Active Site Topologies and Cofactor-mediated Conformational Changes of Nitric-oxide Synthases*

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The active site topologies of neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) nitric-oxide synthases heterologously expressed in Escherichia coli have been examined using three aryldiazene (Ar-N=NH) probes. The topological information derives from (a) the rate and extent of arylation complex formation in the presence and absence of tetrahydrobiopterin (H4B), Ca2+-dependent calmodulin (CaM), and l-arginine, and (b) the N-phenylporphyrin IX regioisomer ratios obtained upon migration of the phenyl of the phenyl-iron complex to the heme nitrogen atoms. The N-phenylporphyrin ratios indicate that the three NOS isoforms have related active site topologies with unencumbered space above all four pyrrole rings but particularly above pyrrole ring D. H4B binds directly above the heme pyrrole ring D or causes a conformational change that constricts that region, because H4B markedly decreases phenyl migration to pyrrole ring D. Small CaM-dependent changes in the nNOS N-phenylporphyrin isomer pattern are consistent with a conformational link between the CaM and heme sites in this protein. The ceiling height directly above the heme iron atom differs among the isoforms and is lower than in the P450 enzymes because only nNOS and iNOS react with 2-naphthyldiazene, and none of the isoforms reacts with p-biphenyldiazene. l-Arg blocks access to the heme iron atom in all three NOS isoforms and nearly suppresses the phenyl-diazene reaction. The data indicate that topological differences, including differences in the size of the active site, are superimposed on the structural similarities among the NOS active sites.

The nitric-oxide synthases (NOS) are homodimeric proteins that oxidize l-arginine (l-Arg) to citrulline and NO (1–5). Three basic types of NOS are known: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS). All three NOS isoforms require heme, FMN, FAD, calmodulin (CaM), and tetrahydrobiopterin (H4B) as cofactors and utilize NADPH and O2 as co-substrates. The NOS are modular hemoproteins in which a heme domain is linked via a CaM-binding sequence to a two-domain, one of which inserts a NADPH domain, the other inserts a hydrophobic domain. CaM is rat neuronal NOS (NOS-1); eNOS, bovine endothelial NOS (NOS-3); iNOS, mouse macrophage-inducible NOS (NOS-2); l-Arg, l-arginine; CaM, Ca2+-dependent calmodulin; H4B, (+)-5,6,7,8-tetrahydrobiopterin; DTT, dithiothreitol; HPLC, high performance liquid chromatography; Nα, Nε, Nγ, and Nδ refer to the N-phenylporphyrin IX regioisomers with the phenyl group on pyrrole rings B, A, C, and D, respectively.

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EXPERIMENTAL PROCEDURES

Materials and General Methods—l-Arg was obtained from Aldrich. Methyl phenyldiazene-carboxylate azo ester was purchased from Research Organics, Inc. (Cleveland, OH). Naphthyl- and biphenyldiazene were synthesized by the procedure of Huang and Kosower (20). H4B was from Alexis Biochemicals (San Diego). H4B solutions were prepared as a 10 mM stock in 100 mM DTT. Negative controls for the effects of H4B in a given assay included the appropriate amount of DTT. Spectra were...
Enzymes—Rat brain nNOS and bovine eNOS were heterologously expressed in *Escherichia coli* and purified as previously reported (19, 21). Murine iNOS was co-expressed with human CaM in *E. coli* and was purified by ADP-Sepharose chromatography. Related systems for the co-expression of CaM and iNOS have recently been reported (22, 23). The three NOS proteins were purified in the absence of H$_4$B and L-Arg. Protein concentrations were determined by the Bradford assay (Bio-Rad) using bovine serum albumin as the standard. Concentrations of heme-containing NOS were determined using the extinction coefficients $e_{400 \text{nm}} = 100 \text{mM}^{-1} \text{cm}^{-1}$ for the ferric enzyme and $D_{444-490 \text{nm}} = 76 \text{mM}^{-1} \text{cm}^{-1}$ for CO-bound NOS (24).

