Spatial Relationship between L-Arginine and Heme Binding Sites of Endothelial Nitric-oxide Synthase*

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Binding of L-arginine and imidazole to the endothelial nitric-oxide synthase (eNOS) was characterized by direct heme spectral perturbation. L-Arginine is competitive with imidazole for binding to eNOS. Both equilibrium binding and kinetic binding were measured at 4 and 23 °C for these two ligands. $K_d$ (imidazole) is 60 $\mu M$ and 110 $\mu M$, $k_{on}$ (imidazole) is $2.5 \times 10^4 M^{-1} s^{-1}$ and $1.3 \times 10^6 M^{-1} s^{-1}$, $k_{off}$ (imidazole) is $118 s^{-1}$ and $116 s^{-1}$ at 4 and 23 °C, respectively. Corresponding values for L-arginine are calculated from the data of binding competition with imidazole and computer modeling. $K_d$ (L-arginine) is 0.5 $\mu M$ and 2.0 $\mu M$, $k_{on}$ (L-arginine) is $2 \times 10^5 M^{-1} s^{-1}$ and $8 \times 10^4 M^{-1} s^{-1}$, $k_{off}$ (L-arginine) is 0.08 $s^{-1}$ and 1.6 $s^{-1}$ at 4 and 23 °C, respectively. It is suggested that binding of both ligands occurs through the same access channel to the heme site based on their similarly slow association rate constants. A series of potential heme ligands and amino acid analogs of L-arginine were evaluated for their binding and their effect on the heme structure. All ligands besides cytidine tested for binding inhibition are competitive with either L-arginine or imidazole. The space for the distal heme ligand was estimated to be $6.3 \times 6.7 \AA$ by three groups of rigid planar ligands: imidazole, pyridine, and pyrimidine. Results of the thiazole and amino acid ligand series permitted the conclusion that the guanidine group of L-arginine is critical for its binding affinity and its specific orientation relative to the heme. Such a specific conformation is essential for the oxygenase mechanism of eNOS.

The biological messenger nitric oxide (NO)† is involved in a plethora of important physiological or pathophysiological processes. Biosynthesis of this simple gaseous molecule was catalyzed by three different isocforms of nitric-oxide synthase (NOS). Although they share only ~60% overall homology for their protein sequences, the reaction mechanisms of each isozyme are rather similar (1). Nitric-oxide synthase catalyzes the five-electron reduction of L-arginine to citrulline and NO in consumption of 1.5 equivalents of NADPH$^+$ + H$^+$ and two molecules of oxygen (1, 2). Substantial knowledge has been accumulated in the aspects of structure-function relationships and reaction mechanism during the last few years (1–5). Successes in the development of overexpression systems for different isocforms of NOS enable a detailed examination of the structural and redox changes within the enzyme molecule during enzyme turnover (6–8). This approach, contrasting with the studies only emphasizing the analysis of the changes of the substrate and products by steady state kinetics, could provide direct and detailed information about the enzyme behavior. For example, $K_m$(L-arginine) values measured from L-citrulline formation by steady state kinetics have been used as a reference for substrate affinity. However, in this case $K_m$ is a complicated combination of many rate constants relating to various intermediate catalytic steps, and its physical meaning cannot be easily interpreted. Using steady state kinetics, Wolff et al. (9) demonstrated that imidazole is a noncompetitive inhibitor to L-arginine of neuronal NOS (9), whereas Mayer et al. (10) showed a competitive inhibition between imidazole and L-arginine. On the other hand, direct binding affinity measurements of substrate or other ligands can be conveniently measured by their perturbation of the heme spectrum as first demonstrated by Master’s group (11). They concluded that the binding of these two ligands as detected by optical difference spectroscopy was “in accordance with the noncompetitive inhibition of citrulline formation reported for imidazole and related compounds (9)” in nNOS (11). This proposal was derived from the observation that the binding of L-arginine to the nNOS appeared to be independent of the presence of imidazole. However, these same investigators later used a competitive inhibition model to analyze their binding competition data (12).

In the present study, we provided a new insight into this issue of ligand binding using equilibrium and kinetic binding measurements as an extension of the approach of spectral perturbation. A series of heme ligands and relevant amino acid ligands have been used in this study to gauge the physical dimension of the heme and L-arginine binding pockets as well as the relative location of these two binding sites.

