Recruitment of governing elements for electron transfer in the nitric oxide synthase family

M. Jáchymová*†, P. Martásek‡, S. Panda*, L. J. Roman*, M. Panda*, T. M. Shea*, Y. Ishimura*, J.-J. P. Kim§, and B. S. S. Masters*¶

*Department of Biochemistry, University of Texas Health Science Center, San Antonio, TX 78229-3900; ‡Department of Pediatrics, First School of Medicine, Charles University, Prague 2 121 09, Czech Republic; and §Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI 53226-4801

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At least three building blocks are responsible for the molecular basis of the modulation of electron transfer in nitric oxide synthase (NOS) isoforms: the calmodulin-binding sequence, the C-terminal extension, and the autoregulatory loop in the reductase domain. We have attempted to impart the control conferred by the C termini of NOS to cytochrome P450 oxidoreductase (CYPOR), which contains none of these regulatory elements. The effect of these C termini on the properties of CYPOR sheds light on the possible evolutionary origin of NOS and addresses the recruitment of new peptides on the development of new functions for CYPOR. The C termini of NOSs modulate flavoprotein-mediated electron transfer to various electron acceptors. The reduction of the artificial electron acceptors cytochrome *c*, 2,6-dichlorophenolindophenol, and ferricyanide was inhibited by the addition of any of these C termini to CYPOR, whereas the reduction of molecular O_2 was increased. This suggests a shift in the rate-limiting step, indicating that the NOS C termini interrupt electron flux between flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) and/or the electron acceptors. The modulation of CYPOR by the addition of the NOS C termini is also supported by flavin reoxidation and fluorescence-quenching studies and antibody recognition of the C-terminal extension. These experiments support the origin of the NOS enzymes from modules consisting of a heme domain and CYPOR or ferredoxin–$NADP^+$ reductase- and flavodoxin-like subdomains that constitute CYPOR, followed by further recruitment of smaller modulating elements into the flavin-binding domains.

flavoprotein | NADPH-cytochrome P450 reductase | protein evolution

Heme enzymes use bound iron protoporphyrin derivatives to catalyze a variety of oxygen-dependent enzymatic reactions. Among them, the cytochromes P450 constitute a vast family of heme thiolate-ligated enzymes with unique redox properties. Many of these enzymes are able to activate molecular oxygen into a reactive oxygenating species that participates in the insertion of oxygen into a large number of substrates (1). Flavin-containing [flavin adenine dinucleotide (FAD) and/or flavin mononucleotide (FMN)] enzymes also play unique roles in biological catalysis in their performance of a wide variety of biochemical processes (2), including single-electron transfer, activation of molecular oxygen, dehydrogenation of various substrates, and others. The diflavin enzyme, NADPH-cytochrome P450 reductase [CYPOR (EC 1.6.2.4)], supplies electrons for eukaryotic cytochrome P450 enzymes (3), heme oxygenase (4), and squalene monooxygenase (5). Mutations in human CYPOR have been linked to Antley–Bixler syndrome, with or without disordered steroidogenesis (6), and CYPOR knockouts in mice are embryonically lethal (7, 8). NADPH transfers two electrons in the form of a hydride ion to the FAD, followed by the transfer of a single electron at a time to FMN, producing $FMNH_2$, which serves as a donor for the protein acceptor. CYPOR is considered to be a product of the fusion of ancestral flavin-containing domains related to flavodoxin and ferredoxin–$NADP^+$-oxidoreductase (9–12) (Fig. 1*a*).

All microsomal human cytochromes P450, as well as heme oxygenase, which is crucial for heme degradation and iron homeostasis, depend absolutely on their interaction with CYPOR for activity, but it is these other enzymes, rather than CYPOR, that regulate electron flux in these systems. For example, the heme of cytochromes P450 will not accept an electron from CYPOR until substrate is bound, and in some cases, an additional protein, cytochrome b5, is required for electron transfer to occur (1). Because CYPOR serves so many different enzymes, and electron transfer needs to be geared toward the catalytic requirements of each individual system, it makes sense for the electron acceptor to impart control over the process.

