errors were less than 10%.
- Estimated from data in Fig. 6.
- ND, not determined.

FIG. 4. Absorption spectral changes at the Soret region of the W676L (A) and W678H (B) mutants of full-length nNOS caused by addition of NADPH in the presence of l-Arg and CO under anaerobic conditions. The solution composed of full-length nNOS (0.5 µM), 50 mM Tris-HCl (pH 7.5), 10 µg/ml CaM, 1 mM CaCl2, 5 µM H4B, 20 µM DTT, 0.1 mM NADPH, 0.1 mM l-Arg, and 1.2 mM CO. Final absorption bands were obtained by adding a trace amount of sodium dithionite. A, red, 8 min; green, 16 min; blue, 20 min; black, with sodium dithionite. B, red, 0.3 min; green, 1 min; blue, 2 min; orange, 6 min; black, with sodium dithionite.

The W676L mutant was very slowly reduced in the presence of l-Arg and H4B, and only a small fraction of the Fe(II)-CO complex was observed even after 20 min of incubation with NADPH. The W678H mutant was quickly reduced similar to that of the wild type enzyme (1.2 min⁻¹) under the same conditions. The heme reduction rates of the W676L and F691L mutants were less than 0.01 min⁻¹. Note that complete removal of l-Arg and H4B from the full-length nNOS does not prevent electron transfer to the heme and the enzyme retains a significant heme reduction rate of 0.08 min⁻¹ upon addition of excess NADPH. Thus, the very slow heme reduction rates observed for those Trp and Phe mutants are mostly due to the effects of the mutation but not to the low affinity of l-Arg and H4B to the aromatic mutants. The mutation of His692 to Leu did not markedly change the heme reduction rate, in accordance with the effect on the NO formation activity. The removal of the hydroxyl group from Tyr706 did not affect the heme reduction rate with NADPH, even though the Y706F mutant had NO formation activity 40% that of the wild type enzyme.

Dimer Formation—It has been suggested that only the dimeric form of NO is catalytically active (9). The dimer formation ability of each mutant enzyme was analyzed in the absence of l-Arg and H4B, or after overnight incubation with l-Arg and H4B, using gel filtration column chromatography, to evaluate whether the mutations influence the monomer-dimer equilibrium. The gel filtration profiles shown in Fig. 5 (A and B) indicate that the full-length wild type and W678H mutant were predominantly oligomers in the absence of l-Arg and H4B. The overnight incubation with l-Arg and H4B resulted in an increase in the fraction of the dimeric form. In contrast, the W676L mutant was mainly oligomeric even after the incubation (Fig. 5C). Interestingly, all mutants studied here always had a proportion of the dimeric form present, with the different percentages summarized in Table II. Thus, it appears that the aromatic mutations of the H4B binding site never completely abolish the dimer formation ability of full-length nNOS. This finding is in contrast with that observed for the oxygenase domain of iNOS, where some aromatic mutations at the H4B binding site dissociate the dimer to the monomer (31). We also estimated the relative NO formation activity for each mutant per dimeric form as shown in Table II. Here again, it appears that the aromatic amino acids such as Trp676, Trp678, and Phe701 are important for NO formation activity and are involved in the electron transfer from NADPH to the heme active site.

