Nitric-oxide Synthase Output State

**DESIGN AND PROPERTIES OF NITRIC-OXIDE SYNTHASE OXYGENASE/FMN DOMAIN CONSTRUCTS**

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Mammalian nitric-oxide synthases are large modular enzymes that evolved from independently expressed ancestors. Calmodulin-controlled isoforms are signal generators; calmodulin activates electron transfer from NADPH through three reductase domains to an oxygenase domain. Structures of the reductase unit and its homologs show FMN and FAD in contact but too isolated from the protein surface to permit exit of reducing equivalents. To study states in which FMN/heme electron transfer is feasible, we designed and produced constructs including only oxygenase and FMN binding domains, eliminating strong internal reductase complex interactions. Constructs for all mammalian isoforms were expressed and purified as dimers. All synthesize NO with peroxide as the electron transfer product for NADPH derived reducing equivalents. FMN is capable of serving as a one electron heme reductant. The nitric oxide (NO) from arginine, requiring 2 mol of O₂ and 1.5 mol of NADPH/mol of NO produced (1–3). The constitutive isoforms, eNOS and nNOS, are regulated by calcium/calmodulin (Ca²⁺/CaM) (4, 5) and additional inputs including phosphorylation of specific residues (6); the NO produced by constitutive isoforms functions as a molecular signal. A cytokine inducible isoform (iNOS) is calcium insensitive, and produces much larger fluxes of NO as a cytotoxin in immune response (4). Eukaryotic NOS isoforms are large modular enzymes. The monomer molecular mass is 120–161 kDa; the dimer is the active form (7), and the dimerization interface includes the tetrahydrobiopterin (H₄B) binding site in the oxygenase domain (8). The common elements are the heme and H₄B containing the oxygenase domain and a complex reductase unit homologous to NADPH P450 reductase that consists of a NAPDH binding domain, a FAD binding domain, and an FMN binding domain (9–11). The reductase and oxygenase regions are linked by a polypeptide segment containing a CaM binding site (12). Evidence suggests that the oxygenase domain of one monomer is reduced by the reductase unit of the other (13) through an oxygenase domain surface that exposes the corner of the heme (8). Ca⁺⁺/CaM controls constitutive isoforms through regulation of electron transfer between NADPH and heme (14). CaM has little or no effect on the thermodynamics of redox processes in NOS, suggesting that regulation is accomplished through modulation of electron transfer (15–18). A 40–50-residue autoinhibitory insertion in the FMN binding domain marks the major difference between cNOSs and iNOS (19), but electron transfer modulation appears also to involve a much smaller insertion present in the subdomain region as well as C-terminal tail differences (20, 21).

Although no solved structure of the holoenzyme exists, both partial structures and structures of close homologs provide considerable information (22–24). In structures of P450 reductase and NOS reductase domains (22–24), the flavin isoalloxazine ring edges are within 5 Å of each other in the interior of the protein. The available structural and evolutionary information strongly suggests that this conformation can serve as the input state from electrons from NADPH but not as the output state to acceptors such as the oxygenase domain or cytochrome c. For example, FMN cannot be docked to within tunneling distance of heme. These enzymes evolved from ancestral systems in which the FMN domain was a separate protein that functioned as a free shuttle before gene fusion events tethered it to the FAD binding domain (25). Available crystal structures show only the input state of the shuttle. Mechanistic information suggests that the limiting step in catalysis is the formation of the output state; NAPDH reduction is 3 orders of magnitude faster than NO production, and cytochrome c reduction is intermediate between the two.

A construct containing just the FMN binding domain and the oxygenase domain would facilitate study of the association of these two domains free of the influence of the FAD and NAPDH binding domains. An analogous construct has been expressed and characterized for cyto-
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chrome P450BM-3 from *Bacillus megaterium*, which has a P450 oxygenase domain unrelated to NOS linked to a reductase unit homologous to the corresponding NOS domains (26). P450BM-3 does not bind calmodulin. The *B. megaterium* construct was used to provide a simpler system for kinetic investigations. This communication describes the design and production of an oxyFMN NOS construct and provides preliminary characterization of its properties. We intend to use this construct to characterize the previously described (25) output state of the FMN binding domain of NOS.

MATERIALS AND METHODS

Design of NOS OxyFMN; Strategy and Bacterial Expression Vectors

The oxygenase and FMN binding domains of mammalian NOS have extended surface charge distributions that appear to define interaction surfaces. The surface of the oxygenase domain has a well defined ring of positive charge surrounding the most exposed edge of the heme. The FMN binding site is surrounded by a group of negatively charged residues, but these surfaces cannot interact when the FMN binding domain is associated with the reductase complex. When the constructs described here were originally designed, the reductase structure was not available, but a good approximation was made by modeling based on the structure of P450 reductase (22). Three logical termination points were selected; the shortest construct terminated directly at the end of the final a helix of the FMN binding domain. The constructs described here were designed to include the initial two acidic residues in cNOS of a C-terminal negatively charged element, which closely wrapped the edge of the FMN binding domain (shown on the left of Fig. 1 terminating as E951) before forming the connector to the FAD binding domain. The extended connector was not included in any of the constructs. The solid green residues are acidic and define two negatively charged surfaces, including the one facing the viewer.