### Aryl Shift and Quantitation of N-Arylporphyrins—

In a microcuvette, one or more of the following were added to 2 nmol of NOS in 600 $\mu$l of 0.1 M Hepes (pH 7.5): 15 $\mu$M H$_4$B, 150 $\mu$M DTT (if no H$_4$B added), 6 nmol of CaM, or 800 $\mu$M L-Arg. The reaction was initiated by addition of 1 $\mu$l of stock aryldiazene solution, and spectra were periodically recorded. The stock diazenes solutions were made by placing 1 $\mu$l of a 125 mM stock solution of the methyl carboxylate azo ester of the diazene in 100 $\mu$l of 1 M NaOH. The reaction of the protein with the diazene was allowed to proceed for 30 min before addition of three 5-$\mu$l aliquots of 20 mg/ml aqueous potassium ferricyanide. Samples were removed 30 and 60 min after the first ferricyanide addition to monitor the formation of the N-aryl isomers. The samples were worked up and chromatographed as previously reported (19).

### Statistical Analysis—

A paired Student's *t* test was carried out to determine whether the difference between any pair of *N*-phenylproto- porphyrin IX regioisomer values was significant. The following equation for *t* was used:

$$t = \frac{x_1 - x_2}{s_p} \left( \frac{N_1 N_2}{N_1 + N_2} \right)^{1/2}$$

(Eq. 1)

where $x_1$ and $x_2$ are the means of each of the two samples being compared, $N_1$ and $N_2$ are the number of measurements carried out on samples 1 and 2, respectively, and $s_p$ is the pooled standard deviation:

$$s_p = \left( \frac{\sum (x_{1i} - x_1)^2 + \sum (x_{2i} - x_2)^2 + \ldots + \sum (x_{ki} - x_k)^2}{N - k} \right)^{1/2}$$

(Eq. 2)

*N* is the total number of measurements and is equal to $(N_1 + N_2 + \ldots + N_k)$, $x_{1i}, x_{2i}, \ldots, x_{ki}$ are the individual values in each set of measurements, and $k$ is the number of sets of analysis (in all cases samples were compared pairwise, therefore $k = 2$). The value obtained for *t* was compared to the tabular value of *t* with the appropriate degrees of freedom at a 95% confidence level. If the value obtained for the two sets of data was larger than the tabulated value at a 95% confidence level, we conclude that there is a significant difference between the two values.

#### Rates of Aryl-Iron Complex Formation—

Incubations (350 $\mu$l) at 25°C in 0.1 M Hepes buffer with 10% glycerol contained 3 $\mu$M NOS and, when indicated, one or more of the following: 6 $\mu$M CaM, 30 $\mu$M H$_4$B, or 1.5 mM L-Arg. Rates of aryl-iron complex formation were determined by fitting the time-dependent changes in the 470 nm absorbance to a single exponential fit using the program Igor (version 1.28, WaveMetrics). The extinction coefficient employed for the phenyl-iron complex was $D_{470}$.

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2 C. R. Nishida and P. R. Ortiz de Montellano, unpublished results.
H4B decreases the rate of phenyl-iron complex formation, as the reaction with phenyldiazene could not be evaluated. However, CaM-free iNOS is not available, and the effect of CaM on its active site topology (21) indicates that the structure of iNOS in the absence of H4B and L-Arg (Fig. 2, Table I). When CaM is bound at the same time as H4B, the extent of nNOS (80%) and iNOS (59%), but is higher for eNOS (88%) when only CaM is bound. CaM generally increases the extent of complex formation, but the effect of H4B is less consistent. It decreases the degree of complex formation with eNOS and iNOS, but with nNOS sometimes increases and sometimes decreases complex formation.

