Crystal Structure of the FAD/NADPH-binding Domain of Rat Neuronal Nitric-oxide Synthase

COMPARISONS WITH NADPH-CYTOCHROME P450 OXIDOREDUCTASE*

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Nitric-oxide synthase (NOS) is composed of a C-terminal, flavin-containing reductase domain and an N-terminal, heme-containing oxidase domain. The reductase domain, similar to NADPH-cytochrome P450 reductase, can be further divided into two different flavin-containing domains: (a) the N terminus, FMN-containing portion, and (b) the C terminus FAD- and NADPH-binding portion. The crystal structure of the FAD/NADPH-containing domain of rat neuronal nitric-oxide synthase, complexed with NADP\(^+\), has been determined at 1.9 Å resolution. The protein is fully capable of reducing ferriyanide, using NADPH as the electron donor. The overall polypeptide fold of the domain is very similar to that of the corresponding module of NADPH-cytochrome P450 oxidoreductase (CYPOR) and consists of three structural subdomains (from N to C termini): (a) the connecting domain, (b) the FAD-binding domain, and (c) the NADPH-binding domain. A comparison of the structure of the neuronal NOS FAD/NADPH domain and CYPOR reveals the strict conservation of the flavin-binding site, including the tightly bound water molecules, the mode of NADP\(^+\) binding, and the aromatic residue that lies at the re-face of the flavin ring, strongly suggesting that the hydride transfer mechanisms in the two enzymes are very similar. In contrast, the putative FMN domain-binding surface of the NOS protein is less positively charged than that of its CYPOR counterpart, indicating a different nature of interactions between the two flavin domains and a different mode of regulation in electron transfer between the two flavins involving the autoinhibitory element and the C-terminal 33 residues, both of which are absent in CYPOR.

Nitric-oxide synthase (NOS) catalyzes the NADPH-dependent conversion of L-arginine to nitric oxide and L-citrulline (for reviews, see Refs. 1 and 2). Three isoforms of NOS have been identified in mammals; neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed, and their activities are Ca\(^{2+}\)/calmodulin-dependent, whereas inducible NOS (iNOS) is independent of intracellular Ca\(^{2+}\) concentration. Although the three isoforms share 50–60% sequence identity, they differ in size, intracellular location, and regulation. nNOS has a molecular mass of 165 kDa, is located in neurons in the brain and at neuromuscular junctions, and is involved in neurotransmission. eNOS has a molecular mass of 133 kDa, is present in vascular endothelial cells, and is involved in vascular homeostasis. iNOS has a molecular mass of 130 kDa, is located in macrophages, and is expressed only in response to endotoxin or inflammatory cytokines. A distinguishing feature of NOS is that the N-terminal half of the enzyme is the heme-binding domain (or oxygenase domain), similar to the cytochrome P450 enzyme family, and also contains pterin- and arginine-binding sites. The C-terminal half is the flavin-binding or reductase domain containing FAD-, FMN-, and NADPH-binding sites, as found in NADPH-cytochrome P450 oxidoreductase (CYPOR) (3). At the junction between these two domains is a calmodulin (CaM)-binding region. Due to the reversible binding of CaM, the constitutive isoforms (nNOS and eNOS) display Ca\(^{2+}\) dependence in their enzymatic activities (4, 5), whereas iNOS has tightly bound CaM, almost independent of the Ca\(^{2+}\) concentration (6). The reductase domain can be further divided into two subdomains, each of which contains one of the two flavins. The FMN-containing subdomain is situated at the N terminus, and its amino acid sequence is similar to that of flavodoxin, whereas the C-terminal portion contains FAD- and NADPH-binding sites and is homologous to ferredoxin NADP\(^+\) reductase (FNR). From the sequence alignments, Salerno et al. (7) showed additional sequences in the middle of the FMN subdomain of the constitutive NOS isoforms that were not present in iNOS, CYPOR, or flavodoxin. It was proposed that this additional sequence of about 40 residues acts as an autoinhibitory element in the constitutive NOS isoforms that is released upon binding of Ca\(^{2+}\)/CaM, as seen in other Ca\(^{2+}\)/CaM-activated proteins, such as myosin light chain kinase. Deletion of this insert in nNOS (8) and eNOS (9, 10) results in enzymes that require significantly lower Ca\(^{2+}\) concentrations for optimal activity. In addition, removal of this insert greatly enhances the maximal activity of eNOS, which is the least active of the three isoforms. Another significant difference between NOS and CYPOR is that the NOS isoforms contain an additional 21–42-amino acid tail in the C terminus, not present in CYPOR and FNR, which shares about 42% sequence homology with the FAD/NADPH-binding domains of CYPOR and NOS. Roman et al. (11, 12)
have shown that the C-terminal tails of all three NOS isoforms control electron flow within and through the reductase domains and modulate the effect of Ca\(^{2+}/\)CaM, although the mode and extent of the modulation appear to be different in each isozyme.