Fig. 1a shows the face of the oxygenase domain in which a corner of the heme, shown in red, is exposed. The dark blue residues are positively charged, the green residues negatively charged, and cysteines are shown in yellow.

We decided to truncate the oxygenase domains N-terminally at residue 66 for eNOS, 65 for iNOS, and 290 for nNOS to minimize N-terminal proteolysis during purification, as previously described for oxygenase constructs (27, 28). Fig. 2 shows a schematic sequence alignment for the three constructs used in this study. The C-terminal residues are

![FIGURE 2. Schematic sequence alignment of oxyFMN constructs showing terminal positions, domains, and some major structural features. Oxygenase domain at the left has binding determinants for Zn (mouse iNOS Cys-104 and -109) and heme (mouse iNOS axial Cys-194) indicated; additional sites are included but not marked. These include other residues involved in heme binding and binding sites for pterin and substrate arginine. FMN binding domain at the right includes all three regions involved in FMN binding (mouse iNOS residues 540, 587, and 625); cNOS constructs include autoinhibitory insertion within this domain, shown as a white diamond. All three constructs include a CaM binding site between the domains (filled black diamond). SwissProt accession numbers are P29474 (human eNOS), P29476 (rat nNOS), and P2477 (mouse iNOS).](image-url)
buffer B containing 4 mM EDTA. The heme-containing fractions were pooled, concentrated, and dialyzed at 4 °C against buffer B containing 0.5 mM L-Arg, 1 mM dithiothreitol, 4 mM H2B, 2 mM FMN, and 5% glycerol and stored in aliquots at −80 °C until further analysis. Murine iNOS oxyFMN was further purified through a Superdex 200-HR (Amersham Biosciences) gel filtration column. For purification of NOS oxygense domain, one-step Ni2+ affinity purification was done as above except both cell lysis and purification were done in the absence of FMN (27).

**NOS Activity**—Both oxyFMN and oxygense domain activity were measured by following H2O2-supported oxidation of NAD-hydroxy-L-Arg (∼NOHA) at 37 °C for 10 min (32, 33). The reaction was carried out in 100 μl of total volume containing 40 mM EPPS, pH 7.6, 150–500 nM oxyFMN or oxygense domain protein, 1 mM L-NOHA, 0.5 mM dithiothreitol, 30 mM H2O2, 10 units/ml superoxide dismutase, 0.5 mg/ml bovine serum albumin, and variable concentrations of H2B (0–1000 μM). Reactions were initiated by 30 mM H2O2 and stopped by the addition of 1500 units of catalase. Nitrite production was measured by the Griess assay as described previously (27, 32, 33).

**Potentiometric Titrations**—Titrations were carried out under argon in a side arm vessel essentially as described by Dutton (34), with minor NOS-specific modifications as described by Gao et al. (15). Titrations were monitored optically with an Aminco DW2000 scanning dual wavelength spectrometer. Mediators were: methyl viologen, 2 μM; benzyl viologen, 2 μM; safranine O, 1 μM; anthroquinone-2-sulfonate, 2 μM; anthroquinone-2,6-disulfonate, 2 μM; pyocyanin, 1 μM; phenazine methyl sulfate, 2 μM.

Simulations of redox titrations were described in Gao et al. (15); here only terms for heme and FMN were retained since the constructs lack an FAD binding domain. Briefly, the Nernst equation describing the redox equilibrium of an electron carrier is

\[ Eh = Em + RT/nF \log_{10} [(\text{oxidized})/(\text{reduced})] \]  

(Eq. 1)

The redox states for FMN are

\[ E1 \quad \text{FMN} \rightleftharpoons \text{FMN}(\text{H})^+ \rightleftharpoons E2 \quad \text{FMNH}_2 \]  

(Eq. 2)

Titrations were simulated using summed contributions from two heme states and three flavin states. At any wavelength these contributions are of the form,

\[ A_{heme \ ox} = \frac{C_{heme} \times E_{heme \ ox} \times 10^{(Em \text{-Em heme/F)/(RT)}}}{1 + 10^{(Em \text{-Em heme/F)/(RT)}}} \]  

(Eq. 3)

\[ A_{heme \ red} = \frac{C_{heme} \times E_{heme \ red} \times 10^{(Em \text{-Em heme/F)/(RT)}}}{1 + 10^{(Em \text{-Em heme/F)/(RT)}}} \]  