The proposal that this diflavin enzyme, CYPOR, is in turn the precursor of further fusion products that gain new functions is a subject of intensive research (13–15). The family of nitric oxide synthases (NOS) combines the unique properties of heme- and flavin-containing proteins into one polypeptide chain to achieve production, by very intricately controlled processes, of the gaseous messenger, NO. NO functions in cell-to-cell signaling in most tissues, whereas deviant production of NOS plays a role in an increasing number of pathologies, including atherosclerosis, septic shock, cancer, and neurodegeneration (16, 17). Therefore, by necessity, synthesis of NO by the family of NOS is well controlled by a variety of complex mechanisms, including protein–protein interactions, cofactor and substrate compartmentalization or availability, and posttranslational modifications. To meet tissue-specific needs for NO production, NOS isoforms are equipped with specific sequences in their N-terminal regions, which differ in neuronal, endothelial, and inducible forms, directing them to specific compartments within cells. In addition, the C-terminal domains containing the flavin prosthetic groups contain unique regulatory sequences. The localization, enzymology, regulation, and structural and functional aspects of the NOS family have been comprehensively reviewed (14, 18, 19). The search for differences in the oxygenase domains among the different NOS isoforms led to the elucidation of the heme domain structures of all three (18, 20–22). The remarkable similarity of these structures has redirected the interest of our laboratory (14, 15, 23–25) and the laboratories of others (26–29) to the role of the flavoprotein domains of NOS isoforms in their intrinsic and extrinsic regulation. Because the proximal electron donor and acceptor are on the same polypeptide chain in the NOSs, and thus each potential acceptor is linked to its

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Abbreviations: NOS, NO synthase; nNOS, neuronal NOS isoform; eNOS, endothelial cell NOS isoform; iNOS, macrophage inducible NOS isoform; CYPOR, cytochrome P450 oxidoreductase; CYPOR-iNOS^T, CYPOR + iNOS C terminus; CYPOR-nNOS^T, CYPOR + nNOS C terminus; CYPOR-eNOS^T, CYPOR + eNOS C terminus; DCIP, 2,6-dichlorophenolindophenol; CaM, calmodulin.

†Present address: Institute of Clinical Biochemistry and Laboratory Medicine, Faculty General Hospital, Prague 2 121 09, Czech Republic.

¶To whom correspondence should be addressed. E-mail: masters@uthscsa.edu.

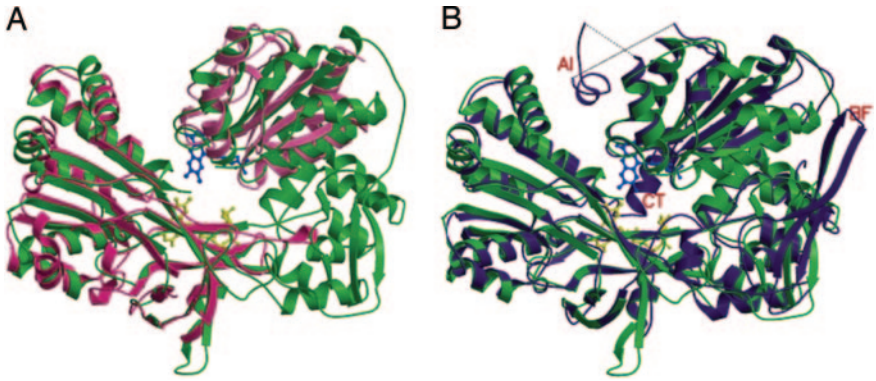

Fig. 1. Ribbon diagrams of flavodoxin (light pink), ferredoxin-NADP+ reductase (FNR; dark pink), CYPOR (green), and the reductase domain of rat nNOS (dark purple). (*A*) Overlay of flavodoxin and FNR onto the CYPOR structure. (*B*) Overlay of CYPOR and the reductase domain of nNOS. The three major structural differences between nNOS and CYPOR are shown: the C-terminal extension of nNOS (CT), the autoregulatory insert (AI), and β-finger (BF). Dotted lines indicate segments that are not visible in the crystal structure of the reductase domain of nNOS.

donor, unlike CYPOR and the cytochromes P450, regulation of electron transfer by the reductase domain is reasonable. In fact, the rate of electron transfer from the reductase to the heme domain determines the turnover rates of NO production in the various NOSs (30), and thus alterations, such as truncations or insertions in the reductase domain, might confer the ability to regulate electron transfer through this domain. The remarkable sequence identity (58%) between the C-terminal 641 amino acids of neuronal NOS and the entire sequence of CYPOR was reported by Bredt *et al.* (31). It is now generally accepted that the CYPOR gene was recruited into or fused with the NOS genes (13, 32), which in prokaryotes produce heme domain-only proteins, resulting in efficient electron flow for NO synthesis. During catalysis by both CYPOR/P450 and NOS isoforms, the electrons flow from NADPH to FAD, to FMN, and finally to the heme iron, where substrate monooxygenation is catalyzed (13, 32–38).

