Crystal Structures of Zinc-free and -bound Heme Domain of Human Inducible Nitric-oxide Synthase

IMPLICATIONS FOR DIMER STABILITY AND COMPARISON WITH ENDOTHELIAL NITRIC-OXIDE SYNTHASE*

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The crystal structures of the heme domain of human inducible nitric-oxide synthase (NOS-2) in zinc-free and -bound states have been solved. In the zinc-free structure, two symmetry-related cysteine residues form a disulfide bond. In the zinc-bound state, these same two cysteine residues form part of a zinc-tetrathiolate (ZnS₄) center indistinguishable from that observed in the endothelial isoform (NOS-3). As in NOS-3, ZnS₄ plays a key role in stabilizing intersubunit contacts and in maintaining the integrity of the cofactor (tetrahydrobiopterin) binding site of NOS-2. A comparison of NOS-2 and NOS-3 structures illustrates the conservation of quaternary structure, tertiary topology, and substrate and cofactor binding sites, in addition to providing insights on isoform-specific inhibitor design. The structural comparison also reveals that pterin binding does not preferentially stabilize the dimer interface of NOS-2 over NOS-3.

Nitric-oxide synthases (NOS) are a family of enzymes that produce nitric oxide (NO) and L-citrulline from L-arginine in the presence of NADPH and O₂. NOS maintains a bidomain structure (2) with the catalytic center residing in the heme domain utilizing electrons derived from the cytochrome P450 reductase-like biflavin domain. The heme domain also contains the binding site for enzyme cofactor, tetrahydrobiopterin (H₂B). The NOS family currently consists of three mammalian isoforms (3, 4). The endothelial (eNOS or NOS-3) and neuronal (nNOS or NOS-1) isoforms are constitutive and are activated under specific physiological conditions in stroke (5) and shock (6), respectively, isoform-specific inhibition of NOS has wide therapeutic potential. As a result, there is considerable interest within the pharmaceutical community to develop isoform-specific NOS inhibitors (7). Crystal structures of the catalytic heme domain for the various NOS isoforms are essential if rational drug design is to be applied to NO up/down-regulation.

Crane et al. (8) solved the crystal structure of the murine NOS-2 heme domain, which established the overall fold of NOS as well as the location and structure of the pterin and L-Arg binding sites. The structure of the NOS-3 heme domain also has been solved (9), which revealed a novel zinc tetrathiolate (ZnS₄) at the bottom of the dimer interface. In the structure of the heme domain of murine NOS-2 (8), a disulfide was identified in the zinc binding site, and no zinc was seen in the structure. To understand the structural consequences of the metal center in other NOS isoforms, we have solved the crystal structure of human NOS-2 in both zinc-free and -bound forms. The availability of a zinc-bound NOS-2 affords direct comparison with NOS-3 and provides a molecular basis for the development of isoform-specific inhibitors.

MATERIALS AND METHODS

Expression and Purification of NOS-2—A cDNA fragment containing the first 703 amino acids of human inducible NOS-2 was obtained by polymerase chain reaction of mRNA made from cytokine-stimulated human colorectal adenocarcinoma DLD-1 cells (11). Primers used were 5'-GATGGCCTGTCCCTGGAAT-3' and 5'-TCCCCAGTTCACATTGGAGGT-3'. This fragment was sub-cloned via intermediate vectors into a pUC18 derivative to place the NOS-2 heme domain under control of the TAC promoter. A 6-histidine tag and stop codon were inserted after residue 504 by mutagenesis to create the expression plasmid pNOS115, which was transformed into Escherichia coli strain W3110. E. coli (strain pNOS115-W3110) cells were grown in "Terror Broth" in a 10 liter fermenter. About 1 h before induction, 0.3 g/liter 6-aminolevulinic acid was added, and the fermenter temperature was reduced to 28 °C. At an A₆₀₀nm of 2–3, the expression of NOS-2 was induced with 0.2 g/liter IPTG. Eighteen to 20 h after the induction, cells were harvested and stored at −20 °C. Seventy-five grams of E. coli cell pellet was thawed, and 500 ml of 40 mM BTP, pH 7.8, 10% glycerol, containing 0.3 g of lysozyme, 80 μl of benzozene, 1 mg of leupeptin, 5 mg of aprotinin, 24 mg of Pefabloc SC, 1 mg of pepstatin, and 0.1 ml of Me₂SO were added. The sample was then frozen and thawed three times and centrifuged at 25,000 rpm (41,000 × g) for 65 min at 4 °C. The supernatant, containing NOS-2, was removed and quick frozen in dry ice-ethanol and then stored at −80 °C.

