Nitric oxide (NO) and l-citrulline are formed from the oxidation of l-arginine by three different isoforms of NO synthase (NOS). Defining amino acid residues responsible for l-arginine binding and oxidation is a primary step toward a detailed understanding of the NOS reaction mechanisms and designing strategies for the selective inhibition of the individual isoform. We have altered Glu-361 in human endothelial NOS to Gin or Leu by site-directed mutagenesis and found that these mutations resulted in a complete loss of l-citrulline formation without disruption of the cytochrome c reductase and NADPH oxidase activities. Optical and EPR spectroscopic studies demonstrated that the Glu-361 mutants had similar spectra either in resting state or reduced form.

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The abbreviations used are: NO, nitric oxide; NOS, nitric-oxide synthase; eNOS, endothelial NOS; iNOS, inducible NOS; nNOS, neuronal NOS; BH₄, (6R)-5,6,7,8-tetrahydro-l-biopterin; CaM, calmodulin.

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amino acid, glycine, to alanine was described elsewhere. The oligonucleotide primer for mutation of Glu-361 → Leu was 5'-CATGAGCA-CAATGATGGCAGAC-3'. This primer introduces an AseI site (underlined) and mutates the GAG codon to TTA (boldface). The primer for the mutation of Glu-361 to glutamine was 5'-AGCAGCTGATCGTAC-CAGGAC-3'. A KpnI site (underlined) was inserted, and GAG codon was changed to CAG (boldface). The basic strategy for polymerase chain reaction-based mutagenesis was described previously (13). The mutations were confirmed by sequencing using the dyeoxy chain termination method and Sequenase 2.0 (U. S. Biochemical Corp.) (21).

Expression and Purification of Wild-type and Mutated eNOS—Wild-type and mutated eNOS cDNAs were inserted into the EcoRI site of the pVL1392 transfer vector and used to generate recombinant cells in Sf9 cells. Procedures for preparation of crude cell homogenates and purification of recombinant proteins were essentially the same as described previously (22). Because the myristoylation site was removed, the proteins were purified directly from the soluble fraction without detergent treatment.

Assays of Enzyme Activity—NOS activity was assayed by measuring conversion of L-[3H]arginine to L-[3H]citrulline (22). NADPH oxidase activity was measured as the decrease in absorbance at 340 nm before and after addition of 0.5 μM calmodulin, using an extinction coefficient of 6.22 mM−1 cm−1. Assays were performed at 37 °C in a cuvette containing 1 ml of 50 mM Tris-HCl buffer, pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM EGTA, 10% glycerol (Buffer A), with 2.5 mM CaCl2, 10 μM BH4, 4 μg of enzyme, and 100 μM NADPH in the presence or absence of 1 mM L-arginine. Cytochrome c reductase activity was determined as the absorbance increase at 550 nm using a ΔA0.550 of 21 mM−1 cm−1 c as described previously (8).

Binding of 3H-Labeled L-Arginine to Wild-type and Mutated eNOS—Assay of L-arginine binding was performed by a previously reported procedure with slight modifications (23). A 100-μl reaction mixture containing Buffer A and 3 μg of purified enzyme was incubated on ice for 15 min with a serial concentration of unlabeled L-arginine (0–20 μM) and a fixed concentration of H-labeled L-arginine (2 μCi). The incubation was stopped by adding 0.15 ml of cold solution of bovine serum albumin prepared in Buffer A (20 mg/ml) and 0.75 ml of a 20% aqueous solution of polyethylene glycol. The mixtures were vortexed, incubated on ice for another 15 min, and then centrifuged at 12,000 × g at 4 °C for 20 min. The supernatant was removed by aspiration, the pellet was dissolved in 100 μl of H2O, and the radioactivity was determined by liquid scintillation counting. Parallel experiments in the presence of 50 μM BH4 were carried out to assess the effects of BH4. Non-specific binding (less than 3%) was measured in the absence of NOS and subtracted from the total binding.

