Effects of Asp-369 and Arg-372 Mutations on Heme Environment and Function in Human Endothelial Nitric-oxide Synthase*

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Eight polar amino acid residues in the putative substrate-binding region from Thr-360 to Val-379 in human endothelial nitric-oxide synthase (eNOS) (Thr-360, Arg-365, Cys-368, Asp-369, Arg-372, Tyr-373, Glu-377, and Asp-378) were individually mutated. Only two of these residues, Asp-369 and Arg-372, were found to be essential for enzyme activity. A further series of mutants was generated by replacing these two residues with various amino acids and the mutant proteins were expressed in a baculovirus system. Mutant eNOS had a very low $\mu$mol association rate (10 mm) except for D369E except R372K, which retained 27% and 44% of the wild-type enzyme activity, respectively. Unlike the wild-type enzyme, all mutants except D369E, R372K, and R372M had a low spin heme (Soret peak at 416 nm). All the Asp-369 mutants had higher $K_D$ values for L-arginine (1–10 mM) than wild-type eNOS (0.4 $\mu$m) and an unstable heme-CO complex, and except for D369E, had a very low (6R)-5,6,7,8-tetrahydro-L-biopterin (BH4) content. In contrast, each of Arg-372 mutants retained a considerable amount of BH4, had a moderate reduction in $\mu$mol affinity, and had a more stable heme-CO complex. 1-Phenylhydrazine did not bind to wild-type eNOS heme, but bound to all Asp-369 and Arg-372 mutants ($K_a$ ranged from 10 to 65 $\mu$m) except R372K. Heme spin-state changes caused by binding of 3,5-lutidin appeared to depend on both charge and size of the side chains of residues 369 and 372. Furthermore, all Asp-369 and Arg-372 mutants were defective in dimer formation. These results suggest that residues Asp-369 and Arg-372 in eNOS play a critical role in oxygenase domain active-site structure and activity.

Nitric oxide (NO) serves as an important signal mediator in diverse pathophysiological processes (1–4). NO is produced together with $\mu$mol through a two-step oxidation of L-arginine by three different NO synthase isoforms (5–7). All isoforms have been identified as cytochrome P450-arginine by three different NO synthase isoforms (5–7). All NOS is thus likely to be the center of oxygen activation and L-arginine oxidation. Defining functional roles for amino acid residues around the distal heme site is a primary step in unraveling the reaction mechanism of NO synthesis.

Our previous results indicated that residue Glu-361 in eNOS is critical for L-arginine binding (16). Another eNOS residue, Asp-369, which corresponds to Asp-379 in mouse iNOS, was also demonstrated to be important in enzyme activity and L-arginine binding (16, 17). These results suggested that the eNOS segment near 361–369 contributed important parts of L-arginine binding site. As L-arginine binding occurs close to the distal heme site in eNOS, mutant proteins with impaired L-arginine binding are likely to provide useful tools to study the relationship between the side chain structure around the substrate-binding region and the distal heme site. To explore this further, eight amino acid residues in the 360–379 segment of eNOS were mutated and their functional effects characterized. Only mutations at residues 369 and 372 abolished eNOS activity. To elucidate the roles of Asp-369 and Arg-372 in maintaining heme environment, we replaced these two residues with a variety of amino acids and characterized the spectral properties of the resulting mutant proteins in complexes with various heme ligands. The results indicate that residues Asp-369 and Arg-372 are critically important in stabilizing eNOS oligomeric structure and heme environment.

EXPERIMENTAL PROCEDURES

Materials—L-[3H]arginine (58 Ci/mmol) was purchased from Amersham Pharmacia Biotech. Tetrahydrobiopterin (BH4) was obtained from Research Biochemical International. AG 50W-X8, cation-exchange resin, Bradford protein dye reagent, and electrophoretic chemicals were products of Bio-Rad. Spodoptera frugiperda (Sf9) cells, baculovirus transfer vector (pVL1392), and Baculovirin DNA were obtained from PharMingen. Grace’s insect cell culture medium was purchased from Life Technologies, Inc. CaM-Sepharose and 2′,5′-ADP-Sepharose were products of Amersham Pharmacia Biotech. NADPH, CaM, and other reagents were purchased from Sigma.

