Three nitric-oxide synthase (NOS) isoforms play crucial, but distinct, roles in neurotransmission, vascular homeostasis, and host defense, by catalyzing Ca^{2+}/calmodulin-triggered NO synthesis. Here, we address current questions regarding NOS activity and regulation by combining mutagenesis and biochemistry with crystal structure determination of a fully assembled, electron-supplying, neuronal NOS reductase dimer. By integrating these results, we structurally elucidate the unique mechanisms for isozyme-specific regulation of electron transfer in NOS. Our discovery of the autoinhibitory helix, its placement between domains, and striking similarities with canonical calmodulin-binding motifs, support new mechanisms for NOS inhibition. NADPH, isozyme-specific residue Arg^{1400}, and the C-terminal tail synergistically repress NO activity by locking the FMN binding domain in an electron-accepting position. Our analyses suggest that calmodulin binding or C-terminal tail phosphorylation frees a large scale swinging motion of the entire FMN domain to deliver electrons to the catalytic module in the holoenzyme.

Nitric oxide (NO) acts in key physiological processes including neurotransmission, blood pressure regulation, and the immune response (reviewed in Ref. 1). NO is transient, small, and easily diffusible; its availability is solely regulated at the synthesis level by the nitric-oxide synthase (NOS) enzymes (EC 1.14.13.39). In mammals, two constitutively expressed NOS isoforms (cNOSs), endothelial (eNOS) and neuronal NOS (nNOS), are Ca^{2+}-responsive and control basal NO levels, whereas the Ca^{2+}-insensitive inducible NOS (iNOS) is expressed in response to specific cytokines or bacterial products (2). Deregulation of NO synthesis is associated with diverse human pathologies including immune-type diabetes, stroke, inflammatory bowel disease, rheumatoid arthritis, hypertension, arteriosclerosis, and infection susceptibility (3).

Each chain of a NOS homodimer (Fig. 1a) contains an N-terminal catalytic oxygenase module (NOSox) and a C-terminal electron-supplying reductase module (NOSred) linked by a 32-residue Ca^{2+}/calmodulin (CaM) binding region (4). In NOSox, the unique winged helix, its placement between domains, and striking similarities with canonical calmodulin-binding motifs, support new mechanisms for NOS inhibition (21). Extensive crystallographic studies of NOSox in complex with substrate, intermediates, and inhibitors in the three isozymes have considerably advanced our understanding of the structural chemistry underlying NOS activity (10–12). NOSox accepts electrons from NOSred to catalyze the sequential monooxygenation of L-arginine into N-hydroxyarginine, then citrulline and NO. NOSred belongs to a large protein family that includes NADPH-dependent cytochrome P450 reductase (CYPOR), sulfite reductase flavoprotein and novel reductase 1. These reductases share a conserved organization of flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and nicotinamide adenine dinucleotide phosphate (NADPH)-binding domains (13–15). The FMN-binding domain is homologous to small electron-carrier flavodoxins (16), whereas the FAD- and NADPH-binding domains associate into a “FNR-like unit,” related to ferredoxin-NADP” reductase (17). An α-helical connecting domain (CD) orients the flavin FMN- and FAD-binding domains to align the two flavins (18). Electron transfer proceeds from NADPH to FAD to FMN to heme (18). This last step is rate-limiting (19), occurs in trans from NOSred of one polypeptide to NOSox of the other, and is uniquely triggered by conformational changes induced by Ca^{2+}/CaM binding (20).