EXPERIMENTAL PROCEDURES

imidazole, 2-methylimidazole, 4-methylimidazole, pyridine, 4-ethylpyridine, 3,5-dimethylpyridine (3,5-lutidine), pyrimidine and 4-methylpyrimidine, aniline, thiazole, 2-aminothiazole, 2-amino-4-methylthiazole, 2-amino-4,5-dimethylthiazole, ethylguanidine, and acetylguanidine are Aldrich products. Nicotinic acid, potassium cyanide, L-arginine, L-lysine, and L-histidine, NADPH, adenosine 2′- and 3′-monophosphate (2′- and 3′-AMP), CHAPS, and calmodulin were purchased from Sigma. L-Citrulline was from ALEXIS Biochemicals, San Diego, CA., L-[2,3,4,5,5-H]$\text{Arginine}$ was obtained from Amersham Corp. (specific activity, 77 Ci/mmol).

Recombinant human endothelial nitric-oxide synthase (eNOS) was prepared using a baculovirus expression system as described previously with slight modification (8, 13). The cell pellets were suspended in buffer A (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 20 mM CHAPS, 10% glycerol, 1 mM antipain, 1 mM leupeptin, 1 mM pepstatin, 1 mM phenylmethylnil fluoride) and sonicated three times for 10 s each. The 100,000 × g supernatant was

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The abbreviations used are: NO, nitric oxide; NOS, nitric-oxide synthase; eNOS, endothelial nitric-oxide synthase; nNOS, neuronal nitric-oxide synthase; 2′- and 3′-AMP, adenosine 2′- and 3′-monophosphate; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; 3,5-lutidine, 3,5-dimethylpyridine.
directly applied to a 2',5'-ADP-Sepharose column (1.5 × 2 cm) pre-equilibrated with buffer A. The column was washed with 25 ml of buffer A plus 0.5 M NaCl, followed with 10 ml of buffer A and then eluted with buffer B (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 5 mM CHAPS, 10% glycerol) plus 30 mM 2'- and 3'-AMP. The eluate was concentrated by Centriprep-100 (Amicon) at 500 g and then applied to a 10-DG column (Bio-Rad). The yield was about 10 mg of purified eNOS from 3 × 10^6 s9 cells. Soluble endothelial nitric-oxide synthase was prepared by specific replacement of the second amino acid, glycine, to alanine through oligonucleotide-directed mutagenesis described elsewhere.\(^2\) Expression and purification of this mutant eNOS were very similar to the wild-type enzyme except for elimination of detergent treatment, and the enzyme was purified directly from the soluble fraction after cell breakage by sonication. Details of the characterization of both wild-type and G2A mutant eNOS were summarized in another manuscript.\(^2\)

Activity of eNOS was measured as the conversion of tritium-labeled L-arginine to L-citrulline according to the published procedures (14). A 3-min incubation of the reaction mixture at 37 °C before quenching was applied because production of L-citrulline is only linear in the first 10-min period.

Binding equilibrium measurements of a specific ligand by the method of spectral perturbation was conducted as described by McMillan and Masters (11) and Roman et al. (12). Optical spectra were recorded on an HP8452 diode array spectrophotometer. The maximal amplitude change in the difference spectrum induced by each tested ligand was analyzed by a double-reciprocal plot as described previously (12) or fit to a hyperbolic one-site binding model. Binding rate constants, \(k_{on}\) and \(k_{off}\), were determined by kinetic measurements on a stopped-flow instrument, Applied Photophysics model SX-17MV, with temperature control by circulating water bath.

