REDOX PROPERTIES OF HUMAN ENDOTHELIAL NITRIC OXIDE SYNTHASE
OXYGENASE AND REDUCTASE DOMAINS PURIFIED FROM YEAST EXPRESSION
SYSTEM

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This work was supported by U.S. Public Health Service Grants GM56818 (A.-L.T.),

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FOOTNOTES

1 The abbreviations used are: NOS, nitric oxide synthase; eNOS, endothelial nitric oxide synthase; eNOS$_{ox}$, eNOS oxygenase domain; eNOS$_{red}$, eNOS reductase domain; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; BH$_4$, (6R)-5,6,7,8-tetrahydro-L-biopterin; 2’ and 3’-AMP, adenosine 2’ and 3’-monophosphate; BCA, bicinchoninic acid; EPR, electron paramagnetic resonance; ICP-MS, Inductively coupled plasma emission mass spectrometry; CPR, cytochrome P450 reductase; CaM, calmodulin; SDS-PAGE, SDS polyacrylamide gel electrophoresis; DCPIP, 2,6-dichlorophenol indophenol.
ABSTRACT

Characterization of the redox properties of endothelial nitric oxide synthase, eNOS, is fundamental to understanding the complicated reaction mechanism of this important enzyme participating in cardiovascular function. Yeast overexpression of both the oxygenase and reductase domains of human eNOS, i.e. eNOS\(_{\text{ox}}\) and eNOS\(_{\text{red}}\), has been established to accomplish this goal. UV-Vis and EPR spectral characterization for the resting eNOS\(_{\text{ox}}\) and its complexes with various ligands indicated a standard NOS heme structure as a thiolate hemeprotein. Two low-spin imidazole heme complexes but not the isolated eNOS\(_{\text{ox}}\) were resolved by EPR indicating slight difference in heme geometry of the dimeric eNOS\(_{\text{ox}}\) domain. Stoichiometric titration of eNOS\(_{\text{ox}}\) demonstrated that the heme has a capacity for 1-1.5 reducing equivalent. Additional 1.5 –2.5 reducing equivalents were consumed before heme reduction occurred indicating the presence of other unknown high-potential redox centers. There is no indication for additional metal center that could explain this extra electron capacity of eNOS\(_{\text{ox}}\). Ferrous eNOS\(_{\text{ox}}\), in the presence of L-arginine, is fully functional in forming BH\(_4\) radical upon mixing with oxygen as demonstrated by rapid-freeze EPR measurements. CaM binds eNOS\(_{\text{red}}\) at 1:1 stoichiometry and high affinity. Stoichiometric titration and computer simulation enabled the determination for three redox potential separations between the four half reactions of FMN and FAD. The extinction coefficient could also be resolved for each flavin for its semiquinone, oxidized and reduced forms at multiple wavelengths. This first redox characterization on both eNOS domains by stoichiometric titration and the generation of high-quality EPR spectrum for the BH\(_4\) radical intermediate illustrated the usefulness of these tools in future detailed investigation into the reaction mechanism of eNOS.
Nitric oxide synthase (NOS) is an uncommon self-sufficient P450-like enzyme catalyzing nitric oxide (NO) biosynthesis from L-arginine (1-4). There are three mammalian NOS isozymes: the constitutive neuronal NOS (nNOS) and endothelial NOS (eNOS) require calmodulin for enzyme activity, whereas the inducible NOS (iNOS) contains tightly bound calmodulin (1-4). All three isozymes have a common bi-domain structure with the reductase domain containing FAD, FMN and NADPH binding sites, and the oxygenase domain harboring the heme center and binding sites for L-arginine and tetrahydrobiopterin (BH₄) (1-4). The main function of the reductase domain is to provide reducing equivalents to the heme center in the oxygenase domain where the key chemistry of L-arginine conversion occurs. Three substrates and four products are involved in NOS catalysis. The overall reaction is a complicated 5-electron oxidation of the key guanidine nitrogen plus three additional electrons from NADPH to reduce 2 molecules of oxygen to water and form the L-citrulline. Several X-ray crystallographic structures for the iNOS and eNOS oxygenase domains have been reported (5-7). The X-ray crystallographic data at 1.9 Å resolution of C-terminal FAD-NADPH binding domain of the nNOS reductase domain was also published recently (8). These data reveal a three-domain modular design. The FAD and NADPH binding subdomains are superimposable on those of cytochrome P450 reductase (CPR) with an rms deviation of 1.3 Å, while the more flexible FMN connecting domain shows a 3.9 Å rms to the α -chain of CPR. The fourth domain that binds FMN is lost during crystallization but the structure is projected to be similar to that of CPR. These crystallographic data give firm support for a modular design of NOS thus provide basis to prepare subdomains for structure/function and reaction mechanism studies. Investigation into individual break down modules could simplify the data interpretation for each redox center and should be a useful approach in elucidation of the
complicated reaction mechanism for NOS.

Overexpression systems for the individual oxygenase and reductase domain of NOS have been developed in bacterial and baculovirus systems including our own group (9 - 17). Only few are related to eNOS (6, 17). Large amount of eNOS were usually obtained by trypsinolysis from intact bovine eNOS (13, 18). Although the baculovirus system is working (17), it is both time-consuming and costly. The bacterial expression system (18), although fast, has unpredictable sudden debilitating mutations in the expression construct and has resisted being scaled up to more than few liters of culture in our hands for unknown reasons. Yeast expression could be an alternative vehicle to generate large amount of active mammalian enzymes (19). Yeast has been shown to be effective in overexpressing eNOS and the reductase domain of nNOS (14, 20). In this study, we report the overexpression in yeast the oxygenase, eNOS\textsubscript{ox}, and reductase, eNOS\textsubscript{red}, domains of human eNOS and the characterization for their oxidation-reduction activities. Both domains show behaviors very similar to the domains present in the whole eNOS and should be useful tools for future biophysical and mechanistic investigations.
EXPERIMENTAL PROCEDURES

**Materials**-- L-[2,3,4,5-3H]Arginine and 2',5'-ADP-Sepharose 4B were purchased from Amersham Pharmacia Biotech. Inc. (17). BH₄ was obtained from Schircks Laboratories (Jona, Switzerland). Plasmids containing human eNOS cDNA in pGEM3Z and human eNOS polyclonal antibodies were kindly provided by Dr. Pei-Feng Chen in our division (21). PCR kits, Expand High Fidelity PCR System were the product of Roche Bioscience. Restriction enzyme PmeI was purchased from New England Biolabs and the other restriction enzymes were from Life Technologies, Inc. All reagents and devices for DNA extraction and isolation were products of Qiagen. Easyselect *Pichia* Expression Kit containing the expression vector pPICZB, *Pichia* strain GS115 and *E. coli* strain TOP10F' was purchased from Invitrogen and used for the expression of both eNOS domains. Reagents for electrophoresis and Western-blot were from Bio-Rad. The other chemicals were from Sigma.

**Expression of human eNOS domains**-- Polymerase chain reaction (PCR) was used to amplify the cDNA product. Human eNOS cDNA in pGEM3Z was used as template and DNA fragments encoding oxygenase (amino acid 1-491) and reductase domain (amino acid 482-1204) were amplified with specific primers. For oxygenase domain, the forward primer was 5’-CGGAATTCAACATG\text{CATCACCATCACCATCACGGCAACTTGAAGAGCGTG-3’}(\text{translation start codon is underlined}), and the backward primer was 5’-GCTCTAGATCA\text{GGTGATGCCGGTGCCCTTGGC-3’}(\text{translation stop codon is underlined}).