NOSred is exquisitely tuned to control NO production (reviewed in Ref. 21). In all isoforms, a 21–42-residue C-terminal tail (CT) represses electron transfer. In addition, the cNOSs contain a 42–45-residue autoinhibitory insert (AH) in the FMN-binding domain that interferes with CaM binding and inhibits both intra- and inter-module electron transfers. Phosphorylation of both of these regulatory elements further modulates cNOS activity. Last, the CD2A regulatory element present in the CD of the cNOSs is predicted to contribute to the observed Ca^{2+}/CaM dependence by interaction with the CaM-binding peptide (22). The recent structure of a proteolytic fragment of NOSred (23), missing the FMN-binding domain and the C-terminal tail, shows the conserved CYPOR fold, but does not address some current major questions about NOS struc-
ture-function relationships including how the unique NOS sequence elements regulate electron transfer. Here, we address these questions by determining and analyzing the x-ray structure of a fully assembled reductase dimer of nNOS. These results provide insights into the molecular interactions that enable a newly identified autoinhibitory helix motif, co-substrate NADPH, the C-terminal tail and its phosphorylation site, to regulate NOS activity. Biochemical data, when combined with these structural results, further suggest a possible model for holoenzyme assembly, in which the FMN domain undergoes large scale movements to shuttle electrons between the two NOS modules.

**EXPERIMENTAL PROCEDURES**

*nNOSred Expression, Purification, Activity Measurements, and Microspectral Characterization—*Rat nNOSred Δ18 (construct 741–1429) was expressed in *Ficha pastoris* (24). nNOSred wild-type and R1400E mutant were overexpressed in *Escherichia coli* BL21(DE3) cells and purified as described (18). Steady-state activities of wild-type and mutant nNOSred were determined at 25 °C as described (18). The redox state of the flavins in the crystal was determined by using a single-crystal microspectrophotometer before and after x-ray diffraction (Supplementary Methods).

Dynamic Light Scattering and Crystallization—Dynamic light scattering was performed with a DynaPro-801 (Protein Solutions) instrument at 20 °C (Supplementary Methods). nNOSred was crystallized in the P1 space group at 20 °C in an anaerobic glove-box using the vapor diffusion method (Supplementary Methods).

Diffraction Data Collection, Processing, and Refinement—X-ray diffraction data for nNOSred were collected at SSRL on beamline 11-1. The structure was solved by molecular replacement, followed by fitting and refinement to current R and free R factors of 24.4 and 27.2%, respectively (Table I and Supplementary Methods). Coordinates for nNOSred are deposited in the RCSB Protein Data Bank with accession code 1TLL.

**RESULTS**

Neuronal NOSred Structure and Architecture—To characterize the molecular basis for NOSred function, we solved the crystallographic structure of a fully assembled nNOSred module at 2.3 Å resolution (Fig. 1b). We used the structure of CYPOR (13), without its FMN domain, as a starting model for molecular replacement and manually built the nNOS FMN...
domain into the electron density. After iterative cycles of fitting and refinement, the final model shows good statistics with R and free R factors of 24.4 and 27.2%, respectively, and good geometry with 99.5% of the residues in the most favored and additional allowed regions of the Ramachandran plot (see Table 1). The structure reveals 4 major domains: FMN binding domain (residues 750–942), connecting domain (943–989 and 1039–1170) with a flexible hinge (943–967) and a β-finger (1059–1078), FAD binding domain (990–1038 and 1171–1231) and NADPH binding (1222–1396) domain (Fig. 2). Unexpectedly, in both our crystallographic structure (Fig. 1c) and in solution, as measured by dynamic light scattering (see Supplementary Methods), nNOSred is a homodimer, suitable for assembly within dimeric holo-NOS (see “Discussion”).

### FMN Binding Sites and X-ray-induced Reduction—FAD, which accepts electrons from NADPH and transfers them to FNM, binds in an elongated conformation (Fig. 3a, center) at one end of the flattened anti-parallel β-barrel fold of the FAD-binding domain (Fig. 1b), as in related FNR (17). The FMN is inserted into the flavodoxin-like FMN-binding domain (16), consisting of a five-stranded parallel β-sheet flanked by five α-helices (Fig. 1b). Extensive networks of hydrogen bonds and hydrophobic stacking interactions (Figs. 2 and 3a), as described for CYPOR (13), stabilize both cofactors.