Equilibrium binding competition between imidazole and L-arginine was conducted in two complementary methods. The first method is a serial optical titration of eNOS by imidazole in the presence of a fixed concentration of L-arginine and the observed optical change, \(\Delta A_{396-430}\), were analyzed by double-reciprocal plot as Roman et al. (12). The complementary method was to monitor the optical changes induced by L-arginine in the presence of a saturating concentration of imidazole. These data were fitted to equation (1) by a nonlinear regression analysis

\[
(\Delta A_\text{on} - \Delta A)/\Delta A_\text{on} = ([\text{Im}]_0 + K_{\text{im}})/[\text{Im}]_0 + K_{\text{im}} (1 + [\text{L-Arg}]/K_\text{d})
\]

where \(\Delta A\) is the measured absorbance change after each addition of L-arginine and \(\Delta A_\text{on}\) is the maximal absorbance change at the end of titration; \([\text{Im}]_0\) is the imidazole concentration before addition of L-arginine, and \(K_{\text{im}}\) and \(K_d\) are the dissociation constants of imidazole and L-arginine, respectively. As for kinetic binding competition between imidazole and L-arginine, eNOS was mixed with imidazole alone or together with a fixed concentration of L-arginine in the stopped-flow apparatus. The kinetic data were simulated by a simple competition model,

\[
E + \text{L-Arg} = E\cdot\text{L-Arg} + \text{Im} \quad \quad \text{I perturbation}
\]

\[
E + \text{L-Arg} = E\cdot\text{L-Arg} + \text{Im} \quad \quad \text{II perturbation}
\]

\[\text{Model 1}\]

using the ScoP program (Simulation Resources Inc., Berrien Springs, MI) to obtain the optimal values for the on and off rate constants.

Estimation of the physical dimension of the heme binding pocket was conducted using a low-level molecular modeling program, Alchemy III (Tripos, Inc., St. Louis, MO). Each potential heme ligand was built and energy-minimized in vacuum and docked on one another, with the atom that directly ligated to the heme-iron overlap right on top of one another. The maximal span at the X and Y directions of these overlapped planar ligands were recorded as the estimation of the pocket size for the distal heme ligand.

RESULTS

Most of the experiments were performed using wild-type eNOS. We found that there is no obvious difference in the binding characteristics evaluated in this study between wild-type and soluble eNOS in which the myristoylation site is removed. Unless specified, the result from the wild-type samples is reported thereafter. To evaluate the spatial relationship between the heme and L-arginine binding sites, we chose to follow the heme spectral changes caused by different heme ligands and amino acid ligands. Both classes of ligands were found to induce either type I or type II spectral changes (11, 21). As illustrated in Fig. 1A, pyridine results in a red shift of the heme Soret band of purified eNOS from 400 to 422 nm (type II perturbation). The difference spectrum exhibits a peak at 430 nm and a trough at 396 nm. In contrast, the homologous 4-ethylpyridine caused a blue shift to 396 nm (type I perturbation). The difference spectrum exhibits a peak at 382 nm and a trough at 418 nm. Similar results were observed for amino acid ligands. Addition of L-lysine caused a red shift of the heme...
Fig. 2. Binding competition between imidazole and l-arginine. One micromolar eNOS was titrated with imidazole at a serial concentration of l-arginine: open diamonds, 0 μM; solid circles, 0.5 μM; solid squares, 10 μM and solid triangles, 50 μM. Absorbance difference between 396 and 430 nm was used for binding calculation. Solid lines are the hyperbolic least square fitting. Inset, double-reciprocal plot of the same data. All titrations were performed at 23 °C.

Soret to 424 nm while a blue shift to 396 nm was observed after treatment by l-histidine (Fig. 1B). The type II difference spectrum induced by l-lysine shows a peak at 430 nm and a trough of 394 nm, whereas the type I difference spectrum generated by l-histidine displays a maximum at 376 nm and a minimum at 418 nm.

The optical spectral changes caused by different ligands have been used to quantify their binding to eNOS protein. A typical equilibrium binding isotherm of eNOS-imidazole formation obtained by optical titrations is shown in Fig. 2 (open diamonds). A \( K_d \) of 150 μM at 23 °C was obtained by hyperbolic fitting to a one-site binding mechanism or from a double-reciprocal plot. The effect of l-arginine on the imidazole binding was measured by the same procedure with eNOS pretreated with a different amount of l-arginine (Fig. 2). As l-arginine concentration increases, there is an apparent increase of \( K_d \) but not the maximal capacity of binding represented as the measured \( \Delta A_{396-430} \) at the end of titration. The double-reciprocal plot gave one y intercept and many x intercepts, indicating a simple competitive inhibition of imidazole binding by l-arginine. The \( K_i \) value calculated from the secondary plot of the double-reciprocal plot or from Equation 1 under “Experimental Procedures” is 2.5 μM, similar to our previous reported value, 0.7 μM, of the dissociation constant (15).