The three-dimensional structures of the oxygenase domains of \(i\)NOS (12, 13), \(e\)NOS (14, 15), and nNOS\(^2\) have been determined. All three structures are very similar to one another but have no similarities to known cytochrome P450 structures. The remarkable similarities among the structures of different NOS isoforms indicate that isoform-specific regulation of NO production is not obvious from the crystal structures of the heme domains alone. Furthermore, it emphasizes the role of the reductase domain in regulation of NO production in each of the three isoforms. To further investigate the isoform-specific modulation of NO production and its mechanism, knowledge of the structure of the reductase domain of all three isoforms is imperative. Although the structure of NADPH-cytochrome P450 oxidoreductase, a close homolog of the NOS reductase domain, has been determined (16), structural determination of the NOS reductase domain has not been successful, presumably due to proteolysis during the crystallization process. To circumvent this problem, we initiated the x-ray structure determination of its most stable domain from proteolysis, which includes the FAD- and NADP(H)-binding subdomains. Here, we report the crystal structure of the FAD/NADP-binding domain of rat nNOS (referred to hereafter as nNOS-FAD/NBD (nNOS FAD- and NADP\(^+\)-binding domain that includes residues 963–1407)) obtained by limited tryptic treatment of the Escherichia coli-expressed holo protein.

**EXPERIMENTAL PROCEDURES**

**Enzyme Purification**—Rat nNOS was overexpressed in \(E\). coli and purified as described previously (17). Briefly, the procedure involved centrifugation of crude cell extract and affinity chromatography on a 2′, 5′-ADP-Sepharose 4B column (Amersham Pharmacia Biotech). The protein was eluted with a buffer solution containing 2 mM 2′-AMP and 0.5 mM NaCl. The heme-containing fractions were pooled and concentrated, and l-arginine and tetrahydrobiopterin were added to final concentrations of 1 mM and 200 \(\mu\)M, respectively. The enzyme was further purified on a gel filtration column (Superose 12/30, Amersham Pharmacia Biotech), and peak fractions corresponding to nNOS dimers were collected. The purified holo nNOS was treated with immobilized trypsin (Sigma), and the cleaved nNOS protein was further purified on a 2′, 5′-ADP-Sepharose column. The flow-through fraction containing the heme domain (red) was saved for other uses. The material bound to the column (yellow; FAD/NADP-binding domain) was eluted with 2′-AMP and further purified on a gel filtration chromatography column. The purified protein, nNOS-FAD/NBD, was subjected to N-terminal sequencing.

**Biochemical Characterization of nNOS-FAD/NBD**—The ability of NOS-FAD/NBD to reduce cytochrome \(c\) and ferricyanide was studied as described previously (18, 19), with the exception that all assays were performed in the presence of 0.25 mM NaCl. The protein concentration was estimated based on the flavin content using an extinction coefficient of 21,600 \(\text{m}^{-1}\). Because of the effect of ionic strength on the reduction rates, parallel determinations were carried out in the presence and absence of 250 mM NaCl. Recombinant rat NADPH-CYPOR was prepared as described previously (20). N-terminal sequencing (Beckman Porton LF 3000) and matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometric analysis (Voyager DEPRO; Applied Biosystems) were performed at the Protein Nuclear Acid Facility at the Medical College of Wisconsin.