(Eq. 4)

\[ A_{FMN} = \frac{C_{FMN} \times E_{FMN} \times 10^{(Em \text{-Em FMN/F)/(RT)}} \times 10^{(Em \text{-Em FMN/F)/(RT)}}}{1 + 10^{(Em \text{-Em FMN/F)/(RT)}} \times 10^{(Em \text{-Em FMN/F)/(RT)}}} \]  

(Eq. 5)

\[ A_{FMNH} = \frac{C_{FMN} \times E_{FMNH} \times 10^{(Em \text{-Em FMNH/F)}}}{1 + 10^{(Em \text{-Em FMNH/F)} \times 10^{(Em \text{-Em FMNH/F)}}}} \]  

(Eq. 6)
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$$A_{\text{FMNH}_2} = \frac{C_{\text{heme}} \times E_{\text{FMNH}_2}}{(1+10^{(Eh-E\text{FMN}F/RT)} + 10^{(Eh-E\text{FMN}F/RT)} \times 10^{(Eh-E\text{FMN}F/RT)})}$$  (Eq. 7)

where $R$ is the gas constant, $F$ is the Faraday constant, $T$ is the Kelvin temperature, and $RT/|F|$ is $\sim$59.6 mV at 25 °C. Variables such as $e_{\text{FMN}}$ represent extinction coefficients for hemes and FMN redox states at specific wavelengths; $A$ values are the contributions of individual components to the optical density. Variables of the form $C_{\text{heme}}$ represent the total concentration of all heme (or, equivalently, FMN) states, including redox, spin, and conformational states, in rapid equilibrium on the time scale of redox equilibration in the experiment. Eh is the standard potential relative to the hydrogen electrode, and midpoint potentials of one electron couples are specified as Emheme, $E1\text{FMN}$, and $E2\text{FMN}$. $E1\text{FMN}$ and $E2\text{FMN}$ are the midpoint potentials for the first and second one-electron reductions of FMN. In some cases multiple heme components differing in concentration, extinction coefficient, and midpoint potential have been introduced for simulation purposes.

Stoichiometric titrations were carried out in the same vessel as the potentiometric titrations. The apparatus was not intended to measure the reducing equivalents accurately, but by making small additions of buffered dithionite while monitoring the flavin absorbance at 455 nm, it was possible to observe intermediate reduction states without using a mediator system.

Spectroscopy—Binding of l-Arg, H$_4$B, and imidazole was monitored by UV-visible spectral perturbation analysis. Light absorbance spectra of the purified NOS oxyFMN proteins were obtained with a Hitachi U2010 spectrometer equipped with computer-assisted data collection software (UV solutions, Wellesley Hills, MA). The NOS-ferrous-CO adduct absorbing at 444 nm was used to quantitate heme protein content by using an extinction coefficient of 74 mm$^{-1}$cm$^{-1}$ (35, 36).

Electron paramagnetic resonance spectra were carried out using a Bruker spectrometer with a flowing helium cryostat essentially as previously described (36). Samples were frozen quickly in cold isopentane or liquid nitrogen and kept on liquid nitrogen until the spectra were recorded.

Flavin Content—Flavin content was determined by measuring the absorbance of the released flavin at 450 nm using extinction coefficients of 17 mm$^{-1}$cm$^{-1}$ for FMN. Twenty μM enzyme in 200 μl of 40 mM Tris-HCl buffer, pH 8.0, was placed in a boiling water bath for 10 min, chilled on ice, and centrifuged at 14,000 rpm for 20 min, and the supernatant used for FMN quantitation using Sigma FMN as standard. The flavin content for all the NOS isoforms mentioned in the results section is an average of the values measured.

RESULTS

The optical spectra of three oxyFMN constructs are shown in Fig. 3, A–C. The heme is primarily in the high spin state after treatment with H$_4$B and arginine during preparation as indicated by the Soret maximum near 400 nm. Features of the oxidized FMN optical spectrum are visible at 450 and 480 nm. The FMN appears to be more oxidized in these constructs than in freshly isolated holoenzyme, probably because the enzyme lacks an NADPH binding site. A band visible near 650 nm is due to the high spin ferriheme charge transfer band. The broad FMN blue neutral band, extending from 550 to 650 nm, that is characteristic of all three mammalian NOS holoenzymes, is very weak or absent. The spectra of corresponding constructs of the nNOS and iNOS are very similar, but in the eNOS oxyFMN construct a significant minority of the ferriheme remains in the low spin state in the presence of arginine. The top trace in Fig. 3D shows electron paramagnetic resonance spectra of nNOS oxyFMN construct replete with arginine and H$_4$B. The high spin features at $g = 7.7, 4.1$, and 1.76 are very similar to the features of the majority species of the arginine and H$_4$B replete holoenzyme. There are very low levels of low spin heme ($g = 2.4$ and 2.2), and almost all the heme is in a single high spin state. In particular, there is almost no $g = 6$ axial ferriheme signal from heme sites that have lost their thiolate ligand. A small amount of adventitious rhombic ferric ($g = 4.3$) iron is overlapped with the $g = 4.1$ feature of the heme. There is no detectable FMNH$^\cdot$ radical in the EPR spectra of the construct as isolated, consistent with the optical spectra. A small amount of copper contaminant is visible just above $g = 2$.