The x-ray structures of CYPOR (11, 39), the NADPH/FAD-binding domain of neuronal NOS (40), and the neuronal NOS reductase domain (41) present the opportunity to address similarities and differences among these enzymes.

All NOS reductase domains are distinct from CYPOR due to longer C-terminal extensions (24, 25), consisting of 21 amino acids in the inducible NOS isoform (iNOS), 33 in the neuronal NOS isoform (nNOS), and 42 in the endothelial cell NOS isoform (eNOS) (Fig. 2). The rate of NO synthesis is inversely related to the length of the C terminus of the respective NOS isoform. It has been proposed by Roman *et al.* (24, 25) that electron flow between the two flavin moieties in NOS is gated by these C-terminal additions. Also, the ability, particularly in eNOS, to control the production of NO through phosphorylation of Ser-1197 in the C-terminal tail (26, 27, 42) is extremely important for function. Recent reports have addressed the consequences of the truncation of the C-terminal sequences of NOS on the various activities of the enzyme (24, 25, 29, 43).

The remarkable sequence homology between the flavoprotein domains of the isoforms of the NO synthases and CYPOR presents a unique opportunity to explore evolutionary mechanisms that produce new functional enzymes from preexisting modules. Therefore, in the present study, the effect of the addition of the C-terminal peptides, specific for each isoform of NOS, on the function and properties of CYPOR was determined, indicating the possible evolutionary origin(s) of NOS and also addressing the effect of recruitment of new peptides in the development of new functions for CYPOR.

Materials and Methods

Chemicals, Enzymes and Plasmids. NADPH, horse heart cytochrome *c,* D(+) glucose, leupeptin, pepstatin A, lysozyme, 2-mercaptoethanol, potassium ferricyanide, and 2,6-dichlorophenolindophenol (DCIP) were purchased from Sigma. Isopropyl β-D-thiogalactopyranoside was from Alexis Biochemical. Hepes, Tris, and agarose were obtained from Fisher. 2′5′-ADP Sepharose 4B was from Amersham Pharmacia Biotech. *Ampli Tag Gold* polymerase and restriction endonucleases were obtained from Roche Applied Science, New England Biolabs, or Promega. The pOR 263 vector, containing the rat CYPOR cDNA, was kindly provided by Charles B. Kasper and Anna Shen (McArdle Laboratory for Cancer Research, University of Wisconsin, Madison). The pCWori+ vector was a gift of Michael Waterman (Vanderbilt University, Nashville, TN).

Molecular Biology. The construct for the *Escherichia coli* expression of the soluble form of rat CYPOR, CYPOR-soluble-pCWori+, was made by truncating the N terminus by 56 amino acids to remove the hydrophobic membrane-binding sequence. The constructs CYPOR-iNOS-pCWori+ (+21 amino acids), CYPOR-nNOS-pCWori+ (+33 amino acids), and CYPOR-eNOS-pCWori+ (+42 amino acids), for the expression of CYPOR/tail chimeric proteins in *E. coli*, are depicted in Fig. 2. See supporting information, which is published on the PNAS web site, for a more detailed description of primers and methods used.

Fig. 2. Alignment of C-terminal amino acid sequences of rat CYPOR, bovine eNOS, rat nNOS and murine iNOS.

Expression and Purification of CYPOR and CYPOR plus C Termini (CYPOR-nNOS^T, -iNOS^T, and -eNOS^T). The expression and purification of the constructs were performed as reported (44, 45), with slight modifications. After the affinity chromatography step (2′,5′-ADP-Sepharose 4B), each protein was further purified by FPLC by using a Superose 6 10/30 column (Amersham Pharmacia), producing a monomeric preparation. CYPOR-soluble, CYPOR-iNOS^T, CYPOR-nNOS^T, and CYPOR-eNOS^T were expressed and purified simultaneously by using identical conditions.

NADPH Oxidation, Cytochrome *c* Reduction, and Ferricyanide Reduction. The ability of CYPOR and CYPOR plus NOS C termini chimeras to reduce artificial electron acceptors, cytochrome *c*, DCIP, and ferricyanide, was studied as described (46, 47),

Reoxidation of Reduced Flavins. The reoxidation of reduced flavins was monitored at 485 nm for all proteins studied, as described (25).