For reductase domain, the forward primer was 5’-CGGAATTCAACATG\text{CATCACCATCACCATCACGGGAGTGCCGCCAAGGGC-3’}, and the backward primer was 5’-GCTCTAGAT\text{TCAGGTGATGCGGTGCCCTTGGC-3’}(\text{translation stop codon is underlined}).
forward primers, the EcoRI site and His6 tag were added, and in each backward primer an XbaI site was added. The correct sequences of the PCR products were confirmed by primer extension sequencing. Both PCR products were double-digested with EcoRI and XbaI and subcloned separately into the corresponding sites of an AOX promoter-driven expression vector pPICZB to obtain a 1.5 kb insert of eNOSox and a 2.1 kb insert of eNOSred. The constructs were linearized with PmeI, transformed into yeast P. pastoris GS115, and selected by growing on the YPDS/Zeocin plates containing 1% yeast extract, 2% peptone, 2% dextrose, 1M sorbitol and 100 μg/ml Zeocin. The colony that grew fastest was inoculated into 25 ml buffered minimal glycerol medium (100 mM potassium phosphate, pH 6.0, 1% yeast extract, 2% peptone, 1.34% yeast nitrogen base with ammonium sulfate without amino acids, 4 x 10^-5% biotin and 1% glycerol) and cultured at 30°C overnight. This culture was then transferred to a 250 ml buffered minimal glycerol medium and grown at 30°C overnight to A600 = 7~10. Cells were harvested and resuspended in 250 ml buffered minimal methanol medium (100 mM potassium phosphate, pH 6.0, 1% yeast extract, 2% peptone, 1.34% yeast nitrogen base with ammonium sulfate without amino acids, 4 x 10^-5% biotin and 0.5% methanol) with 4 mg/liter hemin chloride added and cultured for 72 hrs at 30°C to induce protein expression.

Protein purification--Yeast cells were harvested and washed with buffer 1 (50 mM Tris-HCl, pH 8.0) with protease inhibitors (1 μM Leupeptin, 1 μM Antipain, 1 μM pepstatin A and 1 mM phenylmethylsulfonyl fluoride) and resuspended in equal volume of buffer 1 with protease inhibitors. An equal volume of glass beads (425-600 microns) was added to the suspension. Cells were broken by 10 cycles of 30s vortexing and brief chilling on ice. Cell debris and glass beads were removed by centrifugation at 3400 r.p.m. The supernatant obtained after another
centrifugation at 12,000 r.p.m. in microfuge, was applied to a 2-ml Ni-NTA agarose column. The column was first washed with 50 bed volume of buffer 1 plus protease inhibitors, then ~30 bed volume buffer 1 plus 0.3 M NaCl and 1 mM L-histidine, then ~20 bed volume buffer 1 plus 0.1 M NaCl and 5 mM L-histidine. Finally, buffer 1 plus 40 mM L-histidine was used to elute bound oxygenase domain, and 100 mM L-histidine for the reductase domain. The eluate was concentrated by Centriprep-50, then applied to a 10-DG column (Bio-Rad) and eluted with 50 mM HEPES, pH 7.4, containing 0.1 M NaCl and 10 % glycerol, to remove histidine.

**Biopterin and flavin determination** – The content of BH₄, FAD and FMN of purified eNOS domains was measured as described previously (17, 21) and quantified from a standard curve of authentic BH₄, FAD or FMN, respectively. Biopterin determination was done on eNOSox with or without reconstitution with exogenous BH₄. BH₄ reconstitution was done similar to the published procedures (9, 22) under anaerobic condition. The excess amount of BH₄ was removed by gel-filtration and the amount of bound BH₄ was determined using our HPLC quantitation similar to the published procedure using authentic BH₄ to build a standard curve (23).

**Pyridine hemochromogen Assay** – Heme content was determined by the formation of pyridine hemochromogen as previously described (24). The total heme content was determined from difference spectrum of bispyridine heme (reduced minus oxidized) using $\Delta \varepsilon_{556-538nm} = 24$ mM⁻¹ cm⁻¹.

**Quantification of thiol functional groups** – Surface exposed thiol groups were determined by chemical modification using 4,4′-dithiopyridine to form 4-thiopyridone chromophore with major absorbance at 343 nm. The 4,4′-dithiopyridine itself has almost no absorption at that wavelength (25).
**eNOS<sub>red</sub> activity assay** - Cytochrome c reductase activity was measured as the absorbance increase at 550 nm using $\Delta \varepsilon = 21 \text{ mM}^{-1} \text{ cm}^{-1}$ as described previously (17). Ferricyanide or 2,6-dichlorophenol indolphenol oxidation assay was determined by $\Delta \varepsilon = 1 \text{ mM}^{-1} \text{ cm}^{-1}$ at 400 nm and $\Delta \varepsilon = 21 \text{ mM}^{-1} \text{ cm}^{-1}$ at 600 nm, respectively (14).

**eNOS<sub>ox</sub> Activity in generating biopterin radical.** This activity measurement essentially followed previous published procedure for iNOS<sub>ox</sub> (26, 27). High concentration of BH<sub>4</sub>-reconstituted eNOS<sub>ox</sub> was reduced anaerobically in a tonometer by dithionite titration. The ferrous eNOS<sub>ox</sub> was then reacted with oxygenated buffer using rapid-freeze/EPR technique as we previously published (28). The rapid-freeze apparatus, System 1000, Update Instrument (Madison, WI), was placed inside an anaerobic chamber (Coy Laboratory, MI). The oxygen level was lower than 5 ppm during the whole experiment procedure monitored by an oxygen/hydrogen analyzer (Model 10, Coy Laboratory). One or two push programs were used to obtain samples freeze trapped at different reaction time.

**Spectrometry** - UV-Vis spectra were measured on a HP8453 diode array spectrophotometer with 1 nm spectral bandwidth. EPR was recorded at liquid helium or liquid nitrogen temperature on a Bruker EMX. For liquid helium system, a GFS600 transfer line and an ITC503 temperature controller were used to maintain the temperature. An Oxford ESR900 cryostat was used to accommodate the sample. For liquid nitrogen transfer, a silver-coated double jacketed glass transfer line and a BVT3000 temperature controller was used. Data analysis was conducted using WinEPR and spectral simulation was done using SimFonia programs provided by Bruker. Flavin fluorescence was measured using SLM SPF-500C spectrofluorometer at ratio mode. About 2 µM eNOS<sub>red</sub> in a 1-cm quartz cuvette was excited at 450 nm (5 nm spectral band width) and the
emission spectrum between 450 – 650 nm (7.5 nm spectral band width) was collected at 24 °C.

Stoichiometric titration- The redox capacity of eNOS\text{red} and eNOS\text{ox} were determined by anaerobic stoichiometric titration using sodium dithionite. Stock solution of sodium dithionite was freshly prepared by dissolving powdered reagent in 50 mM, pH 8.2 pyrophosphate buffer pre-saturated with pure nitrogen gas. The concentration of sodium dithionite was standardized by titration against a fixed amount of lumiflavin-3-acetic acid ($\varepsilon_{444} = 1.08 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$) anaerobically before and after individual real sample titration (29). The average concentration was used to calculate the number of reducing equivalents consumed in the titrations. Each protein sample was placed in an anaerobic titrator and made anaerobic by 5 cycles of evacuation (30 sec.) and argon replacement (5 min). Standardized dithionite solution contained in a gas-tight syringe engaged to the side arm of the titrator was quantitatively delivered and mixed with the protein sample under argon atmosphere. Electronic spectrum was recorded on an HP 8452 diode spectrophotometer and to confirm the system was equilibrated after each addition of dithionite reflected by a static absorbance.

