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Review

The journey from NADPH-cytochrome P450 oxidoreductase to nitric oxide synthases ☆

Bettie Sue Siler Masters *

Department of Biochemistry, The University of Texas Health Science Center at San Antonio, Texas 7703 Floyd Curl Drive, San Antonio, TX 78229-3900, USA

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Abstract

This mini-review will reflect the perspective of its author on two fields of research, which have merged as the result of the insights of investigators whose work has influenced both areas immeasurably. It cannot be overlooked, however, that the research activities of many during a period of over five decades have produced the chemical and biological bases for the exciting discoveries now encompassing the cytochromes P450 and their redox partners, and the three isoforms of nitric oxide synthase as they function in their respective biological milieux. Following the remarkable discovery that, indeed, molecular oxygen can be adducted to organic molecules by enzymatic systems and that such processes require a supply of reducing equivalents, it is the purpose of this review to provide a chart, with some of its detours, of the road that followed in the pursuit of interesting biological phenomena involving these two major oxygenation systems. It is not intended to be a balanced review and apologies must be offered in advance to those whose contributions may be overlooked or simply were not directly germane to the development of the author's journey.

Discovery and early history of NADPH-cytochrome *c* (P450) reductase

The discovery of TPNH (NADPH)-cytochrome *c* reductase by Horecker in 1950 [1] set into motion over half a century of studies to determine the intracellular localization and biological function of this enzyme. These early studies by Horecker reported the isolation and purification of this enzyme from a whole liver acetone powder, which did not permit the localization of the enzyme to any subcellular compartment. Later, in 1956 and 1957, Strittmatter and Velick defined NADH-mediated activities in the liver endoplasmic reticulum [2–4] and discovered a separate microsomal fraction that catalyzed cytochrome *c* reduction 20 times faster with NADPH than with NADH, which they suggested could be similar to the enzyme activity demonstrated by Horecker. Not until 1962, when Williams and Kamin [5] and Phillips and Langdon [6] identified and purified these enzymes, using different methods of solubilization, was the identity of their enzymes to Horecker's flavoprotein established. Williams and Kamin [5] based their conclusions on the identity of the flavin prosthetic group, identified by Horecker as FAD, similar specific activities, comparable molecular weights, identical pH optima, reactivation by FAD and FMN, and inhibition by $NADP^+$. Similar conclusions were drawn by Phillips and Langdon [6] and, based on their preliminary experiments, they suggested that NADPH-cytochrome *c* reductase participated in microsomal electron transport activities *not* involving cytochrome *c*.

Interestingly, La Du et al. [7] and Gillette et al. [8] had previously reported that cytochrome *c* inhibited the TPNH (NADPH)-mediated oxidative demethylation of monomethyl-4-aminoantipyrine demethylation catalyzed by liver microsomal fractions. At the time, Gillette et al. [8] did not know the relationship between the NADPH-mediated

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* Fax: +1 210 567 6984.
E-mail address: masters@uthscsa.edu.

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activity of liver microsomes and the NADPH-cytochrome *c* reductase of Horecker [1]. These observations were the first to implicate NADPH-cytochrome *c* reductase in drug metabolism by endoplasmic reticulum but many other studies to follow (vide infra) were to establish this relationship. The photochemical action spectrum of the terminal oxidase, implicating a reduced, CO-binding pigment absorbing at 450 nm, of liver microsomal metabolism of steroids and drugs was yet to be published by Estabrook and colleagues [9].

Kamin's laboratory continued to pursue the purification and characterization of NADPH-cytochrome *c* reductase and published two seminal mechanism papers in 1965 [10,11]. By altering the purification procedure, carefully monitoring the pH during ammonium sulfate fractionation and using new chromatographic procedures, Masters et al. [10,11] were able to determine a minimal molecular weight per flavin of approximately 35,000, indicating that the enzyme contained *two* flavins per mole. Experiments from several laboratories, including those by Horecker [1] and later by Williams and Kamin [5] and Phillips and Langdon [6], had suggested that the enzyme contained only one FAD per mole. Therefore, the actual identity of the second flavin was not determined at that time, despite the fact that FMN seemed to reconstitute its activity more efficiently. In 1973, Iyanagi and Mason [12] reported that NADPH-cytochrome *c* reductase contained equimolar quantities of FAD and FMN. This was subsequently confirmed by other laboratories [13–18] and Yasukochi and Masters [18] also reported that preparations of both NADPH-cytochrome *c* reductase (minus the hydrophobic N-terminus that anchors the enzyme to the endoplasmic reticulum) and NADPH-cytochrome P450 oxidoreductase (CYPOR; containing this amino terminus that renders it capable of reconstituting drug-, fatty acid-, and steroid-metabolizing cytochromes P450) contained *both* FAD and FMN.

Mechanism studies of NADPH-cytochrome *c* oxidoreductase as a flavoprotein

Much of the groundwork for mechanism studies of the NADPH-specific microsomal reductase was laid by the early experiments of Horecker [1], Williams and Kamin [5], and Phillips and Langdon [6]. The catalytically determined K_m values for NADPH closely corresponded with those determined by Cohen and Estabrook [19] for monooxygenase reactions catalyzed by liver microsomes.