Effect of H4B on the Activity—Next we prepared H4B-free enzymes to examine the H4B dependence of the NO formation activity. The resting form of H4B-free wild type enzyme had absorption spectra with a maximum at 418 nm ascribed to the low spin complex (not shown). The H4B-free wild type full-length nNOS did not show any obvious high spin shift on addition of H4B even after 4 h of incubation at 15 °C. However, the addition of l-Arg resulted in a high spin shift within 10 min.
(not shown). This is in contrast to iNOS, where addition of H4B to H4B-free enzyme caused a clear Soret spectral shift from the low spin (420 nm) to the high spin (396 nm) (13). H4B-free W678L, H692L, Y706L, and Y706F mutants of full-length nNOS showed similar spectral shift to the wild type enzyme. All of these H4B-free enzymes did not show any NO formation activity in the absence of H4B as with the wild type enzyme. However, NO formation was observed immediately after addition of the enzyme to the reaction mixture containing H4B with L-Arg. The NO formation rates of the mutants increased with increasing the H4B concentration as with the wild type enzyme (Fig. 6). The EC_{50} values for H4B on NO formation are summarized in Table II. All the mutations studied here resulted in a dramatic increase in the EC_{50} values, indicating a substantial decrease in H4B binding affinity. Note that especially, the mutation of Tyr706 affected H4B binding by increasing the EC_{50} value by 30–40-fold compared with the wild type enzyme.

**R414 Mutants**

Tyr^706 probably interacts with both Arg^414 and the heme carboxylate via hydrogen bonds and is located in close proximity to Trp^678 in full-length nNOS, based on homology-based comparison with the crystal structures of iNOS and eNOS oxygenase-domain dimers (Fig. 7). Arg^414 is well conserved among NOs. To evaluate the role of this residue in the catalytic activity of and electron transfer within full-length nNOS, Arg^414 mutants were prepared and characterized. The resting state of R414Q had a Soret peak at 400 nm, and addition of L-Arg resulted in high spin shift similar to the wild type enzyme. In contrast, R414E and R414L mutants had their Soret peaks at 420 and 441 nm, respectively (not shown). Addition of L-Arg to the resting R414E and R414L mutants did not change the Soret band (not shown), similar to that observed for the W678L, W678L, and W692L mutants. The Fe(II)-CO complexes of all Arg^414 mutants had their Soret absorption bands at around 445 nm. In the case of R414E mutant, the absorption peak of the Fe(II)-CO complex quickly moved from 445 to 420 nm, suggesting that the heme active site of the enzyme was very unstable after the mutation. Table III summarizes various kinetic parameters for the Arg^414 mutants. The NO formation activities of R414E and R414L mutants were less than 0.1 nmol/min/nmol heme, whereas the R414Q mutant had activity comparable with that of the wild type enzyme. The heme reduction rates of the R414E and R414L mutants with excess NADPH were less than 0.01 min^{-1}, whereas the reduction rate of R414Q mutant was similar to that of the wild type enzyme. The EC_{50} values for H4B on NO formation activity for the R414Q mutant was 0.4 \mu M, which is similar to that of the wild type. Thus, it appears that Arg^414 is important in the catalytic activity associated with electron transfer from the reductase domain to the heme active site.

**DISCUSSION**

In the present study, mutational analysis was used to address the specific roles of Arg^414 and the aromatic residues of the H4B binding site in catalysis and electron transfer. Hydrogen bond networks between H4B and the backbone carboxyls of eNOS residues Trp^449 (which corresponds with Trp^678 of nNOS) and Phe^462 (Phe^691 of nNOS), and a water-bridged hydrogen bond to His^463 (His^692 of nNOS) are to be considered (28). In the dimeric interface of nNOS, the H4B ring must be located between Trp^478 of one subunit and Trp^676, Phe^691, and His^692 of the other subunit, based on the crystal structure of eNOS (Fig. 2). The mutation of the aromatic residues Trp^676, Trp^678, and Phe^691 to Leu and the mutation of Arg^414 to Glu or Leu resulted in a clear loss of NO formation activity and a loss of heme reduction ability in the presence of NADPH under anaerobic conditions (Tables I–III).

**Spectral Properties**—The Soret absorption bands of the W678L (Fig. 3D), W678L (Fig. 3A), F691L, and R414L mutants were located at around 438–441 nm. Low spin Fe(III) cytochrome P-450 complexes with oxygen or nitrogen sixth axial ligands have Soret bands at around 416–425 nm (44, 45). Low spin Fe(III) complexes with sulfur as the sixth axial ligand have Soret band positions at around 461–465 nm for cytochrome P-450s (46) or 455–460 nm for NOs (47, 48), respectively. Subunit dissociation or denaturation with urea resulted in a shift in the Soret absorption band to 460 nm (48). The cytochrome P-450-cyanide complex has a Soret absorption band at around 440 nm (46). Therefore, at this point, it is difficult to
assign the 6th axial ligand of these mutants.