Miscellaneous methods--- Protein content was determined by BCA method (30). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10 % Ready-Gels in a Bio-Rad mini-gel apparatus. The gel filtration chromatography was performed on a Sephacryl 200 HR column (1.5 x 5.0cm). The kit for molecular weight 12,000—200,000 (product code: MW-GF-200) was used as the gel filtration marker.

Computer modeling

The SCoP program (Simulation Resources Inc., Redlands, CA) was used for simulating the data obtained from stoichiometric titration, mainly the eNOS\text{red} similar to the method used by
Iyanagi et al. (31). The absorbance changes at different monitoring wavelength during titration were simulated against accumulated reducing equivalents added.

\[ A_\lambda = \varepsilon_1 F_1 + \varepsilon_2 F_1H + \varepsilon_3 F_1H_2 + \varepsilon_4 F_2 + \varepsilon_5 F_2H + \varepsilon_6 F_2H_2 \quad [1] \]

\( A_\lambda \) is the observed absorbance at wavelength \( \lambda \), \( \varepsilon_1 - \varepsilon_6 \) are the extinction coefficients for each flavin redox species: F1, F1H, F1H2 as fully oxidized, semiquinone, fully reduced form of the first flavin, respectively, and F2, F2H, F2H2 are the equivalents for the second flavin. The concentrations of each flavin intermediate during a stoichiometric titration are expressed by:

\[
F_1 = \left(\frac{x_1 \times x_2}{1 + x_2 + x_1 + x_2}\right) f_1 \quad [2]
\]

\[
F_1H = \left(\frac{x_2}{1 + x_2 + x_1 \times x_2}\right) f_1 \quad [3]
\]

\[
F_1H_2 = \left(\frac{1}{1 + x_2 + x_1 \times x_2}\right) f_1 \quad [4]
\]

\[
F_2 = \left(\frac{x_3 \times x_4}{1 + x_4 + x_3 \times x_4}\right) f_2 \quad [5]
\]

\[
F_2H = \left(\frac{x_4}{1 + x_4 + x_3 \times x_4}\right) f_2 \quad [6]
\]

\[
F_2H_2 = \left(\frac{1}{1 + x_4 + x_3 \times x_4}\right) f_2 \quad [7]
\]

Where \( f_1, f_2 \) are the total amount of each flavin and

\[
x_1 = \exp\left(\frac{F}{(R \times T)} \times (Eh - E_1)\right) \quad [8]
\]

\[
x_2 = \exp\left(\frac{F}{(R \times T)} \times (Eh - E_2)\right) \quad [9]
\]

\[
x_3 = \exp\left(\frac{F}{(R \times T)} \times (Eh - E_3)\right) \quad [10]
\]

\[
x_4 = \exp\left(\frac{F}{(R \times T)} \times (Eh - E_4)\right) \quad [11]
\]

\( E_1 - E_4 \) are midpoint potential of the four half reactions of the two flavins in eNOS_{red}. Eh is any measured redox potential value. F is the Faraday constant, 96,485 C mol\(^{-1}\), R is the gas constant, 8.314 J K\(^{-1}\)mol\(^{-1}\), and T is 298K. The total reducing equivalents was simply expressed as:

\[
\text{Equiv.} = 2 \times \left(\frac{F_1H + 2 \times F_1H_2 + F_2H + 2 \times F_2H_2}{f_t}\right) \quad [12]
\]
Where $ft$ is the total flavin, i.e., sum of $f1$ and $f2$.

Simulation was generated by sweeping the Eh values in any desired potential range and seeking optimal values for $E1$ – $E4$ to achieve the best fit to the observed data. It is not possible to achieve a set of absolute midpoint potentials but could converge the relative midpoint potential values. In other words, once one of $E1$ to $E4$ values is fixed the other three can be located by simulation. Absorbance extinction coefficient for the fully-oxidized and fully-reduced flavins are readily available, and those for the flavin semiquinone can be properly estimated from the spectrum at the stage of 1 and 3-electron reduced states. We also let these two coefficients floating in a narrow range and optimized via simulation.
RESULTS

Expression and purification of eNOS subdomains: The yeast expression vector containing AOX promoter is promising in over-expressing both domains of eNOS in *Pichia pastoris*. By introducing His<sub>6</sub> tag in the N-terminus of both domains, purification of target protein can be conveniently done by Ni-NTA agarose column chromatography. The average yields of purified oxygenase domain and reductase domain are ~ 8mg/l and ~22mg/l, respectively. Both purified eNOS<sub>ox</sub> and eNOS<sub>red</sub> run as a single band on SDS-PAGE with apparent molecular weights of 54 kDa and 82 kDa, respectively (Figure 1A) and exhibited immunoreactivity with polyclonal antibodies against eNOS (Figure 1B).

The heme content determined by pyridine hemochromogen assay was 0.93 ± 0.04 (n = 7), almost stoichiometric to the protein subunit (Table 1). Replenish hemin or ?-aminolevulinic acid to the cell medium during yeast growth did not further increase the heme content in purified eNOS<sub>ox</sub>. The purified oxygenase domain also contained endogenous biopterin at a stoichiometry lower than 0.3/monomer. As our sample buffers did not include DTT, most of these biopterin molecules were present as dihydrobiopterin, BH<sub>2</sub>, as analyzed by our HPLC method (data not shown). The functional form of biopterin, BH<sub>4</sub>, can be reconstituted back to the purified eNOS<sub>ox</sub> according to the anaerobic procedure similar to that described by Rusche and Marletta (22). The reconstituted eNOS<sub>ox</sub> has biopterin content as high as 0.72 per monomer (Table 1) and are present as fully reduced form, BH<sub>4</sub>, as analyzed by our HPLC method (data not shown).

The content of both FAD and FMN in purified eNOS<sub>red</sub> is essentially stoichiometric based on our HPLC determination against authentic FAD and FMN standards (Table 1). The ratio of FAD and FMN to that of eNOS<sub>red</sub> monomer is 1.0 and 1.14, respectively, thus further reconstitution of flavins is unnecessary.
Spectroscopic characteristics of eNOS\textsubscript{ox}. UV-Vis spectrum from 250 – 700 nm of purified eNOS\textsubscript{ox} showed a Soret peak at 400 - 404 nm, 81 mM\textsuperscript{-1}cm\textsuperscript{-1}, a broad $\alpha$ / $\beta$ band at 518 nm, 15.4 mM\textsuperscript{-1}cm\textsuperscript{-1}, and a charge-transfer band at 645 nm, 5.8 mM\textsuperscript{-1}cm\textsuperscript{-1}. Treatment with L-arginine shifted the Soret peak to 396 nm with comparable amplitude, 82 mM\textsuperscript{-1}cm\textsuperscript{-1}, and only slight changes in the visible region (Fig. 2). When eNOS\textsubscript{ox} was reduced by dithionite, the Soret band is red-shifted to 413 nm with sizable decrease in intensity, 66.7 mM\textsuperscript{-1}cm\textsuperscript{-1}. The $\alpha$ / $\beta$ band also shifted to 552 nm, 13.0 mM\textsuperscript{-1}cm\textsuperscript{-1}, and the charge-transfer band at about 650 nm is abolished as the lower lying three metal d-orbital are completely filled. Further addition of CO resulted in the hallmark 444 nm Soret band for P450 hemeproteins with an extinction of 91.3 mM\textsuperscript{-1}cm\textsuperscript{-1} with the features at visible region very similar to that of ferrous eNOS\textsubscript{ox}. These spectral behaviors are very similar to our bacterial-expressed eNOS\textsubscript{ox} and other NOS oxygenase domains (9, 13, 17). The ratio of 280 nm to the Soret peak to either the resting or L-arginine-treated eNOS\textsubscript{ox} was ~1.5 which is an index of the purity of the hemeprotein and is a good number compared with other NOS preparations (9, 13, 17).