In the process of studying the steapsin-solubilized, purified reductase, lacking its N-terminal hydrophobic anchor required for binding to the endoplasmic reticulum membrane, Masters et al. [10,11] and Kamin et al. [20,21] determined the spectral species involved in catalytic turnover of NADPH-cytochrome *c* reductase activity with several electron acceptors. Measurement of the oxidation–reduction states of the reductase by stopped-flow kinetic techniques revealed that the reduction of cytochrome *c*, potassium ferricyanide, 2,6-dichlorophenolindophenol, and menadione required a catalytic cycle involving partially reduced forms of the enzyme [10,11,20,21]. A semiquinone form was found to be inert in reducing any of these electron acceptors, and the catalytic cycle involved this partially reduced form as the oxidized redox partner during turnover. The nature of the semiquinone form was not identified until later as a 1-electron-reduced intermediate [12,22–24], and several other flavoproteins were also shown to function by cycling catalytically between partially reduced forms [25–27], including sulfite reductase and adrenodoxin reductase. To clarify the conflicting data on the nature of the semiquinone representing the oxidized redox partner during catalytic turnover for CYPOR, Vermilion and Coon [22] performed very careful spectral and kinetic experiments on preparations of liver microsomal reductase purified by the method of Yasukochi and Masters [18] from phenobarbital-treated rats. This report supported the findings of Iyanagi and Mason [12], who first reported that CYPOR contained both FAD and FMN, and that the semiquinone form identified by Masters et al. [10] was a 1-electron-reduced state. In the Vermilion and Coon publication [22], a footnote documented a personal communication (later published; [24]) by Masters and her colleagues stating full agreement with these findings. Vermilion and Coon [22] also showed unequivocally that although *both* the detergent-solubilized (with N-terminal membrane anchor) and the protease-solubilized (minus membrane anchor) preparations contain FAD and FMN, possess similar spectral properties, and are kinetically similar in their capacities to reduce cytochrome *c* and other artificial electron acceptors, only the detergent-solubilized preparation is capable of interacting with cytochrome P450, so the acronym, CYPOR, will be used for this latter form of the enzyme.

In a later study, Iyanagi et al. [28] identified the redox potentials of three phases of the reduction cycle of trypsin-solubilized NADPH-cytochrome *c* reductase: *System I* (1-electron-reduced) = −109 mV; *System II* (2- and 3-electron-reduced) = −274 mV; *System III* (4-electron-reduced) = −371 mV, determined at pH 7.0 and 25 °C. At this time, identification of these redox intermediates as either FAD or FMN was not performed. In 1978, Iyanagi et al. [29] published a similar study on detergent-solubilized rabbit liver microsomal CYPOR and found similar redox properties for this preparation. These results indicated that the presence of the N-terminal hydrophobic anchor had no effect on the redox properties of the enzyme.

In experiments, designed to determine the roles of the two flavins in catalysis by CYPOR [22,30], Vermilion and Coon specifically removed FMN from CYPOR, showing that removal of FMN impaired its reduction capacity toward the artificial electron acceptors, cytochrome *c*, 2,6-dichlorophenolindophenol, menadione (1,4-naphthoquinone), and its physiological redox partner, cytochrome P450. These experiments produced strong evidence that NADPH reduced FAD (even in the absence of FMN) and that FMN was required for reduction of

the physiological acceptor, cytochrome P450, as well as artificial acceptors. They also reported that ferricyanide reduction could proceed in the absence of FMN. Studies by Alexander et al. [31] confirmed these results but also found that FMN was by far more efficient than FAD in reactivating CYPOR from which FMN had been removed and could do so in stoichiometric amounts. These results also indicated that the FMN-binding site was probably not capable of binding FAD, a much larger molecule, productively.

EPR studies, first reported by Iyanagi et al. [12,29] and later by Yasukochi et al. [24], showed that the spin concentration of the air-stable semiquinone, compared with a flavodoxin semiquinone standard, was 50% of the total flavin concentration. Subsequent stopped-flow studies showed the rapid reduction of either oxidized or 1-electron-reduced CYPOR to more reduced flavin species and slower reoxidation of the latter to the flavin semiquinone form [24]. These experiments showed unequivocally that production of the fully reduced flavin preceded the formation of flavin semiquinone with an estimated rate constant ~7000 times faster than the reoxidation by O_2.

In 1976, Yasukochi and Masters [18] introduced a biospecific affinity chromatography method for purification of the reductase utilizing 2′,5′-ADP-Sepharose 4B prior to its release into the marketplace by Pharmacia. This method exploits the specificity of the NADPH-specific enzyme for the 2′-phosphate group of NADPH and the slightly lower binding affinity of the enzyme for 2′-AMP, compared to NADPH or $NADP^+$, to elute it from this column medium. A similar method was introduced by Dignam and Strobel [17], using NADP-Sepharose. With the introduction of these affinity chromatography techniques, it was possible to produce larger amounts of homogeneous preparations of CYPOR from detergent-solubilized microsomes, which facilitated spectrophotometric titrations, electron paramagnetic resonance spectrometry (EPR), stopped-flow kinetics, and reconstitution studies. This procedure has been used by numerous laboratories for purification of tissue-derived and *Escherichia coli*-expressed CYPOR and, more recently, for the purification of the nitric oxide synthases, first used by Bredt et al. [32].

Discovery of the physiological role(s) of microsomal NADPH-cytochrome *c* oxidoreductase

As previously mentioned, Phillips and Langdon [6], La Du et al. [7], and Gillette et al. [8] had already speculated about the role of microsomal TPNH (NADPH)-cytochrome *c* reductase in other than the reduction of this *mitochondrial* cytochrome. The inhibition of microsomal monomethyl-4-aminoantipyrine demethylation by cytochrome *c* [8] predated the discovery of cytochromes P450, later shown to be involved in the oxidative demethylation (monooxygenation) of this substrate. In 1955, Klingenberg [33] and Garfinkel [34] independently observed a reduced, carbon monoxide-binding pigment, absorbing intensely at 450 nm in rat liver microsomes but it wasn't until 1962 that Omura and Sato [35,36] reported the identification of this pigment and its characterization as a b-type cytochrome. These authors [36,37] then published the properties of the microsomal membrane-bound (P450) and the solubilized (P420) forms and determined their absorption extinction coefficients. At the same time that Omura and Sato [36,37] revealed their purification of microsomal cytochrome P450, Mason and associates (Hashimoto et al. [38]) discovered a new electron paramagnetic resonance spectrum in liver microsomes, later shown to be due to cytochrome P450. Interestingly, this was the same year that NADPH-cytochrome *c* reductase was purified from liver microsomal preparations [5,6].