Kinetic binding of imidazole was also measured by stopped-flow apparatus at 4 °C and 23 °C. eNOS protein was mixed with increasing amounts of imidazole under pseudo first-order conditions. The kinetic change at 428 nm fits nicely to a single exponential function to obtain the observed rate constants. Circles, 4 °C; squares, 23 °C. The vertical bars represent the standard deviation of 10–15 repetitive shots from three different experiments. Essentially same results were obtained at 4 °C using either wild-type enzyme or the G2A mutant eNOS in which the myristoylation site is lost shown. The \( K_f \) value measured at 4 °C is smaller than that obtained at room temperature, indicating the binding of imidazole is an exothermic process.

It is difficult to use a similar method to measure l-arginine binding rate constants. The optical signal change induced by l-arginine is very small and requires an impractically large amount of enzyme to acquire reasonable data. Furthermore, the type I spectral change was found to represent only small percentage of the eNOS molecules (11). We thus decided to use a competitive binding approach. Stopped-flow experiments were conducted at both 4 and 23 °C to follow the binding of imidazole to eNOS in the presence of increasing amounts of l-arginine as shown in Fig. 4. At 4 °C, the observed signal change decreased with increasing amount of l-arginine in the reaction mixture. Computer simulation was performed against a competitive model as described under “Experimental Procedures.” As the binding rate constants for imidazole were obtained and the dissociation constant of l-arginine was available from the equilibrium binding measurement to be 0.5 μM, there is only one independent variable we can change to achieve the optimal fitting. The \( k_{on} \) and \( k_{off} \) converged to the values of 2 × 10^5 m^−1 s^−1 and 0.08 s^−1. Simulation generated by these values (Fig. 4A, bottom) match very well with the data as indicated in Fig. 4A, top. It thus appears that both imidazole and l-arginine have similar rates of association, but once bound to the eNOS protein, l-arginine dissociates slower than imidazole by 100-fold. A similar conclusion was reached with the experimental data obtained at 23 °C (Fig. 4B). In contrast to the low-temperature data, decreasing absorbance at 428 nm was observed in the competition experiments (compare the top panels between Fig. 4A and 4B). The majority of the very fast phase represented as an increase of 428 nm absorbance was lost in the dead time of the stopped-flow as a result of fast association of imidazole at a concentration of 1 mM. The main reaction kinetics we recorded in Fig. 4B is thus the replacement of imidazole ligand by l-arginine binding. The deduced \( k_{on} \) and \( k_{off} \) values for l-arginine from the optimal simulation to the actual data were 0.8 × 10^6 m^−1 s^−1 and 1.6 s^−1, respectively, using a measured \( K_f \) of 2 μM at 23 °C.

The direct competition between imidazole and l-arginine for their binding to eNOS prompted us to evaluate a series of
potential heme ligands for their effect on heme spectral changes and L-arginine binding. We have evaluated the effect of two categories of ligand: small ligands with limited flexibility and amino acid ligands related to the substrate, L-arginine. The results of all tested ligands are summarized in Tables I and II (specifically for amino acid ligands) for their chemical structure, physical dimensions, heme binding and dissociation constant, types and extent of spectral perturbation, as well as the mode of competition with L-arginine, L-lysine, or imidazole.

In general, the ligands which induce type I spectral changes of eNOS heme have absolute spectra with Soret maxima at 396 nm with only one exception, L-arginine which shifted the Soret peak to 394 nm. The corresponding difference spectra display peaks in the range of 380–390 nm and troughs at 418–420 nm. The guanidine series exhibit Soret peaks at exceptionally low wavelengths, ranging from 374 to 376 nm, in the difference spectrum. The extinction coefficients of the difference spectra fall in the range of 10–19 mM$^{-1}$ cm$^{-1}$. These small values are partially due to the fact that the majority of the heme in eNOS was already present as high-spin heme. On the other hand, the ligands that result in type II spectral changes exhibit the Soret peaks in a wider range from 420 to 438 nm. The matching difference spectra show peaks at 432–440 nm and troughs at 394–398 nm. The difference extinction coefficient for the type II perturbation is substantially larger than the type I ligands. Values ranging from 43 to 97 mM$^{-1}$ cm$^{-1}$ were found for these type II ligands. Both types of ligands included in Tables I and II are tested to be competitive with either imidazole or L-arginine with widely different affinities for eNOS.