The first order rate constant for phenyl-iron complex formation, like the extent of the reaction, depends on the presence or absence of CaM, H4B, and L-Arg (Fig. 2, Table I). A very small amount of the phenyl-iron complex is formed in the reaction of nNOS with phenyldiazene if L-Arg is present (Fig. 1C). In general, the binding of L-Arg greatly decreases the extent of phenyl-iron complex formation: almost no complex is formed with eNOS and only minor amounts with nNOS and iNOS. The anomalous finding that L-Arg increases iNOS complex formation in the absence of H4B is not reliable because the spectrum of iNOS in the absence of H4B indicates that the structure of the protein is strongly perturbed. In the absence of L-Arg, the extent of the reaction is highest for CaM- and H4B-bound nNOS (80%) and iNOS (59%), but is higher for eNOS (88%) when only CaM is bound. CaM generally increases the extent of complex formation, but the effect of H4B is less consistent. It decreases the degree of complex formation with eNOS and iNOS, but with nNOS sometimes increases and sometimes decreases complex formation.

Results

The NOS active site topology was examined by a three-step process involving: (a) reaction of the enzyme with aryldiazidines to give aryl-iron complexes, (b) ferricyanide-mediated shift of the aryldiazidines to the heme nitrogens, and (c) HPLC determination of the resulting N-arylprotoporphyrin IX isomer ratios. The nNOS, eNOS, and iNOS employed in the investigation were heterologously expressed in E. coli and were purified by affinity chromatography in the absence of H4B and L-Arg. The recombinant proteins were therefore obtained in an H4B-free state suitable for investigation of the influence of H4B on the active site topology (21).

In the case of iNOS, it was necessary to co-express CaM with the enzyme in order to obtain active iNOS. It was therefore not possible to investigate the effect of CaM on the active site topology of iNOS.

Reaction with Phenyl Diazidene—The three NOS isoforms react with phenyldiazene to form a phenyl-iron complex with an absorption maximum at ~470 nm (Fig. 1), but the rates and extents of the reactions differ. This absorbance maximum is slightly lower than the value of ~480 nm for most P450 aryl-iron complexes but resembles that of the P450 1A1 complex at 474 nm (25). The absorption peak of the NOS phenyl-iron complex is relatively broad.

Effect of Cofactors on the Phenyl Diazidene Reaction—The extent of the phenyl-diazidene reaction depends on the presence or absence of CaM, H4B, and L-Arg (Fig. 2, Table I). A very small amount of the phenyl-iron complex is formed in the reaction of nNOS with phenyldiazene if L-Arg is present (Fig. 1C). In general, the binding of L-Arg greatly decreases the extent of phenyl-iron complex formation: almost no complex is formed with eNOS and only minor amounts with nNOS and iNOS. The anomalous finding that L-Arg increases iNOS complex formation in the absence of H4B is not reliable because the spectrum of iNOS in the absence of H4B indicates that the structure of the protein is strongly perturbed. In the absence of L-Arg, the extent of the reaction is highest for CaM- and H4B-bound nNOS (80%) and iNOS (59%), but is higher for eNOS (88%) when only CaM is bound. CaM generally increases the extent of complex formation, but the effect of H4B is less consistent. It decreases the degree of complex formation with eNOS and iNOS, but with nNOS sometimes increases and sometimes decreases complex formation.

The first order rate constant for phenyl-iron complex formation, like the extent of the reaction, depends on the presence or absence of CaM, H4B, and L-Arg. In general, L-Arg decreases the rate of complex formation (Table I). When CaM is bound, little weight can be placed on the rates of complex formation in its presence. For nNOS, the binding of CaM increases, and the binding of H4B decreases, the rate 2-fold (Fig. 2, Table I). When CaM is bound at the same time as H4B, the effect of the latter agent dominates and the rate is less than that of the protein without CaM (Fig. 2, Table I). A similar pattern is observed for eNOS, except that the increase caused by CaM is smaller and the decrease caused by H4B is larger. Again, the effect of H4B dominates over the effect of CaM. CaM-free iNOS is not available, and the effect of CaM on its reaction with phenyldiazene could not be evaluated. However, H4B decreases the rate of phenyl-iron complex formation, as it does with the other two isoforms. Under any given set of reaction conditions, nNOS reacts most rapidly with phenyldiazene, iNOS at an intermediate rate, and eNOS most slowly.