The soluble cell extract was incubated with 50 ml of Ni-nitrilotriacetic acid resin (Qiagen) for 1 h at 4 °C. The NOS-2-bound Ni-nitrilotriacetic acid resin was placed in a 2.5-cm diameter column and washed with 20 ml imidazole in the column buffer (40 mM BTP, 10% glycerol,
0.3 M NaCl, pH 7.8) and then with 40 mM imidazole in the same buffer until \( A_{280\ nm} \) base line was reached. Elution was carried out with 100 ml of column buffer supplemented with 200 mM imidazole. The purified NOS-2 was concentrated at 4 °C using Centriprep-30 concentrators (Amicon) to \( 10\ mg/ml \) and followed by exhaustive dialysis against 40 mM BTP, 10% glycerol, 1 mM dithiothreitol, 8 mM H4B at pH 7.8.

To generate homogeneous protein for crystallization, limited trypsin digest (500:1, NOS:trypsin, w/w) was performed at 4 °C in 50 mM BTP buffer, pH 7.8, containing 10% (v/v) glycerol and 5 mM GSH. After overnight incubation (15–18 h), the reaction was stopped by adding 0.5 mM PMSF. Two major digested fragments were found on SDS-PAGE (PhastGel, Amersham Pharmacia Biotech). The larger fragment (\( M_r \), 48,000; ~75% of the species) gave a UV-visible absorption spectrum characteristic of a normal high spin heme, whereas the smaller fragment (\( M_r \), 40,000; ~25% of the species) was low spin even in the presence of H4B and L-Arg. The first residue of the larger fragment was identified as Leu74 by N-terminal amino acid sequence analysis. The digested sample was diluted 10-fold with buffer A (50 mM BTP, pH 7.8, 10% glycerol, 5 mM GSH, 5 mM H4B, plus 1 mM L-Arg or 0.5 mM SEITU before loading onto a UNO Q6 (Bio-Rad) anion exchange column controlled by an FPLC system (Amersham Pharmacia Biotech). The larger fragment was eluted with 10 column volumes of a linear NaCl gradient (0–150 mM) made in buffer A, whereas the smaller fragment remained on the column. Fractions with an absorbance ratio \( A_{280\ nm}/A_{395\ nm} \), 1.4 were pooled, concentrated with a Centriprep 30 (Amicon), and stored at -70 °C until usage.

**Crystallization**—Rod-shaped crystals were obtained using sitting drops in a sandwich box setup at 7 °C with a protein concentration of 10 mg/ml. The reservoir solution contained 30 ml of 35% saturated ammonium sulfate, 30% (v/v) glycerol, 50 mM sodium citrate, pH 5.0. The

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**TABLE I**

|                  | ALS \(^a\) | SSRL \(^a\) | Raxis IV |
|------------------|-----------|-----------|---------|
| X-ray wavelength (Å) | 1.000     | 1.080     | 1.542   |
| Cell dimensions (Å)  | \( a = b = 187.35 \) | \( a = b = 188.37 \) | \( a = b = 188.07 \) |
| Data resolution (Å)  | 2.55      | 3.0       | 3.5     |
| Total observations | 477,621   | 355,712   | 360,766 |
| Unique reflections  | 125,717   | 82,161    | 52,422  |
| \( R_{ave} \)         | 0.052     | 0.092     | 0.160   |
| Outer shell          | 0.488     | 0.699     | 0.446   |
| \( <\lambda > \)     | 12.2      | 8.8       | 6.0     |
| Outer shell          | 2.3       | 2.0       | 4.2     |
| Completeness         |           |           |         |
| All                 | 0.959     | 0.989     | 1.000   |
| Outer shell          | 0.712     | 0.984     | 0.999   |
| Reflection used in   | 111,401   | 70,704    | -       |
| refinement (\( F > 2\alpha(F) \)) |           |           |         |
| \( R_{factor} \)     | 0.209     | 0.214     | -       |
| \( R_{free} \)       | 0.243     | 0.238     | -       |
| No. of non-hydrogen atoms | 14,039    | 13,989    | -       |
| No. of waters        | 295       | 0         | -       |
| r.m.s. deviation     |           |           |         |
| Bond length          | 0.007     | 0.007     | -       |
| Bond angle           | 1.317     | 1.183     | -       |