Mutagenesis—The oligonucleotide primers used in preparing Asp-369 and Arg-372 mutants shown in Fig. 1A were synthesized by Genosys Inc. (Woodlands, TX). The strategy for polymerase chain reaction-mediated site-directed mutagenesis was described previously (11). The sequences of the mutated DNA were confirmed using the dyeoxy chain termination method (18). Expression and Purification of the Wild-type and Mutant eNOS—cDNAs for the wild-type and mutant eNOS were inserted into the EcoRI site of the pVL1392 transfer vector, which was used to generate recombinant viruses in the Sf9 cells. Procedures for preparation of crude cell homogenates and purification of recombinant proteins were essentially the same as described previously (19).

Assays of Enzyme Activity—NOS activity was assayed by measuring conversion of L-[3H]arginine to L-[3H]citrulline as described by Bredt and Snyder (6) with slight modification. The reaction mixture containing 20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.05 mM NADPH, and 0.5 mg/ml homogenates and purified recombinant eNOS was incubated at 37 °C for 10 min in the presence of L-[3H]arginine (100 $\mu$m), NADPH, and MgCl2 (2.5 mM). The reaction was stopped by the addition of 1 M HCl, and the samples were extracted with 10% sulfuric acid.

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§ The abbreviations used are: NOS, nitric-oxide synthase; BH4, 6R-5,6,7,8-tetrahydro-L-biopterin; CaM, calmodulin; DTT, dithiothreitol; eNOS, endothelial NOS; iNOS, inducible NOS; nNOS, neuronal NOS.
0.2 μM calmodulin, 1 mM CaCl2, 100 μM b-NADPH, 10 μM BH4, 100 μM L-arginine, and 1 μCi of L-[3H]arginine was incubated with enzyme at 37 °C for 3 min. Cytochrome c reductase activity was determined from the absorbance increase at 550 nm using Dextro-ox of 21 mM 21 cm21 (20).

Biopterin Determination—The reduced form of biopterin was first oxidized by a modified procedure of Schmidt et al. (21). Briefly, the purified wild-type and mutant eNOS proteins (0.3 mg/ml) were oxidized with 1% iodine and 2% KI in 0.25 M H3PO4. After standing for 60 min in the dark, excess iodine was reduced by crystalline ascorbic acid. The sample was centrifuged and analyzed by high performance liquid chromatography using C18 reverse phase column. The column was eluted with 5% methanol, and biopterin was detected by a fluorescence monitor (Shimadzu RF-535) with excitation and emission wavelengths of 350 and 410 nm, respectively. The BH4 concentrations in samples were determined by integrating the peak area and relating it to that of the authentic BH4.

Optical Spectroscopy—Optical spectra were recorded using a Shimadzu-2401 PC or a HP8452 diode array spectrophotometer. The ferrous heme-CO spectrum was obtained by flushing the sample with CO gas, followed by reducing the sample with a few grains of dithionite. The binding affinity for L-arginine and heme ligand was determined by perturbation difference spectroscopy as described by McMillan and Masters (9). The purified enzyme (1.5–2 μM) was directly titrated with various concentrations of L-arginine or heme ligands at room temperature. The absorbance change (ΔA) was generated by subtracting the recorded spectrum at each concentration of ligand from the spectrum of wild-type enzyme.

**TABLE I**

| L-Citrulline forming activities in crude cell homogenates of the wild-type and mutant eNOS |
|-----------------------------------------------|
| % of wild type                  |
| Wild-type                  | 100 |
| T360A                      | 60  |
| T360S                      | 67  |
| R365L                      | 59  |
| C366A                      | 160 |
| D369I                      | 4   |
| R372L                      | 2   |
| Y373F                      | 54  |
| E377I                      | 45  |
| D378E                      | 85  |
| D378L                      | 67  |

L-Citrulline formation was measured at 37 °C as described under “Experimental Procedures.” Values are the means of duplicate experiments agreed within 5% variations. The wild-type activity was 78 pmol/min/mg.