The absorption band of the W678L, W676L, F691L, and R414L mutants did not move on addition of excess L-Arg. This suggests that the substrate binding site of the full-length nNOS was greatly affected and that the affinity of L-Arg may be greatly decreased. It is difficult to examine how the H4B binding site in full-length nNOS was changed by the aromatic and Arg141 mutations. However, the presence of 5 μM H4B markedly stabilized the mutant structures, suggesting that H4B binding is still effective in structuring the active site cavity appropriately.

**Catalytic Properties**—NO formation activities for wild type nNOS correlate with NADPH oxidation rates, because electron transfer is well coupled with the catalytic activity. Accordingly, for the aromatic mutants with low NO formation activities, NADPH oxidation rates are correspondingly low (Table I). However, the F691L, H692L, R414Q, and R414E mutants had relatively high NADPH oxidation rates compared with the wild type, considering their NO formation activities (Tables I and III). It is suggested, therefore, that those amino acid residues are important in the interdomain or intersubunit electron transfer of the nNOS enzyme. When NADPH oxidation and/or electron transfer is not well coupled with the molecular oxygen activation, H₂O₂ and/or H₂O is formed (34, 35, 49–51). Thus, mutants with low NO formation activities produce more H₂O₂ (Table I). Also, it is highly possible that H4B is closely associated with the electron transfer process in this enzyme, because H4B binding in these mutants may be markedly different to that of the wild type enzyme, and appropriate electron transfer involving H4B is no longer feasible in the mutant enzymes.

**Heme Reduction**—The heme reduction rates for mutants with very low NO formation activities (less than 0.1 nmol/min/nmol heme) were also very low (less than 0.01 min⁻¹) (Table II). We also found that H4B-free wild type enzyme showed heme reduction at a significant rate (0.08 min⁻¹) on addition of NADPH even in the absence of L-Arg (not shown). Therefore, the very slow heme reduction rates for the mutants are ascribed to the mutation effect rather than the effect of the substrate or the cofactor. This suggests that the first electron to the heme might come from the reductase domain in an H4B-independent fashion but that the pterin radical, if this exists, may still be involved in the second electron transfer to the oxy-ferrous complex, as proposed by Bec et al. (25). Taken together, these results suggest that the aromatic residues Trp676, Trp678, and Phe691 are important for proper H4B binding and crucial for electron transfer from the reductase domain and for NO formation. If a pterin radical is truly involved in the electron transfer, these aromatic amino acids should help to stabilize the radical during catalysis (25, 26, 28).

**Dimeric Formation Ability**—H4B binding has been suggested to promote dimerization of the enzyme that is thought to be essential for formation of an active enzyme (9–17). A recent report suggested that electrons supplied to the heme of the oxygenase domain originate from the reductase domain of the alternate subunit by intersubunit electron transfer within the dimer (52). Therefore, it is possible that the mutations caused the loss of H4B binding and also affected dimer formation. However, gel filtration analysis of the mutants (Fig. 5) indicates that they did not totally abolish dimer formation, suggesting that H4B binding is not absolutely essential for the dimer formation in full-length nNOS, as has already been suggested by other workers (9, 10, 13, 23, 49). They have reported that dimer formation of nNOS and eNOS is H4B-independent and regulated solely by heme availability. The crystal structural study on iNOS agrees with this suggestion (3), although another group has claimed that H4B binding appears to play a critical role in nNOS dimer formation (11, 12). The present results are consistent with previous reports that full-length nNOS makes dimers in an L-Arg- and H4B-independent fashion (9, 10, 23, 49).