\(^{a}\) ALS, Advanced Light Source, Lawrence Berkeley National Lab; SSRL, Stanford Synchrotron Radiation Lab.

\(^{b}\) \( R_{sym} = \sum |I| - <I>/\sum I \), where \( I \) is the observed intensity and \( <I> \) the averaged intensity of multiple symmetry-related observations of the reflection.

\(^{c}\) \( R_{factor} = \sum |F_o| - |F_c|/\sum |F_o| \), \( F_o \) and \( F_c \) are the observed and calculated structure factors, respectively.

\(^{d}\) \( R_{free} \) was calculated with 5% of reflections set aside randomly.

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**FIG. 1.** The Ca trace of the heme domain of human NOS-2. Helices are represented with cylinders and \( \beta \)-strands with ribbons. The heme, H4B, L-Arg, and zinc are highlighted by the ball and stick models.
solution used for setting up protein drops is identical to the reservoir solution except that 2.0 mM GSH and 10 mM L-Arg (or 1.0 mM SEITU) were also present. The zinc-containing crystals were grown with 5.0 mM ZnSO$_4$, and 2.5 mM TCEP was added to replace GSH in the zinc-free condition. The same drop setup solution also served as the crystal storage and cryoprotectant reagent.

Data Collection—Crystals were flash cooled under a cold nitrogen stream (100 K) for data collection. A 3.5-Å data set was initially collected in-house on an Raxis IV imaging plate equipped with a copper Kα rotating anode x-ray source and double focusing mirrors. Higher resolution data were later collected using a Mar Research imaging plate on beamline 7–1 at SSRL (Stanford Synchrotron Radiation Lab.) or Quantum4 CCD detector on beamline 5.0.2 at ALS (Advanced Light Source, Lawrence Berkeley National Lab.). Raw data were integrated and scaled with DENZO and SCALEPACK (10). The complete outlier rejection was achieved by running ENHKL (Louis Sanchez, California Inst. Tech.). Crystals were indexed in a primitive tetragonal space group P4$_3$2$_1$2 (Table I).

Structure Determination—An initial phase set was obtained using molecular replacement with the program AMoRe (11) in the 20.0–5.0 Å resolution range. The search model consisted of the dimeric bovine NOS-3 structure (9) reduced to polyalanine. Based upon the estimated
Matthews coefficient (12), eight subunits were expected in each asymmetric unit. The dimeric search model could, however, provide only two solutions, which already gave a high correlation coefficient of 0.60 and low R factor at 0.44. The crystals were later confirmed to have a very high (78%) solvent content. The translational search also aided in determination of the space group as P43212 instead of P41212. Because of the nonglobular shape of NOS monomer and the extensive dimer interface (~2700 Å² per monomer), the monomeric search model failed to locate the other subunit within the dimer. The correctness of the molecular replacement solutions was checked by locating the heme iron and/or zinc positions with the Bijvoet difference Fourier synthesis using the molecular replacement phases.

Phases obtained from the NOS-3 polyalanine model were further improved by 4-fold noncrystallographic symmetry averaging and solvent flattening using the program DM (13). The quality of the resultant electron density map allowed the assignment of amino acid side chains for NOS-2. The iterative rounds of model building and refinement were accomplished with TOM (14) and X-PLOR (15). The noncrystallographic symmetry restraint was imposed between two independent dimers during the full course of refinement for the 3.0-Å data but was released at the final stage of refinement for the 2.55-Å data. A bulk solvent correction was implemented in the model so that the reflections down to 30.0 Å are included in the refinement. The Ramachandran plots for the final structures were calculated with PROCHECK (16) with no residue found in the disallowed region for the zinc-bound structure and only Ser-118 with disallowed backbone torsion angles because of the peptide flip of Gly-117 in the zinc-free structure. Solvent molecules, including sulfate anion, glycerol, and water, are found in the 2.55-Å structure, whereas no water was modeled in the 3.0-Å data. The refinement statistics are detailed in Table I.