0.2 μM calmodulin, 1 mM CaCl2, 100 μM β-NADPH, 10 μM BH4, 100 μM L-arginine, and 1 μCi of L-[3H]arginine was incubated with enzyme at 37 °C for 3 min. Cytochrome c reductase activity was determined from the absorbance increase at 550 nm using Δεred-ox of 21 mM cm−1 (20).

**FIG. 1.** A, nucleotide sequences of primers used to generate various eNOS mutants. The mutated residues are highlighted by a large font. The silent mutations to create restriction sites are underlined and annotated. B, alignment of amino acid sequences around the L-arginine binding region. Amino acid residues 360–379 for human eNOS (31), 596–615 for human nNOS (32), and 376–395 for human hepatocyte iNOS (33) are aligned. Boldface letters denote residues that are mutated in eNOS isoform.

**FIG. 2.** Absorbance spectra of the wild-type and mutant eNOS. Solid lines denote the resting forms; dotted lines, imidazole-enzyme complexes; and dashed lines, L-arginine-enzyme complexes. For wild type (A), the resting enzyme (1.5 μM) was first incubated with 0.5 mM imidazole and 1 mM L-arginine was then added; for D369I (B) and R372L (C), the resting enzymes (2 μM) were first incubated with 20 mM L-arginine and 2 mM imidazole was then added.

**FIG. 3.** Absorbance spectra of the wild-type and mutant eNOS. Solid lines denote the resting forms; dotted lines, imidazole-enzyme complexes; and dashed lines, L-arginine-enzyme complexes. For wild type (A), the resting enzyme (1.5 μM) was first incubated with 0.5 mM imidazole and 1 mM L-arginine was then added; for D369I (B) and R372L (C), the resting enzymes (2 μM) were first incubated with 20 mM L-arginine and 2 mM imidazole was then added.
the resting enzyme. Because $K_d$ of the wild-type eNOS for L-arginine binding was close to the enzyme concentration, the free L-arginine was obtained by correction for the bound L-arginine from the total L-arginine based on the following formula: $[L]_{\text{free}} = [L]_{T} - \frac{[E]_{L}}{D_{A}} \cdot [L]_{T}$, where $[L]_{\text{free}}$ is the concentration of free ligand, $[L]_{T}$ is the concentration of total ligand, $[E]_{L}$ is the ligand-enzyme complex, $D_A$ is the absorbance change at indicated concentration of ligand, $D_{A,\text{max}}$ is the maximum absorbance change at the saturated concentration of ligand, and $[E]_{T}$ is the total enzyme concentration. Binding isotherms were constructed by plotting the difference absorbance ($D_A$) versus the free ligand concentration. Dissociation constant ($K_d$) values were estimated by fitting the data to a hyperbolic one-site binding model.

Size Exclusion Chromatography—Wild-type and mutant eNOS proteins were analyzed by fast protein liquid chromatography using a BioSelect 250 gel filtration column (Bio-Rad). The column was pre-equilibrated with phosphate-buffered saline and calibrated with the following protein molecular mass standards: thyroglobulin, 675 kDa; $\gamma$-globulin, 158 kDa; bovine serum albumin, 67 kDa; ovalbumin, 44 kDa; and myoglobin, 17 kDa.