Flavin Fluorometry. Fluorescence spectra were recorded by using a Fluorolog-3 (Jobin Yvon Spex, Edison, NJ) spectrofluorometer. The intensities recorded in the instrument are linear up to 4.0×10^6 counts per sec^{-1} (cps). However, in our experiments, we have limited our investigation to $\approx 1.0 \times 10^6$ cps to avoid any nonlinearity in the intensities. The excitation was at 457 nm (path length 5 mm, 2-nm band pass), and emission was recorded in the range 470–800 nm (path length 2 mm, 5-nm band pass). The buffer was 50 mM Tris·HCl/100 mM NaCl, pH 7.4, at 25°C, and was filtered by using 0.2-μm Corning filters and degassed.

Relative concentrations of the total flavins present in the proteins were estimated by using a method similar to that reported by Faeder and Siegel (48). Protein samples (1 μM) in 1 ml of 50 mM Tris·HCl/100 mM NaCl, pH 7.4 buffer were placed in aluminum-foil-covered centrifuge tubes and heated over boiling water for 10 min. This released all of the bound flavins. Tubes were cooled rapidly and centrifuged by using an Eppendorf 5415C centrifuge at $14{,}000 \times g$ for 20 min to remove denatured protein. The fluorescence intensities of the supernatants were measured by using a Fluorolog-3 spectrofluorometer with 450 nm excitation. The λ_{max} values of the emission spectra were at 523 nm, at which the intensities were measured.

Western Blotting. SDS/PAGE (49) and electrotransfer to a 0.2-μm poly(vinylidene difluoride) membrane (50) were performed as described. Detection was performed with a monoclonal antibody to bovine eNOS (Sigma, N9532), which recognizes the C-terminal epitope (amino acids 1185–1205), and visualized with an alkaline phosphatase color reagent.

Results

Fig. 1*B* shows a comparison of the structures of CYPOR and the reductase domain of nNOS to illustrate the remarkable similarity in the 3D structures of these FAD-/FMN-binding proteins (11, 41). The similarities between the FAD- and NADP$^+$-binding domains of these enzymes have been discussed in detail (40), and previous work from this laboratory has shown that all three NOS isoforms contain from 21 to 42 additional residues in their C termini extending from the glycine residue homologous to serine 677 in CYPOR that modulate electron flux (24, 25). It was not possible to examine the structure of the C terminus of the nNOS FAD/NADPH-binding domain due to proteolytic clipping of the 22 terminal residues in this protein (40). Furthermore, the remaining 10 residues extending from valine 1397 are disordered in the structure, possibly indicating a certain flexibility in this region. It was possible, however, to suggest that these C termini form a "separate structural domain" that most likely "lies near the interface between the two flavin domains." A recent publication (41) in which the FMN-/FAD-/NADPH-binding domain (so-called $nNOS_{red}$) structure was reported, confirms this suggestion: "The regulatory C-terminal tail similarly contains an α-helix (residues 1401–1412), which fits within a negatively charged groove across the FAD/FMN interface." The structural comparisons shown in the present report also confirm that these residues have the potential to confer additional functional control on the NOS isoforms. This was demonstrated amply by the work of Roman and colleagues (24, 25) prior to any structural information and was confirmed and extended by other laboratories (29, 43).

Table 1. Reduction of artificial electron acceptors by CYPOR-soluble, CYPOR-eNOS^T, CYPOR-nNOS^T, and CYPOR-iNOS^T

Enzyme constructs	Turnover numbers, min^{-1}		
	Cytochrome *c*	DCIP	Ferricyanide
CYPOR-soluble	3,273 ± 201	1,669 ± 349	6,563 ± 607
CYPOR-iNOS^T	2,613 ± 133	1,415 ± 236	6,381 ± 479
CYPOR-nNOS^T	2,428 ± 211	1,246 ± 135	5,352 ± 860
CYPOR-eNOS^T	1,884 ± 96	1,030 ± 215	5,403 ± 934

These data are expressed as mean ± SD. At least triplicate determinations were performed with each of a minimum of five different batches of enzymes for each data set.

The sequence alignments of the C-terminal amino acids of NOS isoforms and CYPOR are shown in Fig. 2. Considering the possible convergent evolution of function of the NOS isoforms from simpler proteins (10, 13, 31), such as CYPOR, these C termini were added to the CYPOR structures, as detailed in supporting information. The purity of the preparations of these constructs and their virtually identical spectral properties are also detailed in supporting information.