The three imidazole-based ligands, imidazole, 2-methylimidazole, and 4-methylimidazole, ligated to heme iron directly and produced a low-spin heme spectrum with $K_d$ in the millimolar range. All three imidazole ligands are competitive with L-arginine (Fig. 2 and Table I). On the other hand, among the four pyridine-based ligands: pyridine, 2-amino pyridine, 4-ethylpyridine, and 3,5-lutidine, only pyridine induced a type II spectral change with binding $K_d$ of 0.2 mM. The other three ligands, 2-amino pyridine, 4-ethylpyridine, and 3,5-lutidine produced type I spectral changes and binding dissociation constants of 0.1, 0.1, and 0.2 mM, respectively. Pyridine is also competitive with L-arginine. A similar trend was found for the two pyrimidine ligands: pyrimidine and 4-methylpyrimidine. Pyrimidine caused a type II spectral change with eNOS heme and is competitive with L-arginine as shown in Fig. 1. Binding of pyrimidine is less tight than pyridine ($K_d = 5.2$ mM versus 0.2 mM). In contrast, the bulkier 4-methylpyrimidine caused a type I spectral change at similar concentration. This relationship between the physical size of the ligand and the type of spectral changes observed for the pyrimidine/pyridine series is reversed, however, in the series of thiazole-based ligand (Table I). Thiazole by itself generated a type II spectral change of the eNOS heme with a dissociation constant of 2.2 mM, while 2-amino thiazole, 2-amino-4-methylthiazole, and 2-amino-4,5-dimethylthiazole showed type I spectral perturbations and the affinity increased with the physical size of the ligand ($K_d = 125, 13, and 1.2$ mM for 2-amino thiazole, 2-amino-4-methylthiazole, and 2-amino-4,5-dimethylthiazole, respectively). This series of amino thiazole ligands was determined to be competitive with imidazole. The other two bulkier strong field ligands, aniline and nicotinic acid, caused type II spectral changes only at...
### Table I

Spectral perturbation by different potential heme ligands and their binding characteristics