RESULTS AND DISCUSSION

Overall Structure—The tertiary topology and quaternary structure of the dimeric zinc-bound human NOS-2 heme domain (Fig. 1) is indistinguishable from bovine NOS-3 (9) and is similar to murine NOS-2 (8). The root mean square (r.m.s.) deviation of main chain atoms between the human NOS-2 and bovine NOS-3 heme domains is 1.06 Å, while the primary sequence identity between the two heme domains is 59.5% for the 420 residues compared. Residues 74–82 are disordered and not visible in the electron density map. Residues 83–502 form a continuous stretch of electron density in the zinc-bound structure, including the loop region (Pro122–Pro135) preceding the α helix that is disordered in the NOS-3 structure.

Zinc Tetrathiolate Center—As in the murine NOS-2 structure (8), the human NOS-2 structure also reveals a disulfide bond formed between symmetry-related Cys115 residues at the dimer interface (Fig. 2). In sharp contrast, NOS-3 has a zinc ion tetrahedrally coordinated to pairs of symmetry-related Cys96 and Cys101 residues (9). The zinc binding motif, Cys-(X)4-Cys, conserved in all known NOS isoforms to date, suggests that the physiologically relevant NOS structure has the zinc ion bound. After the submission of the present work for publication, we became aware of the work by Fischmann and colleagues (17) which has independently identified the structural zinc in both human NOS-2 and NOS-3, respectively. We have also con-
firmed that zinc ion is bound in the structure of neuronal NOS (NOS-1). Thus, the use of E. coli recombinant expression systems and purification protocols utilized in the production of both murine (8) and human NOS-2 (this work) may have resulted in the loss of zinc and subsequent formation of a disulfide bond.

If the zinc site is the “natural” form, then it should be possible to reconstitute the zinc site in NOS-2. This was accomplished by simply adding Zn\(^{2+}\) in the presence of a strong reducing agent directly to the crystallization mixture. The metal incorporation strengthened the crystal lattice as indicated by a decrease in cell dimensions and better diffraction (Table I). The topology of the metal binding motif (Fig. 2) is nearly identical to that found in bovine NOS-3. The zinc is located along the dyad axis of symmetry and is tetrahedrally coordinated to pairs of symmetry-related Cys residues. The zinc is equidistant from both H\(_B\) molecules (\(-12\) Å) and both heme iron atoms (21.8 Å). The strategically located metal center underscores its important structural roles (9) that include maintaining the integrity of the pterin-binding pocket and possibly providing a docking surface for the reductase domain.

The electrostatic potential in the vicinity of the ZnS\(_4\) of NOS-2 is positive and similar to that observed in NOS-3 (9). The reductase domain of NOS shares high sequence similarity with the microsomal cytochrome P450 reductase (18). The FMN probe, Fig. 4). Compared with NOS-3, bulky amino acid substitutions in NOS-2 (Leu\(^{108}\), Ile\(^{462}\), Phe\(^{488}\)) and the alteration in the backbone corresponding to the Ala\(^{104}\) to Leu\(^{108}\) region, decreases the cavity volume. This internal cavity no longer exists in zinc-free NOS-2 because of the loss of inter-subunit hydrogen bonds and disordering which lead to opening of the cavity to bulk solvent. The function of this cavity at the bottom of the dimer interface, and particularly in the immediate vicinity of the zinc center, is unknown. The cavity is filled with solvent molecules and, considering its large size, may be a binding site for an as yet to be identified ligand. Alternatively, the cavity may be involved in mediating inter-subunit motions (26) that could play a role in regulating NOS function.