With this background, in 1963, Estabrook et al. [9] and Cooper et al. [39] employed the photochemical action spectrum technique, reported by Warburg [40] for the identification of cytochrome oxidase as the terminal oxidase in mitochondrial respiration, to implicate cytochrome P450 as the oxygenase involved in the monooxygenation of both steroids and drugs. These experiments established unequivocally the role of cytochrome P450 in the catalysis of these reactions, but the link was still not made between NADPH-cytochrome *c* reductase and cytochrome P450 in microsomal reactions, although such a role was suspected from various observations. For example, Omura and Sato [37] showed a proportional loss of cytochrome P450 reduction and aniline hydroxylation upon solubilization of the reductase, and Ernster and Orrenius [41] reported a parallel *increase* in microsomal cytochrome P450 and NADPH-cytochrome *c* reductase by phenobarbital treatment of rats. The most definitive evidence implicating NADPH-cytochrome *c* reductase in microsomal drug and steroid monooxygenation reactions was based on immunochemical and reconstitution experiments. Antibodies prepared against purified trypsin-solubilized reductase inhibited both solubilized and membrane-bound (microsomal) reduction of cytochrome *c*, reported by Kuriyama et al. [42], and Omura [43] demonstrated the inhibition of aniline hydroxylation by this antibody. This immunochemical approach was utilized by Wada et al. [44], Masters et al. [45,46], and Glazer et al. [47] to demonstrate inhibition of other NADPH-dependent microsomal monooxygenase reactions, including drugs and carcinogens. Masters et al. [45] were able to show *concomitant* inhibition of NADPH-mediated cytochrome *c* reduction, cytochrome P450 reduction and aminopyrine demethylation in the same microsomal fraction by anti-reductase immunoglobulin, showing the dependence of these activities on the enzyme activity, NADPH-cytochrome *c* oxidoreductase, later to be called NADPH-cytochrome P450 reductase (CYPOR). Masters' laboratory also used this immunochemical approach to demonstrate, for the first time, the role of CYPOR in the microsomal oxygenation of heme in spleen and liver microsomes by heme oxygenase [48].

Reconstitution of the cytochrome P450-mediated monooxygenation systems in vitro

Although other studies had strongly implicated the interaction between CYPOR and microsomal cytochrome P450 in situ, very convincing evidence for this relationship was obtained with the reconstitution experiments of Lu and Coon [49] in which they were able to demonstrate the ω-hydroxylation of lauric acid with purified components. This report led the way to reconstitution of other systems when it became obvious that there were many cytochromes P450, even in liver microsomes, which needed to be characterized at the molecular level. The system was composed of three fractions separated from a microsomal extract, cytochrome P450, NADPH-cytochrome P450 reductase, and a "heat-stable factor." This report, however, was not able to identify the properties of the heat-stable factor. A later study from Strobel et al. [50] demonstrated reconstitution of these activities with dilauroylphosphatidylcholine.

Evidence for convergent evolution of the diflavin enzymes from ferredoxin-NADP$^+$ reductase and flavodoxins

It was first proposed by Porter and Kasper [51,52] that NADPH-cytochrome P450 oxidoreductase (CYPOR) evolved from the ancestral genes coding for bacterial flavodoxins (FMN) and ferredoxin-NADP$^+$ reductase. Although at the time of this work, no structure of CYPOR was available, the authors compared the sequences of ferredoxin-NADP$^+$ reductase and NADH-cytochrome b_5 reductase to glutathione reductase, of which the crystal structure was known, for FAD and cofactor binding similarities. The conservation of sequence between CYPOR residues (77–228) and ferredoxin-NADP$^+$ reductase (FAD) and CYPOR residues 267–678 and bacterial flavodoxins (FMN) presented compelling evidence for this hypothesis. The subsequent crystallization and atomic structural determination of CYPOR by the laboratories of Kim and Masters [53] further solidified this proposal (Fig. 1). It is apparent that three major domains of the reductase are involved in its function as a flavoprotein. The FAD-NADPH-binding domain and FMN-binding domain are joined by a connecting domain that allows some flexibility of movement between them. The interatomic distances between FAD and FMN in the CYPOR structure are 3.5 and 4.5 Å between the C_7M atoms and C_8M atoms of the xylene rings and allow direct electron transfer between flavins. However, in the NADP$^+$-bound structure [53], the distances between the C_4 atom of NADP$^+$ and the N_5 atom of the FAD isoalloxazine ring were too great (9 and 14 Å, respectively) to allow for direct hydride transfer in either of two conformers of the crystalline structure. By changing the dihedral angles of both sides of the bridging pyrophosphate oxygen between the phosphates of the nicotinamide and adenine rings of the NADP$^+$ molecule, the nicotinamide ring could be positioned where the penultimate residue, W677, lies in the CYPOR structure. In studies by Hubbard et al. [54], the movement of the indole ring of W677 upon the binding of NADPH was directly demonstrated. Karplus et al. [55] previously proposed this positioning for ferredoxin-NADP$^+$ reductase. So, the theme for flexibility between the cofactor-binding domains of several flavoproteins was already in the literature and would become important in the consideration of structure–function relationships in the nitric oxide synthases later on.

The emergence of the nitric oxide synthases and their physiological significance

As early as 1916, Mitchell et al. [56] found that the diets of rats, pigs, and humans could not account for the amount of nitrogen oxides (NO_3^-) excreted in urine but there was no proof presented for metabolic production of such products. When Tannenbaum and colleagues [57,58] showed that ^{15}N-labeled precursors were converted to labeled nitrate metabolites, this proved that such metabolism occurred. These workers [58] showed that the source of these labeled metabolites was not bacterial by using germ-free compared to conventional rats. Then, Marletta and colleagues [59] reported macrophage synthesis of nitrite, nitrate, and *N*-nitrosoamines from L-arginine and other arginine-containing compounds, including peptides, and Hibbs et al. [60] also showed that L-arginine was the source of the nitrogen produced in these nitrogen oxides.

Meanwhile, the search for the endothelium-derived relaxing factor reported by Furchgott and Zawadski [61] culminated in pioneering work by the laboratories of Ignarro [62], Furchgott [63], and Moncada [64] identifying gaseous nitric oxide, NO$^\bullet$, as that factor. Earlier, in 1977, the stimulation of guanylyl cyclase by NO$^\bullet$ had been reported by Murad's laboratory [65], which found increased levels of guanosine 3′,5′-cyclic monophosphate in various tissue preparations.

