Structural Basis for the Specificity of the Nitric-oxide Synthase Inhibitors W1400 and N°-Propyl-L-Arg for the Inducible and Neuronal Isoforms*

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The high level of amino acid conservation and structural similarity in the immediate vicinity of the substrate binding sites of the oxygenase domains of the nitric-oxide synthase (NOS) isoforms (eNOSoxy, iNOSoxy, and nNOSoxy) make the interpretation of the structural basis of inhibitor isoform specificity a challenge and provide few clues for the design of new selective compounds. Crystal structures of iNOSoxy and nNOSoxy complexed with the inhibitors W1400 and N°-propyl-L-arginine provide a rationale for their isoform specificity. It involves differences outside the immediate active site as well as a conformational flexibility in the active site that allows the adoption of distinct conformations in response to interactions with the inhibitors. This flexibility is determined by isoform-specific residues outside the active site.

NOS are multidomain proteins consisting of a heme containing catalytic oxygenase domain, a calmodulin binding linker, and a NADPH reductase domain that catalyze the formation of NO using l-arginine, oxygen, and electrons (1). In mammals, three NOS isoforms have been identified sharing 50–60% sequence identity, that differ in cellular distribution, regulation, and activity (1). Endothelial NOS (eNOS) regulates vascular tone and smooth muscle tension. Neuronal NOS (nNOS) produced NO functions as a diffusible neurotransmitter, whereas NO generated by inducible NOS (iNOS) binds calmodulin irreversibly and is transcriptionally regulated in response to cytokines (2). In contrast, both constitutive isoforms (nNOS and eNOS) are regulated by intracellular Ca\(^{2+}\)/CaM and generate trace amounts of NO as an intercellular messenger (3). It activates soluble guanylate cyclase in target cells such as smooth muscle. Although this NO signal channel has received the most attention (4), NO affects many other sites in the cell. In line with the central biological role of NO, there are a number of pathological processes associated with its overproduction or underproduction. For example, nNOS is implicated in stroke and migraine and iNOS is implicated in septic shock, arthritis, and multiple sclerosis. The possibility of treating these and other conditions by inhibiting NOS has elicited intense efforts to identify or design NOS inhibitors (5, 6). Because the three isoforms of NOS have unique roles in separate tissues, selective inhibition of one isozyme over the others is essential. In particular, it is important not to inhibit eNOS because of its critical role in maintaining vascular tone. Thus, there was great anticipation that the crystal structures of the oxygenase domains of iNOS and eNOS might guide the rational design of isoform specific inhibitors. Indeed, the high level of amino acid conservation and high structural similarity in the immediate vicinity of the substrate binding sites (7–11) readily explained the difficulty in finding selective NOS inhibitors. Nevertheless, selective inhibitors exist such as N-(3-aminomethyl)benzylacetamidine (W1400) (12) and N°-propyl-L-Arg (NPA) (13) that are specific for iNOS and nNOS, respectively, implying that there are significant differences in the active site geometries. Unraveling these differences has been hindered by the fact that although there are a number of structures of the oxygenase domains of iNOS, eNOS, and nNOS available (7–11, 14–19), no structures of complexes of different isoforms and of a specific inhibitor have been determined. Therefore, we have determined the crystal structures (see Table I for details) of the oxygenase domains of murine iNOS and rat nNOS complexed with the specific inhibitors W1400 and N°-propyl-L-Arg, respectively and compared them with the complexes with the substrate L-arginine. Our study implies that the immediate vicinity and periphery of the NOS active site contain isoform-specific regions or residues that may confer differential modes of inhibitor binding, which may be used for isoform specific inhibitor design.

MATERIALS AND METHODS

Protein Cloning, Purification, and Crystallization—The heme oxygenase domains of murine iNOS (residues 65–498) and rat nNOS (residues 49–231) were expressed in Escherichia coli and purified as described (12). The heme oxygenase domain of rat iNOS was expressed in yeast and purified as described (15). The refolding and crystallization of the oxygenase domain of rat iNOS (residues 50–604) was achieved using Mosquito (Berdnik et al., manuscript in preparation). The crystal structures were determined by molecular replacement using Phaser (McRee, 2000), and phenix (Adams et al., 2002) and their high-resolution structures were determined using SHELX and SHELXL (Sheldrick, 2008). The structures were refined using PHENIX crystallographic refinement software (Adams et al., 2002) and the atomic coordinates and structure factors (code 1QW4, 1QW5, 1QWC, 1QW6, and 1QWD) have been deposited in the Protein Data Bank, in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Crystal Structures of NOSoxy Inhibitor Complexes