| Structure                  | Dimension X x Y (Å) | λ max (cm⁻¹) | Difference Spectrum / λ max-min | Δ g nm² cm⁻¹ | Inhibition of L-argin / imidazole | K₄ |
|----------------------------|---------------------|--------------|---------------------------------|-------------|----------------------------------|----|
| Imidazole                  | 4.2 x 2.8           | 428          | Type II 432-394                 | 97          | competitive to L-arginine         | 0.15 mM |
| 2-Methyl imidazole         | 5.2 x 2.8           | 426          | Type II 432-396                 | 52          | competitive to L-arginine         | 17.5 mM |
| 4-Methyl imidazole         | 4.2 x 4.2           | 426          | Type II 432-396                 | 60          | competitive to L-arginine         | 0.87 mM |
| Pyridine                   | 4.2 x 3.9           | 422          | Type II 430-396                 | 52          | competitive to L-arginine         | 0.20 mM |
| 2-Amino pyridine           | 5.2 x 3.9           | 396          | Type I 382-418                  | 10          | ND                               | -0.1 mM |
| 4-Ethyl pyridine           | 4.2 x 6.3           | 396          | Type I 382-418                  | 19          | competitive to imidazole          | 0.10 mM |
| 3,5-Lutidine               | 0.3 x 3.9           | 396          | Type I 382-418                  | 11.7        | ND                               | 0.20 mM |
| Aniline                    | 4.2 x 5.7           | ND*          | Type II 432-396                 | ND*         | ND                               | >100 mM |
| Nicotinic Acid             | 5.4 x 3.9           | ND*          | Type II 432-396                 | ND*         | ND                               | >100 mM |
| Pyrimidine                 | 4.2 x 4.8           | 420          | Type II 432-394                 | 43          | competitive to L-arginine         | 5.25 mM |
| 4-Methyl pyrimidine        | 4.2 x 4.7           | 396          | Type I 382-418                  | 19          | ND                               | 1.80 mM |
| Thiazole                   | 4.6 x 3.7           | 422          | Type II 428-394                 | 82          | competitive to L-arginine         | 2.20 mM |
| 2-Amino thiazole           | 5.4 x 3.7           | 396          | Type I 382-418                  | 12          | ND                               | 125 μM |
| 2-Amino-4-methyl-thiazole  | 5.4 x 4.5           | 396          | Type I 382-418                  | 14.5        | ND                               | 13.0 μM |
| 2-Amino-4-5-dimethyl-thiazole | 6.3 x 4.5   | 396          | Type I 382-418                  | 14          | competitive to imidazole          | 1.20 μM |
| 2-Aminoox-4-chlorophenylthiazole | 5.4 x 9.2 | 396          | Type I 382-418                  | 17.5        | ND                               | 300 μM |
| Amino-guanidine            | 3.8 x 4.3           | ND**         | Type I 376-418                  | 11          | competitive to imidazole          | 3.0 mM |
| Acetyl-guanidine           | 3.9 x 5.5           | 396          | Type I 376-418                  | 14          | competitive to imidazole          | 0.45 μM |
| Ethyl-guanidine            | 3.9 x 5.5           | 396          | Type I 374-418                  | 16          | competitive to imidazole          | 20.0 μM |
| Cyanoide                   | 2.25                | 438          | Type II 440-398                 | 97          | not compete with L-arginine       | 100 mM |
| Acetonitrile               | 4.3                 | 396          | Type I 380-416                  | ND*         | ND                               | >100 mM |
concentrations higher than 100 mM, indicating very weak affinity. Moderate to weak-field ligand such as halides, dimethyl sulfoxide, dimethyl sulfide, and thiocyanate, did not have any effects on the heme spectrum at millimolar concentration. Acetonitrile only yielded type I spectral perturbation at concentrations as high as 100 mM. The strong field ligand cyanide bound to eNOS heme and generated a low-spin heme complex, but the affinity is rather weak ($K_a = 10 \text{ mM}$). The unique feature of cyanide binding to the eNOS heme is its independence of L-arginine binding as demonstrated in Fig. 5, a distinctive behavior from all the other ligands.

In the realm of the amino acid ligands, we evaluated the effects of L-citrulline, L-lysine, L-histidine, and L-ornithine in addition to the substrate, L-arginine. L-Citrulline, the final product of eNOS, showed very poor affinity with a $K_a$ estimated to be greater than 200 mM. The spectral change caused by L-citrulline is a surprising type II change, in contrast to that by L-arginine. L-Lysine also gave an unexpected type II spectral perturbation (Fig. 1). This type II spectral change represented by the absorbance difference between 430 and 398 nm was used to evaluate the influence of L-arginine on its binding. As shown in Fig. 6, the apparent $K_a$ value but not the maximal binding capacity increases with increasing amount of L-arginine. The double-reciprocal plot between the absorbance change and the concentration of L-lysine displayed the typical behavior of a competitive inhibition (Fig. 6, large inset). The $K_a$ value obtained from the slope in the secondary plot for L-arginine, 1.1 $\mu$M, is very similar to its $K_a$ value obtained previously (Fig. 6, small inset) (15). L-Lysine was tested to be competitive with L-histidine also. L-Histidine, which has an imidazole moiety, yielded a dramatic type I spectral change rather than a type II change found for other imidazole derivatives (Fig. 1 and Table I). Spectral perturbation was hardly observed even at 100 mM of L-ornithine. The affinities for both L-lysine and L-histidine are similar, with a $K_a$ in the mM range, and are three orders of magnitude weaker than that of L-arginine.

**DISCUSSION**

Binding competition between two low-spin heme ligands or high-spin heme ligands cannot be demonstrated easily by spectral perturbation due to small signal changes. It is straightforward, however, that those ligands causing type II spectral changes of eNOS are competitive to one another due to a direct ligation to the heme iron. Those ligands producing type I spectral changes could be mutually competitive, also, due to overlapping with L-arginine for their binding sites.