**Crystallization and Data Collection**—The purified nNOS-FAD/NBD protein was concentrated to 10 mg/ml in 30 mM potassium phosphate buffer, pH 7.6, containing 0.5 mM NADP\(^+\). Crystals were grown by the hanging drop vapor diffusion method at 4 °C by mixing 1.5 \(\mu\)l of the protein solution with 1.2 \(\mu\)l of a precipitant solution (9% polyethylene glycol 4000, 0.1 mM Hepes, pH 7.5, and 0.4 mM sodium formate) and equilibrating against the precipitant solution. Small thin plate crystals appeared within 24 h and grew to their maximum size over 2 weeks. Crystals of size 0.2 × 0.3 × 0.01 mm were soaked in a cryoprotectant solution (20% glycerol in 11% polyethylene glycol 4000, 0.1 mM Hepes, pH 7.5, and 0.3 mM sodium formate) for about 3 min before flash-freezing under a liquid nitrogen stream. The diffraction data were collected at 100 K on a charge-coupled device detector (CCD Quantum 4) at beamline BioCARS 14BM-C at the Advanced Photon Source at Argonne National Laboratory, Argonne, IL. The crystal-to-detector distance was 160 mm, and the oscillation angle for the data collection was 0.5°. A total of 960 frames were recorded covering 180° oscillation range, and the exposure time was 8 s/frame. The crystal diffracted to 1.9 Å resolution, and the data were processed with the DPS-MOSFLM (21) and Dares 2.1 (22). The crystal structure was solved by molecular replacement with the MOLREP program (23), using the structure of the corresponding domain of rat CYPOR (16) (Protein Data Bank code, 1AMO) as the search model. The initial phases were obtained by the molecular replacement method with the Expert model program package (23), using the structure of the corresponding domain of rat CYPOR (16) (Protein Data Bank code, 1AMO) as the search model. The initial rotation/translation search gave an \(R\) value of 52.4% and a correlation coefficient of 18.4 for the data between 10.0 and 4.0 Å resolution. The subsequent rigid body refinement lowered the \(R\) value to 51.4% and increased the correlation coefficient to 31.4. This model was subsequently refined using the CNS program package (24). Each cycle of refinement included maximum likelihood simulated annealing, conjugate-gradient minimization, and individual B-factor refinement. Bulk solvent correction was applied throughout the refinement process. All data within the resolution range of 30 to 1.9 Å were used for the refinement, excluding 8% of reflections randomly extracted for cross-validation.

| Table I | Data collection statistics |
|---------|--------------------------|
| Resolution range (Å) | 32.0–1.9 (2.0–1.9)* |
| No. of observations | 233747 |
| No. of unique reflections | 41027 |
| Data completeness (%) | 84.5 (69.7) |
| \(\langle R \rangle\) | 17.5 (3.6) |
| \(R_{sym}\) | 0.067 (0.405) |
| Crystal parameters | |
| Unit cell dimensions (Å) | a = 59.07, b = 64.37, c = 139.43 |
| No. of molecules/asymmetric unit | 1 |
| \(V_c (\text{Å}^3/\text{Da})\) | 3.13 |
| Solvent content (%) | 60.65 |

* Numbers in parentheses are for the highest resolution shell.