**H4B and Aromatic Character**—Several reports have suggested that H4B functions commonly in all NOS isoforms as an electron supplying cofactor and an allosteric effector (9–17). A comparison of the iNOS and eNOS crystal structure reveals the striking conservation of the H4B binding site (27–30). As mentioned above, from the eNOS crystal structure, a catalytic model was proposed involving a pterin cation radical based on the affinity of L-Arg to the H4B binding site (28). Also, analysis of the nNOS reaction at ~30 °C suggested that a pterin radical may deliver an electron to the oxy-ferrous intermediate complex generated during catalysis (25). The pterin radical was recently detected with EPR under specific turnover conditions, although the specific nature of the radical could not be determined (26). If that is the case, aromatic residues in the dimer interface could stabilize the radical intermediate with aromatic stacking interactions. In fact, the W676L, W678L, and F691L mutants, which have both lost the aromatic character, did not have any NO formation activity, and heme reduction was extremely slow for these mutants in the presence of NADPH. On the other hand, the W678H mutant that still conserves the aromatic stacking showed normal spectral properties, NO formation activity, and heme reduction rate with NADPH in the presence of excess L-Arg and H4B, although its H4B affinity was greatly reduced (EC₅₀ value of H4B increased 18-fold of wild type). These results suggest that the aromatic character of Trp676, Trp678, and Phe691 are essential for efficient electron transfer to the heme, probably by stabilizing the pterin radical during catalysis (26, 28).

**Hydrogen Bonding Network Involving the Heme Carboxylate**—The mutation of Tyr706 dramatically increased the EC₅₀ value for H4B (40-fold that of wild type) on NO formation activity, although the same mutations did not significantly
affect the heme reduction rate with NADPH (Table II). The replacement of Arg414 with Glu (acidic residue) or Leu (neutral residue) resulted in a clear loss of NO formation activity and an extreme decrease in the rate of heme reduction with NADPH (Table III). Interestingly, the mutation of Arg414 to Gln had little effect on NO formation activity, rate of heme reduction, and H4B binding, despite resulting in a loss of positive charge. It appears, therefore, that the hydrogen bonding role of Arg414 is more important than its charge. Tyr706 and Arg414 are well conserved among all NOSs. In the eNOS dimer structure (28), Tyr706 is located at the side of the heme plane and interacts with the carbonyl moiety of heme propionate as shown in Fig. 7. Tyr706 may also interact via a hydrogen bond with Arg414 which is the adjacent residue to the axial ligand, Cys415 (with RasMac 2.6-uch1.0 software) (Fig. 7). Hydrogen bonds between the heme and Tyr706 and between Tyr706 and Arg414 could stabilize the active conformations of the heme environment. The other propionate group of heme is hydrogen bonded to the pterin and simultaneously interacts with the amino group of the substrate l-Arg. Thus, several hydrogen bond networks appear to keep the structure of the heme active site in an optimal conformation for maximal electron transfer and NO formation to occur. The charge balance between H4B, Trp676, Trp678, and Phe691 in the H4B binding site of the iNOS oxygenase domain, which correspond to Trp676 and Phe691 in nNOS, respectively, dissociated the dimeric form of the oxygenase domain. The dissociated monomer did form heterodimers with a full-length iNOS subunit. Interestingly, these heterodimers retained partial NO formation activities. Ala mutation of Trp555 and Phe570 of the iNOS oxygenase domain, which correspond to Trp676 and Phe691 in nNOS, respectively, dissociated the dimeric form of the oxygenase domain of iNOS. The dissociated monomer did form heterodimers with a full-length iNOS subunit. Interestingly, these heterodimers retained partial NO formation activities. Ala mutation of Trp555 of iNOS, which corresponds to Trp676 in nNOS, markedly reduced the H4B binding affinity, probably via its influence on the other propionate which interacts directly with H4B. Arg414 is also crucial for NO formation activity and electron transfer through its involvement in this key hydrogen bonding network in the heme active site.

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