Another role for the ZnS\(_4\) is in maintaining dimer stability. In going from the zinc-free to the -bound state, there is a net gain of eight hydrogen bonds (Table II) which would contribute favorably to the free energy of dimer stabilization. Even though the zinc-free NOS-2 heme domain dimer has an intermolecular disulfide bond, the key hydrogen bonds required to stabilize the structural elements in this region are absent. This argues for

![Image](255x500 to 554x729)

**Fig. 4. Cavities and pockets in NOS-2.** The large internal cavity in the vicinity of the ZnS\(_4\) is shown with the atoms lining the concave surface as spheres. The cavity volume corresponds to ~750 Å\(^3\). In the zinc-free structure, the internal cavity opens to the bulk solvent and becomes part of the neighboring pocket. The cavity adjacent to His\(^{336}\), His\(^{337}\) pair at the dimer interface is also defined (purple). The orange and green sphere covered regions define the ligand binding pockets (~4000 Å\(^3\) per subunit, corresponding to heme, 1-Arg, and pterin binding sites) in the two subunits, respectively.

![Image](255x500 to 554x729)

**Table II**

| Donor            | Acceptor       |
|------------------|----------------|
| A-Leu\(^{108}\)N | B-Ser\(^{112}\)O |
| B-Leu\(^{108}\)N | A-Ser\(^{112}\)O |
| A-Cys\(^{115}\)N | B-Asn\(^{482}\)O |
| B-Cys\(^{115}\)N | A-Asn\(^{482}\)O |
| A-Asn\(^{482}\)N | B-Cys\(^{115}\)O |
| B-Asn\(^{482}\)N | A-Cys\(^{115}\)O |
| A-Ser\(^{112}\)OG| B-Met\(^{480}\)O |
| B-Ser\(^{112}\)OG| A-Met\(^{480}\)O |

\(^{a}\) Donor-acceptor distance of ≤3.1 Å.

The latter should not contain the ZnS\(_4\) center and accordingly shows a weaker binding to pterin (\(\Delta G\) 1.1 kcal mol\(^{-1}\) in the presence of 0.1 mM substrate). Therefore, it is not too surprising that replacement of the zinc Cys ligands with other residues leads to altered pterin binding (20–23), most likely because of changes in pterin-protein interactions similar to that found by comparing the NOS-2 zinc-free and -bound structures.

Interestingly, in both NOS-2 and NOS-3 structures, the ZnS\(_4\) is surrounded by a large inter-subunit cavity (~750 Å\(^3\) molecular surface volume; calculated (Refs. 24, 25) using a 1.4-Å probe, Fig. 4). Compared with NOS-3, bulky amino acid substitutions in NOS-2 (Leu\(^{108}\), Ile\(^{462}\), Phe\(^{488}\)) and the alteration in the backbone corresponding to the Ala\(^{104}\) to Leu\(^{108}\) region, decreases the cavity volume. This internal cavity no longer exists in zinc-free NOS-2 because of the loss of inter-subunit hydrogen bonds and disordering which lead to opening of the cavity to bulk solvent. The function of this cavity at the bottom of the dimer interface, and particularly in the immediate vicinity of the zinc center, is unknown. The cavity is filled with solvent molecules and, considering its large size, may be a binding site for an as yet to be identified ligand. Alternatively, the cavity may be involved in mediating inter-subunit motions (26) that could play a role in regulating NOS function.

\(^{a}\) C. S. Raman, H. Li, P. Martásek, B. S. S. Masters, and T. L. Poulos, unpublished observations.
the inability of a disulfide to substitute for the zinc at the NOS dimer interface. In addition, strongly reducing conditions of the cytosol do not favor disulfide formation (27), further supporting the view that NOS utilizes zinc binding to amplify the conformational stability of the dimer interface.

Inhibition of NOS-1 activity by zinc (Ki; 30 μM) has been reported (28, 29) and has been thought to result from altered reduction of the heme iron. Crystallization of NOS-2 heme domain has been carried out in the presence of 2.5 mM ZnSO4, and zinc is incorporated exclusively at the ZnS4 site. Bijvoet difference Fourier calculations did not reveal the presence of additional zinc site(s). This suggests that zinc-mediated inhibition of NOS may be achieved by binding to the reductase domain or directly to calmodulin.