Our direct binding measurements by heme spectral perturbation concluded that L-arginine and imidazole are competitive in their binding to eNOS. The different types of spectral perturbation produced by L-arginine and imidazole derive directly from their different binding modes. Imidazole acts as a true heme ligand and thus leads to the formation of a low-spin heme complex. The type I spectral change caused by L-arginine is the change from a mixture of 5- and 6-coordinate heme to a pure 5-coordinate heme upon arginine binding (16). Competition between imidazole and L-arginine in their binding to eNOS simply indicates an overlap of their binding domains. Binding equilibrium measurements of imidazole at different levels of L-arginine similar to that shown in Fig. 2 and kinetic measurements as shown in Fig. 4 might be useful to resolve the controversy of whether these two ligands are competitive or non-competitive in their binding to nNOS (9–10).

The binding rate constants of either L-arginine or imidazole to NO-s have not been reported. Our data obtained by stopped-flow indicate a rather slow binding process for both ligands. Very similar values of the on rate constants of these two ligands and their temperature dependence gave implication that both ligands access their binding sites, which are deeply buried inside the protein molecule, through a common passage. A similar substrate access channel was found for cytochrome P450BM3, P450terp, and P450cam and revealed in their crystal structures (18–20). For the smaller imidazole molecule, it is easy to reach the heme iron and form coordination, while the larger L-arginine apparently cannot bind directly to the heme iron due to steric limitation. Further reduction of the size of the heme ligand finally resulted in a binding independent of L-arginine. Cyanide is the only such example we have found thus far (Fig. 5) and not too many small anionic ligands will satisfy these criteria due to the highly electronegative thiolate ligand at the 5th position. It is evidenced further by the low affinity of cyanide for eNOS (Table I) and other P450 enzymes (21).

The imidazole ligand series all produced type II spectral changes, indicating they are strong heme ligands and are small enough to fit into the distal ligand pocket. The less strong
binding of the 2-methylimidazole is most likely attributed to the steric strain by the methyl residue (22). When the size of the ligand is increased from imidazole to pyridine or pyrimidine ligand series, bimodal behavior on the heme spectral perturbation was noticed (Table I). These results indicate that the physical dimensions of the distal heme ligand pocket are on the borderline of the size of these pyridine and pyrimidine ligand series. The parent molecules, pyridine and pyrimidine, can still bind to heme iron directly and cause type II spectral changes, whereas those bulkier homologs cannot form direct coordination with Fe(III). Simple molecular modeling as stated under “Experimental Procedures” allows an estimation of the size of the distal heme site as shown in Fig. 7. The bulkier 3,5-lutidine and 4-methylpyrimidine serve as the delimiters to gauge the horizontal and vertical dimensions using an average bond length of 2.0 Å between Fe(III) and the ligand. Validity of this estimation of a pocket dimension is enhanced by the observation that aniline and nicotinic acid, which have sizes on this borderline, can barely squeeze in the pocket and form low-spin heme complexes, although the affinities for both these two ligands are rather low.

The type I spectral changes caused by these bulkier rigid ligands can represent direct interactions with the heme edge or the distal ligand or indirect effects mediated by the protein amino acid residues. We should not treat the type I spectral changes as the consequence of a single mechanism, namely repulsion of the original distal ligand, implied by the much narrower range of spectral perturbations induced by type I ligands than those generated by the type II ligands (Table I and Ref. 23). However, the EPR spectral changes caused by L-arginine in nNOS (16) and our EPR studies using various type I ligands similar to those in this study have revealed quite different spectral perturbations of the high-spin heme of eNOS (23). We do not exclude the possibility that binding of a type I ligand could cause perturbations stemming from both a common mechanism and a ligand-specific mechanism. The large differences among the binding affinities between the pyridine and pyrimidine ligand series gave indication that the parent ligand most likely binds at different domains as their substituted homologs.