\(\text{R}_{sym} = \frac{\sum_i |I_i| - \langle I_i \rangle/\sum_i I_i|}{|I_i|} \), where \(h\) are unique reflection indices, and \(i\) indicate symmetry equivalent indices.

| Table II | Refinement and final model statistics |
|---------|--------------------------------------|
| Resolution range (Å) | 32.0–1.9 |
| No. of reflections used | 41269 |
| No. of nonhydrogen atoms | 4099 |
| Non-protein molecules | |
| FAD/NADP\(^+\) | 1/1 |
| Glyceraldehyde/formate | 4/4 |
| Water | 462 |
| \(R_{cryst} (%)\) | 18.8 |
| \(R_{free} (%)\) | 20.9 |
| Average B-factors (Å\(^2\)) | |
| Main chain atoms | 21.7 |
| Side chain atoms | 26.2 |
| FAD/NADP | 21.2 |
| Water molecules | 34.7 |
| Glyceraldehyde/formate | 29.6 |

* \(R_{free}\) is equal to \(R_{cryst}\) for a randomly selected 8% subset of reflections not used in the refinement.
results indicate that nNOS-FAD/NBD retains its ability to accept reducing equivalents from NADPH and to transfer them to FAD and to ferricyanide.

The Overall Structure—The final model of nNOS-FAD/NBD contains 435 residues starting from Ser963 to Val1397, and the last 10 C-terminal residues are disordered, suggesting the flexible nature of this terminus. The overall polypeptide fold is, as expected, essentially the same as that of the corresponding domain of CYPOR. The structure is composed of three subdomains, as in the case of the CYPOR structure (16), and these subdomains, from N to C terminus (Fig. 2, from bottom to top), are as follows: (a) the connecting domain, (b) the FAD-binding domain, and (c) the NADP(H)-binding domain. The connecting domain is not contiguous in the linear amino acid sequence, as it is in the CYPOR structure. The root mean square (rms) difference for the Cα atoms in the FAD-binding and NADP+ -binding domains is 1.3 Å between the structures of the two enzymes, excluding the insertion/deletion regions and the entire connecting domain. Fig. 3 shows a ribbon diagram of a superposition of the two structures. The NADP+ -binding domain is shown on the right, the FAD-binding domain is shown in the middle, and the connecting domain is shown on the left. The connecting domain shows the most difference from the structure of the corresponding domain of CYPOR (rms difference, 3.9 Å), reflecting the lower sequence homology in this part of the molecule. The structure of the two nucleotide-binding domains, the FAD- and NADP(H)-binding domains, is also very similar to that of FNR (27), phthalate dioxygenase reductase (28), and cytochrome b5 reductase (29). A structure-based amino acid sequence alignment of nNOS-FAD/NBD, CYPOR, and FNR together with the sequences of the corresponding domains of eNOS and iNOS is shown in Fig. 4.

FAD-binding Site—As in the FAD domains of CYPOR, FNR,
...also indicated. This figure was prepared with Molscript (34) and rendered with Raster3D (35).

The isoalloxazine ring of FAD is bound at the interface between the FAD and NADPH domains (Fig. 2). Just as in the structure of CYPOR, the pyrimidine side of the isoalloxazine ring has extensive hydrogen bonding interactions with the polypeptide and with two tightly bound water molecules (Fig. 5). The O2 atom of the isoalloxazine ring makes hydrogen bonds with the main chain atoms of Ala1193 (amide nitrogen) and Thr1191 (carbonyl oxygen), the latter of which, in turn, makes a hydrogen bond with the N3 atom of FAD. The N1 atom of the FAD ring is involved in this hydrogen-bonding network via a water molecule (W1 in Fig. 5) to the carbonyl oxygen of Ala1193, the O2 atom, and the 4′-OH group of the ribityl chain. The O4 atom of the isoalloxazine ring is also linked to Thr1191 via another water molecule (W2). This intricate network of hydrogen bonds must play an important role in FAD binding/orientation and is likely to be crucial in stabilizing semiquinone/hydroquinone forms of FAD during electron transfer from NADPH to FAD and from FAD to FMN. The residues implicated in the hydride transfer reaction in CYPOR (30) are also structurally conserved in nNOS-FAD/NBD; the hydroxyl group of Ser1176 (corresponding to Ser457 of CYPOR) makes hydrogen bonds to Asp1393 (Asp675 of CYPOR) and the O4 atom of the flavin ring, located near the N5 atom of FAD (3.7 Å), and is coplanar with the isoalloxazine ring. The carboxylate of Asp1393 makes a hydrogen bond with His1032 (3.8 Å; His319 in CYPOR) and is near Cys1349 (3.7 Å; Cys630 in CYPOR). On the other hand, the xylene ring side of FAD is exposed to the surface of the molecule, strongly suggesting that in the holoenzyme, the FMN domain would interact through this side and receive the reducing equivalents from FAD. The FAD ring is sandwiched between two aromatic residues, Phe3495 at its re-face and Tyr1175 at the si-face, serving as stacking residues.