The second trace in Fig. 3D shows a similar spectrum of iNOS oxyFMN construct. Small differences in the $g$ values of the heme spectra are not detectable in this wide scan (but see Fig. 9). The lower trace shows the corresponding spectrum of a nNOS oxyFMN preparation that has been passed through a cycle of reduction with a small amount of dithionite and air oxidized, producing an air stable FMN semiquinone, visible at $g = 2.1$, and regenerating the high spin ferriheme. UV-visible spectra of a corresponding experiment are shown in Fig. 6.

All preparations of the eNOS, nNOS, and iNOS oxyFMN constructs contained between 0.9 and 1 heme/mol based on the CO difference spectra (not shown but indistinguishable from the CO difference spectra of holoenzyme and oxygenase domains); all of the heme content measured was in the native thiolate heme complex absorbing near 445 nm, with no detectable 420-nm band. The FMN content measured after extraction from the protein was in all cases at least 90% of the heme content, a difference that could result from loss of FMN during the process. The iNOS construct can be purified in higher yield, probably because it lacks an autoinhibitory element and is less likely to be destroyed by proteolysis. The high level of incorporation of the two major prosthetic groups suggests proper folding during expression.

Because there is no NADPH binding site, none of the oxyFMN constructs exhibit either NO production with NADPH as electron donor or any of the common NADPH reductase activities of NOS holoenzymes. However, oxyFMN constructs of all three isoforms are capable of NO synthesis from N-hydroxarginine (l-NOHA) with peroxide as the electron donor. Fig. 4 shows the ability of all three NOS oxyFMN constructs and their oxygenase domain counterparts to catalyze the H$_2$O$_2$-supported nitrite formation from l-NOHA as a function of H$_4$B concentration (0–1000 μM). The activity of both murine and human eNOS and iNOS oxyFMN is similar to their oxygenase domain activities, whereas nNOS oxyFMN showed increased activity compared with that of its oxygenase counterpart. The dependence of NO formation on H$_4$B concentration is substantially the same for oxyFMN and oxygenase domain preparations. H$_4$B is not a reactant, since it is substoichiometric with NO production, but the requirement for full activity is greater than expected from the $K_a$, which is very low. H$_2$O$_2$-supported nitrite formation from l-NOHA by NOS oxyFMN also depends on H$_4$B concentration at similar H$_4$B levels, as reported earlier for the oxygenase domain and holoprotein (32, 33). The requirement for H$_4$B has not been elucidated but appears to be related to the use of peroxide as reductant. The activity measured by this method reflects the full functionality of the oxygenase domain catalytic site after expression.

Purified NOS oxyFMN derived from all three isoforms migrates as a dimer during gel filtration analysis (data not shown) without significant aggregation or monomer formation. The 280-nm/Soret absorbance ratio is $\sim 2.2–2.5$ in 80–90% pure preparations and serves as a routine
purification index. The iNOS construct migrates as a single majority band at 69 kDa on SDS-PAGE, with a minority band at 50 kDa due to a cleavage product and a 15.7-kDa band from co-expressed CaM. The eNOS and nNOS oxyFMN preparations have additional minor bands at 50–55 kDa (Fig. 4, inset). The estimated molecular mass for nNOS oxyFMN is 75 kDa.

In addition, all these spectra showed typical flavin (FMN) peaks at 450 and 480 nm and a characteristic high spin ferriheme charge transfer band at 650 nm. Panel D (top trace) shows the electron paramagnetic resonance spectrum of nNOS oxyFMN construct at a concentration of 45 μM recorded at 10 K. Instrument settings were: microwave power, 2 milliwatts; modulation amplitude, 10 gauss; modulation frequency, 100 kHz; microwave frequency, 9.47 GHz; magnetic field scan, 0.4 millitorr; field center, 0.23 millitorr. The spectrum is dominated by a single high spin species with features at g = 7.7, 4.0 and 1.76. Axial high spin heme at g = 6 and low spin heme at g = 2.4 are almost undetectable, and there is very little free radical at g = 2. The conditions for the lower two traces are the same except that the temperature is 15 K, and the gain has been increased. The middle trace shows a spectrum of oxidized iNOS oxyFMN construct; the sample concentration was ~15 μM. The lower trace shows a second spectrum of nNOS oxyFMN complex after reduction with dithionite and partial reoxidation by exposure to air; the heme is only about 50% in the ferric state. An FMN radical is visible at g = 2.