RESULTS

Our previous studies demonstrated that Glu-361 of eNOS is critically involved in L-arginine binding and enzyme activity (16). To study the role of the region around Glu-361, we mutated eight additional polar residues in the segment from Thr-360 to Val-379, which are conserved among all three human NOS isoforms (Fig. 1B) and expressed these mutants in Sf9 cells. The crude cell homogenates were prepared, and the correct size of the expressed full-length mutant proteins was confirmed by Western blot analysis (data not shown). All mutants retained much of the L-citrulline formation activity (45% of the wild type) with the exception of D369I and R372L, which lost essentially all activity (Table I). These two mutant proteins were purified for spectral studies. The purified wild-type eNOS exhibited a Soret peak at 398 nm, and addition of imidazole shifted the Soret peak to 428 nm, typical for a six-coordinate low spin imidazole complex (Fig. 2A). This complex was converted back to a five-coordinate high spin species, with a Soret peak at 402 nm by mixing with excess dithionite and bubbling with CO.

| Table II | Catalytic activities and BH$_4$ content of the purified wild-type and mutant eNOS |
|----------|--------------------------------------------------|
| L-Citrulline formation$^a$ | Cytochrome c reductase$^a$ | BH$_4$ content$^b$ |
| nmol/min/mg | eq/subunit | % of wild type |
| Wild-type | 82.3 | 1430 | 0.55 | 100 |
| D369E | 22.4 | 1500 | 0.22 | 40 |
| D369I | 1.3 | 1260 | 0.03 | 5 |
| D369N | 1.4 | 1310 | 0.03 | 5 |
| D369V | 2.3 | 1200 | UD$^c$ | |
| D369G | 1.8 | 1120 | UD$^c$ | |
| R372K | 36.3 | 1240 | 0.42 | 76 |
| R372M | 3.4 | 1460 | 0.34 | 62 |
| R372L | 2.2 | 1360 | 0.3 | 55 |
| R372V | 3.7 | 1250 | 0.28 | 51 |
| R372G | 3.8 | 1430 | ND$^c$ | ND$^c$ |

$^a$ L-Citrulline formation and cytochrome c reduction activities were measured at 37 °C as described under “Experimental Procedures.” Values are the means of duplicate experiments, which agreed within 5%.

$^b$ BH$_4$ content was determined as described under “Experimental Procedures.”

$^c$ UD, undetectable.

$^d$ ND, not determined.

| Table III | Soret absorption characteristic peaks of wild-type and mutant eNOS |
|----------|--------------------------------------------------|
| Soret peak position (nm) |
| Resting state | +L-Arginine$^e$ | +1-PhIm$^b$ | +3,5-Lutidine |
| Wild-type | 398 | 395 | 399 | 395 |
| D369E | 407 | 395 | 424 | 395$^b$ |
| D369I | 411 | 398 | 424 | 395$^b$ |
| D369N | 413 | 398 | 424 | 420 |
| D369V | 416 | 398 | 424 | 420 |
| D369G | 416 | 398 | 424 | 422 |
| R372K | 402 | 395 | 403 | 395 |
| R372M | 403 | 395 | 426 | 395 |
| R372L | 416 | 395 | 426 | HS/LS$^e$ |
| R372V | 416 | 395 | 426 | 421 |
| R372G | 416 | 395 | 426 | HS/LS$^e$ |

$^a$ All mutants except R372K and R372M, exhibited a broader peak at 398 nm after addition of 20 mM L-arginine.

$^b$ 1-Phenylimidazole.

$^c$ The amplitude of the Soret peak was reduced and its position shifted to 398 nm.

$^d$ The Soret peak was shifted to 395 nm with a shoulder at 411 nm.

$^e$ Mixed high and low spin states.

BioSelect 250 gel filtration column (Bio-Rad). The column was pre-equilibrated with phosphate-buffered saline and calibrated with the following protein molecular mass standards: thyroglobulin, 675 kDa; $\gamma$-globulin, 158 kDa; bovine serum albumin, 67 kDa; ovalbumin, 44 kDa; and myoglobin, 17 kDa.
peak at 395 nm upon addition of L-arginine (Fig. 2A). In contrast, both mutant proteins exhibited a Soret peak at 416 nm with a slight shoulder at 390–397 nm and a less prominent charge-transfer band at 467 nm, indicating a mixture of high and low spin states with the majority present as the low spin form (Fig. 2, B and C). Imidazole induced a shift of the Soret peak from 416 to 428 nm in both mutants. However, it is difficult to convert this imidazole-heme complex back to the high spin form even with addition of a high concentration of L-arginine (≥50 mM). L-Arginine was thus first added to both mutant proteins and shifted the Soret peak of each sample from 416 to 398 nm. Further addition of imidazole shifted the Soret peak to 428 nm, indicating the formation of the imidazole-heme complex (Fig. 2, B and C). Wild-type eNOS and the D369I and R372L mutants exhibited similar Soret peaks at 445 nm in the reduced CO-heme complexes (Fig. 3). However, the 445-nm peak of D369I mutant was unstable and quickly disappeared with concomitant formation of a 420-nm peak (Fig. 3B).