The NADP⁺-binding Domain—Similar to the NADP⁺ domains of CYPOR and FNR and the NAD⁺ domains of cytochrome b₅ reductase and phthalate dioxygenase reductase, the nNOS NADP⁺ domain has the αβα structural motif, with a five-stranded β-sheet sandwiched between α-helices. Unlike the CYPOR and FNR structures, where the ribose-nicotinamide ring of NADP⁺ is disordered, the current structure clearly shows that the nicotinamide moiety of NADP⁺ is bound at the surface of the molecule, and the cofactor has a bent conformation in which the nicotinamide and adenine rings point to the same side. Fig. 6A shows the vicinities of the NADP⁺-binding site. A distinct feature in this binding site is the presence of three arginine residues interacting with the phosphate groups of the cofactor: Arg1010 makes a salt bridge with the phosphate group, Arg1284 (lying in the middle of and parallel to the two other guanidinium side chains) balances the negative charges of the phosphates. The interaction between Arg1314 and the 2′-phosphate group of the AMP moiety of the cofactor is most likely a major discriminating factor between NADPH and NADH as a substrate. All three Arg residues are...
conserved in CYPOR and in the other two NOS isozymes. Although the ribose-nicotinamide moiety is well-ordered in this structure, it is located at the surface of the molecule and does not involve any strong interactions with the polypeptide chain (Fig. 6A), implying that its binding is not as tight as that of the 2H11032-ADP portion of the cofactor. As shown in the mutant studies with both CYPOR (30) and FNR (31), it is most likely that during hydride transfer, the phenyl ring of Phe1395 (Trp677 of CYPOR), which covers the re-face of the isoalloxazine ring of FAD, swings away to make room for the nicotinamide moiety to bind at the re-face of the isoalloxazine ring. Fig. 6B shows a comparison of the nNOS-FAD/NBD-NADP/H11001 structure with that found in the mutant CYPOR structures. Minor dihedral angle changes at the pyrophosphate linkage would transform the bent conformation of NADP/H11001 to the extended conformation in which the nicotinamide ring can replace the phenyl side chain of Phe1395 and directly interact with the flavin ring for the hydride transfer reaction. The exact mechanism and energetics of this conformational change are not clear at present.

The Connecting Domain and the FMN Domain Binding Sites—Approximately 150 residues of nNOS-FAD/NBD constitute the connecting domain, consisting mainly of α-helices (Figs. 2 and 4). This domain is unique among CYPOR family members that have both FAD- and FMN-binding sites in a single polypeptide chain, which include NOS, CYPOR, bacterial P450-BM3, methionine synthase reductase (32), and the α subunit of sulfite reductase (33). The connecting domain is not conserved in CYPOR and in the other two NOS isozymes. Although the ribose-nicotinamide moiety is well-ordered in this structure, it is located at the surface of the molecule and does not involve any strong interactions with the polypeptide chain (Fig. 6A), implying that its binding is not as tight as that of the 2H11032-ADP portion of the cofactor. As shown in the mutant studies with both CYPOR (30) and FNR (31), it is most likely that during hydride transfer, the phenyl ring of Phe1395 (Trp677 of CYPOR), which covers the re-face of the isoalloxazine ring of FAD, swings away to make room for the nicotinamide moiety to bind at the re-face of the isoalloxazine ring. Fig. 6B shows a comparison of the nNOS-FAD/NBD-NADP/H11001 structure with that found in the mutant CYPOR structures. Minor dihedral angle changes at the pyrophosphate linkage would transform the bent conformation of NADP/H11001 to the extended conformation in which the nicotinamide ring can replace the phenyl side chain of Phe1395 and directly interact with the flavin ring for the hydride transfer reaction. The exact mechanism and energetics of this conformational change are not clear at present.