If the C termini act as regulatory appendages of the NOS flavoprotein domains (24, 25), one would expect consequences in catalytic function from their attachment to the CYPOR progenitor protein. Therefore, the flavin-mediated reduction of artificial electron acceptors was determined for each of these constructs (Table 1). Measurement of NADPH-cytochrome *c* and DCIP reductase activities of these preparations revealed increasing inhibition of CYPOR activity (also shown for comparison) with increasing length of the C termini, exactly correlating with the relative reductase activities of the NOS isoforms themselves. In the case of cytochrome *c* and DCIP reduction, it has been shown that these activities are mediated through the FMN domain of CYPOR (34). Separate roles for FMN and FAD in catalysis by liver microsomal NADPH-cytochrome P450 reductase and NOS (32, 51, 52) have already been proposed and demonstrated, so the addition of the C termini had the predicted effect. The reduction of potassium ferricyanide, however, has been shown to be mediated through the FAD domain of these enzymes (15). As a result, the addition of the shortest (iNOS) C terminus had no effect on ferricyanide reduction, whereas the addition of either the nNOS or eNOS C terminus produced minimal effects. Due to the small molar extinction coefficient for potassium ferricyanide, the statistical significance of these effects is questionable. Thus, as shown by Roman *et al.* (24, 25) for NOSs, the C-terminal tails have little effect on the first step of electron transfer from NADPH to FAD but exert more influence on electron transfer between the flavins or to the final acceptor. These results are in full agreement with the regulatory role proposed for the C termini of NOS isoforms by Roman *et al.* (24, 25).

The interaction of the C termini with the interface between the flavin-binding domains could also be expected to affect the interaction of the flavins with O_2 as an electron acceptor (24, 25, 41). In fact, attachment of these residues resulted in the doubling of NADPH oxidation (1.3 ± 0.1 min^{-1} for wild-type CYPOR as opposed to 2.4 ± 0.2, 2.4 ± 0.1, and 1.9 ± 0.2 min^{-1} for CYPOR-iNOS^T, CYPOR-nNOS^T, and CYPOR-eNOS^T, respectively, in the presence of 50 μM NADPH), indicating that the presence of these C termini somehow alters the exposure to and/or reduction of

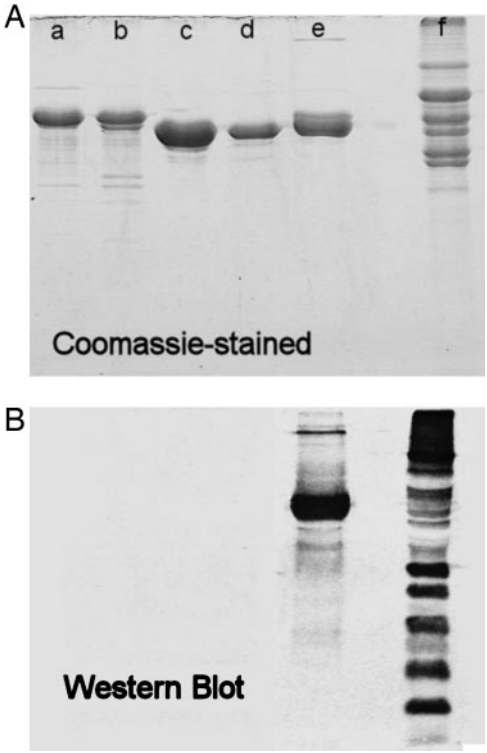

Fig. 3. Detection of eNOS C terminus by a monoclonal antibody, directed to the intact enzyme tail. Enzyme preparations (lanes a–f) were subjected to 10% SDS/PAGE and either Coomassie-stained (*A*) or subjected to Western analysis (*B*), where immunoreactive protein was visualized via alkaline phosphatase reagent. Lanes: a and b, full-length CYPOR; c, CYPOR-iNOS^T; d, CYPOR-nNOS^T; e, CYPOR-eNOS^T; f, eNOS, which was partially degraded so that sensitivity of the antibody to protein fragments could be assessed. Only CYPOR-eNosT reacted with the monoclonal antibody.

molecular O_2. To examine the redox states of the flavins during the reoxidation phase, the three chimeric constructs were compared with CYPOR after reduction by NADPH and subsequent reoxidation in air. CYPOR exhibits a very stable semiquinone state, which serves as the oxidized partner in its catalytic cycle (53, 54). The semiquinone states appear to be equally stable for all three chimeras of CYPOR containing the NOS C termini (data not shown).