Thiazole, with a physical size similar to imidazole, as expected, caused a type II spectral change as a result of formation of a low-spin heme complex. To our surprise, 2-aminothiazole, which is similar to 2-methylimidazole in size, generated a type I spectral change and a substantially increased binding affinity from thiazole. Realizing that 2-aminothiazole and its substituted congeners all share with L-arginine a common guanidine structure, a specific binding competition of this ligand series with L-arginine becomes the most natural explanation for our data. These thiazole ligands were determined to be strong inhibitors of eNOS and nNOS for L-citrulline formation (24). Our data corroborate Garvey et al. (24) in that the 2-aminothiazole ligands behave as cyclized isothioureas and compete with L-arginine for its guanidine binding site. The critical role of the guanidine group in the affinity of L-arginine is exemplified further by the strong affinity of acetyl guanidine and ethylguanidine. These substituted guanidines bind to the same site as the guanidino moiety of L-arginine is supported by the fact that these substituted guanidines are directly competitive with imidazole (Table I). Frey and Griffith (25) also found that acyl guanidines are potent competitive inhibitors for nNOS. It is worth noting, however, that guanidine at micromolar levels does not perturb the heme spectrum, but behaves as a protein denaturant at millimolar concentrations.

L-Lysine, which has one methylene unit shorter than L-arginine on its side chain, formed a low-spin complex with eNOS, indicating that the side chain of lysine is in a more stretched conformation than L-arginine to reach the heme iron. In contrast, the guanidine head group of L-arginine interacts specifically with its partner amino acid through hydrogen bonding and polar-polar interactions prohibits its side chain to be fully extended (1, 26). The importance of the secondary amine and the imino group in the guanidine for the binding affinity of L-arginine is again illustrated by the relative low affinity of L-lysine. The eNOS product, L-citrulline, showed extremely weak binding, indicating that a change from an imino group (a hydrogen bond donor) to a keto group (a hydrogen bond acceptor) is a disaster. The type II spectral change observed at very high concentrations is also due to the increased flexibility of the head group of the L-citrulline side chain as L-lysine. Similar contrasting spectral changes by homologous ligands were also

![Fig. 6. Binding competition between L-lysine and L-arginine.](image-url)

Similar to the procedure described in the legend to Fig. 2, 1 μM eNOS was titrated with L-lysine at several different concentrations of L-arginine: 0 μM (open circles); 5 μM (open squares), 7.5 μM (open triangles), 10 μM (open inverted triangles), and 15 μM (open diamond). Lines are the hyperbolic fittings. Inset, double-reciprocal plot of the main figure with matching symbols and the secondary plot of the slope values (solid circles) calculated from each straight line in the double-reciprocal plot against L-arginine concentrations.

![Fig. 7. Schematic representation of the spatial relationship between the heme and the arginine binding site.](image-url)

The porphyrin plane is shown as two segments of thick line with the iron atom sitting at the center. L-Arginine is accessible to the heme through a defined channel and is independent of the binding of cyanide located at the distal site of the heme. The dimension of the distal heme pocket was estimated to be 6.3 × 6.7 Å delimited by two rigid planar ligands: 3,5-lutidine and 4-methylpyrimidine.
demonstrated by Frey et al. (27) for the binding of thiocitrulline and homothiocitrulline to nNOS. L-Histidine and L-ornithine, both having potentially strong field nitrogen ligands, are simply too short to reach the heme iron when occupying the L-arginine binding site. The type I spectral change caused by L-histidine most likely originates from indirect perturbation of the heme structure. Both L-histidine and L-ornithine are, however, too bulky to fit in the distal heme ligand pocket to form a low-spin heme complex. Neither of these amino acid ligands bind as strongly as L-arginine due to the lack of the guanidino head group.

In summary, L-arginine binds to the vicinity of the heme iron and is ready to interact with the heme distal ligand. Only small ligands such as cyanide could bind freely without any steric conflict with L-arginine while most other heme ligands tested are competitive with L-arginine due to a direct steric effect. An overall picture of the spatial relationship between the heme and L-arginine binding sites can be represented in Fig. 7. In this representation, the L-arginine molecule was aligned in its binding domain, which also serves as the end of the access channel for other ligands. The estimated dimensions of the distal heme site only serve as an average of many dynamic protein conformations in response to the binding of various ligands. Such a topology fits in nicely with the monooxygenation reaction mechanism. The guanidino nitrogen orients toward the 6th heme ligand site and ready to react with the active heme intermediate, [Fe-OH]1+, for a hydrogen abstraction and latter rebound of the hydroxyl group to form N-hydroxyl arginine.

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