In the nNOSred structure (Fig. 3a), extended hydrogen-bonding networks incorporating the flavins reveal that: (i) FMN N5 is a hydrogen bond donor to the main chain carbonyl of Gly310 (“O-up” conformation (25)), and is thus protonated; (ii) FMN N1 has no hydrogen-bonding partner; (iii) FAD N5 makes a long hydrogen bond (3.6 Å) to Ser1176 side chain hydroxy; and (iv) FAD N1 hydrogen bonds to a conserved water molecule, proposed to be important for catalysis in CYPOR (26). These structural results reveal that the FMN is likely present as the semiquinone species, consistent with the spectral properties of the resting state of NOS in solution. To unambiguously determine the FAD redox state, we used single-crystal microspectrophotometry (Supplementary Methods). Reduction of the flavins occurs quickly in the intense synchrotron x-ray beam, and the disappearance of the absorption band at 457 nm unequivocally shows that no oxidized species is present after 2 h exposure (Fig. 3c). Our combined structural and microspectral studies thus show that the structure (determined from 17 h of data collection) represents the one-electron-reduced FMN semiquinone and either the FAD doubly reduced hydroquinone or a mixture of semiquinone/hydroquinone species, corresponding to one of the proposed (27) catalytic intermediates.

### The FMN/FAD Domain Interface and Rotational Flexibility of the FMN Domain—The FMN/FAD interface is created by plugging the flavin-containing face of the FMN-binding domain into the deep cup-shaped surface (Fig. 1b) formed by the FAD,
FIG. 2. Structure-based sequence alignment of nNOSred, eNOSred, iNOSred, and CYPOR. The alignment was carried out using SEQUOIA (56). The secondary structure elements of nNOSred are indicated above the sequence and colored according to Fig. 1a. Unstructured regions are indicated by dashed lines and the regulatory elements by AH, CD2A, and CT (CTN and CTc). Key residues are indicated as follows: N and C for the first and last ordered residues in the structure; @ and $, FMN and FAD aromatic stacking residues, respectively; phosphorylation site residues Thr495 (only in eNOS), Ser847 and Ser1412 (both in cNOSs) are represented by asterisks. Salt bridges at the FMN/FAD (blue circles) or the dimer (green triangles) interfaces, respectively, are indicated by SB1 and SBa, and SBb. The purple diamonds indicate hydrophobic residues of the autoinhibitory helix.
NADPH, and connecting domains (referred to here as the FNR/CD unit). At the center of this interface (burying a total surface of 2500 Å²), the FMN- and FAD-binding domains interact through a double salt bridge between residues Glu816 and Arg1229, which are both conserved in cNOSs (Fig. 2). Other interactions include hydrophobic contacts and hydrogen bonds (Supplementary Materials Table S1). On one side of the FNR/CD “cup,” the autoinhibitory insert of the FMN domain contacts the FMN- and NADPH-binding domains (Supplementary Materials Table S2). On the other side, the CD braces the FMN- and FAD-binding domains, as seen in CYPOR, to help align the alloxazine rings of the two flavin cofactors (Fig. 1b). In fact, the xylene ring methyl groups of the cofactors are only 4.8 Å apart (Fig. 3a), suggesting direct electron transfer from FAD to FMN in this electron-accepting position. The FMN is completely buried in this FMN/FAD interface, inhibiting subsequent flow of electrons from FMN to the heme or other electron acceptors.

The two FMN-binding domains of the two crystallographically independent NOSred modules exhibit 4° rotations relative to each other, mimicking an elbow joint within the concave interface of the FNR/CD unit (Fig. 4a). These rigid-body rotations are unexplained by crystal packing. Conformational diversity in NOSred is further evident from weakly defined electron density for parts of the CD, including the hinge (residues 943–967) linking the FMN domain to the CD, and the protruding β-finger (1059–1078) containing the CD2A regulatory element. The two FMN-binding domains have also higher (71 Å²) than average (57 Å²) overall crystallographic temperature factors. The observed rotational flexibility of the FMN domains has important functional implications (see “Discussion”).

**Unique NOSred Regulatory Elements**—This fully assembled NOSred structure extends the structure of the NOSred proteolytic fragment (23) to include not only the missing FMN-binding domain and the dimer assembly but also key parts of three unique regulatory elements: the FMN domain autoinhibitory insert, the C-terminal tail, and its Ser1412 phosphorylation site.