Discovery of the sequence similarity of the C-terminal flavoprotein domain of neuronal nitric oxide synthase with NADPH-cytochrome P450 reductase

When Solomon Snyder's laboratory reported the 58% sequence similarity between CYPOR and the C-terminal 641 amino acids of rat neuronal nitric oxide synthase [32], a new *mammalian* family of FAD- and FMN-containing enzymes was introduced. Other laboratories reported that nitric oxide synthases (NOSs) contained stoichiometric amounts of both FAD and FMN [66–69] but no one had pursued the possibility of sequence similarity to known enzymes. In addition, because nitric oxide synthases were found to require tetrahydrobiopterin [32,70,71] for activity, a cofactor that could serve as the site of oxygenation required for the formation of L-citrulline and NO, there had been little consideration of the possibility of another prosthetic group in these enzymes.

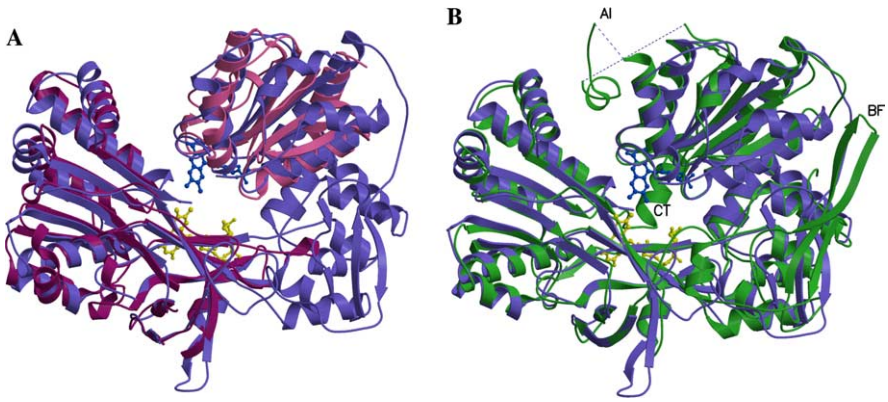

Fig. 1. Comparisons of the ribbon structures of: (A) NADPH-cytochrome P450 oxidoreductase (CYPOR) in blue with overlays of *D. vulgaris* flavodoxin (rose pink) from Watenpaugh et al. [119] and ferredoxin-$NADP^+$ reductase (maroon) from Karplus et al. [55]; and (B) CYPOR in blue with an overlay of the structure of the reductase domain of neuronal nitric oxide synthase (green) from Garcin et al. [112]. The dashed lines represent the autoregulatory (or autoinhibitory) insert, described by Salerno et al. [92], that was unresolved in the structure. The beta finger (BF) was first described by Zhang et al. [101] for the FAD-/NADPH-binding domain structure of nNOS and the C-terminal (CT) extensions of all three NOS isoforms were first recognized and studied by Roman et al. [102,103].

Discovery of heme in the oxygenase domains of nitric oxide synthases

Masters and her colleagues, having obtained the stably transfected clone of rat neuronal nitric oxide synthase (nNOS) in human kidney 293 cells from Solomon Snyder's laboratory, invested the effort to isolate the enzyme from these cells in large quantities. In the meantime, other laboratories were conducting similar experiments on neuronal and/or inducible NOS enzymes. As a result, four different laboratories simultaneously demonstrated that these isoforms of NOS contained iron protoporphyrin IX as a prosthetic group [72–75]. While delivering the Federation of American Societies for Experimental Biology Excellence in Science Award lecture in April, 1992, the author announced her laboratory's findings. Amazingly, its spectral properties identified it as an iron protoporphyrin IX in a b-type cytochrome quite similar to the cytochromes P450, i.e., exhibiting reduced, carbon monoxide difference spectra with absorption maxima at ~450 nm [72–75]. Although database searches had failed to reveal sequence homologies between cytochromes P450 and NOS isoforms, McMillan et al. [72] suggested a consensus sequence surrounding a cysteinyl peptide in the NOS sequences: rat neuronal NOS (nNOS; [32]), $RNASRC^{415}VGRIQW$; murine macrophage inducible NOS (iNOS; [76]), $RNAPRC^{194}IGRIQW$; and bovine endothelial NOS (eNOS; [77]), $RNAPRC^{186}VGRIQW$. Based on this information, site-directed mutagenesis was performed by McMillan and Masters [78], and the proximal thiolate ligand of the heme of nNOS, expressed in *E. coli*, was determined to be C^{415}. Studies were also performed by Marletta's laboratory [79] to determine the effect of mutagenesis of C^{415} in a preparation of nNOS from a baculovirus overexpression system. Drawing on experience with cytochromes P450, which exhibit two major optical difference spectra, so-called Type I and Type II depending upon the mode of binding of ligands to residues in the vicinity of the heme-binding site *or* directly to the heme Fe, respectively, McMillan and Masters [80] reported the spectral perturbation of nNOS by various ligands, including L-arginine, *N*-hydroxy-L-arginine, N^G-methyl-L-arginine, and imidazole. L-Arginine and *N*-hydroxy-L-arginine were shown to produce Type I binding spectra, while imidazole produced Type II spectral shifts. Pufahl and Marletta [81] produced a Type I binding spectrum with macrophage NOS, induced by lipopolysaccharide and interferon-γ in RAW 264.7 cells, by the addition of *N*-hydroxy-L-arginine. Both studies established the perturbation of the prosthetic heme upon the binding of substrates (or inhibitor) and provided comparisons with the cytochromes P450, which also contain a cysteine thiolate-bound heme. Judging from the nature of the substrate, McMillan et al. [72] had already suggested that the heme-binding cleft of the nitric oxide synthases would differ significantly from the cytochromes P450. This turned out to be true.