Liquid helium temperature EPR of the resting eNOS\textsubscript{ox} showed mixture of high-spin and low-spin heme structures (Fig. 3, spectrum A). The rhombic high-spin heme has g tensor values of 7.53, 4.23 and 1.83 (the $g_{\text{min}}$ was only observable at somewhat lower temperature, ~ 4K) and the low-spin heme show conspicuous rhombic g values at 2.43, 2.28 and 1.90. Both sets of parameters are typical for NOS and other P450 type hemeproteins containing a cysteine thiolate proximal heme ligand (32, 33). Addition of excess amount of L-arginine essentially wiped out the low-spin heme signals and substantially increased the high-spin heme signals (Fig. 3, spectrum B). The g values of the high-spin heme shifted to 7.56, 4.17 and 1.82, corresponding to a small rhombicity shift from 20.6 to 21.1 %. On the other hand, imidazole converted eNOS\textsubscript{ox} to fully low-spin heme.
complex (Fig. 3, spectrum C). There are two well-resolved rhombic low-spin heme complexes with g values of 2.71/2.29/1.75 and 2.60/2.29/1.81. Resolution in EPR spectrum of the two imidazole low-spin heme is even better than that found for whole eNOS (33).

Stoichiometric titration of the eNOS\textsubscript{ox}. To determine the redox capacity of the purified eNOS\textsubscript{ox}, a stoichiometric titration was conducted using standardized dithionite solution. L-arginine was added to make the heme homogeneously high-spin. The spectral conversion from ferric to ferrous heme during the course of titration appeared to involve only one simple redox reaction as evidenced by the isosbestic points at 410, 489, 532, 615, and 678 nm for both the absolute and difference spectra relative to the resting eNOS\textsubscript{ox} spectrum. An isosbestic point at 338 nm was slightly perturbed by dithionite whose absorption peaks at 314 nm (Fig. 4A and Inset). However, the optical amplitude changes at 444 – 388 nm showed a long lag for 1.5-2.5 (in three separate titrations) reducing equivalents before a sharp rise. 1-1.5 reducing equivalents were needed to completely reduce eNOS\textsubscript{ox} (Fig. 4B). The additional 1.5-2.5 reducing equivalents consumed during titration were not due to oxygen contamination as assessed by lumiflavin titration using the same titration vessel and conditions. Furthermore, similar stoichiometric titrations performed on eNOS\textsubscript{red} did not show an initial long lag (see below). The extra reducing equivalents used to titrate eNOS\textsubscript{ox} could be due to oxidized biopterin or free sulfhydryl group at the protein surface. The former may be a consequence of autooxidation of BH\textsubscript{4} and the latter could be due to the loss of the zinc cluster, which coordinate with four cysteine ligands with two cysteines from each monomer (2, 5, 6). However, the samples used in these titrations are not BH\textsubscript{4}-replenished. The content of biopterin was as low as 0.2-0.3/heme and was preset as BH\textsubscript{2}, which is not reducible by dithionite. This left the zinc loss as the most possible cause of the additional consumption of dithionite in the titration. Three experiments were conducted to assess this hypothesis. Zinc analysis by ICP-mass was
carried out using either eNOS$_{ox}$ or purified whole eNOS. Sufficient amount of zinc was determined by ICP-MS analysis (data not shown). Gel-filtration chromatography was conducted to determine the population of eNOS$_{ox}$ monomer and dimer. Molecular sieving using five molecular weight standards and purified eNOS$_{ox}$ indicated that the whole population is present as a dimer with a molecular weight greater than 100 kDa (Fig. 5). Titration of free thiol by 4,4’-dithiopyridine was also conducted on eNOS$_{ox}$ using free L-cysteine as positive control. Time-dependent modification of the thiol was monitored in parallel with urea-treated eNOS$_{ox}$ and a bovine eNOS$_{ox}$ predetermined to have zinc and present as a dimer (5) (Fig. 6). Both yeast expressed human eNOS$_{ox}$ and eNOS$_{ox}$ trypsinolyzed from bacterial expressed bovine eNOS exhibited almost identical kinetics of chemical modification (Fig. 6). Two to three thiol groups were easily modified in both protein samples at a rate of 0.5 min$^{-1}$, but the next six residues were modified much slower at 0.02 min$^{-1}$. Pre-treatment of 5 M urea significantly enhanced the extent of chemical modification in the fast phase as a result of exposure of about additional 2-3 thiol groups. The rates of the two phases remain similar, 0.5 and 0.02 min$^{-1}$, respectively, but the contribution of each phase shifted from 2:5 to 4:2.5 after urea treatment. Moreover, the overall extent of modification after 2-hr period remained the same as eNOS$_{ox}$ sample not treated by urea. In contrast, free L-cysteine followed simple modification kinetics and the rate, 8 min$^{-1}$, is even faster than the fast phase of that observed for eNOS$_{ox}$ samples.

_Tetrahydrobiopterin radical formation of eNOS$_{ox}$._ BH$_4$-reconstituted eNOS$_{ox}$ prepared at a concentration of ~300 $\mu$M was premixed with excess L-arginine and reacted with oxygenated buffer anaerobically at room temperature and freeze trapped at several time points. EPR spectrum corresponding to 100 ms reaction time is given in Fig. 7. EPR recorded at 11K between 200 - 4200
G revealed both the heme component and the radical component (Fig. 7A). EPR spectrum of a control L-arginine-treated eNOS_{ox} was also recorded under the same EPR conditions. Two spectra are normalized to the same concentration of heme. Approximately 50% of the BH_{4} is converted to BH_{4} radical and other diamagnetic heme intermediates estimated from the decrease of the high-spin heme signal amplitude. The radical signal observed at g = 2 region was remeasured at 120K (Fig. 7B). The hyperfine feature pertaining to nitrogen and proton splittings are clearly revealed. The biopterin radical centered at g = 2.002 and an overall line width of 39 G. Microwave power dependence indicates a strong magnetic interaction with the heme center with a P^{1/2} as high as 14 mW at 120 K (data not shown). Spin concentration was estimated by double integration of the EPR signal, using a copper standard, to be ~ 20 µM. After correction for the ~ 4x dilution factor during rapid freeze, we essentially observed ~ 80 µM radical, equivalent to about 40% of the total biopterin as BH_{4}/heme ratio was ~ 0.7. This radical EPR could be closely simulated by including one strongly-coupled nitrogen at N5, one alpha-proton at N5 and one beta proton at C6 (dashed line in Figure 7 B).

Enzyme activities of eNOS_{red}. Cytochrome c reductase activity, DCPIP and ferricyanide reduction activities were evaluated for eNOS_{red} expressed in yeast (Table 2). Cytochrome c reductase activity was 70.3 mols/min/mol and was increased about 2-3 fold, 194.3 mols/min/mol, in the presence of Ca^{2+}/CaM. This effect of CaM was less than we previously observed for baculovirus-expressed eNOS_{red} (17). However, the absolute activity, both in the presence and absence of CaM, are higher than the values reported for sf9-expressed eNOS_{red} and eNOS, i.e. 13.8/20.9 and 138/224 without and with Ca^{2+}/CaM, respectively. Both ferricyanide and DCPIP reductase activities were greater than that of cytochrome c reductase. Ferricyanide reductase activity increased from 3220 to 4480
min\(^{-1}\) by Ca\(^{2+}\)/CaM whereas DCPIP reductase activity was increased from 400 to 800 min\(^{-1}\) by adding Ca\(^{2+}\)/CaM (Table 2). Our data are compatible with literature data for eNOS\(_{\text{red}}\) or full eNOS (Table 2). Although there are some variations of cytochrome c and ferricyanide reductase activities among different eNOS or eNOS\(_{\text{red}}\) preparations, most of them are within the same order of magnitude (Table 2). Differences in assay temperature, expression system and buffer composition could be the reasons that resulted in these variations.