The FMN domain autoinhibitory insert unexpectedly contains an α-helix (residues 840–848), sequestered into a hydrophobic pocket formed between FMN-binding domain helices α4 and α6, and NADPH-binding domain helix α24 (Fig. 1b). Conserved residues from these α-helices make hydrophobic and hydrogen-bonding contacts with the autoinhibitory insert (Supplementary Materials Table S2). Ser840 N-caps the helix by hydrogen bonding to the Lys842 backbone amide. Surprisingly, the ordered region (residues 836–849) of the autoinhibitory insert is flanked by 5 unstructured N-terminal residues and 23 unstructured C-terminal residues. To make the covalent connections to the FMN domain, these disordered N- and C-terminal regions must span 9 and 29 Å distances, respectively.

The regulatory C-terminal tail similarly contains an α-helix (residues 1401–1412), which fits within a negatively charged groove across the FAD/CD interface, shielding the flavins from solvent (Fig. 4, a–c). Ordered C-terminal tail residues (1397–1413) extend from the last β-strand of the NADPH-binding domain and form a right-angle turn at Arg1400 followed by an α-helix (1401–1412), which is terminated by the regulatory Ser1412 phosphorylation site (Figs. 1b and 4c). C-terminal tail residues 1397–1400 are stitched to the FMN- and NADPH-binding domains via hydrogen bonds with residues Tyr929 and Asp1351, respectively, and ionic interactions with NADP(H), all of which help to “lock” the FMN domain into its electron-
Fig. 4. Structural features implicated in the regulation of electron transfer. a, superposition of the nNOSred modules A-red (magenta), B-red (blue), and CYPOR W677G mutant (green), showing a very good agreement for the FN/R/CD units (root mean square deviations of 0.6 Å for 1,732 main chain atoms). The FMN domains (root mean square deviations of 0.3 Å for 572 main chain atoms) differ in orientation; the hinge and the β-finger differ in position and conformation (black arrows). b, stereo view of the FMN/FAD interface in the NADPH-locked NOSred structure. Key interactions of CTn residues (magenta) with NADP(H) (orange) and NADPH- and FMN-binding domains (orange and yellow, respectively) are indicated. The phosphorylation site on the penultimate residue (Ser1412) is in close proximity to conserved acidic residues. c, C-terminal Ser1412 phosphorylation site. The electrostatic potential (contoured at ±2 eV and colored in blue and red for positive and negative, respectively) displayed on the surface of NOSred reveals a patch of negatively charged residues near Ser1412. The FAD and NADP(H) are colored in gray and orange, respectively.

accepting position (Fig. 4b). Extensive hydrophobic interactions with FMN-binding domain and CD residues (Supplementary Materials Table S3) further anchor the C-terminal tail.

At the end of the C-terminal α-helix, the phosphorylatable O-γ atom of Ser1412 is directed toward negatively charged FMN-binding domain residues Glu916 (Gln in eNOS) and conserved Asp918 (Figs. 2 and 4c). This structure thus suggests a mechanism for phosphorylation-induced NOS activation by electrostatically induced conformational changes.

DISCUSSION

Quaternary Structure of NOSred—Our structural and biochemical studies reveal that NOSred is dimeric (Fig. 1c). This is in contrast with earlier studies suggesting that NOSox alone mediates dimerization (28). The extensive (3200 Å² buried surface), nearly symmetric, dimer interface is consistent with yeast two-hybrid studies (29) that indicate significant dimer association in nNOSred and eNOSred, but not in iNOSred. The iNOSred sequence lacks the ability to make all salt bridges and 5 of 8 hydrogen bonds found across the nNOSred dimer interface (Fig. 2). Because dimerization is required for NOS activity (30), this distinction among the NOS isoforms may be of considerable biochemical and therapeutically important.