The ability to separate the oxygenase (heme-binding) domain from the reductase (flavin-binding domain) of neuronal NOS was illustrated first by Sheta et al. [82] using immobilized trypsin proteolysis. These experiments showed that the domains of nNOS can be cleanly separated and remain functionally active. This meant that the heme domain bound ligands, producing spectrally identifiable complexes and undergoing reversible redox changes, and that the flavoprotein domain contained both flavins and could reduce the artificial electron acceptors, cytochrome *c*, 2,6-dichlorophenolindophenol, and ferricyanide with electrons donated from NADPH. These experiments led McMillan et al. [78] to express these domains separately in a prokaryotic system to facilitate their biophysical characterization of these domains in order to understand the structure–function relationships of the holoenzyme. Other laboratories also utilized this approach and the crystal structures of the heme domain dimers of two isoforms of NOS

appeared in 1998 from the laboratories of Tainer and Stuehr [83], and Poulos and Masters [84]. Crane et al. [83] determined the structure of inducible NOS heme domain (residues 66–498), expressed in *E. coli*, and fused in the C-terminus with a hexahistidine tag for purification on a Ni–NTA Sepharose 4B column. Raman et al. [84] purified *E. coli* endothelial NOS heme domain (residues 39–482), proteolyzed from a holo eNOS construct missing 75% of its calmodulin-binding region, and purified on a 2′,5′-ADP-Sepharose 4B column originally developed for CYPOR purification [18]. These structures presented the same global heme domain dimer structures, and subsequent reports by Li et al. [85] and Fischmann et al. [86], confirmed these remarkable similarities in the heme structures of these two isoforms. In fact, Fischmann et al. [86] also confirmed the presence of zinc in a tetrathiolate center reported first by Raman et al. [84]. The ligands for zinc are contributed by two cysteine residues five positions apart in the sequences of each monomer of both eNOS [84,86] and iNOS [86], and represent a beautifully symmetrical structure. The zinc tetrathiolate center in eNOS, for example, is equidistant (21.6 Å) from both hemes, which are 34 Å apart from each other, and 12 Å from each of the tetrahydrobiopterin cofactors. It is interesting to note the sequence proximity of the ZnS_4 to the Ser^{104} residue in eNOS and Ser^{112} in iNOS, which are within H-bonding distance of the dihydroxypropyl side chain of tetrahydrobiopterin. A later publication of the nNOS heme domain crystal structure [87] revealed a similar structure. All of these observations with the various NOS isoforms further substantiated the similarities in the heme domain structures and directed the attention of the author's laboratory again to the flavin-binding (reductase) domain of these enzymes for the identification of unique structural motifs and regulatory sequences.

Cloning and expression of intact NOS isoforms in heterologous expression systems and subsequent purification and characterization

In order to pursue structure–function and biophysical experiments on the NOS isoforms, without the labor-intensive and expensive handling of mammalian cells in culture, several laboratories attempted to express the NOS isoforms in heterologous expression systems. Several publications appeared in the literature from the Moncada [88] and Marletta [79] laboratories describing the heterologous expression of nNOS cDNA in *Spodoptera frugiperda* (Sf-21) insect cells using a baculovirus vector but the activities and/or heme content of these preparations were not equal to those of the enzyme purified from rat neuronal tissue. The first successful effort in producing high yields of enzyme was made in 1995 by Roman et al. [89] for the cloning and expression of neuronal NOS in *E. coli* and its subsequent purification. Using the pCW_{ori} vector, successfully employed in the expression of cytochromes P450 in *E. coli*, Roman et al. [89] took advantage of a protease-deficient *E. coli* cell line, BL-21, and co-expressed these cDNAs with another vector containing the coding sequence for *E. coli* chaperonins, groEL and groES. These preparations possessed high activity, producing a 2.7-fold stimulation of activity in the presence of tetrahydrobiopterin to a specific activity of 435 nmol/min/mg. The expression system produced yields of 20–24 mg of nNOS per liter of *E. coli* culture. Ortiz de Montellano's laboratory [90] simultaneously published a successful expression system for the production of intact nNOS using the same pCW_{ori} vector but not including the protease-minus BL-21 *E. coli* cells or co-expression with *E. coli* chaperonins. It was not possible to compare yields between the Roman et al. [89] and Gerber et al. [90] preparations, since these were stated in entirely different units but these two publications proved that intact NOS isoforms could be expressed effectively in a prokaryotic system producing adequate yields for biophysical studies. In 1996, Martásek et al. [91] characterized a highly active preparation of intact eNOS overexpressed in *E. coli* using a system similar to that described by Roman et al. [89] for nNOS. This procedure provided the starting material for the crystallographically pure preparations used in determining the eNOS heme domain dimer structure [84].

Exploration of the roles of the cofactor-binding domains of nitric oxide synthase isoforms in the regulation of activity: intrinsic regulation within the flavoprotein C-termini

Comparison of the sequences of the NO synthases by Salerno et al. [92] revealed very striking and important differences between the constitutive NOS isoforms and the inducible isoform (iNOS) and CYPOR. By sequence alignment, the insertion of approximately 45 residues, found to be located within the FMN-binding domain of both nNOS and eNOS, is notably absent in dozens of flavodoxins to which they were compared [92]. In order to demonstrate the ability of these additional residues to act as regulatory elements, varying length peptides (hexamer to 33-mer) were generated from the sequence of this insert in endothelial NOS (eNOS) and tested in catalytic assays for NO formation and cytochrome *c* reduction. The longer peptides were effective in competing with ^{125}I-labeled calmodulin binding to nNOS demonstrated, for example, in the presence of Ca^{2+} and, $eNOS^{607–634}$ peptide, which inhibited the production of NO catalyzed by nNOS, and was competitively displaced by increasing Ca^{2+}/calmodulin concentrations [92]. These studies also showed the exposure of a particular lysine residue in both nNOS and eNOS structures upon the binding of calmodulin and the production of different fragments of the cNOSs. It was also found that the limiting peptide that could inhibit the intact protein was a hexamer consisting of the residues, WRRKRK, found in the eNOS sequence, whereas a jumbled hexapeptide could not. In this publication and in unpublished observations from the Masters laboratory, this hexapeptide inhibits the reduction of artificial electron acceptors, such as cytochrome *c*, and