Reaction with 2-Naphthyl- and p-Biphenyl Diazidenes—To examine the NOS active site at greater distances above the heme iron than are sampled by the phenyl probe, the reactions of the NOS isoforms with 2-naphthyl diazidene and p-biphenyl diazidene in the absence of H4B and L-Arg were investigated. 2-Naphthyl diazidene does not form an aryl-iron complex with eNOS, but 60% of the nNOS and 30% of the iNOS readily form the complex with first order rate constants of 0.08 and 0.20 min⁻¹, respectively (Table I). These extents of complex formation are comparable to those obtained with phenyldiazene even though the naphthyl diazidene reaction rates are slower. p-Biphenyl diazidene does not detectably form a p-biphenyl-iron complex with any of the NOS isoforms. The differences in rates for the larger aryldiazidenes could be due to differences in the steric and/or hydrophobicity requirements for the reaction, but the fact that the extent of naphthyl-iron complex formation is eventually the same as phenyl-iron complex formation is more consistent with steric than hydrophobicity constraints in determining whether the aryl group is bound to the iron.
to more lipophilic regions, as expected if steric rather than entropic effects dominate the rearrangement process. The N-arylporphyrin ratios thus provide direct information on the active site topology.

The reactions of the NOS isoforms with the aryldiazenes were monitored at 470 nm until maximum complex formation was observed. Ferricyanide was then added, and the N-arylprotoporphyrin IX adducts were isolated and quantitated by HPLC (Fig. 3). The isomers elute from the HPLC in the order NB:NA:NC:ND, where the subscript denotes the pyrrole ring bearing the aryl group. The regioisomer patterns were obtained for the two constitutive NOS isoforms in the presence and absence of H4B and CaM and for iNOS in the presence and absence of H4B (Fig. 3, Table II). In those cases where the extent of phenyl-iron complex formation is low, the yields of N-phenylprotoporphyrin IX isomers are also low, and the integrated ratios are less reliable. The phenyl-iron shift was therefore not investigated in the presence of l-Arg due to the very small extent of phenyl-iron complex formation.

The binding of CaM to nNOS in the absence of H4B causes a small (9%) but reproducible decrease in the proportion of the N5 regioisomer and a compensatory increase (12%) in the proportion of the N4 isomer (Table II). Both of these changes are statistically significant at the 95% confidence level by a paired Student’s t test (Table III). The binding of CaM in the absence of H4B also appears to decrease when CaM binds to eNOS, but the difference is not statistically significant. The effect of CaM on the iNOS active site topology could not be evaluated because the CaM-free protein is not available. The CaM-mediated nNOS regioisomer changes provide the first experimental data suggesting that CaM binding alters the NOS active site topology.

The binding of H4B to NOS in the absence of CaM alters the N-phenylprotoporphyrin IX ratios more than the binding of CaM in the absence of H4B (Table II). The proportion of the N5 isomer decreases in nNOS from 74 to 41% and in eNOS from 81 to 53% when the aryl shift is carried out in the presence versus absence of H4B. The decrease in the N5 isomer is compensated for by increases in the other regioisomers. All of the following changes are statistically significant by a paired Student’s t test (Table III): the decreases in the N5 isomer in nNOS and eNOS, the increases in N6 and N7 in nNOS, and the increases in N6 and N7 in eNOS. These patterns suggest that a decrease in the space above pyrrole ring D due to the binding of H4B increases the shift of the phenyl toward the other pyrrole nitrogens.