A Swinging FMN Domain Mechanism for Electron Shuttling—The intact NOSred structure provides unique insights into the mechanism of inter-module electron transfer (FMN to heme), which is the rate-limiting step (19) in NO synthesis by CaM-bound full-length NOS. The observed buried “electron accepting” (from FAD) position for the FMN cofactor, which is sandwiched between the FMN-binding domain and the FN/R/CD unit (Fig. 1b), raises an important question: how will the electrons get transferred from FMN to heme? No arrangement of NOSox and NOSred will overcome the problem of the buried FMN cofactor, but mobility of the FMN-binding domain provides a solution. Conformational diversity of the FMN-binding domain in reductase modules is observed not only between the two NOSred monomers, but also by comparison with CYPOR (Fig. 4a). Furthermore, in several CYPOR mutants (26), and sulfite reductase flavoprotein (14) structures, the electron density for the FMN domain is completely missing. Fluorescence increases in nNOS upon CaM binding or addition of chaotropic agents also indicate that conformational changes are limited to the FMN-binding domain (18, 31). These combined results all support a mechanism for electron transfer in which the conformational mobility of the FMN-binding domain is a key component of catalysis and a common feature among members of this family of reductases.

We evaluated structurally allowed pathways for intermodule electron transfer from FMN to heme within holo-NOS. An experimentally determined structure of full-length NOS remains elusive because of difficulties in obtaining suitable crystals, consistent with our results indicating functionally important flexible components. To make a model for the homomeric holoenzyme (without the PDZ domains), we used known biochemical data to assemble the structures of its components parts: the dimeric NOSox and NOSred modules, and a CaM: NOS-peptide complex (32). We rotated and translated the NOSox module and CaM: NOS-peptide complex with respect to the NOSred module to fit the constraints required by the short covalent connections from NOSox to NOSred through the CaM-binding region. Families of models with the C-terminus of NOSox and the N terminus of NOSred at opposite sides of the molecule were ruled out because the large separation (distance...
between these termini (Fig. 5a, model 1, top to bottom; model 2, back to front) is incompatible with the short length (~30 Å) of the intervening mostly α-helical CaM-binding linker. Only families of models in which the NOSox C terminus is oriented facing the NOSred N terminus (Fig. 5a, model 3) had an intervening distance (~47 Å) that is easily compatible with the expected length of the intervening nNOS CaM-binding linker. The flexible nature of this linker is consistent with biochemical data indicating that this region is susceptible to proteolytic cleavage (33). In this family of models, the polypeptide chains cross each other, and the short CaM-binding linker brings together opposing NOSox and NOSred modules (Fig. 5a, right). The known transfer of electrons in trans from NOSred to NOSox across the dimer (20) imposes further constraints on possible rotations of the two modules along a vertical axis. In our proposed assembly, the shortest distance between the FMN and heme cofactors is ~70 Å, which is substantially too long (34) for direct intermodule electron transfer.

Together, the structural and modeling results predict that the entire FMN domain serves as a one-electron shuttle by swinging back and forth between its two redox partners. The flexible hinge region (residues 943–967) serves as a pivot point for this motion (Figs. 1b and 4a), consistent with the proposed role of this region in related cytochrome P450-BM3 (35). Others have suggested small scale oscillations of the FMN-binding domain to expose the FMN for electron transfer in NOS and related reductases (13, 36, 37). In contrast, we propose a much larger motion, reminiscent of those found in multiple redox centers containing proteins (38–40), whereby the rotation of the FMN domain around the hinge region brings the FMN within 15 Å of the heme cofactor. Such large scale movement is not unprecedented in other multidomain proteins like pyruvate
orthophosphate dikinase (41), where a central domain swivels around a hinge region between the two active sites separated by $-45 \AA$, or methionine synthase, where the cobalamin-bind-
ing domain must swing back and forth between two active sites located $-50 \AA$ apart (42). In NOS, this swinging FMN domain mechanism would account for the slow rate of inter-module electron transfer, expected to be conformationally gated (43), and explain previous experimental results (44), in which FMN-associated fluorescence anisotropy decay within holo-NOS occurred on a time scale ($\Phi_2 = -3 ns$) consistent with such motions of the entire FMN domain.