To further understand the involvement of Asp-369 and Arg-372 in distal heme function, a series of mutants was generated by replacing these two residues with a variety of amino acids: D369E, D369N, D369V, D369G, R372K, R372M, R372V, and R372G. All the mutants were expressed in a baculovirus system and purified to apparent homogeneity, as judged by SDS-polyacrylamide gel electrophoresis (data not shown). The optical absorption spectra, the L-citrulline formation, and cytochrome c reduction activities of all purified mutant proteins were determined. As shown in Table II, all mutants retained essentially all the NADPH-cytochrome c reductase activity, but had a very low L-citrulline formation activity with the exception of mutants D369E and R372K, which retained 27% and 44% of the wild-type enzyme activity, respectively.

The BH4 content of mutant proteins was determined and shown in Table II. All Asp-369 mutants except for D369E, which retained 40% of the wild-type BH4 content, had a very low BH4 content (less than 5% of the wild type). In contrast, mutation of Arg-372 to either charged or neutral residues preserved more than 50% of the wild-type BH4 content. The Soret absorption characteristics of the wild-type and mutant eNOS are summarized in Table III. Instead of the 398-nm peak found for the wild-type eNOS, most mutants exhibited a Soret peak at 416 nm. On the other hand, absorbance in the D369E, R372K, and R372M mutants peaked at 407, 402, and 403 nm, respectively. L-Arginine converted all mutants back to the high spin state (Table III). The Kd value for L-arginine determined by optical perturbation for the wild-type enzyme was 0.4 μM, but for mutants was higher. Asp-369 mutants had Kd values in the range of 1–10 mM. R372K, R372M, and R372L had Kd values between 5 and 20 mM, and R372V exhibited a Kd of 140 μM (Table IV). Replacement of Arg-372 with glycine greatly weakened the L-arginine binding (Kd ~ 11 mM), suggesting a dramatic change in the enzyme structure.

1-Phenylimidazole is frequently used as a heme ligand for P450 proteins. Binding of 1-phenylimidazole to human iNOS shifted the Soret peak to 428 nm, with a Kd ~ 28 μM (22). However, addition of 1-phenylimidazole to the wild-type eNOS did not change the spin state, even at 100 mM concentration (Fig. 4), indicating a lack of binding to this isoform. Similar behavior was observed for the R372K mutant (Table IV). In contrast, addition of 1-phenylimidazole to all other mutant proteins shifted the Soret peak to 424–426 nm. This is exemplified by the specific mutants D369I and R372L shown in Fig.

### Table IV

| Binding affinities of wild-type and mutant eNOS for L-arginine and heme ligands |
|--------------------------|-----------------|-----------------|
|                          | Kd (μM)         |                 |
| L-Arginine               | 1-PhIm          | 3,5-Lutidine    |
| Wild-type                | 0.4             | >100,000        | 200 (HS)       |
| D369E                    | 1100            | 26              | —              |
| D369I                    | 4700            | 26              | —              |
| D369N                    | 9100            | 31              | —              |
| D369V                    | 5350            | 20              | —              |
| D369G                    | 9800            | 65              | —              |
| R372K                    | 15              | >100,000        | 190 (HS)       |
| R372M                    | 17              | 16              | 198 (HS)       |
| R372L                    | 10              | 10              | (HS)(LS)       |
| R372V                    | 140             | 22              | 160 (LS)       |
| R372G                    | 1,500           | 17              | (HS)(LS)       |