Optical and EPR Spectroscopy—Optical spectra were recorded by using a Shimadzu-2101 PC spectrophotometer. The ferrous heme-CO spectrum was obtained by flushing the sample with CO gas, followed by reducing the sample with dithionite solution. Spectral perturbation by L-arginine or imidazole was conducted as described by McMillan and Masters (24). Titration of the enzyme with imidazole was carried out by stepwise additions of a stock solution of imidazole. Binding isoformers were constructed by plotting the difference in absorbance at 432 and 394 nm as a function of imidazole concentration. Dissociation constant (Kd) values were estimated by fitting the data to a hyperbolic one-site binding model. EPR spectra were recorded on a Varian E-6 spectrometer with an Air Products liquid helium transfer line (25). A Hewlett-Packard HP5342 frequency counter was used to monitor the microwave frequency. Progressive power saturation of the flavin radical was detoured elsewhere (26). Half-saturation power, P50, was obtained by non-linear regression to an equation of the form,

\[ \log(S/P0^{50}) = -b/2 \log (P_e + P) + b/2 \log (P_c) + 1 \log (K) \]  

where b is set to 1 for nonhomogeneous saturation behavior, as found for most hemoproteins, and K is a proportionality factor (27).

### Table I

| Human eNOS | Human nNOS | Human iNOS |
|------------|------------|------------|
| 33RNLLEIGGLEFPAPFSGWMS 34 | 35EIEGRNLCDPHERNYLEDV | 34EIGRNLCDPHERNYLEDV |
| 35NLLEIGGLEFSCPGSWM 36 | 37EIGRNLCDPHERNYLEDV | 36EIGRNLCDPHERNYLEDV |
| 36NLLEVGGLEFGCPHGWM 37 | 37EIGRNLCDPHERNYLEDV | 37EIGRNLCDPHERNYLEDV |

### Table II

| L-Citrulline formation | pmol/min/mg | % |
|------------------------|-------------|---|
| Wild type | 62 | 100 |
| E342I | 53 | 85.5 |
| E347L | 1.5 | 2.4 |
| E361L | UD | <1 |
| E361Q | UD | <1 |
| D369I | 2.6 | 4.2 |
| E377I | 45 | 73 |

*L-Citrulline formation was measured at 37 °C as described under "Experimental Procedures." Data were the mean values of duplicate experiments with less than 5% variations.

UD, denotes undetectable.

Size Exclusion Chromatography—Gel filtration chromatography was carried out using a Superdex 200 column (Pharmacia). The column was pre-equilibrated with phosphate-buffered saline. Thryglobulin, γ-globulin, bovine serum albumin, ovalbumin, and myoglobin were used as molecular weight standards.

**RESULTS**

Activity of Wild-type eNOS and the Glu-361 Mutants—Sequence homology comparisons revealed that the putative L-arginine binding regions are highly conserved among the three human NOS isoforms (Table I). Several anionic residues in the region from residue 338 to 379 of human eNOS were mutated, and these mutants were expressed in Sf9 cells. The crude cell homogenates were prepared for L-citrulline formation assay and Western blotting. When the same amount of expressed proteins was used in assays, E342I and E347I retained most of L-citrulline forming activity as the wild-type enzyme. In contrast, E347L, E361L, and D369I mutants were almost inactive (Table II). These three eNOS mutants were overexpressed and purified. The optical spectra of purified protein were determined, and only the Glu-361 mutant preserved the native heme spectra. We thus further characterized the Glu-361 mutant. Besides E361L, E361Q was also constructed, and the mutant protein was purified. L-Citrulline formation, cytochrome c reductase, and NADPH oxidase activities of both purified Glu-361 mutants are summarized in Table III. Mutation of Glu-361 to Leu or Gln resulted in a complete loss of L-citrulline formation, but retained essentially all of the NADPH-cytochrome c reductase and NADPH oxidase activities. Similar to that of the wild type, the cytochrome c reduction rate of the Glu-361 mutants was enhanced 10-fold by the presence of Ca2+/CaM. NADPH oxidation was observed only in the presence of CaM for all these recombinant enzymes. When l-arginine was added to the reaction mixture, the NADPH oxidation rate was doubled for the wild type but was not increased for the two mutants. L-[3H]Arginine Binding—The rate of NADPH oxidation by NOS directly reflects the rate of electron flux to the heme center (10, 11). The two Glu-361 mutants exhibited similar cytochrome c reductase and NADPH oxidase activities as the wild type, indicating that electron transfer from flavin to cytochrome c or to the heme center was not affected by mutation of Glu-361 to Leu or Gln. Results from L-[3H]arginine binding studies indicated that L-arginine binding to wild-type eNOS was concentration-dependent with a Kd of ~1.2 μM. The bind-
ing was not affected by the presence of 50 μM BH4. In contrast, no l-arginine binding was detected with either of the Glu-361 mutants (Fig. 1).