In the absence of a crystal structure of the CYPOR/NOS constructs, it was necessary to affirm that a wild-type epitope of the C terminus of a NOS isoform was preserved in these new constructs, i.e., that structural elements of this region were maintained in both NOS and CYPOR. To this end, wild-type CYPOR and the three constructs containing the C termini of each of the isoforms were probed with a monoclonal antibody that recognizes exclusively the eNOS C terminus. Fig. 3 illustrates the specificity of this antibody to the C terminus of eNOS, demonstrating that the 3D epitope remained intact, even when the C terminus was added to another protein.

Both NADPH and oxidized flavins exhibit intrinsic fluorescence (55). When flavins in aqueous solutions are excited at 450 or 370 nm, they exhibit fluorescence maxima ≈520 nm (56). Flavins bound to a protein can exhibit either enhanced or quenched fluorescence to a degree that is both specific and characteristic of the particular protein. The fluorescence scans of CYPOR and CYPOR with nNOS, eNOS, and iNOS tails are shown in Fig. 4. A dramatic change in the fluorescence intensities between CYPOR and those with different NOS tails can be noted from the observed spectra recorded under identical reaction conditions, indicating that the tail regions have an effect on CYPOR conformation.

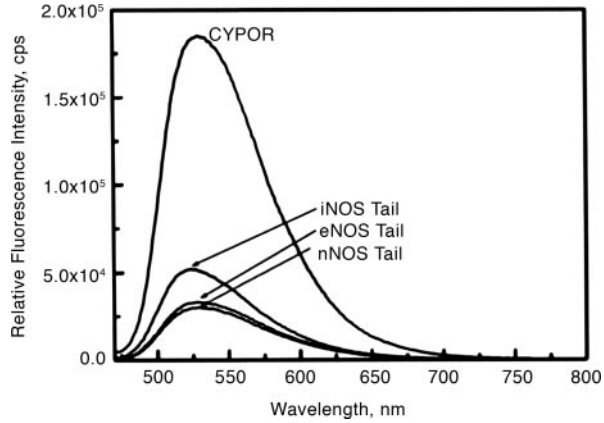

Fig. 4. Fluorescence emission spectra of CYPOR, and CYPOR with nNOS, eNOS, and iNOS tails. The relative emission intensities at the λ_{max} of the spectra were 185,280, 52,140, 33,130, and 30,070 counts per second for CYPOR, CYPOR-iNOS^T, CYPOR-eNOS^T, and CYPOR-nNOS^T tails, respectively. The λ_{max} values for the maxima were 530 nm (CYPOR), 523 nm (CYPOR –iNOS^T), 528 nm (CYPOR-eNOS^T), and 530 nm (CYPOR-nNOS^T). The protein concentrations were 1 μM each, and reactions were performed as described in *Materials and Methods*.

Discussion

The molecular basis of the differences in electron transfer among the NOS isoforms resides in the calmodulin (CaM)-binding sequence, the C-terminal extensions, and the autoregulatory inserts in the FMN-binding module of the reductase domain. In this manuscript, we have examined the functional effects of attaching the C termini of the three isoforms, respectively, to CYPOR, which does not contain any of these regulatory elements. The C termini of NOS isoforms down-regulate their respective catalytic activities (24, 25). These C termini, varying in length from 21 to 42 residues, extend beyond the glycine residue homologous to the C-terminal serine in CYPOR. The rates of electron transfer catalyzed by the three NOS isoforms are directly related to the lengths of the C termini attached to their respective flavoprotein domains, i.e., endothelial NOS, with a 42-residue C terminus is the slowest and iNOS with a 21-residue C terminus is the fastest, with nNOS falling in between these rates (24, 25). In a study in which the flavin-binding domains of nNOS and CYPOR were swapped (15), the FAD/NADPH-binding domain of CYPOR could substitute completely in nNOS, producing a chimera that is more active in both flavoprotein-mediated electron transfer and in NO production. As in the C-terminal deletion mutant, cytochrome *c* reduction by this chimera is inhibited by Ca^{2+}/CaM binding but activated toward NO production. However, a chimera containing the FMN-binding domain of CYPOR, with the remainder of the molecule being nNOS-derived, was not competent in producing an active neuronal NOS and was not modulated by CaM.