TABLE I
Data and refinement statistics

| nNOS in complex with N'-Propyl-l-Arg | W1400 | iNOS in complex with N'-Propyl-l-Arg | W1400 |
|------------------------------------|-------|------------------------------------|-------|
| 1QW6<sup>a</sup>                  | 1QWC<sup>a</sup> | 1QW<sup>a</sup>                   | 1QW<sup>a</sup> |
| Crystal parameters                 |       |                                   |       |
| Group                               | C222  | C222                               | P6<sup>b</sup>,2<sup>c</sup> | P6<sup>b</sup>,2<sup>c</sup> |
| Cell parameters: alpha, beta, gamma (°) | 45.0, 108.9, 164.8 | 44.8, 108.5, 164.3 | 214.6, 214.6, 117.4 | 214.6, 214.6, 117.4 |
| Data collection                     |       |                                   |       |
| Beams                              | ID4–1, ESRF | ID4–1, ESRF | ID4–1, ESRF | X13, EMBL/DESY |
| Detector                           | ADSC Q4 | ADSC Q4 | ADSC Q4 | MARCCD |
| Wave length (Å)                     | 0.933 | 0.933 | 0.934 | 0.902 |
| Resolution of data (Å)              | 2.1 | 2.7 | 2.3 | 2.7 |
| All unique reflections              | 160168/23389 | 125740/17658 | 350863/128211 | 223915/42993 |
| Completeness (%)<sup>b</sup>        | 96.8/91.0 | 96.2/93.1 | 95.4/89.4 | 97.5/95.8 |
| (I/|I|) (total/high)                     | 17.1/6.5 | 15.8/5.1 | 9.8/2.6 | 15.4/4.2 |
| Refinement statistics               |       |       |       |       |
| Resolution range (Å)                | 8.0–2.1 | 8.0–2.3 | 8.0–2.4 | 8.0–2.7 |
| Included amino acids                | 297–716 | 297–716 | 2 x (77–495) | 2 x (77–495) |
| No. of waters                       | 280 | 206 | 353 | 263 |
| R<sub>work</sub>,R<sub>free</sub> (%) | 19.5/24.3 | 19.32/25.1 | 21.29/21.1 | 21.29/21.1 |
| R.m.s.d. bonds (Å/Angles (°))        | 0.007/1.3 | 0.009/1.4 | 0.008/1.3 | 0.009/1.3 |

<sup>a</sup> Completeness, R<sub>work</sub>, and (I/|I|) are given for all of the data and for the highest resolution shell.
<sup>b</sup> R<sub>work</sub> = Σ|F<sub>obs</sub> - |F<sub>calc</sub>|/Σ|F<sub>obs</sub> |. 5% of randomly chosen reflections were used for the calculation of R<sub>work</sub>.
<sup>c</sup> These are Protein Data Bank codes.

291–722<sup>b</sup> were cloned, expressed, and purified as described previously (20–22). The resulting 3290 nNOSoxy represents the catalytic core of the nNOS oxygenase domain similar to the d65 murine and 780 human iNOS oxygenase domains (7, 21) and retains essentially all of the properties characteristic of the native nNOS oxygenase domain. All of the materials were of the highest purity available and were obtained from Sigma, Fluka, and Alexis. nNOSoxy and nNOSoxy crystals were grown in the dark at 4 °C and 20 °C, respectively, by vapor diffusion using the hanging drop geometry. For iNOSoxy, 1 µl of protein (15 mg/ml in 40 mM Na<sup>+</sup>-EPPS, pH 7.6, 150 mM NaCl, 5% glycerol, 4 mM H<sub>4</sub>B<sub>8</sub>, 2 mM β-mercaptoethanol, 50 mM β-octyl-glycoside, and 1–10 mM of inhibitor) and reservoir solutions were mixed, the latter contained 100 mM Na<sup>+</sup>-MES buffer, pH 6.5, and 0.7 M Na<sup>+</sup>–malonate (22). Prior to flash-cooling, crystals were rinsed in a cryoprotectant consisting of the reservoir solution supplemented with 50 mM β-octyl-glycoside and 20% glycerol. For nNOSoxy crystallization, 1 µl of protein (15 mg/ml in 40 mM Na<sup>+</sup>-EPPS, pH 7.6, 150 mM NaCl, 5% glycerol, 4 mM H<sub>4</sub>B<sub>8</sub>, 2 mM β-mercaptoethanol, and 1–10 mM of inhibitor) and reservoir solutions were mixed, the latter contained 100 mM Na<sup>+</sup>–HEPES, pH 7.0, 5 mM diithiothreitol, 130 mM Ca<sup>2+</sup>–acetate, 30 mM spermine tetrahexadecyl-, and 20% (w/v) polyethylene glycol 3350. Rod-shaped crystals (20 x 20 x 50 µm<sup>3</sup>) appeared within a day. Prior to flash-cooling, crystals were rinsed in a cryoprotectant solution consisting of the reservoir solution supplemented with 11.5% (w/v) polyethylene glycol 10,000.