The site of interaction on NOSOx for the negatively charged FMN-binding domain (Fig. 5b, middle) was suggested (7) to be the sequence-conserved positively charged surface patch favoring the back face of the heme (Fig. 5b, top), rather than the zinc-binding region strongly argued for by others (8). Mutational studies (18, 43, 45) further implicate NOSOx residue Lys$^{423}$, located on the back face of the heme and NOSSred acidic cluster 1 residues (Asp$^{918}$–Glu$^{919}$) of the FMN-binding domain, in electron delivery from NOSSred to NOSSox. Likewise, in the electron-accepting position, the interacting FMN-bind-
ing domain and FNR/CD unit surfaces have complementary negative (Fig. 5b, middle) and positive (Fig. 5b, bottom) electrostatic potentials, respectively. FMN domain-related flavodoxin (46) similarly uses overlapping interaction sites surrounding the exposed FMN to interact with both its redox partners. The remarkable parallel positions of the NOSSox heme back faces (Fig. 5c, left) and the NOSSred FMN-binding domains (Fig. 5c, right) on opposite faces of each NOS dimer are likewise suitable for a swiveling motion of the FMN domain shuttle from FAD to the heme. These results are all consistent with the swinging FMN domain mechanism in which the FMN-binding domain uses the same face, containing the exposed edge of the FMN cofactor, to form mutually exclusive complexes with both the FNR/CD unit and NOSSox.

In both the crystallographically observed electron-accepting position (Figs. 1b and 5d) and the modeled electron-donating position (Fig. 5d) for the FMN domain shuttle, direct electron transfer is facilitated (34). The FAD to FMN distance is less than 5 Å, and the FMN to heme distance is $-15 \AA$ in the electron-accepting and electron-donating positions, respectively. We therefore predict that sequential interaction of the FMN domain with each redox partners, guided by analogous complementary electrostatic interactions, are required to shuttle electrons from NOSSred to NOSSox.

Structural Determinants Regulating Electron Transfer in NOS—By revealing the position and conformation of key parts of the NOS-specific regulatory elements, the NOSSred structure provides novel insights into the molecular mechanisms that control both the intra-module electron transfer from NADPH to the flavins and the inter-module electron transfer from FMN to heme.

The Autoinhibitory Helix—The NOSSred structure reveals the most sequence-conserved portion (encompassing residues 836–849, Fig. 2) of the FMN domain autoinhibitory insert unique to cNOSs. The N-terminal segment $\Delta^{540} SYKVRFSV$ of this insert, including the Ser$^{847}$ phosphorylation site, folds into an $\alpha$-helix. Three conserved hydrophobic residues (Tyr$^{841}$, Phe$^{845}$, and Val$^{848}$) preceded by two basic residues (Arg$^{838}$ and Lys$^{839}$) form a new motif, referred to here as the AH, which shares striking similarities with the canonical “1-5-8-14” Ca$^{2+}$-dependent CaM binding motif (47). It is notable that the eNOS amino acid sequence contains an additional hydrophobic Leu residue in position 14 (Fig. 2). Previous studies emphasize the role of a non-conserved C-terminal patch of basic residues in the eNOS insert and suggest that the autoinhibitory insert interferes with CaM binding by interacting with the CaM-binding linker (48). From the AH motif and its position in the NOSSred structure between the FMN- and NADPH-binding domains, we propose two additional hypotheses for its role in NOS regulation: (i) AH binds CaM and acts as a “spoon” that inhibits CaM binding to the CaM-binding linker; (ii) interactions of the AH with the FMN- and NADPH-binding domains contribute to the “locked” electron-accepting position of the FMN domain. At elevated concentrations of Ca$^{2+}$, CaM would bind to both the AH and CaM-binding regions and release the FMN domain for inter-module electron transfer. These alternative molecular mechanisms require direct binding of the AH to CaM, as suggested previously (49), and thus provide an informed basis for future experiments.