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A closer examination of the connecting domains of nNOS-FAD/NBD and CYPOR reveals small but significant structural differences between the two molecules. The two most significant differences lie in the first 27 residues at the N terminus and the loop between strands $\beta 6$ and $\beta 7$ (Glu1077 to Asp1091, Fig. 3). Fig. 4, both of which are located at the surface that might interact with the FMN domain. The N-terminal 27 residues (Ser1063-Lys1068), 12 residues shorter than the corresponding region in CYPOR (Ser241-Lys252), share no obvious sequence homology to CYPOR, and, accordingly, their secondary structures also differ. In nNOS, residues Asp979-His988 form a regular $\alpha$-helix, whereas the corresponding part of CYPOR adopts a loop structure. On the other hand, region Glu1077-Asp1091 in nNOS contains 8 extra residues compared with the CYPOR molecule. These residues form a long $\beta$-stranded finger, extruding out of the molecule into the solvent region, whereas the corresponding region in CYPOR makes a short loop that is tightly bound to the rest of the molecule. At the tip of the finger lies a hydrophobic loop with residues 1072-Ala-Lys-Gly-Val-Ile, suggesting that this loop is most likely buried in the presence of the FMN domain and possibly CaM. Interestingly, a structure-based sequence alignment (Fig. 4) shows that this loop is also short in iNOS as in CYPOR. The distance between the tip of this loop and the insertion point of the putative autoinhibitory element described by Salerno et al. (7) is about 27 Å. Considering the size of the autoinhibitory loop (~45 residues), it is possible that these two loops interact with each other in response to Ca$^{2+}$/CaM binding. It should be noted that the autoinhibitory domain, inserted in the middle of the FMN domain, is present only in the constitutive NOSs and absent in iNOS and CYPOR, both of which do not respond to Ca$^{2+}$/CaM concentration.

Superposition of the nNOS-FAD/NBD with the CYPOR structure (Fig. 3) also reveals the binding site for the FMN domain, the location of the autoinhibitory loop insertion, and their relative positions to the rest of the structure. The putative FMN-domain binding site lies mainly on the connecting domain. Fig. 7 shows the electrostatic surface potentials of the interface of nNOS-FAD/NBD that might interact with the FMN domain, along with the corresponding surface of CYPOR and the complementary surface of its FMN domain. In the case of CYPOR, the interaction between the two domains (the FMN and FAD/NBD domains) is highly electrostatic, with the former being negative, and the latter being positive (Fig. 7, D and C, respectively). The corresponding surface of nNOS-FAD/NBD is less charged (Fig. 7B), suggesting that the complementary surface of the nNOS FMN domain must be correspondingly more neutral. There are five salt bridges in the CYPOR structure that link the two domains: Arg578/Asp555, Glu992/Lys985, Glu147/Arg514, Glu209/Arg634, and Glu213/Arg382. From the structure-