Diffraction Data Collection and Refinement—Diffraction data were collected at DESY and the ESRF using MAR and ADSC-Q4 CCD detectors (see Table II for details) and reduced with the XDS program package (23). Structure determination was initiated by a round of rigid body refinement using the protein part of the iNOSoxy. l-Arg coordinates (Protein Data Bank code 1NOD) as an initial model. Refinement was continued with CNS (24) and included simulated annealing and individual B-factor refinement. During cyclic rounds of refinement and manual rebuilding zinc ions, solvent molecules, and ligands were included in the models. The final models display good stereochemistry (Table I). Structures were overlaid with the program SAP (25) and adjusted manually by superposing structures. Cavities, volumes, and masks were calculated with VOIDOO (26) using a probe radius of 0.5 Å.

RESULTS AND DISCUSSION

Structures of the N'-propyl-l-Arg Complexes—NPA belongs to the class of N-alkyl-l-arginine inhibitors (13) and is highly selective for nNOS (Table II). The complexes with the oxygenase domains of murine iNOS and rat nNOS were obtained by co-crystallization. There is good electron density for all of the parts of NPA bound to nNOSoxy (Fig. 1A) and iNOSoxy (Fig. 1B), respectively.

As predicted previously (27), NPA binds similarly to the substrate l-Arg (11) and forms a number of slightly different interactions with the oxygenase domains that are listed in Table II and shown in Fig. 2, A and B. There is one qualitatively different interaction in the nNOSoxy complex (N<sup>2</sup>-Gln-478–NPA-O1, 3.0 Å) that originates ultimately from Asn-498 located in the substrate access channel (Fig. 2A). iNOS has a threonine (Thr-277) at this position that is too short to interact with the guanidinium group of the adjacent Arg-260. Thus, its side chain orients itself to interact with Gln-257 (N<sup>2</sup>-Arg-260–O<sup>1</sup>-Gln-257, 2.7 Å), which therefore cannot interact with O1 of the NPA carboxylate. In nNOSoxy, Asn-498 forms a hydrogen bond with Arg-481 (O<sup>4</sup>l-Asn-498–N<sup>2</sup>l-Arg-481, 3.2 Å), resulting in an orientation of the guanidinium group that is perpendicular to the one of Arg-260 in iNOSoxy (Fig. 2A). Therefore, the interaction with Gln-478 is not possible and Gln-478 is free to interact with the carboxylate of NPA or the substrate l-Arg (11).<sup>4</sup>

The additional H-bond between rat nNOS and NPA compared with murine iNOS may contribute to the selectivity of NPA against murine iNOS, consistent with the structure-activity-relationships (SAR) described for bovine nNOS (assuming that bovine nNOS has an asparagine at the position corresponding to rat nNOS Asn-498 as all of the known nNOS sequences from mammals do) and murine iNOS (Table II). However, the co-structures with NPA reported here suggest that the compound may not be as selective against human iNOS. The human isozymes have an asparagine at the position corresponding to rat nNOS Asn-498 and murine Thr-277 (human eNOS Asn-286, iNOS Asn-283, and nNOS Asn-502). Therefore, all of the human isozymes may potentially form a hydrogen bond between the residue homologous to NOS Gln-478 and the O1 atom of NPA. Indeed, the K<sub>i</sub> values for NPA

<sup>4</sup> R. Fedorov, E. Hartmann, D. K. Ghosh, and I. Schlichting, unpublished data.
determined by competition analysis with imidazole are 0.6, 23.5, and 49.0 \( \mu \text{M} \) for rat nNOS and human and murine iNOS, respectively.\(^3\) Thus, additional factors contribute to isoform specificity such as the size of the ligand binding cavity. More structural and biochemical analysis including ligand binding affinity coupled with enzyme inhibition studies using the human isozymes is needed to assess the selectivity of NPA for nNOS against human iNOS.