Concerted Repression by NADPH, Phe$^{1305}$, Arg$^{1400}$, and the C-terminal Tail—In the NOSSred structure (Figs. 3a and 4b), the NADP(H) ribityl-nicotinamide moiety is prevented from productive $\pi$-stacking with the FAD alloxazine ring by an ar-
omatic side chain (Phe$^{1305}$), similarly to other NADPH-containing flavoproteins (13, 14, 17). The conformations of the NADP(H) ribityl-nicotinamide moieties are distinct from that described in the NOSSred fragment structure (23), probably because of the presence of the C-terminal tail. Recent studies on CYPOR (26) and nNOS (50) highlight the importance of Phe$^{1305}$ for NADP$^+$ release, and the possibility that this resi-
due acts in concert with the C-terminal tail in nNOS to repress electron transfer. To model NADPH in a productive conformation for hydride transfer to FAD, we superimposed the structures of nNOSred and the CYPOR W677G mutant (26). This superposition (Fig. 4a) suggests that, in nNOS, structural re-
arrangements necessary for productive electron transfer include significant displacements of residues 1351–1353, Gly$^{1396}$, and the first 2 residues of the regulatory NOSS-specific C-terminal tail (1397–1398), as well as movement of the preceding Phe$^{1396}$ aromatic side chain (Fig. 4b).

From the structure-based sequence alignment of NOS isoforms (Fig. 2), we define two distinct regions of the C-
terminal tail: the N-terminal segment (CTN, residues 1397–1413) is sequence conserved (59% identity in the cNOSs and 23% homology with iNOS) and the C-terminal segment (CTC, residues 1414–1429) is variable in length (25 in eNOS and 4 in iNOS) and sequence (12.5% identity in the cNOSs) among isoforms. Previous biochemical data indicate that CaM-bound trun-
cation mutants of eNOS (CA27), lacking the CT$C$ (and preceding two residues) mimic the NO synthesis activity of full-length nNOS (51), whereas cNOSs mutants lacking both the CT$N$ and the CT$C$ synthesis less than half as much NO (52). In this nNOSred structure, CT$N$ adopts a well ordered $\alpha$-helical conformation whereas CT$C$ is poorly ordered. In con-
trast to earlier suggestions (52), CT$C$ does not fit between the FAD and FMN cofactors. Strong, but disconnected, peaks in difference electron density maps suggest that this peptide ex-
tends across the FMN domain toward its N terminus, directly adjacent to the CaM-binding region. This position is consistent with the proposal (51) that CT$C$ interferes with CaM binding.

The well ordered CT$N$ residues in the NOSSred structure hydrogen bond to the NADP(H) 2’ phosphate as well as resi-
dues from both the FMN- and the NADPH-binding domains (Fig. 4b). Selective recognition for NADPH versus NADH in-
volves interactions between the NADP 2’-phosphate and conserved residues (Ser$^{1315}$-Arg$^{1314}$ in nNOS) in related reducta-
ses (26, 53). In the non-stacking conformation found in the NOSSred structure, NADPH binding and selectivity also ap-
pear to rely on interactions between its 2’-phosphate group and CT$N$ residue Arg$^{1400}$. We tested and confirmed this hypothesis with the R1400E mutant, which exhibits a 4-fold increase in
the $K_m$ value for NADPH when compared with wild type. Recent studies further show that NADPH binding to CaM-free NOS paradoxically “locks” the FMN domain and prohibits inter-module electron transfer (27). Our results provide a structural basis that helps resolve this paradox. We propose that, in the absence of CaM, the ionic interactions between NADPH and the cNOSs-conserved Arg$_{1400}$ residue (Ser in iNOS) help to orient the C-terminal tail, and thus repress NOS by locking the FMN domain into the observed electron-accepting position. We tested this hypothesis with the charge-change mutant R1400E, in which Glu$_{1400}$ should repel rather than attract NADPH, thus destabilizing the observed CT$_N$ position and relieving repression. Indeed, rates of electron transfer from NADPH through nNOS-red structure were markedly (5-fold) increased in the absence of Ca$_2^+/$/CaM, showing significant relief of repression. CaM-free activity of this mutant is two-thirds that of CaM-bound wild-type holo-NOS (Fig. 3b), despite decreased affinity for NADPH. We therefore conclude that cNOS-specific Arg$_{1400}$ helps maintain the repressed or locked electron-accepting position of the FMN domain observed here. Together with other biochemical data (50, 54), the structural and mutational studies presented here suggest that NADPH, Ph$_{3}^{1395}$, the newly identified Arg$_{1400}$ and the C-terminal tail function synergistically to repress NOS activity in the absence of CaM.