As judged by the effect of arginine on the heme Soret band, all three constructs bind H₄B and arginine. Arginine- and H₄B-replete iNOS and nNOS preparations are essentially 100% high spin by optical criteria, consistent with a high level of intactness of the cofactor and substrate binding sites and with accessibility of all sites to arginine and H₄B, a low spin minority species persists in eNOS preparations. The dissociation constant of L-arginine (K_d) for all three NOS oxyFMN constructs, as
determined by spectral perturbation analysis in presence of imidazole (progressive titration spectra is not shown, but titration spectra reflect low to high spin transitions as in the difference spectrum shown in the inset of Fig. 5), indicates a high affinity site ($K_d \approx 0.5–1.0 \mu M$), confirming proper folding and dimerization of the oxygenase domain in these constructs.

Fig. 6 shows spectra of the iNOS oxyFMN construct recorded during stoichiometric titration with small aliquots of sodium dithionite solution. After stepwise addition of enough reductant to bleach the FMN bands at 455 and 480 nm, UV-visible spectra were recorded, and additional dithionite was added until FMN and heme were fully reduced, as judged by the spectra. The first electron into the construct reduces FMN to the blue neutral semiquinone state with loss of the oxidized FMN bands at 455 and 480 nm; at the same time, the weaker semiquinone bands can be observed near 560 and 600 nm. An additional band at 480 nm is difficult to resolve because it is overlapped by the stronger oxidized FMN band at 480 nm, but the broader semiquinone absorbance contributes additionally near 520 nm.

Further addition of reductant causes parallel bleaching of the semiquinone bands and conversion of the heme to the ferrous state. Heme reduction is visible as a red shift in the Soret and as loss of the high spin ferric charge transfer band at 650 nm; the latter is overlapped by the semiquinone bands. No red anionic semiquinone was detected. An additional cycle of oxidation and reduction produced spectra nearly identical to the original oxidized and reduced spectra, indicating that the construct was stable during the experiment. This method can be used to generate flavin semiquinone, whereas the heme is completely oxidized, but heme reduction and reduction of FMN to FMNH$_2$ cannot be separated in the iNOS construct, which has calmodulin tightly bound. This is probably due to equilibration of heme and FMN faster than the time scale of the experiment (about 1 min). The stability of the construct to cycles of oxidation/reduction is critical to the design of kinetics experiments to demonstrate rapid heme/FMN electron transfer.

Fig. 7 shows selected UV-visible spectra of iNOS FMN domain during a potentiometric titration. The midpoint potential for heme reduction is about $-180 \text{ mV}$, slightly higher than observed in titrations of the holoenzyme or the independently expressed oxygenase domain. This can be readily observed in the Soret region; the peak position shifts from 390 nm to about 407 nm, and the band sharpens as the heme is reduced to the ferrous state. The charge transfer band at 650 nm disappears as the heme is reduced, and the ferrous a band region increases in intensity. Very similar results were obtained for the eNOS and nNOS constructs.

The oxidized FMN bands at 450 and 480 nm decrease in intensity as FMN is reduced to the semiquinone state. The blue neutral semiquinone is again most easily observed as a group of broad bands centered near 560, 590 and 620 nm and, in addition, by increased absorbance at 520 nm. The inset shows an expanded view of the titration of the 600 – 650-nm region, showing initially increasing intensity at 620 nm followed by loss in intensity at 620 and 650 as the potential is lowered further. The initial reduction of FMN occurs at approximately $+70 \text{ mV}$; semiquinone reduction is essentially complete by $-260 \text{ mV}$.

Fig. 8a shows a plot of the absorbance at 620 and 650 nm as a function...
of potential for spectra collected during the iNOS oxyFMN titration. The simulations are composed of two components, a ferriheme \( n = 1 \) component and an FMN semiquinone titrating as a thermodynamic transient. The potentials used to fit the 3 one-electron couples are \(-180 \text{ mV for the one-electron heme couple (} E_{\text{heme}} \text{), } 70 \text{ mV for the FMN/FMNH}^+ \text{ couple (} E_{\text{FMN}} \text{), and } -180 \text{ mV for the FMNH}^+/\text{FMNH}_2 \text{ couple (} E_{\text{FMNH}} \text{).}

Similar simulations of data collected at 455 and 520 nm are shown in Fig. 8b. The major contributions to the changes in absorbance at 455 nm are the heme Fe\(^{2+}/\text{Fe}^{3+}\) couple and the FMN/FMNH\(^+\) couple. The data can be fit well with the potentials used to fit the data of Fig. 8a, but a slightly better fit was obtained with an \( E_{\text{FMNH}} \) of +50 mV. This difference is not significant. Because FMNH\(^+\) does not contribute significantly here, the FMNH\(^+/\text{FMNH}_2\) couple cannot be accurately determined at this wavelength, but it must be significantly lower than the FMN/FMNH\(^+\) couple.