The propyl chain of NPA points into the heme cavity and lines up at its "back wall" made up of \(-\) strand S15 (Fig. 2B). The active sites differ significantly in the iNOSoxy, nNOSoxy, and eNOSoxy complexes with the active site of the iNOSoxy complex being smaller by 13 \( \AA^3 \) compared with nNOSoxy and 8.4 \( \AA^3 \) compared with eNOS, which is reflected by the distance of 1.1 \( \AA \) between the C\( \beta \) atoms of the homologous residues Gly-365 (murine iNOS) and Gly-586 (rat nNOS) (Fig. 2C–E). This is caused by the presence of an asparagine residue (Asn-364) interacting with the backbone carbonyl oxygen atom of Ala-237 (3.1 \( \AA \)) located in the adjacent \(-\) strand S4 (10) and thereby acting as a "spacer" (Fig. 2B). nNOSoxy and eNOSoxy have a serine at this position (Ser-585/356, respectively) that forms in eNOSoxy a hydrogen bond with the N\( ^2 \) imidazole nitrogen atom of His-216 located in helix H7 (10). Thus, the structures suggest that discrimination of iNOSoxy against iNOSoxy.

| Inhibitor | Atoms | nNOS | iNOS molecule 1 | iNOS molecule 2 |
|-----------|-------|------|-----------------|-----------------|
| **a) W1400** | | | | |
| N1 | O\(^{\varepsilon2} \)-E592 (3.0 \( \AA \)) | O\(^{\varepsilon1} \)-E371 (3.2 \( \AA \)) | O\(^{\varepsilon1} \)-E371 (3.1 \( \AA \)) |
| N2 | O\(^{\varepsilon1} \)-E592 (2.9 \( \AA \)) | O\(^{\varepsilon1} \)-E371 (2.8 \( \AA \)) | O\(^{\varepsilon1} \)-E371 (2.7 \( \AA \)) |
| N3 | O1A-Heme (2.8 \( \AA \)) | O1D-Heme (3.1 \( \AA \)) | O1A-Heme (2.8 \( \AA \)) |
| C1 | CHC-Heme (4.0 \( \AA \)) | CHC-Heme (4.0 \( \AA \)) | CHC-Heme (3.9 \( \AA \)) |
| Fe-Heme (4.1 \( \AA \)) | Fe-Heme (4.3 \( \AA \)) | Fe-Heme (4.0 \( \AA \)) |
| NB-Heme (3.8 \( \AA \)) | NB-Heme (3.6 \( \AA \)) | NB-Heme (3.5 \( \AA \)) |
| b) N\(^{\varepsilon2} \)-propyl-L-arginine | | | | |
| O1 | N\(^{\varepsilon2} \)-Q478 (3.0 \( \AA \)) | O\(^{\varepsilon1} \)-Y367 (2.8 \( \AA \)) | O\(^{\varepsilon2} \)-D376 (2.9 \( \AA \)) |
| O2 | O\(^{\varepsilon2} \)-D597 (2.8 \( \AA \)) | O\(^{\varepsilon2} \)-D376 (2.9 \( \AA \)) | |
| N1 | O\(^{\varepsilon2} \)-E592 (3.2 \( \AA \)) | O\(^{\varepsilon2} \)-E371 (2.7 \( \AA \)) | O\(^{\varepsilon2} \)-E371 (2.6 \( \AA \)) |
| O1A-Heme (3.2 \( \AA \)) | O1A-Heme (3.2 \( \AA \)) | O1A-Heme (3.2 \( \AA \)) | O1A-Heme (3.0 \( \AA \)) |
| N2 | O\(^{\varepsilon1} \)-E592 (3.0 \( \AA \)) | O\(^{\varepsilon1} \)-E371 (2.9 \( \AA \)) | O\(^{\varepsilon2} \)-E371 (2.8 \( \AA \)) | O\(^{\varepsilon2} \)-E371 (2.8 \( \AA \)) |
| N3 | O\(^{\varepsilon2} \)-E592 (2.6 \( \AA \)) | O\(^{\varepsilon2} \)-E371 (3.1 \( \AA \)) | O\(^{\varepsilon1} \)-E371 (2.9 \( \AA \)) | O\(^{\varepsilon2} \)-W366 (3.6 \( \AA \)) |
| O\(^{\varepsilon1} \)-W587 (3.0 \( \AA \)) | O\(^{\varepsilon1} \)-W366 (3.7 \( \AA \)) | O\(^{\varepsilon1} \)-E371 (2.9 \( \AA \)) | O\(^{\varepsilon2} \)-W366 (3.6 \( \AA \)) |