These studies indicate that the C termini of NOS isoforms regulate electron transfer to various electron acceptors catalyzed by these CYPOR/NOS chimeras. The rates of reduction of cytochrome *c* and DCIP were decreased proportional to the length of the C terminus attached to CYPOR, and reduction of molecular O_2 was increased in these chimeras, suggesting a shift in the rate-limiting step of the flavoprotein-catalyzed activities. An interruption of the flow of electrons between FAD and FMN in the CYPOR/NOS chimeras containing these C-terminal tails could explain these results, because the reduction of both cytochrome *c* and DCIP requires FMN, and molecular O_2 is reduced by FAD in CYPOR (34, 57). There was little or no effect on ferricyanide reduction, also mediated by FAD, catalyzed by the CYPOR/NOS chimeras. These data explain to some degree the differences

observed among the NOS isoforms with respect to NO production and artificial electron acceptor reduction.

Catalysis of electron transfer by CYPOR involves shuttling of electrons between the reduced and semiquinone states of FMN during catalysis (58). Contrary to the situation in NOS or CYP102, in which the flavoprotein and heme protein are components of a single polypeptide chain, microsomal CYPOR supplies electrons as a partner in a multienzyme system. Regulation of the cytochrome P450 multienzyme systems depends upon the levels of the components and the interactions between them. CYPOR is not modulated except at the transcriptional level, e.g., by phenobarbital pretreatment (59). The data support the hypothesis that, to confer intrinsic regulation to the NOS isoforms, additional residues have been inserted into the structures of their progenitors.

In the two published structures showing the FAD-binding domain of nNOS (40, 41), the C-terminal tail, although truncated, lies across the FAD/FMN interface. Zhang *et al.* (40) proposed that the C-terminal subdomain "modulates the interflavin distance and, in response to Ca^{+2}/CaM concentration, regulates the electron flow between the two flavins and from FMN by controlling the access to FMN of various electron transfer partners, including cytochrome *c* and the oxidase domain." More recently, Garcin *et al.* (41) reported that the N-terminal segment of the C terminus of nNOSred, containing both FAD/NADPH- and FMN-binding domains, contains a well ordered α-helical conformation that lies across the FAD/FMN interface. In the experiments reported here, the ability of the eNOS tail to be recognized by a monoclonal antibody raised to this region, when attached to the CYPOR construct, indicated that the wild-type epitope has been preserved in the CYPOR-eNOS^T construct and that the 3D structure of the C terminus of the CYPOR-eNOS^T construct is similar, if not identical, to that attached to wild-type eNOS.

The penultimate and ultimate residues of CYPOR, Trp/Ser, perform important functions (11). The conformation of the nicotinamide-ribose moiety of bound $NADP^+$ was determined to be different in two different molecules of CYPOR in the asymmetric unit (11). However, in either of these conformations, the C_4 atom of the nicotinamide (the hydride ion donor) and the N_5 atom of the isoalloxazine ring of FAD (the hydride ion acceptor) are too distant from each other to accommodate hydride transfer. Because the structures were obtained with $NADP^+$ bound, it was proposed that they represented the enzyme–product complex (11). By rotation of the nicotinamide moiety around the pyrophosphate bond, it can effectively displace Trp-677 during catalysis, permitting transfer of the hydride ion from NADPH to the N_5 position of FAD (39), similar to ferredoxin-$NADP^+$ reductase (60). This action requires flexibility of the CYPOR molecule and, presumably, for the NOS isoforms as well.

The present studies address an intraprotein interaction that influences CYPOR catalysis by adding the C termini of NOS isoforms to the CYPOR structure at the Ser-678 residue. Examining the sequence homologies of the known NOS enzymes across phylogeny, one finds an absolute conservation of both Phe and Gly, without exception, in the C terminus.

Phe is a stacking residue on the *re* face of the FAD isoalloxazine ring characteristic of all known NOSs (40). Mutations of the homologous residue (F1395) in nNOS (62) to the nonaromatic residue serine, but not to the aromatic residue tyrosine, affected cytochrome *c*, DCIP, and ferricyanide reduction and flavin reduction kinetics, confirming that the aromaticity of the residue in this position is essential. In addition, and unlike wild-type nNOS, the Phe-1395-Ser mutant increased its activity with NADH, which eliminates the 2′-phosphate as a factor in binding interactions. Reports that NADPH or $NADP^+$ must be bound for electron flow to be repressed in CaM-free nNOS provide a clue to Phe-1395 function in nNOS (61). Adak *et al.* (62) suggest that the phenyl side chain of Phe-1395 can flip away from FAD to accommodate NADPH or $NADP^+$, and that the phenyl side chain movement is a component of a conformational trigger mechanism that regulates electron transfer out of the nNOS reductase domain, similar to the mechanisms described for CYPOR (39) and ferredoxin-$NADP^+$ reductase (60). Such use of the conserved aromatic residue would be in keeping with other unique aspects of the nNOS flavoprotein and certainly deserves further investigation. In one mechanism described by Adak *et al.* (62), the C-terminal element would stabilize the Phe-1395-FAD stacking interaction, making the formation of a productive nicotinamide-FAD stacking interaction (requiring the phenyl group to move away) rate-limiting. In an alternative mechanism, the phenyl side chain of Phe-1395 could stabilize a stacking geometry between FAD and nicotinamide in concert with the C terminus that was not optimal for electron transfer in CaM-free nNOS. The binding of CaM would then move the C-terminal control element and relieve the negative modulation of NOS activity, as originally proposed by Roman *et al.* (24, 25).