**Flavin fluorescence of eNOS\(_{\text{red}}\) and its interaction with calmodulin, CaM.** The emission spectrum of isolated eNOS\(_{\text{red}}\) shows a broad band from 470 to 650 nm peaked at ~ 528 nm (Fig. 8). The intensity of the fluorescence was increased by ~ 30% in the presence of Ca\(^{2+}\)/CaM, but there was no obvious shift of the peak. Excess EDTA could only reverse ~ 80% of the fluorescence change caused by Ca\(^{2+}\)/CaM (Fig. 8). This residual fluorescence increase, which is not reversed by EDTA, could be simply the slight increase of intensity caused by Ca\(^{2+}\) alone (data not shown). This fluorescence change provided a nice index to determine the CaM binding. Titration of micromolar level of eNOS\(_{\text{red}}\) with CaM in the presence of Ca\(^{2+}\) showed a sharp breaking point at 1:1 ratio of CaM to eNOS\(_{\text{red}}\) (Inset of Fig. 8). This result indicated that the K\(_d\) of CaM is significantly lower than micromolar and each reductase domain binds one CaM.

**Stoichiometric titration of eNOS\(_{\text{red}}\).** Identical anaerobic titration procedure as eNOS\(_{\text{ox}}\) titration was applied here to determine the redox capacity in eNOS\(_{\text{red}}\) using dithionite as titrant. A total of four reducing equivalents were required to fully reduce eNOS\(_{\text{red}}\) accounted exactly for the capacity of two flavin cofactors (Figure 9). No additional redox centers other than the FAD and FMN was disclosed by this titration. There are three stages of reduction observed in the dithionite titration. The first stage took one reducing equivalent and the electronic spectra showed isosbestic points (~}
366 and ~ 508 nm) in this process (Figure 9A), indicating a single redox transformation step. The absorbance decrease at 456 nm and the corresponding increase at 600 nm are attributed to the formation of a neutral flavin semiquinone. The second stage took two reducing equivalents. It appeared to show one isosbestic point (~342 nm) but is not conclusive indicating that this stage is likely involved at least two chemical reaction steps (Figure 9B). There was a large decrease of 456 nm absorbance accompanied by a small change at 600 nm. The last stage involved one-electron reduction to reach fully reduced state (Figure 9C). The further bleaching of 456 and 600 nm absorbance evidenced disappearance of both the oxidized flavins and flavin semiquinone. The general titration profile is very similar to that published for microsomal CPR and nNOS reductase domain (14, 31, 34).

Computer simulations for the reductive titration data. The data shown in Figure 9D at three different wavelengths were simulated by SCoP program according to Eqs. 1 – 12 to obtain three redox potentials gaps, \( \Delta E_1 - \Delta E_3 \), between four half reactions of two flavins in eNOS\(_{\text{red}}\) (31). Computer simulation for the data at 456, 508 and 600 nm was successful as indicated by the close match of the simulations and the actual data except the initial < 0.3 reducing equivalents (Fig. 9D). The initial short lag was probably due to residual amount of oxygen in the titrator. This simulation process was tested using any arbitrary absolute midpoint potential value for one of the four half reactions and to zoom in the values for \( \Delta E_1 - \Delta E_3 \). The variation for each of the redox potential gap is not significant as shown in Table 3. The optimal value for \( \Delta E_1 \) is the biggest, 180 mV, and a clean conversion from one oxidized flavin to the semiquinone form is expected in the first stage of titration. In contrast, \( \Delta E_2 \) is almost zero indicating that the second stage of reduction consisted of two almost parallel half reactions. The value of \( \Delta E_3 \), 73 mV, is in the middle and could be used
to estimate the cutoff point to obtain the absorbance contribution from only one specific half-reaction. The extinction coefficients at three different wavelengths were also converged by several simulation cycles and were shown in Table 4. In principal, we could conduct this simulation cycles on any wavelength between 300 and 700 nm and to reconstruct the spectrum for each of the six flavin redox species.

**DISCUSSION**

We have successfully prepared both eNOS$_{ox}$ and eNOS$_{red}$ using yeast expression system. Purified eNOS$_{ox}$ domains appear to have most of the heme characteristics in intact eNOS. The optical spectra of ferric and ferrous eNOS$_{ox}$ and its ferrous-CO complex and the spin-state change by L-arginine are all typical for eNOS and eNOS$_{ox}$ we observed previously (17, 21, 24). The purity index expressed as the ratio of $A_{280}/A_{396}$ was as good as 1.3-1.6 compared to 1.5–1.7 in our previous baculovirus expressed recombinant protein (17). This ratio is not as good as recent iNOS$_{ox}$ preparation expressed from E.coli (9) but our oxygenase domain does not have the N-terminal heterogeneity as observed for iNOS$_{ox}$ as only one single peptide band was observed on SDS-PAGE at ~50 kDa region (9).

EPR spectrum of the isolated eNOS$_{ox}$ showed dominant low-spin P450-type heme. Using the low-field $g = 7.5$ signal amplitude to estimate the spin-state population against the L-arginine-treated eNOS$_{ox}$, about 75% of the heme was present as low-spin. This high proportion of low-spin heme is at odds with the room temperature optical data (Fig. 2 vs. Fig. 3) judging from the similar amplitude of the charge-transfer band at 650 nm of the resting eNOS$_{ox}$ and eNOS$_{ox}$ treated with L-arginine. Optical data implied that majority of the heme was present as high-spin, thus a
temperature-dependent heme spin-state change likely occurred with the low-spin heme electronic configuration as the ground state. Truth diagram analysis for the correlation between heme rhombicity and axial ligand field strength for the low-spin heme component put the eNOS\textsubscript{ox} at the lower right edge of the P zone, indicating a P450-like protein (33). The rhombic distortion (V), axial perturbation (\(\Delta\)) and heme rhombicity (%) obtained were 2.09, 5.75 and 36.4, respectively. There might be another low-spin component present as indicated by the shoulder at the \(g = 2.43\) signal. However, this additional component is not as visible as that observed for full eNOS and only the low-rhombicity low-spin heme component appears present in the isolated eNOS\textsubscript{ox} (33). In contrast, imidazole low-spin heme complex showed two better-resolved EPR species than the corresponding imidazole complex of full eNOS (compare Fig. 3, spectrum C with Fig. 6a in 33) with very similar g values for the two sets of low-spin heme complexes. The calculated values of \(V\), \(\Delta\) and % rhombicity for the two low-spin species are 2.52/4.53/56\% and 2.63/4.05/65\%, respectively, and places these two heme complex at the center of the P zone. Among the entire low-spin imidazole heme complex, there was a reciprocal relationship between the heme rhombicity and the tetragonality, indicating that the heme rhombic distortion attenuate the axial ligand intensity, possibly via a lengthening of the Fe-S bond.