**Implications for Ser$_{1412}$ Phosphorylation in cNOSs—**The nNOS-red structure provides an informed basis to evaluate the consequences of the regulatory phosphorylation of Ser$_{1412}$ on NOS activity. cNOSs contain a C-terminal Akt/PKB-dependent phosphorylation site $$(^{1407}RXXX(S/T)^{1412})$$ In the structure, Ser$_{1412}$ is positioned at the end of the CT$_N$ helix, which lies in a negatively charged groove at the surface of NOS-red (Fig. 4c). The negative charge introduced by the phosphate group or the Ser $\rightarrow$ Asp mutation (55), to mimic the phosphoserine, would repel both the partial negative charge of the $\alpha$-helix dipole and the nearby conserved negatively charged residues (Fig. 4, b and c). Thus, Ser$_{1412}$ phosphorylation would destabilize and displace the regulatory C-terminal tail to relieve repression of NO synthesis. Indeed, mutation of this site to Asp increases electron transfer rates both into and out of NOS-red in CaM-free NOS (55). Our structure-based interpretation provides an attractive molecular mechanism whereby phosphorylation of a single residue induces large conformational changes that de-repress NOS.

**Concluding Remarks—**The results presented here change current understanding of NOS structure-function and regulation and provide testable hypotheses for new lines of experiments. The crystallographic structure of the nNOS-red dimer reveals the conformations and positions for the FMN-binding domains in electron-accepting positions, as well as key parts of NOS-specific regulatory elements, including the autoinhibitory insert, the C-terminal tail, and its phosphorylation site. The striking resemblance of the newly identified autoinhibitory helix with canonical CaM-binding motifs suggests that cNOS inhibition may occur through direct CaM binding. Our structural and mutagenesis results identify a new isozyme-specific residue (Arg$_{1400}$) involved in the repression of NOS activity. Importantly, the nNOS-red structure helps resolve the paradox of the “NADPH-induced lock,” by revealing how co-substrate NADPH, Ph$_{3}^{1395}$, Arg$_{1400}$, and the CT$_N$ link together the FMN- and NADPH-binding domains and ultimately lock the FMN-binding domain into its electron-accepting position. At the end of the CT$_N$, the cNOS-specific Ser$_{1412}$ phosphorylation site is strategically positioned to repel nearby negatively charged groups from the FMN-binding domain.

In light of biochemical results, we assembled the structures of the NOSox and NOS-red dimers and a CaM: NOS-peptide complex to model the holo-NOS enzyme. In NOS and likely in related reductases, our analysis suggests a swinging FMN-domain mechanism, whereby electron shuttling occurs through large scale rearrangements of the two FMN domains, alternating between electron-accepting and electron-donating positions. In NOS, where the two redoxt partners of the FMN shuttles are located on adjacent polyproline, dimerization provides a means for fine-tuning the electron transfer mechanism. In cNOSs, our results argue that both Ca$_2^+/$/CaM binding and Ser$_{1412}$ phosphorylation de-repress NO synthesis by displacing the C-terminal tail, thus unlocking the FMN-binding domain to promote inter-module electron transfer.

Significant progress in understanding the structural chemistry of NOSox has revealed the striking similarity of the three NOS isoforms. This poses a challenge for the design of isozyme-specific inhibitors for the treatment of NOS-related pathologies, including stroke, rheumatoid arthritis, or hypertension. The structural and mutagenesis studies on nNOS-red reveal significant isozyme-specific features involved in regulating rate-limiting electron transfer for NO synthesis, and suggest an alternative design strategy. Ligands that favor (or disfavor) the electron-accepting (or electron-donating) position of the FMN-binding domain hold the potential to slow or preclude NO synthesis in an isozyme-specific manner by slowing or stopping electron transfer.

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