the production of NO, which can be overcome by increasing the Ca^{2+}/calmodulin concentration. The possibility that Ca^{2+}/calmodulin binding can influence either the transfer of electrons between NADPH and FAD or from FAD to FMN was studied by Matsuda and Iyanagi [93] who found that electron flux between the flavin prosthetic groups was activated by Ca^{2+}/calmodulin binding. These authors proposed that, in its absence, interflavin electron transfer is rate-limiting but, upon addition of Ca^{2+}/calmodulin, the rate-limiting step is switched to the reduction of FAD by NADPH, as in CYPOR. Other laboratories have reported the stimulation of electron transfer by Ca^{2+}/calmodulin [94–96] but the first demonstration of a conformational change was published by Sheta et al. [82] using fluorescence spectroscopy. The exact relationship of this autoregulatory element to the Ca^{2+}/calmodulin binding site is unknown as there are no known structures of intact NOS isoforms that encompass this binding site and the FMN-binding domain structure but many models have been proposed [92–94,97–100]. Craig et al. [100] proposed a "conformational lock" induced by NADPH binding to nNOS, restricting access to FMN by external electron acceptors through a conformational change in the reductase domain. These authors suggest that calmodulin binding reverses this locked conformation, producing a 30-fold increase in the second-order rate constant for cytochrome *c* reduction by nNOS. At the time of this publication, the Masters and Kim laboratories had published the crystal structure of only the FAD-/NADPH-binding domain of nNOS [101]. Due to the indication from this structure that the truncated C-terminus ended in proximity to the FAD and FMN interface and could interfere with NADP/H binding, Craig et al. [100] suggested that their discovery that NADP/H binding controls nNOS reductase domain conformation, in the absence of calmodulin, aided in explaining how the various autoinhibitory inserts in constitutive NOS isoforms might function. These data will be discussed later in the context of the structural determinations of nNOS reductase domains.

Upon further examination of the sequences of the various NOS isoforms, Roman et al. [102,103] discovered that the C-termini of all known NOS enzymes are extended beyond the homologous C-terminal residue of CYPOR in the FAD-/NADPH-binding domain. These extensions are 21, 33, and 42 residues in length in the mammalian iNOS, nNOS, and eNOS isoforms, respectively. By removing these C-termini from iNOS [102], and nNOS and eNOS [103], Roman et al. demonstrated a 7- to 10-fold increase in cytochrome *c* reduction. In the presence of Ca^{2+}/calmodulin, these enhanced rates in the constitutive NOS isoforms, nNOS and eNOS, returned to that of wild type enzyme. Ca^{2+}/calmodulin is bound with very high affinity in iNOS during biosynthesis of the protein, so the effects on catalytic activity in its absence could not be measured. It was possible to determine in stopped-flow experiments that the flow of electrons from NADPH to FAD and between FAD and FMN was not rate-limiting for heme reduction in the iNOS holoenzyme. In these experiments, in which both the fast and slow phases of flavin reduction were measured, these rates were 2- and 5-fold faster with truncated iNOS than the wild type enzyme [102], leading to the proposal that comproportionation of electrons between FAD and FMN was influenced by the C-terminus. Since Dr. Jung-Ja Kim's and the author's laboratories, in collaboration, have shown that the truncated C-terminus in the crystal structure of the FAD-/NADPH-binding domain of nNOS [101] and the C-terminus of CYPOR [53] lie near the junction of the FAD/FMN interface, Roman et al. [102,103] suggested that the longer C-termini of NOSs regulate electron flux between the flavins and also between FMN and the heme. Roman et al. [103] showed with nNOS and eNOS truncated forms that cytochrome *c* reduction was increased 7- and 21-fold, respectively, over wild type isoforms in the absence of Ca^{2+}/calmodulin but remained very similar to their wild type counterparts in the presence of Ca^{2+}/calmodulin. These authors proposed [103] that, in the absence of Ca^{2+}/calmodulin, "the C-terminal tail is located between the prosthetic flavins and/or between FAD and the NADPH, slowing down electron transfer between them. When calmodulin is bound and the autoinhibitory loop swings away, this change in the protein conformation realigns the flavins and the NADPH-binding site such that the C-terminus is less of a barrier." At this point, there was convincing evidence that at least two autoregulatory loops existed in the flavoprotein domains of the mammalian constitutive NOS isoforms. A subsequent report from Gross's laboratory [104] reported the properties of a truncated form of eNOS (Δ27) in which the S^{1179} phosphorylation site, found by Fulton et al. [105], Dimmeler et al. [106], Chen et al. [107], and Hayashi et al. [108] to be involved in the activation of eNOS, was the last residue removed. This phosphorylation site was also removed in the Roman et al. [103] constructs but the constitutive NOSs, in this case, were constructed to resemble CYPOR at the C-termini, resulting in much shorter enzymes. Lane and Gross [104] reported a 2-fold *increase* in NO synthesis and a 3- to 4-fold *increase* in cytochrome *c* reduction in their truncated mutant and, interestingly, a 5-fold *decrease* in the EC_{50} for Ca^{2+}, while no difference in affinity for calmodulin was observed. The authors invoked the observation of Persechini et al. [109] that the C-terminus of calmodulin binds Ca^{2+} at 80 nM and the N-terminus binds at 800 nM but that binding of the N-terminus of calmodulin is *required* for activation of nNOS, to explain their results. It would follow that at the basal physiological levels of Ca^{2+} (~100 nM), eNOS would contain CaM bound at its C-terminus. However, since the Ca^{2+}-dependence curves for activation and binding were superimposable in the truncated eNOS, the authors concluded that the C-terminal tail of eNOS interferes with the binding of the N-terminal lobe of calmodulin, thus preventing activation. In the absence of the C-terminus in their construct, the obstacle to the binding of calmodulin is removed resulting in the concomitant activation of eNOS and binding of

CaM. Roman et al. [102,103], and Lane and Gross [104] concluded that a correlation must exist between the length of the C-terminus among the NOS isoforms and the relative rates of electron flux through the flavoprotein domain as well as the rate of heme reduction.