The major contributions to the absorbance changes at 520 nm are the transient formation of semiquinone and the slightly greater contribution of ferric heme. The data have been fit with the same potentials as in Fig. 8a, except that the FMN/FMNH\(^+\) couple is again better fit at +50 mV. Similar results were obtained in titrations of nNOS (not shown).

Spectra taken during titration of eNOS and nNOS constructs are included in supplementary information. For eNOS and nNOS, calmodulin effects were examined by adding calmodulin and taking spectra at a few selected potentials. The results for both isoforms were consistent with a shift of about 20 mV to higher potential for both the heme and FMNH\(^+/\text{FMNH}_2\) couples, an effect at the edge of resolution in our experiments. An effect large enough to be responsible for the activation
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FIGURE 6. UV-visible spectra taken during stepwise reductive titration of 8 μM iNOS oxyFMN construct replete with H4B and arginine at pH 7.5 in 50 mM MOPS, 100 mM NaCl, 10% glycerol. Reduced species were generated by adding continuous small aliquots of concentrated sodium dithionite solution (in 100 mM Tris base) until changes in absorbance at 480 nm were visible; spectra were then collected, and further additions were made. A, oxidized construct with no additions. The heme spectrum is indicative of almost completely high spin population (Soret and 650-nm charge transfer bands), and FMN is fully oxidized (strong 450- and 480-nm bands and no semiquinone features), consistent with epr results. B, oxyFMN iNOS partially reduced with a small aliquot of dithionite solution. Most of the change in the Soret region is due to the loss of the flavin band at ~348 nm; note that most of the heme charge transfer band at 650 is still present. C, fully reduced with additional dithionite; note the loss of flavin bands and reduced Soret position. D, reoxidized with potassium ferricyanide; spectrum is nearly identical to A. E, re-reduced with excess dithionite; spectrum is almost identical to C.

FIGURE 7. Spectra taken during titration of iNOS oxyFMN construct showing oxidized FMN bands at 450 and 480 nm, red shift of heme Soret during reduction, and charge transfer and blue neutral semiquinone bands near 650 nm. The inset shows details of charge transfer and blue neutral semiquinone band changes in the 600-650-nm region.

FIGURE 8. a, simulation of potentiometric titration of 620 and 650-nm absorbance of iNOS oxyFMN construct. Parameters for 620-nm simulation were: EmFMN = +70 mV, Emheme = -180 mV. For the 650-nm simulation, the maximum contribution of the semiquinone was 0.018 and of ferriheme, 0.02 units, relative to the absorbance of the reduced enzyme. simheme represents the contribution of the heme to the absorbance at 650 nm. b, simulation of titration of 455- and 520-nm absorbance with potential. Parameters for 520-nm simulation were: EmFMN = +70 mV, Emheme = -180 mV, EmFMN = -170 mV. For the 455-mV simulation, the total contribution of ferriheme and semiquinone relative to reduced enzyme was ~0.09 units, and the contribution of oxidized FMN was 0.1 units.

TABLE 1
Midpoint potentials at pH 7.5 of electron carriers in oxyFMN constructs

| Construct   | FMN sq/hq | FMN sq/hq | Heme   |
|-------------|-----------|-----------|--------|
| eNOS        | -210 mV   | -210 mV   | -210 mV|
| nNOS        | -180 mV   | -180 mV   | -180 mV|
| iNOS        | -180 mV   | -190 mV   | -190 mV|

of electron transfer is ruled out, consistent with previous results with holoenzyme (15).

The results for titrations of iNOS, eNOS, and nNOS are summarized in Table 1. The potentials are generally similar, although the details are slightly different. In general, the first one-electron reduction of the FMN occurs at high potential as in holoenzyme and in reductase constructs. The second single electron reduction of FMN and heme reduction are nearly isopotential. The eNOS construct contains a significant minority species that is low spin in the presence of arginine.