Dimer Interface—There is controversy in the literature about the differences in the dimer stability between the three NOS isoforms (30, 31). Thus, it is important to carry out a detailed comparison between dimer interfaces. Calculation of the solvent-accessible surface of both dimers using a 1.4-Å (24) probe (Table III) reveals that the dimer interface in both proteins is extended and corresponds to roughly 14% of the monomer-accessible surface area (ASA). Whereas nearly 60% of the atoms comprising the interface are hydrophobic in both NOS-2 and NOS-3 (9), it should be stressed that the higher resolution structure of NOS-3 (9) reveals over twenty water-bridged interactions at the dimer interface. Thus, the make-up of the NOS dimer interface resembles two-thirds of all known dimer interfaces in which a combination of hydrophobic, polar,
FIG. 6. Structural basis for zinc-mediated conformational stability. Murine NOS-2 missing both zinc and pterin is susceptible to cleavage by trypsin (20) at the solvent-exposed Lys117 (equivalent to Lys123 (yellow)) in human NOS-2. H4B protects against this cleavage (20). In zinc-free human NOS-2, Ile112-Ser114 are disordered, resulting in the loss of eight hydrogen bonds (Table II) involving residues 108, 112, 115, and 118 which are necessary to maintain the structural integrity of this region. In addition, the backbone C=O of Leu112 makes a close (−3.1 Å) van der Waals contact with a small 3_{10} helix in which Lys123 resides. Thus, zinc is the key determinant of conformational stability in the region corresponding to 107–124, and pterin binding only plays a secondary role via a hydrogen bond to Ser118 and van der Waals contacts with Met120. Some of the side chains are not shown for clarity.

and solvent-bridged interactions dominate (32). Residues that comprise the dimer interface of NOS-2 arise from six different segments composed of β-sheet region 85–91 and 101–121 and of α-helical segments 380–392, 405–427, 435–441, and 461–483. A similar pattern is observed in the NOS-3 dimer interface. The complementarity of the NOS dimer interface, calculated by estimating the volume of the gaps between the interacting subunits (gap volume index = 2.5; Ref. 33), is closer to the mean value seen in heterodimeric protein interfaces. In both NOS-2 and NOS-3, the complementarity of the dimer interface is intermediate between those seen in protease-protease inhibitor interactions and antibody-protein complexes. This suggests a strong affinity between the two subunits. Calculating a best fit plane (34) through the atoms that make up the interface of NOS-2 and NOS-3 also suggests that the interface is relatively flat in both isoforms but at the borderline (r.m.s. = 5.9 Å) of being considered as nonplanar (33). Also, an inventory of cavities at the dimer interface of both NOS-2 and NOS-3 reveals that, in NOS-2, most cavities have a reduced volume because of cavity-minimizing amino acid substitutions. One exception to this involves a cavity in the vicinity of the His437-His437 pair (Fig. 4). Overall, the similarities between the NOS-2 and NOS-3 dimer interface are striking, including the intersubunit salt bridge(s), and any differences in their stability must reside in the small number of amino acid substitutions between the two isoforms at the interface region.

Tetrahydrobiopterin Recognition—One proposed role for H4B is in stabilization of the NOS-2 dimer, whereas the NOS-1, and particularly NOS-3, dimers are stable in the absence of pterin (35, 36). H4B must be tightly bound in the human NOS-2 structure because pterin was only supplemented in the protein purification buffer and not in the crystallization mixture. The residues that interact with H4B in the human NOS-2 structure fall within the region correctly identified by Cho et al. (37) via site-directed mutagenesis. As expected, the interactions at the pterin site of human NOS-2 revealed in this work are the same as those described for murine macrophage NOS-2 (8). A comparison between the H4B site in NOS-3 and NOS-2 reveals only one significant difference. Met120 substitutes for a valine in NOS-3 (Val106), with its bulkier side chain making a tighter 4-Å contact with the pterin N-5 atom (Fig. 5A). All other critical interactions between the pterin and protein observed in NOS-3 are conserved. These include aromatic stacking between the pterin ring and Trp163, a hydrogen bond between pterin N-3 and the heme propionate group, and a hydrogen bond between the dihydroxypropyl side chain of H4B and the backbone carbonyl of Ser118. Because NOS-1 has a Met residue at the position equivalent to Met120 in NOS-2, this difference alone is not expected to play a key role in differentiating the NOS-2 and NOS-3 pterin sites. The striking similarity between the NOS-2 and NOS-3 pterin binding sites suggests a similar function for H4B in both isoforms. Whereas the structure stabilization mediated by H4B can be equally accomplished by H4B and other pterin analogs (38), only the former can sustain catalytic activity, suggesting a redox role for the cofactor. Based on the ability of L-Arg to bind at the pterin site of NOS-3, we proposed (9) a reaction mechanism involving the trihydrobiopterin radical cation. A similar scenario is expected for NOS-2 as well.