More recent experiments have been performed by Jáchymová et al. [110] in the author's laboratory in which the C-termini of all three NOS isoforms have been attached to CYPOR by recombinant DNA techniques. These experiments clearly show that CYPOR constructs containing the C-termini of NOSs exhibit inhibited electron transfer activities by CYPOR to the artificial electron acceptors, cytochrome *c*, and 2,6-dichlorophenolindophenol, but do not affect ferricyanide reduction. Fluorometric evidence was presented for conformational changes in each of these constructs as a result of the attachment of the C-termini of varying lengths. These results again suggest interference with electron transfer *between* FAD and FMN by these extensions in NOS isoforms.

In an attempt to examine the interchangeability of the FAD- and FMN-binding domains between CYPOR and nNOS with respect to the reconstitution of NO production or the reduction of artificial electron acceptors, Roman et al. [111] generated a series of chimeric enzymes. These chimeras consisted of nNOS holoenzyme structures substituted by the FMN- and/or FAD-binding domains of CYPOR. These constructs are shown in Fig. 2. Using

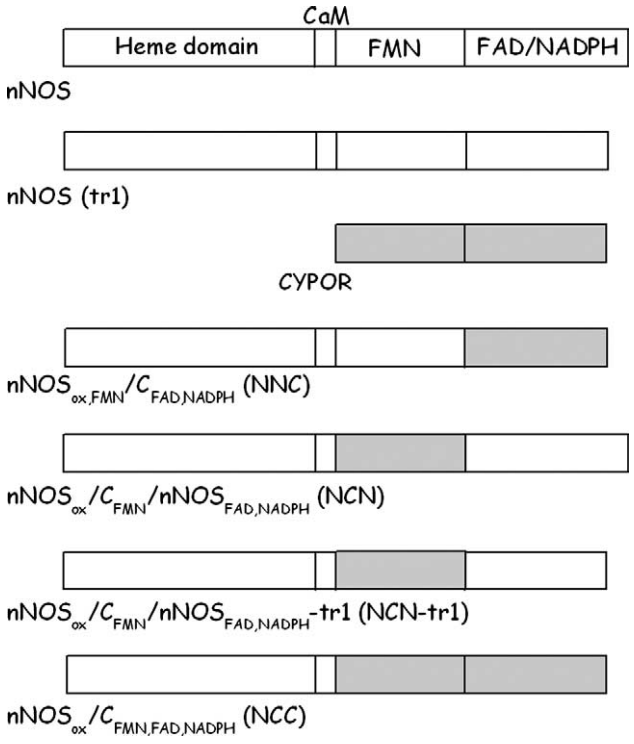

Fig. 2. Scheme of the various constructs of Roman et al. [111] in which chimeras of CYPOR and nNOS were expressed in *E. coli*, extracted, and purified for characterization studies. The open boxes represent all domains derived from nNOS (designated by the N) and the shaded boxes represent domains derived from CYPOR (designated by C). These constructs were assayed for various activities as described in the text.

spectral analyses, enzymatic assays for the reduction of artificial electron acceptors, as well as the production of NO, and stopped-flow, rapid reaction kinetics, it was shown that the FMN domain of nNOS contains unique sequences required for the reconstitution of NOS activity (production of NO) but that the CYPOR FMN- and FAD-binding domains can substitute for the reduction of artificial electron acceptors, such as cytochrome *c*. As previously reported by Roman et al. [102], the removal of the C-terminus of nNOS resulted in >20-fold increase in cytochrome *c* reduction in the absence of Ca^{2+}/calmodulin that was dampened to the level of wild type activity in its presence. The construct containing nNOS heme and FMN-binding domains with CYPOR FAD-binding domain (NNC) produced the same level of cytochrome *c* reduction as the C-terminal truncated nNOS. Those constructs containing either the heme domain of nNOS, the FMN-binding domain of CYPOR, and the FAD-binding domain of nNOS (NCN) or its truncated counterpart (NCN_{tr1}) exhibited 1/4 to 1/8 the activity of wild type nNOS, respectively. Interestingly, the construct containing nNOS heme domain and both flavin-binding domains of CYPOR (NCC) lost all semblance of calmodulin control. Comparison of the rates of NO production showed that the NNC construct actually catalyzed increased production of NO compared to wild type nNOS ($88 \pm 1\ min^{-1}$ vs. $71 \pm 6\ min^{-1}$). None of the other constructs produced NO, illustrating the necessity for additional regulatory elements in the FMN-binding domain of nNOS to reconstitute this activity.

Recent publications of the crystal structures of the nNOS FAD-/NADPH-binding domain by the Kim and Masters laboratories in 2001 [101] and the nNOS reductase domain dimer containing the FMN-/FAD-/NADPH-binding sites by the Getzoff/Tainer and Stuehr laboratories in 2004 [112] have presented new insights into the functions of these enzymes. The studies by Zhang et al. [101] noted the similarities between the overall polypeptide folds of their structure containing, from N- to C- terminus, the connecting, the FAD-binding, and the NADPH-binding domains and those of CYPOR [53] and the strict conservation of the FAD-binding site and mode of $NADP^+$ binding. However, the FAD-binding domain of nNOS presents a less positively charged surface to its FMN-binding counterpart than CYPOR, suggesting a different mode of interaction between FAD and FMN in the nNOS isoform. Two regions in the nNOS sequence [101] showed major departure from the CYPOR structure: the N-terminal β-loop-helix (residues 963–989) and a β-finger (residues 1070–1080). The connecting domain (~150 residues in an α-helical structure), unique to the CYPOR family members that contain both FAD- and FMN-binding in a single polypeptide chain, including CYPOR [53], NOS isoforms [101,112], bacterial cytochrome P450 BM-3 [113], methionine synthase reductase [114], and the α-subunit of sulfite reductase [115], revealed the most differences between CYPOR and the nNOS structure, as pointed out by Zhang et al. [101]. Within the FAD-/NADPH-binding

domains of CYPOR and nNOS, neglecting the connecting domain and the insertion/deletion regions, the root mean square (rms) difference between the C_α backbones is 1.3 Å but the connecting domains show a 3.9 Å rms difference [101]. These differences may account for how the FAD- and FMN-binding domains interact differently with one another in the holoenzyme structure and/or may influence the interactions of the FMN domains with their redox partners in the respective enzymes.