The present results show that, with tryptophan (Trp-677) being the stacking residue on the *re* side of CYPOR, attaching the C termini of the NOS isoforms individually to the C terminus of CYPOR inhibits the rates of electron transfer to cytochrome *c* and DCIP. On the other hand, NADPH oxidation was increased, and ferricyanide reduction was only minimally affected, indicating that these effects are manifest differentially depending upon which flavin, FAD or FMN, was mediating the electron transfer. It is obvious that the large extensions, from 21 to 42 additional residues, could influence the movement of the Trp-677 stacking residue and therefore inhibit NADPH-cytochrome P450 reductase, which is not affected by CaM binding or additional autoregulatory loops in its structure.

It is tempting to speculate that the helical C-terminal extension, as seen in the structure of the nNOS reductase domain (41), is in a position to influence the interaction or alignment of the NOS prosthetic flavins through interaction with other residues in proximity to the flavins. In the case of CYPOR, the aromatic residue at the C terminus (Trp-677) produces significant conformational changes upon binding of NADPH, and there is significant mobility between the two flavin domains during catalysis (39). It is reasonable to assume that the role of Phe-1395 in nNOS is analogous to that of Trp-677 of CYPOR, i.e., regulating NADPH binding. Furthermore, our results here strongly suggest that, in the NOS isozymes, the movements between the two flavin domains are further regulated by their respective C termini, and that the extent of the movements is greater than that of CYPOR.

CYPOR is a very weak NADPH oxidase, catalyzing the oxidation of NADPH with O_2 as receptor at a rate 2 orders of magnitude slower than that of cytochrome *c* reduction. On the other hand, the NOS isoforms catalyze NADPH oxidation somewhat faster (1 or 2 orders of magnitude less than cytochrome *c* reduction in the presence and absence of Ca^{+2}/CaM, repectively), and these rates are influenced by the presence of autoregulatory elements and the binding of Ca^{+2}/CaM (24, 25). In addition, alteration in interflavin distance and the relative orientation between the two flavin rings, negatively modulated by the respective C terminus, might be another mechanism by which electron transfer between the two flavins is regulated (24, 25). Thus, the shorter C termini, i.e., in CYPOR and iNOS, produce tighter electron flow between the flavins and weaker NADPH oxidase activity. nNOSred and eNOSred, which have relatively longer C termini, behave as though FAD acts more independently of its redox partner, thereby producing the "uncoupled" oxidation of NADPH and the production of reduced O_2 species at a more rapid rate. The attachment of the C termini of NOS isoforms to CYPOR suggests that the interaction of these C termini with residues in the vicinity of the interface between the flavins slows electron flux between them, as revealed in both cytochrome *c*

and DCIP reduction, but the reduction of ferricyanide does not require electron flow between the flavins, and the reduction of O_2, in the absence of artificial electron acceptors, can proceed at a more rapid rate.

Regarding the conserved glycine residue, the flexibility of the bond around iNOS Gly-1123, nNOS Gly-1396, and eNOS Gly-116 presumably permits the C termini to move relatively freely. The presence of the serine residue in CYPOR confers less flexibility to the C termini of the CYPOR/NOS chimeras. The C termini attached to the highly conserved glycine also possess interesting sequences. Comparison of the constitutive NOS C termini reveals remarkable similarities among those residues immediately adjacent to the conserved glycine:

1161 LTLRTQEVTSRIRTQS..... human eNOS
1397 VTLRTYEVTNRLRSES..... rat nNOS
This similarity is not shared in the iNOS C terminus:
1124 AVFSYGAKKGSALEEP..... murine iNOS.

It is probable that these residues play an important differential functional role in each of these isoforms. Further studies, including the crystal structures of the C terminus-intact flavoprotein domains of NOS isozymes, will reveal the roles of these C termini in regulating the activities of NOS.

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