Fig. 9 shows effects of interdomain interactions on EPR signals of the high spin ferriheme. The three top traces are the gmax features of nNOS constructs; the asymmetry is due to the details of strain broadening. The central peak is the gmax signal from a nNOS oxygenase preparation replete with arginine and H4B; gmax ~ 7.72. The left peak is the corresponding signal from a nNOS oxyFMN preparation. gmax for this peak is ~7.72. Although the peak position of the strain broadened line is only slightly shifted to high field relative to the oxygenase construct, the line is significantly sharpened, and the low field edge is noticeably shifted to the left (see "Discussion"). The right hand peak is the gmax signal from the same nNOS oxyFMN preparation in the presence of Ca2+/CaM. The peak has shifted downfield to g ~ 7.69, and the low field side of the line is broader. We attribute this shift in peak position and line shape to the effect of calmodulin-induced association of the FMN binding domain on the ligand geometry of the heme. This is the first report of a calmodulin effect on the spectrum of heme in any NOS construct.

The two lower spectra show the gmax features of iNOS oxyFMN

The absorbance at 480 nm were visible; spectra were then collected, and further additions were made. A, oxidized construct with no additions. The heme spectrum is indicative of almost completely high spin population (Soret and 650-nm charge transfer bands), and FMN is fully oxidized (strong 450- and 480-nm bands and no semiquinone features), consistent with epr results. B, oxyFMN iNOS partially reduced with a small aliquot of dithionite solution. Most of the change in the Soret region is due to the loss of the flavin band at ~348 nm; note that most of the heme charge transfer band at 650 is still present. C, fully reduced with additional dithionite; note the loss of flavin bands and reduced Soret position. D, reoxidized with potassium ferricyanide; spectrum is nearly identical to A. E, re-reduced with excess dithionite; spectrum is almost identical to C.

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The results for titrations of iNOS, eNOS, and nNOS are summarized in Table 1. The potentials are generally similar, although the details are slightly different. In general, the first one-electron reduction of the FMN occurs at high potential as in holoenzyme and in reductase constructs. The second single electron reduction of FMN and heme reduction are nearly isopotential. The eNOS construct contains a significant minority species that is low spin in the presence of arginine.

Fig. 9 shows effects of interdomain interactions on EPR signals of the high spin ferriheme. The three top traces are the gmax features of nNOS constructs; the asymmetry is due to the details of strain broadening. The central peak is the gmax signal from a nNOS oxygenase preparation replete with arginine and H4B; gmax ~ 7.72. The left peak is the corresponding signal from a nNOS oxyFMN preparation. gmax for this peak is ~7.72. Although the peak position of the strain broadened line is only slightly shifted to high field relative to the oxygenase construct, the line is significantly sharpened, and the low field edge is noticeably shifted to the left (see "Discussion"). The right hand peak is the gmax signal from the same nNOS oxyFMN preparation in the presence of Ca2+/CaM. The peak has shifted downfield to g ~ 7.69, and the low field side of the line is broader. We attribute this shift in peak position and line shape to the effect of calmodulin-induced association of the FMN binding domain on the ligand geometry of the heme. This is the first report of a calmodulin effect on the spectrum of heme in any NOS construct.

The two lower spectra show the gmax features of iNOS oxyFMN...
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construct. Because the iNOS construct must be coexpressed with a calmodulin, we couldn’t perform the analogous experiment to demonstrate the change in the heme environment triggered by CaM binding. The solid trace at $g_{\text{max}} \sim 7.72$ is very close in peak position to the nNOS oxyFMN construct position after the addition of CaM. The dashed line shows the $g_{\text{max}}$ feature of the same preparation after the addition of 2 μl of saturated dithionite solution to the 250-μl sample. This generated a FMNH$_2$ radical in about ½ of the enzyme molecules. The high field side of the $g_{\text{max}}$ line is broadened in this sample. To produce an effect of this magnitude in a sample with only partial conversion to the radical state, a magnetic interaction of 5–10 gauss would be required. The spectrum of the flavin radical at $g = 2$ is also broadened; we hope to present an analysis of the magnetic interactions in a subsequent communication.

DISCUSSION

The results presented here demonstrate that two-domain oxyFMN constructs of all three mammalian NOS isoforms can be expressed in E. coli and are well folded and bind functional prosthetic groups. The oxyFMN construct is designed to be the "output state" counterpart of the three-domain reductase construct and as such should be of value in probing the mechanism of the enzyme. The purpose of this study was to establish that such constructs could be expressed and purified in qualities and quantities suitable for detailed structural and functional studies. In addition, characterization of the constructs was undertaken to determine some critical parameters for internal electron transfer reactions.

It is clear from the spectroscopic and enzymology results that the oxygenase domain is stable and functional. By spectroscopic criteria, preparations of all three constructs are superior to most holoenzyme preparations in that they are homogeneous, bind substrate and cofactors stoichiometrically, and do not contain significant amounts of denatured material.