* Purified enzyme (1.5 μM) was directly titrated with various concentrations of L-arginine or heme ligands. When the Kd value is close to the enzyme concentration, correction for bound ligand was done using the following formula: [L]_total = [L]_T - [EL] = [L]_T - ΔA/ΔA_max [EL]/[L]_T, where [L]_total is the concentration of free ligand, [L]_T is the concentration of total ligand, [EL] is the ligand-enzyme-complex, ΔA is the absorbance change at indicated concentration of ligand, ΔA_max is the maximum absorbance change at the saturated concentration of ligand, and [EL]/[L]_T is the total enzyme concentration.

* 1-Phenylimidazole.

* Kd not determined due to a slow equilibration.

* High spin state.

* Mixed high and low spin states.

* Low spin state.

![Fig. 4](http://www.jbc.org) Effects of 1-phenylimidazole on the absorption spectra of wild-type eNOS (A), the D369I mutant (B), and R372L mutant (C). Solid lines denote the resting forms, and the dashed lines, the spectra after addition of 5 mM 1-phenylimidazole. The insets in B and C show the spectra ΔA432–394 nm as a function of the ligand concentration.
4 (B and C) and is consistent with formation of a six-coordinate low spin heme complex with a N-ligand. The $K_d$ values for 1-phenylimidazole calculated from optical titrations were in the $10^{-6}$–$10^{-5}$ M range for these mutants (Table IV).

We also used another heme ligand, 3,5-lutidine, which has been employed to probe width of the eNOS heme pocket in our previous study (23), to determine the role of the side chains of Asp-369 and Arg-372 on the eNOS heme environment. As shown in Fig. 5A, binding of this heme ligand to the wild-type eNOS shifted the Soret peak from 398 to 395 nm. The difference spectrum exhibited a peak at 390 nm and a trough at 420 nm (Fig. 5A, inset), a change typical for a type I perturbation. Similar to the wild type, binding of 3,5-lutidine to R372K and R372M shifted the Soret peak to 395 nm and caused a type I difference spectrum (Fig. 5, B and inset; Table III). In contrast, binding of 3,5-lutidine to the R372V mutant shifted the Soret peak to 421 nm (Fig. 5C), yielding a type II difference spectrum with a peak at 434 nm and a trough at 412 nm (Fig. 5C, inset), indicating that the nitrogen atom of 3,5-lutidine directly coordinates to the heme iron (24, 25). Binding of 3,5-lutidine to the R372L and R372G mutants produced broad absorbance peaks centered at 416 nm with shoulders at 400 nm and 421 nm, indicating a mixture of high and low spin states (spectra not shown). As with wild-type eNOS, the $K_d$ values of the R372K and R372M mutants derived from the type I difference spectra were $200 \mu$M. The $K_d$ value for R372V derived from the type II difference spectrum was $160 \mu$M. The Soret peak shifts caused by binding of 3,5-lutidine to the Asp-369 mutants were similar to those of the Arg-372 mutants. Binding of 3,5-lutidine to D369I caused a mixed spin state with a predominant high spin form (Fig. 6A). Binding of 3,5-lutidine to D369E reduced the amplitude of the Soret peak and shifted
its wavelength to 398 nm (Fig. 6B), indicating a spin mixture with predominant high spin. Binding of 3,5-lutidine to the D369N, D369V, and D369G mutants shifted the Soret peak to 420–423 nm, consistent with formation of a six-coordinate nitrogen-based ligand (Fig. 6, C–E; Table III).

The quaternary structure of wild-type eNOS and several mutants was analyzed by gel filtration chromatography. The wild-type eNOS had a single major peak with molecular mass estimated to be ~280 kDa, consistent with a dimeric structure (Fig. 7A). Mutant D369E had a broader peak with elution position in the range of the dimeric and monomeric species, indicating a mixture of dimeric and other forms (Fig. 7B). Two separate peaks with elution volume corresponding to both monomeric and dimeric forms, respectively, were shown in all other mutants such as D369V, R372K, and R372L (Fig. 7, C–E). The monomeric species of R372K is eluted before other mutant monomers, possibly due to the difference in shape as well as size of these monomer species. A minor fraction of the protein eluted in the void volume for all mutants, indicating formation of high molecular mass aggregates.