The report by Garcin et al. [112] of a nNOS reductase dimer crystal structure elucidates the interactions that are possible within the structure of the flavoprotein domain of nNOS, containing FMN- and FAD-/NADPH-binding domains joined by the connecting domain, but the structure of an *intact* NOS isoform, containing *both* oxygenase (heme-containing) and reductase (flavin-containing) domains, remains to be solved for the determination of the relative positions of these domains to each other in the dimeric structure. Such a structure will provide very important information about the interaction between the reductase and the heme domains. There are data from solution-based techniques, such as analytical ultracentrifugation,[1] that do not support the existence of a reductase domain dimer but there have been no studies reported to date, performed under conditions reproducing the high concentrations required for crystallography for comparison purposes. Garcin et al. [112] proposed from their structure that, since the FAD to FMN distance is less than 5 Å and the FMN to heme distance is ~15 Å in the electron-accepting and electron-donating positions, respectively, sequential interactions of the FMN must occur with each of its redox partners guided by electrostatic interactions.

In view of the proposal of Craig et al. [100] for a "conformational lock," induced by NADPH binding, that precludes interdomain electron transfer between the flavoprotein and heme domains of NOS, Garcin et al. [112] have provided a structural explanation. These authors propose that, in the absence of calmodulin binding, ionic interactions between the pyridine nucleotide and nNOS- and eNOS-conserved Arg^{1400} serve to orient the C-termini, resulting in repression of activity by "locking" the FMN into an "electron-accepting" conformation. To test this hypothesis, these authors mutated Arg^{1400} to Glu^{1400} to produce a charge change, which resulted in a 5-fold increase in calmodulin-free activity. While this experiment supports their hypothesis, the mechanism assumes a cause-effect with the Arg^{1400} determining the effect of the C-terminal tail. The report of Jáchymová et al. [110] sheds some light on this conclusion in that CYPOR with the attached C-termini from each of the three NOS isoforms was inhibited by their presence and CYPOR does *not* contain a calmodulin-binding site and, yet, its activity is regulated by the C-terminal extensions from the NOS isoforms alone. While these charge interactions are likely important, their primary role is yet to be established. The field eagerly awaits the structural determination of an intact NOS isoform in order to clarify the relative positioning of the various domains of these enzymes and the possible influence their spatial relationships have on function.

Several recent reviews have been written that address structure–function relationships and protein–protein interactions and these serve as views of the field at this stage, while not always being in complete agreement [116–118]. For a comprehensive coverage of the structure–function relationships and cofactor functions of the nitric oxide synthases, a review written by Raman et al. [118] addresses the chemistry of these systems with scholarly thoroughness and poses appropriate questions for research of the future.

Summary and conclusions

The ribbon structure of CYPOR from Wang et al. ([53]; Fig. 1A; blue) can be represented as a convergence of the structures of plant ferredoxin-$NADP^+$ reductase (FNR) containing the FAD- and NADPH-binding sites (Fig. 1A; lower left, maroon) and bacterial flavodoxin (Fig. 1A; upper right, rose pink), as first proposed by Porter and Kasper [51,52] based on sequence comparisons. In the development of mammalian enzymes, the utilization of the basic FAD- and NADPH-binding motifs of plant FNR and the FMN-binding motif of bacterial flavodoxin can be confirmed by the presence of these structurally similar domains. Recent studies by Jáchymová et al. [110], from the author's laboratory, have produced more evidence for the utilization of basic motifs into which sequences have been inserted for regulatory purposes by demonstrating that the addition of the C-termini of NOS isoforms from 21 to 42 residues in length to CYPOR produce chimeras that are downregulated, as demonstrated by Roman et al. [102,103] in the NOS isoforms. The removal of these C-termini [102,103] produced 7- to 21-fold increases in iNOS-, eNOS-, and nNOS-mediated cytochrome *c* reduction over wild type enzymes in the absence of calmodulin, indicating that their presence downregulates these activities. However, truncated eNOS (33%) and nNOS (45%) were both less active than their wild type counterparts in the production of NO, whereas the truncated and wild type iNOS preparations exhibited comparable NO production. The construction by Roman et al. [111] of chimeras of nNOS (Fig. 2), containing various combinations of the domains of CYPOR, provided further proof that the FMN-binding domain of nNOS containing the autoregulatory insert (AI) was absolutely essential for the reconstitution of NO formation. The absence of the C-terminus in the nNOS chimera, containing the CYPOR FAD-/NADPH-binding domain, produced the highly stimulated activities observed in the nNOS construct from which the C-terminus had been removed [103,111]. These studies provide strong evidence for the utilization of basic motifs with the insertion

[1] Pavel Martásek, Borries Demeler, Satya Panda, and Bettie Sue Masters, unpublished observations.

of regulatory sequences in the phylogenetic development of new enzymes.

Fig. 1B shows the overlay of the ribbon structures of the diflavin enzymes, CYPOR [53] and $nNOS_{red}$ [112]. In this figure, similarities and differences become obvious. It is important to point out the autoinhibitory loop (labeled AI in the figure), the so-called β-finger (BF), first identified by Zhang et al. [101], and the extended C-terminus on the nNOS structure. The C-terminus was not fully visible in this preparation, probably due to the flexibility of this extension from the stacking residue, Phe^{1395}, in NOSs homologous to the penultimate Trp^{677} residue in rat CYPOR.

The recent reports from Miller's laboratory [120,121] of the role of CYPOR missense mutations/polymorphisms in human craniofacial and skeletal abnormalities observed in patients with disordered steroidogenesis and/or Antley-Bixler syndrome have spurred efforts to identify the molecular basis of these dysfunctional enzymes. In addition, the nitric oxide synthases have been shown to play key roles in the production of NO for neurotransmission, vasodilation, and inflammation/cytotoxicity [97]. The dysfunction of these enzymes has been implicated in a number of diseases including ischemic injury, cardiovascular diseases, diabetes, multiple sclerosis, to mention only a few. The quest for understanding the structure–function relationships of these diflavin enzymes continues and promises not only to provide insights into the development of disease but, perhaps, some basis for therapeutic approaches.

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