The extra 1.5 – 2.5 reducing equivalents required to initiate the heme reduction in the stoichiometric titration is puzzling (Fig. 4). These additional equivalents are not originated from biopterin as the amount of pterin was too low to account for this amount of reducing equivalents and dithionite does not reduced BH\textsubscript{2} to BH\textsubscript{4} due to unfavorable redox thermodynamics (35). The zinc loss is not the reason as there is plenty of zinc present in isolated eNOS\textsubscript{ox} assessed by ICP mass analysis. The isolated eNOS\textsubscript{ox} is a perfect dimer as analyzed by gel-filtration. Monomeric
form was not even detected. Furthermore, the titration for the surface exposed thiol function group showed identical modification kinetics as another bovine eNOS$_{ox}$ whose crystallographic data indicates the presence of zinc cluster. Thus the hypothesis that a zinc loss leading to surface exposed disulfide linkage as that found in the iNOS$_{ox}$ crystallographic data does not apply to our isolated eNOS$_{ox}$ (7). Furthermore, the possibility for propensity of zinc loss in recombinant eNOS$_{ox}$ but not in intact eNOS may be unfounded in our yeast expression system. We do not see additional metal redox centers such as heme, iron-sulfur cluster or copper center by optical or EPR spectroscopy, thus leave us with no explanation for the extra reducing equivalents which show much higher redox potential than the heme center. Reduction of the heme center appeared to consume more than one reducing equivalent (1-1.5 in three experiments). A similar case in iNOS$_{ox}$ was also observed recently (9). The sharp increment of absorbance change at the beginning of the titration and a curvature and even tailing approaching the end of the titration seems to indicate that the titrant may not have electron donating power strong enough to completely reduce all the heme molecules. Considering the very negative midpoint potential of thiolate-ligated heme, it is possible in the later portion of the titration that only part of the reducing equivalents from dithionite were donated to the heme center dictated by the midpoint potential difference between the heme and dithionite (36). We thus put more emphasis in using the initial linear sharp rise to estimate the end point of titration. By doing this, we get a stoichiometry closer to 1 rather than 1.5.

The biological activity of our eNOS$_{ox}$ was demonstrated by its capability in forming biopterin radical (Fig. 7). We chose this method as it is directly linked to the redox function of the protein and provide detailed information regarding the reaction mechanism of eNOS. In our study, the radical signal plateaued at ~100 ms at room temperature. The line shape, intensity and initial
kinetics of biopterin radical formation appeared very similar to those published for iNOS\textsubscript{ox} (26, 27). Computer simulation for the BH\textsubscript{4} radical indicates a minimal requirement for one nitrogen and two proton nuclei to match the EPR data. As N5 (or its 4a carbon) is para to the electron-releasing amino group at C2 and thus has high electronegativity, it is thus more favorable than N8 to give the first electron. N8 (or its 8a carbon) is meta to C2 and C4; thus electron withdrawal can only occur via conjugation with 4-oxo group. The pKa of the N5 proton is much higher than neutral and is not dissociated easily, thus a hyperfine interaction of this proton with the unpaired electron at N5 is expected. The second proton has to come from the C6 beta proton. Such initial trial of simulation appears fairly promising. Our observed biopterin radical is thus likely a BH\textsubscript{4}\textsuperscript{+} cation radical (37, 38). Although N8 nuclei and its associated proton have been proposed to involve in the unpaired spin system (37), it remains to be clarified by further spectroscopic studies using isotope replacement. Nonetheless, we presented here the first high quality EPR spectrum of eNOS biopterin radical and will pursue the mechanistic role of biopterin using rapid-freeze EPR approach in parallel with stopped-flow and other kinetic methods.

eNOS\textsubscript{red} activity was assessed by three different assays. The cytochrome c reduction and the DCPIP reduction assays require the participation from both flavins and ferricyanide reduction activities were believed to involve only FAD (39). In all cases, CaM enhanced the activity and the enhancement for cytochrome c reduction and DCPIP reduction activity to a similar extent. Why we only see ~ 3 \times activity increase for the cytochrome c reductase activity by CaM by our yeast protein and 10 \times increase in our previous sf9 expressed eNOS\textsubscript{red} is unclear (Table 2). Nonetheless, CaM appeared to interact both between FMN and the heme as well as between FAD and FMN as initially observed in nNOS (40). There are many factors that enhance the reductase activity of eNOS including the CaM binding, the removal of the autoinhibitory peptide and the
phosphorylation of the C-terminus of the reductase (1, 39, 41, 42). Furthermore, the presence of DTT, EDTA and variation in ionic strength during different stage of the purification also affect the sensitivity of the reductase domain activity to Ca\(^{2+}\)/CaM (17, 39, 41, 42). Compare eNOS\(_{\text{red}}\) activity values determined from different laboratories does not yield straightforward conclusions. The effect of CaM on nNOS\(_{\text{red}}\) does not appear to shift the midpoint potential of either half reactions of the two flavins (43) as addition of CaM caused only marginal change on the FMN/FMNH midpoint potential and essentially no change on the other three half-reactions.

The only redox centers present in eNOS\(_{\text{red}}\) are the two flavins as exactly four reducing equivalents were consumed in the stoichiometric titration (Fig. 9D). Optical changes of the flavins occurring almost immediately after dithionite addition contrasts with the data for eNOS\(_{\text{ox}}\) which required 1.5 – 2.5 additional reducing equivalents before reduction of heme and supports that additional redox centers were present in eNOS\(_{\text{ox}}\) (Fig. 4B). In addition to quantifying the redox capacity of eNOS\(_{\text{red}}\), stoichiometric titration also enabled determination of the relative redox potentials between different half reactions as illustrated in this study (Fig. 9D and Table 3). Successful simulation in data of all three wavelengths using the same set of difference midpoint potential values attested to the utility of this approach. The redox potential gap between the first and second half-reaction was 170 - 190 mV, thus a complete separation of the first half-reaction from the other three is expected. The optical spectrum at the point of addition of one reducing equivalent should contain one intact flavin (FAD) and one flavin semiquinone (FMNH\(^{-}\)) (Fig. 9A, heavy line). The extinction coefficient for the FMNH\(^{-}\) semiquinone could thus be unambiguously determined. The spectral change at 600 nm is completely due to semiquinone forms of the flavins as the fully reduced and fully oxidized sample are silent at this region. The titration data indicate
that the second semiquinone was gradually reduced to its hydroquinone at the addition of the fourth reducing equivalent. However, $\Delta E_3$ is only 56 – 90 mV and prohibits clean separation of the last half-reaction from the other three. As $10^{(\Delta E_3/0.059)} = (\text{Ox}_1 \cdot \text{Red}_2)/(\text{Ox}_2 \cdot \text{Red}_1)$ for the last two overlapping half reactions:

\[
\begin{align*}
\text{Ox}_1 + e^- &= \text{Red}_1 \\
\text{Ox}_2 + e^- &= \text{Red}_2
\end{align*}
\]

We expect that only 70 – 80 % of the reaction after addition of the 4th electron is only contributed by FADH- semiquinone reduction to FADH$_2$. Simulation for the 600 nm data here is very useful to define the extinction coefficient for the second semiquinone species as the trapezoidal titration profile is very sensitive to the difference midpoint potential as well as the extinction coefficient (31) thus greatly assisted in converging the value of the $\Delta E$ values and the extinction for the second flavin semiquinone by simulation. There is a 20 % difference in the extinction coefficient at 600 nm for these two flavin semiquinones and almost 3 $\times$ difference at 456 nm and $\sim$50 % difference at 508 nm with the FMNH- having the higher values (Table 4). The middle two half-reactions, corresponding to that after addition of the 2nd and 3rd reducing equivalents, attributable to the formation of FAD semiquinone and FMNH- transformation into the fully reduced form are almost equipotential and were titrated together. The absolute values of the midpoint potential for all four half-reactions will be determined by potentiometric titration. Once these values are available, they will be used to validate the $\Delta E$ values obtained in this study and to refine the accuracy of the extinction coefficient derived in this study. The spectral contribution from each half reaction at every single wavelength can be reliably determined and will be useful in future mechanistic studies by stopped-flow measurements.
Comparing the ΔE values with other diflavin reductase proteins (Table 3) eNOS_{red} resembles closely with rabbit P450 reductase in all three potential gaps. Our results differ from nNOS_{red} for its 40 mV larger ΔE_1 and 40 mV smaller ΔE_2, and differs from P450BM3 from its very small ΔE_1 and 80 mV larger ΔE_2, and also differs from sulfite reductase for the 55 mV larger ΔE_2 and 130 mV smaller ΔE_3. These differences have major impact on the thermodynamic control on electron transfer and the nature of the half-reaction that couples with the heme reduction (30, 43 – 45).