**Optical and EPR Spectroscopic Characterization**—Optical spectroscopy was utilized to examine the heme environment of the E361L mutant. The resting E361L mutant exhibited a Soret peak at 397 nm, a charge-transfer band at 467 nm, and a flavin absorbance shoulder between 450 and 475 nm (Fig. 2A). The dithionite-reduced, CO-bound E361L displayed spectral peaks at 445 nm and 550 nm (Fig. 2A). These characteristics are similar to those observed for the wild-type enzyme.2 As shown in Fig. 2, B and C, addition of imidazole shifted the Soret peak to 428 nm for both the wild-type and the E361L enzymes. The Kd values for imidazole were calculated from the data of optical titration to be 102 and 50 μM for the wild-type enzyme and the E361L mutant, respectively (Fig. 2, and inset). A similar heterogeneity was observed in the E361L-imidazole complex. A similar heterogeneity was also seen in the imidazole complex of wild-type eNOS (26). The sharp free radical signals observed in Fig. 3A are enlarged in Fig. 3B, inset. This free radical displayed an overall line width of 20 G and was centered at a g value of 2.004, typical for a neutral flavin semiquinone radical (26, 31). A power saturation study of the flavin radical gave the same P1/2 value (50 micro-watts) for both wild-type eNOS and the E361L mutant. This result indicated that mutation of Glu-361 did not change the dipolar interaction between the heme center and the flavin radical (Fig. 3B).

**Dimeric Structure of the Wild-type eNOS and the Glu-361 Mutant—**l-Arginine binding has been reported to influence the dimeric assembly of macrophage iNOS (32). The quaternary structures of wild-type eNOS and the Glu-361 mutant were analyzed by gel filtration chromatography. The two recombinant enzymes had indistinguishable elution volumes (Fig. 4) with molecular mass estimated to be 280 kDa. Given the subunit molecular mass of 135 kDa deduced from SDS-polyacrylamide gel electrophoresis,2 the chromatographic results indicate that both recombinant enzymes were homodimeric.

**DISCUSSION**

Understanding of the NOS interaction with its substrate, l-arginine, is important not only for developing highly selective inhibitors, but also for elucidating the catalytic mechanism. l-Arginine analogs in conjunction with spectroscopic studies to determine the factors contributing to the Arg-NOS interaction yielded conflicting results. Some reports suggested that positive charge is required for the tight binding of l-Arg to NOS (33, 34). Others showed that positive charge may not be critical for the Arg-NOS interaction (35, 36). Studies with the Glu-361 mutant in this report has provided a clearer insight into the nature of the interaction of eNOS with arginine. Results described above indicate that the Glu-361 residue is specifically

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**TABLE III**

| l-Citrulline formation | Cytochrome c reduction | NADPH oxidase activity |
|------------------------|------------------------|------------------------|
|                        | Without Ca2+/CaM       | With Ca2+/CaM           | Without Arg | With Arg |
|                        | nmol/min/mg            | nmol/min/mg             | nmol/min/mg | nmol/min/mg |
| Wild type              | 106 ± 8                | 177 ± 14                | 1656 ± 91   | 71 ± 4     | 140 ± 13   |
| E361L                  | UD*                    | 164 ± 10                | 1529 ± 73   | 92 ± 6     | 98 ± 9     |
| E361Q                  | UD*                    | 178 ± 13                | 1568 ± 106  | 109 ± 11   | 108 ± 9    |