The titration results are of special interest. The FMN semiquinones are stable and are the blue neutral forms as in the holoenzymes. The midpoint potentials of the heme and the FMNH$_2$/FMH$_2$ couple appear, however, to be slightly above $-200$ mV in all three cases. This is 60 mV or more greater than the potentials obtained using individual domains and holoenzyme preparations, a modest but significant difference corresponding to a 1 order of magnitude change in equilibrium constants for electron transfer reactions. For example, in holoenzymes, typical values for arginine-replete heme and FMN are $-240$ mV for FeII/FeIII, 0 mV for FMN/FMNH$_2$, and $-250$ mV for FMNH/FMH$_2$ (15). The FMN couples were reported by Noble et al. (17) to be about $-50$ and $-275$ mV in isolated reductase constructs.

In summary, as in holoenzyme the FMNH/FMH$_2$ and heme couples are an approximately isopotential group, whereas the FMN/FMH$_2$ couple is too high in potential to be an effective heme reductant in steady state experiments. This need not rule out the possibility of generating a transient one-electron reduced FMN species that would effectively reduce the heme by rapid electrochemical or photochemical methods.

Observation of higher potentials in a construct in which the electron carriers may be more exposed to solvent should not be surprising. The heme in these constructs, however, has a potential higher than that reported for individually expressed oxygenase domains ($-220$ mV in the presence of arginine) (38). This appears to be a special property of the construct and may reflect interactions between the domains. In nNOS and iNOS constructs, arginine generates nearly complete high spin ferriheme; small amounts of low spin sometimes persist. As Fig. 3 indicates, the eNOS construct is less homogeneous spectrally and contains a significant low spin component corresponding to about 30% of the total heme in the presence of arginine.

Some differences in opinion exist concerning the effects of arginine. Presta et al. (38) report little or no effects of arginine on apparent midpoint potentials of iNOS oxygenase domain constructs. We found that arginine shifted the midpoint potential of heme in iNOS and nNOS holoenzymes by at least 60 mV; preparations free of H$_2$B did not titrate as a single component, suggesting a range of conformations and/or dimerization states with different properties (15, 41).

It is particularly interesting to note that the potentials of both the heme and the FMNH/FMH$_2$ couple in the iNOS and nNOS constructs, which are the most homogeneous and native of the three expressed so far and are both slightly higher than the potentials of these carriers in holoenzymes but remain approximately isopotential. This suggests the possibility that the $-60$-mV shift we observe reflects the effects of FMN/oxygenase domain interactions and that these interactions developed to retain the reversibility of the FMN/heme electron transfer in the absence of a reaction (e.g. NO production) pulling the equilibrium toward the oxygenase site. Potential differences are not strong evidence of FMN-heme interaction, but interactions between the two domains are a possible explanation for small differences in the observed potentials.

In contrast, the spectral evidence for interactions between the domains is very strong. The calmodulin-driven shifts in the EPR peak position and line shape of the nNOS oxyFMN complex shown in Fig. 9 indicate changes in the ratio of the rhombic and axial zero field splitting parameters E and D. As E/D increases, $g_x$ and $g_y$ split about 6; $g_z$ and $(g_x + g_y)/2$ decrease as a second-order effect. High spin ferrithemins are well described by the $S = 5/2$ spin Hamiltonian $D(S^z - \frac{1}{3}(S(S+1)) + E(S^x^2 - S^y^2)$, where $E$ and $D$ reflect differential admix-
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ture of low-lying states into the ground state $^6A_1$ sextet by spin-orbit coupling because of tetragonal and rhombic splitting of the $t_{2g}$ d orbitals (see for example Ref. 37).

The strain-broadened line shape is produced by distributions of E/D centered around 0.083 that inherently produce a sharpen high field side to the line because the dependence of the field position on E/D becomes weaker as E/D increases. All NOS EPR spectra observed so far result from E/D ratios in the range 0.058–0.088. In this case E/D is probably close to the upper limit which can be obtained with thiolate ligation and the underlying molecular geometry.

It is not surprising that CaM-driven association of the FMN and oxygenase domains affects the distribution of E/D at the heme iron. Interaction of the surface charge distributions adjacent to the prosthetic groups is the likely source of the observed small distortion of the heme environment. CaM does not produce a comparable effect in holoenzyme, probably because the fraction of molecules in which the FMN environment. CaM does not produce a comparable effect in holoenzyme domains affects the distribution of E/D at the heme iron.

The strain-broadened $g_{max}$ line produced by formation of FMN radical in iNOS oxyFMN is less obvious than the broadening of the radical because the heme line width at $g_{max}$ is about twice that of the FMN radical in iNOS oxyFMN is less obvious than the broadening of the domain associates with the heme site is too small. 

In conclusion, the oxyFMN constructs of all three mammalian NOS isoforms have been expressed as stable proteins with activity and interesting properties. We have observed the direct effects of CaM on NOS heme spectra and the first indications of FMN-heme magnetic coupling. They appear to be good candidates for further kinetic and structural studies of the NOS output state.

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