**Inhibitor binding affinity**

| Inhibitor | IC\(_{50}\) (\( \mu \text{M} \)) | nNOS | iNOS | eNOS |
|-----------|----------------|------|------|------|
| W1400 \(^c\) | | 7.3 | 0.23 \(^c\) | 1000 |
| K\(_i\) | 2 / 2 | 0.14 \(^d\) / K\(_i\)=7nM, k\(_{\text{satt}}\)=0.028s\(^{-1}\) | 75 / 50 |
| NPA \(^e\) | | 0.057 / 0.11 | 180 / 80 | 8.5 / 10 |

\(^a\) O\(^{\varepsilon1} \) stands for main chain carbonyl oxygen atom.
\(^b\) Included only for comparison with nNOS.
\(^c\) IC\(_{50}\) values are from Ref. 1. IC\(_{50}\) values from Collins et al. (34) and Garvey et al. (12).
\(^d\) Inhibition by iNOS is time-dependent (12); the values are therefore underestimated.
\(^e\) K\(_{i}\) values for N\(^{\varepsilon2} \)-propyl-L-arginine are from Zhang et al. (13) and Huang et al. (35).
hibitors with large or inflexible groups at the position corresponding to $N^\omega$ is because of the asparagine residue. In addition, in nNOSoxy, there is a hydrogen bond between the unsubstituted terminal nitrogen (N3) of NPA and the Trp-587 carbonyl oxygen atom (Table II). Because there is no structure of a NPA complex of eNOSoxy, we compared the structures of the L-Arg complexes of the three isoforms to obtain insight into the active site geometries. In complex with L-Arg, the eNOSoxy active site is larger than the one of iNOSoxy (by 8.4 Å$^3$) but smaller than the one of nNOSoxy (by 4.6 Å$^3$) (Fig. 2, C–E). This is a likely reason for relatively similar binding affinity of eNOSoxy and nNOSoxy for inhibitors with a group extending from the $N^\omega$ position. The sequence differences leading to the differences in the cavity volume (Asn-364/Ser-585/Ser-355 in murine iNOSoxy/rat nNOSoxy/bovine eNOSoxy) are also found in human isozymes (Asn-370/Ser-590/Ser-353 in iNOSoxy/nNOSoxy/eNOSoxy). Furthermore, the three-dimensional structures around this part of $N^\omega$-strand S15 of human iNOSoxy (Protein Data Bank code 4NOS) and eNOS (Protein Data Bank code 3NOS) align nicely over murine iNOSoxy and nNOSoxy, respectively, after superposition of the dimers (Fig. 2F and Table III). Hence, these structural differences may be relevant to drug design.

To summarize, the co-structures with NPA presented here provide an important guideline for rational design of nNOS inhibitors specific against iNOS. However, the critically important issue of avoiding eNOS inhibition requires further investigation to fully understand the SAR.