The changes in the regioisomer ratio when both CaM and H4B are bound are similar to those obtained with H4B alone (Table II). The binding of CaM if H4B is already present does not significantly alter the regioisomer ratio, but the ratio is altered when H4B is added to the CaM-bound enzyme. The changes observed in N6, N5, and N7 in nNOS, and all the changes observed in eNOS, are statistically significant by a paired Student’s t test (Table III). This also appears to be true for iNOS, although the change due to CaM alone cannot be determined. In the case of nNOS, the binding of H4B brings

**TABLE I**

| Effector(s) | nNOS | eNOS | iNOS |
|------------|------|------|------|
|            | Rate | Reacted | Rate | Reacted | Rate | Reacted |
| None       | 0.44 | 52 %   | 0.32 | 78 %   | 0.50 | 40 %   |
| CaM        | 0.95 | 55 %   | 0.41 | 88 %   | 0.71 | 40 %   |
| H4B        | 0.20 | 40 %   | 0.10 | 39 %   | 0.24 | 59 %   |
| l-Arg      | 0.21 | 13 %   | 0.32 | 05 %   | 0.28 | 01 %   |
| CaM/H4B    | 0.30 | 80 %   | 0.08 | 47 %   | ND  | ND    |
| l-Arg/H4B  | 0.43 | 10 %   | 0.28 | 01 %   | ND  | ND    |
| l-Arg/CaM  | 0.37 | 27 %   | 0.28 | 01 %   | 0.23 | 12 %   |
| l-Arg/H4B/CaM | 0.55 | 17 %   | ND  | ND    | NR  | NR    |
| Naphthalenediazene | 0.08 | 60 %   | NR  | NR    | NR  | NR    |

* a ND, not determined. Spectroscopic changes were too small to firmly attribute them to aryl-iron complex formation.

* b No other reagents were present. The percent of the protein forming the complex is calculated on the assumption that the observed spectral changes are due to phenyl-iron complex formation.

* c NR, no reaction.

**FIG. 3.** HPLC traces of the separation of N-phenylprotoporphyrin IX isomers on a C18 column to determine the ratio of the isomers present. A, nNOS without CaM or H4B; B, nNOS plus CaM and H4B; C, eNOS without CaM or H4B; D, eNOS plus CaM and H4B. The isomers were eluted isocratically with 50:20 A:B (A = 6:4:1 MeOH: H2O:AcOH; B = 10:1 MeOH:AcOH) at 1 ml/min. The identities of the four isomers based on comparison of their retention times with authentic standards are indicated in A and are the same for the other panels.**
Nitric-oxide Synthase Active Site Topology

N-Phenylprotoporphyrin IX regiosomer ratios obtained from the sequential reaction of NOS with phenyldiazene and ferricyanide

\[ V = \text{vinyl, } P = \text{propionic acid} \]

**Table II**

**Statistical significance of the differences in the individual N-phenylprotoporphyrin IX regiosomer values for comparisons of NOS forms**

| Effector(s) | nNOS | eNOS | iNOS |
|-------------|------|------|------|
| None        | 11 ± 2.5:12 ± 1.03 ± 2.5:74 ± 1 | 04 ± 3.04 ± 3.11 ± 3.81 ± 6 | NP |
| CaM         | 07 ± 5.5:24 ± 4.04 ± 4.65 ± 6 | 08 ± 15.07 ± 3.13 ± 2.72 ± 5 | 10:17:08:65 |
| H4B         | 15 ± 3.25 ± 6.19 ± 2.41 ± 3 | 11 ± 2.20 ± 3.16 ± 4.53 ± 6 | NP |
| CaM/H4B     | 17 ± 2.20 ± 3.22 ± 3.41 ± 6.5 | 13 ± 2.20 ± 3.20 ± 3.47 ± 6 | 22:18:20:40 |

\( \text{NB:NA:NC:ND} \)

\( n \) indicates the number of independent experiments on which the averages are based; NP indicates experiment not possible.