ACKNOWLEDGMENTS

We want to thank Dr. Susan Cates at Rice University, Department of Biochemistry and Cell Biology, for initial efforts in measurement of eNOS_{ox} molecular weight using equilibrium centrifugations. We also thank Dr. Robert E. Serfass at University of Texas Medical Branch at Galveston, Department of Preventive Medicine and Community Health to run ICP-mass analysis for zinc in our eNOS_{ox} and eNOS proteins. A gift of bovine eNOS_{ox} sample provided by Dr. C.S. Raman here at UTHSC, Department of Biochemistry and Molecular Biology for our chemical modification studies is very much appreciated.
REFERENCES

1. Roman, L.J., Martásek, P. and Masters, B.S.S. (2002) Chem. Rev. 102, 1179-1189.
2. Raman, C.S., Martásek, P., and Masters, B.S.S. (2000) in The Porphyrin Handbook, Kadish, K.M., Smith, K.M., and Guilard, R. eds. Acad. Press, p. 293-327.
3. Feldman, P.L., Griffith, O.W. and Stuehr, D.J. (1993) Chem. Eng. News 71, 26-38.
4. Alderton, W.K., Cooper, C.E., and Knowles, R.G. (2000) Biochem. J. 357, 593-615.
5. Raman, C.S., Li, H., Martásek, P., Král, V., Masters, B.S.S., and Poulos, T.L. (1998) Cell, 95, 1-20.
6. Fischmann, T.O., Hruza, A., Xiao, D.N., Fossetta, J.D., Lunn, C.A., Dolphin, E., Prongay, A.J., Reichert, P., Lundell, D.J., Narula, S.K., and Weber, P.C. (1999) Nature Struct. Biol. 6, 233-242.
7. Crane, B.R., Arvai, A.S., Ghosh, D.K., Wu, C., Getzoff, E.D., Stuehr, D.J., and Tainer, J.A. (1998) Science, 279, 2121-2126.
8. Zhang, J., Martásek, P., Paschke, R., Shea, T., Masters, B.S.S., and Kim, J.J. (2001) J. Biol. Chem. 276, 37506-37513.
9. Hurshman, A.R., Marletta, M.A. (2002) Biochemistry 41, 3439-3456.
10. Perry, J. M., Moon, N., Zhao, Y., Dunham, W. R., and Marletta, M. A. (1998) Chem. & Biol. 5, 355-364.
11. McMillan, K., Masters, B.S. (1995) Biochemistry 34, 3686-3693.
12. Abu-Soud, H. M., Gachhui, R., Raushel, F. M., and Stuehr, D. J. (1997) J. Biol. Chem. 272, 17349-17353.
13. Ghosh, D. K., and Stuehr, D. J. (1995) Biochemistry 34, 801-807.
14. Gachhui, R., Presta, A., Bentley, D.F., Abu-Soud, H.M., McArthur, R., Brudvig, G.,
Ghosh, D.K., Stuehr, D.J. (1996) J. Biol. Chem. 1996 271, 20594-20602.
15. Bec, N., Gorren, A.C.F., Voelker, C., Mayer, B., and Lange, R. (1998) J. Biol. Chem. 273
13502-13508.
16. Mayer, B., Klatt, P., Harteneck, C., List, B.M., Werner, E.R., and Schmidt, K. (1996)
Methods Neurosci. 31, 130-139.
17. Chen, P.-F., Tsai, A.-L. Berka, V., and Wu, K.K. (1996) J. Biol. Chem. 271, 14631-14635.
18. Martásek, P., Liu, Q., Liu, J., Roman, L. J., Gross, S. S., Sessa, W. C., and Masters, B. S.
(1996) Biochem. Biophys. Res. Commun. 219, 359-365.
19. Romanos, M.A., Scorer, C.A., and Clare, J.J. (1992) Yeast 8, 423-488.
20. Leber, A., Hemmens, B., Klosch, B., Goessler, W., Raber, G., Mayer, B., Schmidt, K.
(1999) J. Biol. Chem. 274, 37658-37664.
21. Chen, P.-F., Tsai, A.-L., and Wu, K.K. (1995) Biochem. Biophys. Res. Commun. 215,
1119-1129.
22. Rusche, K.M., Marletta, M.A. (2001) J. Biol. Chem. 276, 421-427.
23. Lunte, C.E. and Kissinger, P.T. (1983) Anal. Biochem. 129, 377-386.
24. Berka, V., Palmer, G., Chen, P.-F., and Tsai, A.-L. (1998) Biochemistry, 37, 6136-6144.
25. Grassetti, D.R., Murray, J.F. Jr. (1996) J. Chromatogr. 41, 121-123.
26. Hurshman, A.R., Krebs, C., Edmondson, D.E., Huynh, B.H., and Marletta, M.A. (1999)
Biochemistry. 38, 15689-15696..
27. Wei, C.C., Wang, Z.Q., Wang, Q., Meade, A.L., Hemann, C., Hille, R., and Stuehr, D.J.
(2001) J. Biol. Chem. 276, 315-319.
28. Tsai, A.-L., Wu, G., Palmer, G., Bambai, B., Koehn, J.A., Marshall, P.J., and Kulmacz, R.J. (1999) J. Biol. Chem. 274, 21695-21700.
29. Foust, G. P., Burleigh, Jr., B. D., Mayhew, S. G., Williams, D. H., and Massey, V (1969) Anal. Biochem. 27, 530-535.
30. Smith, P.K., Krohn, R.L., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. (1985) Anal. Biochem. 150, 76-85.
31. Iyanagi, T., Makino, N., and Mason, H.S. (1974) Biochemistry, 13, 1701-1710.
32. Stuehr, D.J., and Ikeda-Saito, M. (1992) J. Biol. Chem. 267, 20547-20550.
33. Tsai, A.-L., Berka, V., Chen, P.-F., and Palmer, G. (1996) J. Biol. Chem. 271, 32563-32571.
34. Gachhui, R., Presta, A., Bentley, D.F., Abu-Soud, H.M., McArthur, R., Brudvig, G., Ghosh, D.K., and Stuehr, D.J. (1996) J. Biol. Chem. 271, 20594-20602.
35. Gorren, A.C.F., Kungl, A.J., Schmidt, K., Werner, E.R., and Mayer, B. (2001) Nitric Oxide: Biol. and Chem. 5, 176-186.
36. Presta, A., Weber-Main, A.M., Stankovich, M.T., and Stuehr, D.J. (1998) J. Am. Chem. Soc. 120, 9460-9465.
37. Schmidt, P.P., Lange, R., Gorren, A.C., Werner, E.R., Mayer, B, and Andersson K.K. (2001) J. Biol. Inorg. Chem. 6,151-158.
38. Archer, M.C., Vonderschmitt, D.J., and Scrimgeour, K.G. (1972) Can. J. Biochem. 50, 1174-1182.
39. Roman, L. J., Martásek, P., Miller, R. T., Harris, D. E., de la Garza, M.A., Shea, T.M., Kim, J.-J., and Masters, B.S.S. (2000) J. Biol. Chem. 275, 29225-29232.
40. Abu-Soud, H.M., Yoho, L.L., Stuehr, D.J. (1994) J. Biol. Chem. 269, 32047-32050.
41. Chen, P.-F., and Wu, K.K. (2000) J. Biol. Chem. 275, 13155-13163.
42. Nishida, C. R., Montellano, P. R., (1999) J. Biol. Chem. 274, 14692-14698.
43. Noble, M.A., Munro, A.W., Rivers, S.L., Robledo, L., Daff, S.N., Yellowlees, L.J.,
Shimizu, T., Sagami, I., Guillemette, J.G., and Chapman, S.K. (1999) Biochemistry. 38,
16413-16418.
44. Daff, S. N., Chapman, S. K., Turner, K. L., Holt, R. A., Govindaraj, S., Poulos, T. L., and
Munro, A. W. (1997) Biochemistry 36, 13816-13823.
45. Zeghouf, M., Fontecave, M., Macherel, D., and Coves, J. (1998) Biochemistry 37, 6114-
6123.
46. Tsai, A.-L., Berka, V., Kulmacz, R.J., Wu, G., and Palmer G. (1998) Anal. Biochem. 264,
165-171.
Table 1: Stoichiometry of the cofactors of purified eNOS subdomains (n = 7)