**Structures of the W1400 Complexes**—W1400 belongs to the class of carbamidine-containing NOS inhibitors and is highly selective for iNOS (Table II). The complexes with the oxygenase domains of murine iNOS and rat nNOS were obtained by co-crystallization. There are two crystallographically independent molecules in the asymmetric unit of the iNOSoxy crystals that have slightly different conformations in the W1400 complexes (Figs. 1 and 3). There is strong electron density for all parts of the inhibitor in the iNOSoxy and nNOSoxy structures (Fig. 1B). The benzyl ring of W1400 is positioned atop the heme pyrrole A, and the 3-aminomethyl group of W1400 interacts with both propionic acid groups of the heme. The latter group is held in place by interactions with the hydroxyl groups of Tyr-485/706 (iNOSoxy/nNOSoxy) and the cofactor $H_4B$, respectively. The absence of $H_4B$ in the structure of the eNOS W1400 complex (17) might be the reason why there is only some residual electron density for the 3-aminomethyl group (17). Based on the available data, it
FIG. 2. A, comparison of the structures of the iNOSoxy (green) and nNOSoxy (gray) NPA complexes. There is one qualitatively different interaction in the nNOSoxy complex that originates ultimately from Asn-498 located in the substrate access channel. iNOSoxy has a threonine (T277) at this position. B, the view onto the heme plane shows the difference in the positioning of β-strand S15 (F363 to W366, iNOS numbering) that makes up the back wall of the heme cavity. Asn-364 limits the space available for the propyl chain of NPA in iNOSoxy. C–E, for illustration, the cavity between the backwall and N° of the bound 1-Arg is shown (green mesh) and the NPA coordinates are superimposed on the corresponding 1-Arg complexes. For eNOSoxy (Protein Data Bank code 4NSE), the coordinates of NPA of the nNOSoxy-NPA complex were used. In contrast to iNOSoxy (E), the cavity is rather large in nNOSoxy (C) and eNOSoxy (D), explaining the latter’s good acceptance of inhibitors with bulky groups at the N° position. F, β-strand S15 in murine iNOSoxy (green), human iNOSoxy (gray), bovine eNOSoxy (violet), and human eNOSoxy (red) structures.
is not possible to decide whether the missing interaction between the 3-aminomethyl group and the heme propionate groups in eNOS is caused by the absence of H$_4$B or whether it is characteristic for this complex, explaining the low inhibition constant of W1400 for eNOS (Table II).

The W1400 amidine group mimics the guanidinium group of the substrate l-arginine and forms hydrogen bonds with the carboxylate oxygen atoms of the catalytic Glu-371/592 in iNOSoxy/nNOSoxy, respectively, as listed in Table II. What is

![Figure 3](image)

**Table III**

Average root mean square coordinate differences between the main-chain atoms of the β-strand S15 in NOS isoforms from different organisms

|                | iNOSoxy murine | iNOSoxy human | eNOSoxy bovine | eNOSoxy human |
|----------------|----------------|---------------|----------------|---------------|
| iNOSoxy murine | 0.0            | 0.2           | 0.8            | 0.8           |
| iNOSoxy human  | 0.0            | 0.9           | 0.3            |               |
| eNOSoxy bovine | 0.0            | 0.0           | 0.3            |               |
| eNOSoxy human  | 0.0            | 0.0           | 0.3            |               |
not apparent from this table is the conformational flexibility of the active site of iNOSox which is the slightly different binding modes of W1400 to the two iNOSox molecules present in the asymmetric unit of the unit cell and, more importantly, when comparing iNOSox with nNOSox. In order for all three nitrogens of W1400 to interact strongly with the carboxylate oxygens of the heme and Glu-371, W1400 moves toward the propionates and Glu-371 follows (Cα–distance 0.6 Å compared with the L-Arg complex in molecule 1/2, respectively), causing a shift of helix H11 (Fig. 3). In nNOSox this shift is not possible due to Tyr598 (instead of Phe977/Leu969 in iNOSox/eNOSox) in H11 that forms a hydrogen bond (3.2 Å) with the carbonyl of Lys5–60, thereby fixing the helix position. The movement of W1400 in iNOSox results in different positions of the C1 methyl group relative toward the propionates and Glu-371 follows (Cboxylate oxygens of the heme and Glu-371, W1400 moves when comparing iNOSoxy with nNOSoxy. In order for all inhibitors targeted against the active site may be achieved only with compounds that are large enough to interact with isoform-specific residues in the substrate access channel or dimer interface and that fill the active site, thereby locking its conformation.

**CONCLUSIONS**

The crystal structures presented suggest a rationale for isoform specificity that is rooted in isoform-specific residues outside the immediate active site but that nevertheless contribute to its shape. In particular, the active site is not rigid but displays some ligand induced conformational flexibility. Moreover, the exact positioning of the cofactor H4B, located at the isoform-specific interface between oxygenase monomers, seems to play an important role since it is involved in positioning the heme propionates, which may interact with functional groups of inhibitors. These findings on top of the high level of amino acid conservation and overall structural similarity (7, 8, 10) identify a further difficulty in the rational design and analysis of the structure-activity relationships of isoform-specific NOS inhibitors. It seems that prediction and thus rational design of inhibitors targeted against the active site may be achieved only with compounds that are large enough to interact with isoform-specific residues in the substrate access channel or dimer interface and that fill the active site, thereby locking its conformation.

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