In light of these structural comparisons as well as the zinc center in NOS, it is worthwhile to re-examine the conclusions drawn from biochemical data relevant to the role of H4B in dimer stability. For example, Ghosh et al. (20) have shown that Lys137 in the murine NOS-2 heme domain is susceptible to trypsinolysis, whereas H4B protects against this cleavage. Lys137 (equivalent to Lys123 in murine NOS-2) in human NOS-2 is fully exposed and does not interact with pterin (Fig. 6). It is conceivable that the loss of the ZnS4 and not the lack of pterin significantly increased the flexibility of this region, leading to proteolytic attack. This is underscored by the missing hydrogen bonds at the human NOS-2 dimer interface in the absence of zinc (Table II). Binding of pterin (as would zinc) most likely decreases the conformational mobility of this region (Fig. 6), resulting in a protection from trypsinolysis. An Arg residue takes the place of Lys137 in NOS-3, and this region of the protein is disordered in both pterin-free and -bound states. NOS-3, however, does not undergo proteolysis at this site in the pterin-free state. Moreover, there is no difference at the pterin site or elsewhere in the structure when the pterin-free and -bound NOS-3 structures were compared (9). Apparently all NOS isoforms share a similar pterin binding pocket with comparable H4B binding affinity (39). Thus, whereas pterin binding enhances the dimer stability in both NOS-2 and NOS-3 by mediating inter-subunit contacts, there is no structural evi-
Zinc-free and -bound Human NOS-2

Substrate Binding Site and Design of Isoform Specificity—The substrate binding site in human NOS-2 bears a striking similarity to that in the bovine NOS-3 structure and agrees with the results from resonance Raman spectroscopic studies (40). Most of the amino acid residues that make direct contact with L-Arg are conserved, except Asp382 which is Asn368 in NOS-3. The extensive hydrogen-bonding network observed in NOS-2 between L-Arg and amino acid side-chains is also retained in NOS-2 (Fig. 5B). In addition, the α-amino group of the substrate hydrogen bonds with the same propionate that hydrogen bond-donating group at the Cα position of L-Arg interacts with the heme propionate, that and the glycol moiety extends into the polar binding pocket where the carbonylate of L-Arg binds. The extensive hydrogen bonding network thus formed between the inhibitor and NOS-2 is schematically illustrated in Fig. 7. The critical interactions are those involving the terminal hydroxyl group of inhibitor, which accepts a hydrogen bond from Tyr373 side chain while donating hydrogen to the carboxylate of Asp382 in NOS-2. However, the homologue to Asp382 is Asn368 in NOS-3, and Asn368 cannot accept a hydrogen bond from the inhibitor as can Asp382 because the amide N of the Asn368 side chain is oriented toward the inhibitor hydroxyl (Fig. 7). The approximately 700-fold greater affinity for NOS-2 corresponds to 3.9 kcal mol⁻¹ difference in free energy, which is not unreasonable for a single hydrogen bond difference, and may account for the isoform-specific nature of the inhibition. Finally, some of the amino acid side chains that do not make direct contact with the substrate but line the periphery of the active site are different between NOS-2 and NOS-3 and can be utilized in the design of isoform-specific inhibitors for controlling NO generation in pathological states. Fischmann et al. (17) have independently addressed the issue of utilizing this key difference in designing isoform-specific inhibitors.

In summary, we have described the crystal structures of human NOS-2 in zinc-free and -bound states that reveal the importance of a ZnS₄ center in maintaining the stability of the dimer interface and molecular recognition of the pterin cofactor. In addition, direct comparison of the NOS-2 and NOS-3 structures reveals a remarkable conservation of substrate and pterin binding interactions. However, subtle differences between NOS isoforms exist that can be used to direct rational design of second-generation isoform-specific NOS inhibitors with broad therapeutic potential.

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