**Table III**

| NOS forms compared | \( \Delta N_b \) | \( \Delta N_A \) | \( \Delta N_c \) | \( \Delta N_d \) |
|--------------------|----------------|----------------|----------------|----------------|
| nNOS CaM vs. nNOS | x              | x              | x              | x              |
| nNOS H4B vs. nNOS  | x              | x              | x              | x              |
| nNOS H4B/CaM vs. nNOS CaM | x | x | x | x |
| eNOS H4B vs. eNOS  | x              | x              | x              | x              |
| eNOS H4B/CaM vs. eNOS CaM | x | x | x | x |
| eNOS H4B vs. eNOS H4B | x | x | x | x |
| eNOS vs. nNOS      | x              | x              | x              | x              |
| eNOS H4B vs. nNOS H4B | x | x | x | x |
| eNOS CaM vs. nNOS CaM | x | x | x | x |
| eNOS H4B/CaM vs. nNOS CaM | x | x | x | x |

about a structural change in the same region that is perturbed by the binding of CaM. This change involves a diminution of the available space above pyrrole ring D, a finding consistent with the decrease in both the rate and the extent of phenyl-iron complex formation due to the binding of H4B to nNOS and eNOS. In the presence of CaM and H4B, all three NOS active sites appear to have similar active site topologies (Table II).

**Discussion**

The active sites topologies of the three NOS isoforms differ, but the differences are superimposed on what appear to be global topological similarities. The similarities are most evident in the N-phenylprotoporphyrin IX isomer ratios for the CaM- and H4B-saturated enzymes (\( N_b, N_A, N_c, N_d \)); nNOS (17:20:22:41), eNOS (13:20:20:47), and iNOS (22:18:20:40) (Table II). The small differences among these ratios indicate that the active sites of the three NOS isoforms are similarly structured with respect to the prosthetic heme group. In all three active sites, the region above pyrrole ring D is the most open, but none of the pyrrole rings is completely masked. However, significant differences in the dimensions of the active site cavity are revealed by the reaction with larger aryldiazenes. Differences are also found in the topological alterations caused by the binding of CaM, H4B, and L-Arg.

The reactions of the three NOS isoforms with 2-naphthyldiazene and p-biphenyldiazene indicate that the head space directly above the heme iron atom is limited. In contrast to the P450 enzymes, no p-biphenyl-iron complexes are formed in the NOS reactions with p-biphenyldiazene. A difference in ceiling height among the NOS isoforms is evident from the reaction with 2-naphthyldiazene, which yields a 2-naphthyl-iron complex with nNOS and iNOS but not with eNOS. The formation of the 2-naphthyl-iron complex is -6 times slower with nNOS, and 3.5 times slower with iNOS, than the formation of the phenyl-iron complex (Table I). These rate differences reflect the differences in the lipophilicities of the phenyl (log \( p = 2.1 \)) and 2-naphthyl (log \( p = 3.3 \)) groups as well as differences in their size. Models of the 2-naphthyl- and p-biphenyl-iron complexes based on the crystal structure of the P450cam phenyl-iron complex indicate that the 2-naphthyl ligand extends to a height of \( \text{about 7.1 Å} \) above the iron and the p-biphenyl to a height of \( \text{about 9.9 Å} \) (27). Formation of the nNOS and iNOS 2-naphthyl but not p-biphenyl complexes thus suggests that a protein residue closes the active site directly above the iron atom at a distance of 8–10 Å. The capping residue in eNOS, which forms the phenyl but not 2-naphthyl complex, is presumably lower than that in the other two isoforms. The NOS ceiling directly above the iron is lower than that in myoglobin or P450 because myoglobin and most P450 enzymes form both the 2-naphthyl- and p-biphenyl-iron complexes (16, 28).

Clear differences in the NOS active sites are evident in the differential effects of CaM, H4B, and L-Arg on the phenyldiazene reaction. L-Arg decreases the extent (from 80 to 17%) but modestly increases the rate (from 0.30 to 0.55 min\(^{-1}\)) of the reaction of phenyldiazene with CaM- and H4B-saturated nNOS (Table I). This result provides quantitative support for the earlier qualitative observation that L-Arg inhibits the reaction of CaM- and H4B-saturated NOS with phenyldiazene (19). Indeed, L-Arg decreases the rate, and particularly the extent, of
the phenyldiazene reaction under most conditions, although the iNOS reaction is less sensitive to l-Arg (Table I). The binding of l-Arg appears to close the active site of the enzyme, leaving only a small population of the protein with an active site that is still able to react with phenyldiazene. The nature of this small population is unclear. Evidence that the binding of l-Arg to NOS causes a low- to high-spin shift suggests that l-Arg binds close to the heme (21, 29–31), as it must if it is oxidized by a heme-bound species. The finding that the reaction of phenyldiazene with the enzyme is largely suppressed by l-Arg is consistent with binding of the natural substrate near the heme.