![Fig. 6. A, stereo diagram showing residues near the NADP$^+$-binding site. The conventional color scheme is used: oxygen, red; nitrogen, blue; carbon, yellow; sulfur, green; and phosphorous, orange. The cofactor is shown in red, with its nitrogen atoms in blue. NADP$^+$ is bound at the surface of the enzyme molecule and adopts a bent conformation. The 2'-phosphate makes salt bridges with Arg1314 and hydrogen bonds with Tyr1322 and Ser1313; the pyrophosphate group makes salt bridges with Arg1314 and a hydrogen bond with Thr1325. The plane of Arg1314 is parallel to and sandwiched between the planes of Arg1010 and Arg1314. Whereas the phosho-AMP moiety makes extensive interactions with the polypeptide, the ribose-nicotinamide portion is loosely bound, suggesting conformational flexibility of the cofactor during catalysis. B, superposition of the NADP$^+$ structure observed in nNOS-FAD/NBD (dark blue) with that of a productive mode found in the mutant CYPOR (red) (30). The 2'-AMP moiety common to both conformations is shown in green. In order for the hydride transfer reaction to occur, the nicotinamide group must replace the phenyl side chain of Phe1395, which occupies the re-face of the FAD ring. Minor adjustments of dihedral angles at the pyrophosphate linkage transform a resting conformation (blue) to an active (or productive) conformation (red).]
domain (and presumably the other NOS isozymes). It also confirms that the structure of the FAD/NADPH domain is very similar to those of the members of the FNR superfamily, including FNR, cytochrome $b_5$ reductase, and phthalate dioxygenase reductase. A closer comparison between the structures of nNOS-FAD/NBD and CYPOR, however, reveals not only important similarities but also some significant differences between the two enzymes (Figs. 3 and 4). The residues shown to be catalytically important for the hydride transfer mechanism in CYPOR (Ser$^{457}$, Cys$^{630}$, Asp$^{675}$, and possibly His$^{319}$) are all structurally conserved in nNOS-FAD/NBD (Ser$^{1176}$, Cys$^{1349}$, Asp$^{1393}$, and His$^{1032}$, respectively) (Fig. 4). In addition, the tightly bound water molecules involved in the hydrogen bonding network at the pyrimidine side of the isoalloxazine ring of FAD and an aromatic residue stacking at the re-face of the flavin ring (Phe$^{1395}$ in nNOS and Trp$^{677}$ in CYPOR) are also conserved. This constellation of residues in the vicinity of the isoalloxazine ring of FAD strongly suggests that the hydride transfer mechanism of nNOS is very similar to that of CYPOR suggested by Hubbard et al. (30). Also, this hydrogen bonding network is most likely responsible for stabilization of the semiquinone/hydroquinone form of FAD during catalysis, and a water molecule (W2 in Fig. 4) might act as a proton donor/acceptor during hydride transfer. Ser$^{1176}$ is in close proximity to the N5 atom of the flavin ring, and the arrangement of Ser$^{1176}$, Asp$^{1393}$, and Cys$^{1349}$ (or His$^{1032}$) resembles the catalytic triad of the serine protease family, suggesting a proton relay during catalysis. Furthermore, the mode of NADP$^−$ binding is also similar in the two enzymes; the 2'-ADP moiety is tightly bound to the enzyme, yet the nicotinamide side of the cofactor is either disordered (in CYPOR) or loosely bound (in nNOS) at the surface of the molecule. Recently, Hubbard et al. (30) have shown with CYPOR mutants in which the aromatic residue (Trp$^{677}$) was either deleted or mutated to glycine that the nicotinamide moiety of NADP$^−$ binds at the re-face of the FAD ring in a manner suitable for hydride transfer from the nicotinamide ring to FAD, and they suggested that a conformational change occurs during catalysis involving Trp$^{677}$ and the pyridine nucleotide. Modeling of NADPH in a productive conformation similar to the one observed in the mutant CYPOR structure (Ref. 30; Fig. 5B) suggests that Ser$^{1176}$ and Asp$^{1393}$ are involved in interactions with the nicotinamide ring, thereby orienting and stabilizing the binding of the pyridine nucleotide for the hydride transfer reaction. Site-specific mutagenesis studies are in progress to confirm the role of these residues in the catalytic reaction. Although the FMN domain is not included in the nNOS-FAD/NBD structure, the current structure reveals the putative binding site for the FMN domain and the relative locations of various elements of the reductase domain that control/modulate different catalytic steps involved in cytochrome c reduction and NO production. The exact binding sites of the C-terminal and autoinhibitory domains and their interactions with the rest of the reductase domain and Ca$^{2+}$/CaM must await the crystal structure determination of the entire reductase module, including the calmodulin-binding region.

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