DISCUSSION

Results presented here indicate that among polar residues in the segment 360–379, Asp-369 and Arg-372 are important in several aspects of eNOS structure and activity. The role of these two residues is primarily involved in the oxygenase domain function. Replacement of Asp-369 and Arg-372 with either charged or neutral amino acid residues preserved essentially all NADPH-cytochrome c reductase activity, whereas the oxygenase domain activity was greatly reduced except for two charge-conserved mutants, D369E and R372K. These results suggest that residues 369 and 372 participate in charge interactions that are crucial for eNOS oxygenase domain catalysis (Table II).

Asp-369 and Arg-372 are also important in the binding of L-arginine and of heme ligands. Mutations in these two residues substantially decreased the L-arginine binding affinity (Table IV). 1-Phenylimidazole, a poor ligand for the wild-type eNOS, exhibited high affinity for all Asp-369 and Arg-372 mutants except R372K, and shifted the Soret peak to 424–426 nm, indicating that these changes enable a direct ligation of the 1-phenylimidazole N-1 nitrogen to the heme iron. Binding of another heme ligand, 3,5-lutidine, was found to be sensitive to alteration in both charge and size of the side chains of Asp-369 and Arg-372 (Figs. 5 and 6), suggesting an important role for these two residues in modulating ligand access to the distal heme.

The general structural integrity of the oxygenase domain active site was altered to varying degrees by Asp-369 and Arg-372 mutations. All mutants had normal heme incorporation, although most mutants had a heme present in the low spin rather than the high spin state found in wild-type eNOS (Table III). In addition, the reduced CO-heme complexes of the Asp-369 mutants were unstable and rapidly converted to a species with 420-nm Soret peak (Fig. 3). These findings indicate that Asp-369 and Arg-372 are essential for maintaining a proper heme environment. Furthermore, mutation of Asp-369 or Arg-372 caused derangement in dimer formation as shown in Fig. 7. A conservative change in these two residues (such as D369E and R372K) influenced the dimer stability, suggesting a strict requirement for eNOS quaternary structure and a critical role of Asp-369 and Arg-372 in NOS dimerization.

While this manuscript was under review, Crane et al. (26) and Masters et al. (27) reported the x-ray crystallographic structures for complexes of murine iNOS and bovine eNOS oxygenase domains with L-arginine, respectively. These results indicate that a hydrogen-bond network is of critical importance in formation of the oxygenase domain active site. Dimers of NOS create a deep active-site channel with several components located at the dimer interface including L-arginine-binding helix (α7a helix, residues 360–368 in eNOS) and BH4, and exposes the heme edge on the side opposite to the active-site channel, which provides a surface for interaction with reductase domain. BH4 sits proximal and perpendicular to the heme, and participates in the extensive hydrogen-bond network for
the formation of active site via interactions with heme propionate A, l-arginine-binding helix, and residues of the second subunit. Helix α7 itself (residues 360–379) traverses the active site and supplies residues that interact with l-arginine, BH₄, and heme. Our previous results implicated residue Glu-361 in BH₄ binding (26, 27). The structural basis for this is unclear. A multitude of functional and spectral changes caused by mutations of these two residues suggests that they are involved in several aspects of oxygenase domain structure and activity. Data from x-ray crystallographic structures of iNOS and eNOS oxygenase domains (26, 27) indicate that residues Asp-369 and Arg-372 occupy important positions at oxygenase domain active site in stabilizing the hydrogen-bond network. A multitude of functional and spectral changes caused by mutations of these two residues has confirmed that the extensive hydrogen-bond network at NOS active site is crucial for maintaining the interconnections among BH₄ cofactor, l-arginine binding, dimer formation, and heme stability.

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