* UD, undetectable.
involved in binding of L-arginine to eNOS. 1) Neither E361L nor E361Q mutant binds to L-[3H]arginine (Fig. 1). 2) In contrast to the wild type, imidazole-derived low spin complexes of Glu-361 mutants were insensitive to L-arginine and other type I ligands, such as 2-aminothiazole and acetylguanidine (Figs. 2 and 3), consistent with a defective L-arginine binding. 3) The NADPH oxidase activity for wild type was increased 2-fold upon addition of L-arginine, but no such change was observed for either mutant. Arginine binding changed the heme spin state and likely increased the redox potential. This change will facilitate the electron transfer to heme center and increase \( \text{NADPH oxidation rate} \) (11). The lack of change of \( \text{NADPH} \) will facilitate the electron transfer to heme center and increase NADPH oxidation rate (11). The lack of change of \( \text{NADPH} \) will facilitate the electron transfer to heme center and increase NADPH oxidation rate (11). The lack of change of \( \text{NADPH} \) will facilitate the electron transfer to heme center and increase NADPH oxidation rate (11). The lack of change of \( \text{NADPH} \) will facilitate the electron transfer to heme center and increase NADPH oxidation rate (11). The lack of change of \( \text{NADPH} \) will facilitate the electron transfer to heme center and increase NADPH oxidation rate (11). The lack of change of \( \text{NADPH} \) will facilitate the electron transfer to heme center and increase NADPH oxidation rate (11). The lack of change of \( \text{NADPH} \) will facilitate the electron transfer to heme center and increase NADPH oxidation rate (11). The lack of change of \( \text{NADPH} \) will facilitate the electron transfer to heme center and increase NADPH oxidation rate (11). The lack of change of \( \text{NADPH} \) will facilitate the electron transfer to heme center and increase NADPH oxidation rate (11). The lack of change of \( \text{NADPH} \) will facilitate the electron transfer to heme center and increase NADPH oxidation rate (11).

than the wild type, this was probably attributed to a slight difference in the solvent accessibility of the heme in that the Glu-361 mutant had more molecules of \( \text{H}_2\text{O}/\text{OH}^- \) at the sixth position. Furthermore, the Glu-361 mutant retained a homodimeric structure just like the wild type (Fig. 4). On the other hand, mutations of other anionic residues in the vicinity of the Glu-361 residue either had no effect or showed multiple effects on eNOS. Mutations of residues Glu-347 and Asp-369 also led to a complete loss of L-citrulline formation; however, the heme environment of these two mutants was disturbed. In contrast, mutation of Glu-361 preserved the native heme spectra and other functional characteristics. Loss of enzymic activity mainly resulted from the defective L-arginine binding. These results strongly indicate that Glu-361 plays an important role in L-arginine binding. Glu-361 residue interacts with L-arginine probably by ionic interaction. 2-Aminothiazole and acetylguanidine, which share with L-arginine a common guanidine structure but lack the amino acid function, could convert the low spin imidazole complex to a high spin state in the wild type but not in the Glu-361 mutant, indicating a direct interaction of the guanidine moiety of L-arginine with Glu-361 residue. We have also shown that mutation of Glu-361 to Gln destroyed the L-citrulline formation and L-[3H]arginine binding, indicating that the negative charge
on γ-carboxylate of Glu-361 residue is required for the interaction with L-arginine. In addition, $K_v$ values of L-arginine binding to wild-type eNOS determined in the buffers containing 0, 1, and 2 mM NaCl are 1.1, 9.0, and 10.5 μM, respectively, implicating that L-arginine binding affinity decreased by increasing ionic strength. The result substantiates the importance of charge interaction in L-arginine binding and confirms our EPR studies (26) which imply that the distal heme pocket of eNOS contains a much more polar surrounding than other P450s. In studies (26) which imply that the distal heme pocket of eNOS

In summary, this work has demonstrated that Glu-361 residue in human eNOS is critically involved in the interaction with L-arginine. Mutation of this residue specifically impaired the L-arginine binding without disruption of other characteristics. Our study has provided important information about the nature of Arg-NOS interaction and will shed light on the substrate binding mechanism of eNOS and the other two isoforms.

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Scheme I. Model for L-arginine binding to eNOS. The porphyrin plane is shown as two segments of thick line, with the heme iron in the center of the porphyrin plane ligated with a cysteine thiolate group. L-arginine is accessible to the heme through a defined channel. One of its guanidinium nitrogens directly interacts with γ-carboxylate of Glu-361 via charge paring.