The binding of CaM to nNOS and eNOS modestly increases the rate and extent of the phenyldiazene reaction, with the exception of the eNOS rates in the absence of either H4B or l-Arg (Table I). The changes in the nNOS N-phenylprotoporphyrin IX isomer patterns obtained after migration of the phenyl group from the iron to the porphyrin nitrogens indicate that CaM causes a small but statistically significant (Table III) decrease in the proportion of the Np isomer in the absence, but not presence, of H4B. A similar small, but in this case statistically insignificant, change is observed in the proportion of the Np isomer when CaM binds to eNOS. These CaM-mediated changes in the nNOS active site topology suggest that CaM binding has at least a small direct effect on the active site conformation. It is not known if this small conformational change has any relation to the role of CaM in facilitating electron transfer from the flavins to the heme iron atom.

The effects of H4B on the rate and extent of the phenyldiazene reaction vary. H4B decreases the rate of the nNOS reaction except when l-Arg is present, and decreases the extent except when CaM is also bound (Table I). H4B decreases both the rate and extent of the phenyldiazene reaction with CaM-bound eNOS and (rate only) iNOS. These rate decreases are consistent with constriction of the active site in a manner that interferes with the phenyldiazene reaction. More direct evidence for topological alteration of the NOS active site is provided by the phenyl shift data (Table II). The binding of H4B causes the largest change in the N-phenylprotoporphyrin IX patterns. With all three NOS isoforms, H4B binding results in a major statistically significant decrease (Table III) in migration of the phenyl group to the nitrogen of pyrrole ring D with compensatory increases in its shift to the other pyrrole ring nitrogens, particularly pyrrole ring B. These changes in isomer ratio indicate that H4B binding increases the steric constraints above pyrrole ring D. The simplest interpretation of this finding is that H4B binds above pyrrole ring D, an interpretation supported by independent evidence that H4B binds near the heme group (32, 33). However, the possibility cannot be excluded that the decrease in space above pyrrole ring D is the result of a conformational alteration associated with the binding of H4B at a distal site. In either case, the results clearly demonstrate that binding of H4B specifically decreases the available space above pyrrole ring D.

In summary, the active site topologies of nNOS, eNOS, and iNOS are similar, although the ceiling height directly above the iron atom differs. The differences in these ceiling heights, in the rates of the phenyldiazene reactions with the three isoforms, and the ability of the isoforms to react with naphthyl-diazene, suggest that the size of the active site decreases in the order nNOS > iNOS > eNOS. In all three active sites, the binding of H4B markedly decreases the space above pyrrole ring D, suggesting that the cofactor binds, or causes a conformational constriction, in that region. The available space in the active site is further curtailed by the binding of l-Arg, which severely impairs the phenyldiazene reaction by physically blocking access to the heme. Despite an overall similarity, the NOS active sites exhibit topological differences that can possibly be exploited for the development of isoform-specific inhibitors.

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REFERENCES

1. Griffith, O. W., and Stuehr, D. J. (1990) Annu. Rev. Physiol. 57, 707–736
2. Marletta, M. A. (1994) Cell 78, 927–930
3. Masters, R. S. S. (1984) Annu. Rev. Nutr. 14, 131–145
4. Knowles, R. G., and Moncada, S. (1994) Biochem. J. 296, 249–258
5. Brent, D. S., and Snyder, S. H. (1994) Annu. Rev. Biochem. 63, 175–195
6. Brent, D. S., Huang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R., and Snyder, S. H. (1991) Nature 351, 714–718
7. Sessa, W. C., Harrison, J. K., Barber, C. M., Zeng, D., Durieus, M. E., D’Angelo, D. B., Lynch, K. R., and Peach, M. J. (1992) J. Biol. Chem. 267, 15274–15276
8. Lowenstein, C. J., Glatt, C. S., Brent, D. S., and Snyder, S. H. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 6711–6715
9. Mayer, B., and Werner, E. R. (1995) Naunyn-Schmiedeberg’s Arch. Pharmacol. 351, 453–463
10. Brent, D. S., and Snyder, S. H. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 682–685
11. Busse, R., and Mulisch, A. (1990) FEBS Lett. 265, 133–136
12. Cho, H. J., Xie, Q., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., and Nathan, C. (1992) J. Exp. Med. 176, 599–604
13. Punting, C. P., and Phillips, C. (1995) Trends Biochem. Sci. 20, 102–103
14. Liu, J., and Sessa, W. C. (1994) J. Biol. Chem. 269, 11691–11694
15. Robinson, L. J., Busconi, L., and Michel, T. (1995) J. Biol. Chem. 270, 995–998
16. Ortiz de Montellano, P. R. (1995) Biochimie 77, 581–593
17. Ringe, D., Plesko, G. A., Kerr, D. E., and Ortiz de Montellano, P. R. (1984) Biochemistry 23, 2–4
18. Raag, R., Swanson, B. A., Poulos, T. L., and Ortiz de Montellano, P. R. (1990) Biochemistry 29, 8119–8126
19. Gerber, N. C., and Ortiz de Montellano, P. R. (1995) J. Biol. Chem. 270, 17791–17796
20. Huang, P. C., and Kosower, E. M. (1968) J. Am. Chem. Soc. 90, 2354–2362
21. Rodriguez-Crespo, I., Gerber, N. C., and Ortiz de Montellano, P. R. (1996) J. Biol. Chem. 271, 11462–11467
22. Passer, J. D., Niu, X. D., Lunn, C. A., Zavordov, P. J., Narula, S. K., and Lundell, D. (1996) FEBS Lett. 379, 135–138
23. Wu, C., Zhang, J., Abu-Soud, H., Ghosh, D. K., and Stuehr, D. J. (1996) Biochem. Biophys. Res. Commun. 222, 439–444
24. Sono, M., Stuehr, D. J., Ikeda-Saito, M., and Dawson, J. H. (1995) J. Biol. Chem. 270, 19945–19948
25. Swanson, B. A., Dutton, D. R., Lunetta, J. M., Yang, C. S., and Ortiz de Montellano, P. R. (1991) J. Biol. Chem. 266, 19258–19264
26. Fruetel, J. A., Mackman, R. L., Peterson, J. A., and Ortiz de Montellano, P. R. (1994) J. Biol. Chem. 269, 28815–28821
27. Tuck, S. F., and Ortiz de Montellano, P. R. (1992) Biochemistry 31, 6911–6916
28. Tuck, S. F., Graham-Lorence, S., Peterson, J. A., and Ortiz de Montellano, P. R. (1993) J. Biol. Chem. 268, 269–275
29. Salerno, J. C., Frey, C., McMillan, K., Williams, R. F., Masters, B. S. S., and Griffith, O. W. (1995) J. Biol. Chem. 270, 27423–27428
30. McMillan, K., and Masters, B. S. S. (1995) Biochemistry 34, 9875–9880
31. Sennequier, N., and Stuehr, D. J. (1996) Biochemistry 35, 5883–5892
32. Klatt, P., Schmid, M., Leopold, E., Schmidt, K., Werner, R. R., and Mayer, B. (1994) J. Biol. Chem. 269, 13861–13866
33. Chabin, R., McCaulley, E., Calaycay, J., Kelly, T., MacNaul, K., Wolfe, G., Hutchinson, N., Madhusudanaraju, S., Schmidt, J., Kozarich, J., and Wong, K. (1996) Biochemistry 35, 9567–9575