|        | Heme | FAD     | FMN     | BH4      |
|--------|------|---------|---------|----------|
|        | 0.93 +/- 0.04\(^a\) | 1.0 +/- 0.33\(^b\) | 1.14 +/- 0.21\(^b\) | 0.72 +/- 0.12\(^a\) |
|        | 0.85 +/- 0.09 (A\(_{398}\)) | 0.56 +/- 0.06 | 0.79 +/- 0.08 | ND |
|        | 0.71 +/- 0.02 (HPLC) |              |          |          |

\(^a\) This study, for eNOS\(_{ox}\).
\(^b\) This study, for eNOS\(_{red}\).
\(^c\) Data from Ref. 20 determined for whole eNOS.

Table 2: Catalytic activity of eNOS\(_{red}\) and eNOS

| Sample | Cytochrome c reductase (mol/min/mol) | Ferricyanide reductase (mol/min/mol) | DCPIP reductase (mol/min/mol) | Comments |
|--------|-------------------------------------|--------------------------------------|-------------------------------|----------|
|        | [−CaM] | [+ CaM] | [−CaM] | [+ \(Ca^{2+}/CaM\)] | [−CaM] | [+ \(Ca^{2+}/CaM\)] | | |
| eNOS\(_{red}\) | 70.3 +/- 8.0\(^a\) | 194.3 +/- 11.5 | 3220 +/- 520\(^b\) | 4480 +/- 760 | 400 +/- 60\(^b\) | 800 +/- 80 | This study 24°C, with 0.1 M NaCl |
| eNOS\(_{red}\) | 13.8 +/- 1.0 | 137.8 +/- 6.9 | | | | | Ref. 17 24°C, with 0.1 M KPi |
| eNOS | 20.9 +/- 1.4 | 224.4 +/- 14.96 | | | | | Ref. 17 24°C, with 0.1 M KPi |
| eNOS\(_{red}\) | 237.8 +/- 32.8 | 795.4 +/- 172.2 | 1878 +/- 37 | 2526 +/- 49 | | | Ref. 43 37°C, with 0.1M NaCl |
| eNOS | 72.1 +/- 1.4 | 299.2 +/- 54.4 | 924.8 +/- 54.4 | 3332 +/- 136 | | | Ref. 43 37°C, with 0.1M NaCl |
| eNOS | ND | 373.7 +/- 18.6 | | | | | Ref. 20 37°C |
| eNOS | 173 | 478 | 3860 | 4604 | | | Ref. 44 23°C, with 0.25M NaCl |
| eNOS | ~ 54 | ~ 700 | 482 | 2250 | | | Ref 45 37°C |

\(^a\) S.D. (n = 3).
\(^b\) S.D. (n = 5)
Table 3. The three difference potentials of the four half-reactions of flavins of eNOS$_{\text{red}}$ and related redox systems.

|                    | $\Delta E_1$   | $\Delta E_2$ | $\Delta E_3$ | ref    |
|--------------------|----------------|---------------|---------------|--------|
| diflavin reductase |                |               |               |        |
| eNOS$_{\text{red}}$ | 0.180 $\pm/-$ 0.012 | $\sim$0       | 0.073 $\pm/-$ 0.017 | this study |
| nNOS$_{\text{red}}$ | 0.225           | -0.042        | 0.048         | 39     |
| P450BM3            | 0.020           | 0.079         | 0.080         | 40     |
| rabbit P450        | 0.170           | -0.005        | 0.097         | 30     |
| sulfite reductase  | 0.175           | 0.055         | -0.060        | 41     |

Table 4. Extinction coefficients of the different redox states of flavins in eNOS$_{\text{red}}$ at different wavelengths.

| Wavelength /nm | Component         | $\varepsilon_{\text{FMN}}$/M$^{-1}$cm$^{-1}$ | $\varepsilon_{\text{FAD}}$/M$^{-1}$cm$^{-1}$ |
|----------------|-------------------|---------------------------------------------|---------------------------------------------|
| 600            | semiquinone       | 5670 $\pm/-$ 290$^a$                          | 4430 $\pm/-$ 310                           |
| 456            | fully oxidized    | 12100 $\pm/-$ 790                            | 11300                                       |
|                | semiquinone       | 6000                                         | 2030 $\pm/-$ 400                           |
|                | fully reduced     | 2570 $\pm/-$ 110                            | 2600 $\pm/-$ 100                           |
| 508            | fully oxidized    | 3670 $\pm/-$ 670                            | 3670 $\pm/-$ 670                           |
|                | semiquinone$^b$   | 3670 $\pm/-$ 670                            | 2300                                        |
|                | fully reduced     | 1270 $\pm/-$ 210                            | 1270 $\pm/-$ 210                           |

$^a$ S.D. n = 3.

$^b$ Only refer to FAD semiquinone.
FIGURE LEGENDS

**Figure 1.** Homogeneity of purified eNOS$_{ox}$ and eNOS$_{red}$. Purified eNOS$_{red}$ (Lane 2) and eNOS$_{ox}$ (Lane 3) after Ni-chelate column chromatography were analyzed by SDS-PAGE/Coomassie Blue staining (A) and Western blotting (B). Molecular weight markers are shown in Lane 1. Approximately 1 µg samples were used for each run.

**Figure 2.** UV-Vis spectra of eNOS$_{ox}$ (solid line) and its L-arginine complex (long dash), ferrous form (medium dash-dot) and ferrous CO complex (short dash). The spectra between 500 – 700 nm are enlarged in the INSET. Spectra for a typical preparation were given. Some eNOS$_{ox}$ preparations show a purity index, i.e. A$_{280}$/A$_{396}$ in L-arginine complex, as good as 1.3.

**Figure 3.** EPR spectra of eNOS$_{ox}$ (A), L-arginine complex (B) and imidazole complex (C). The concentration for eNOS$_{ox}$ was 25 µM in 50 mM HEPES, pH 7.4, with 10 % glycerol and 0.1M NaCl, L-arginine was 100 mM and imidazole was 40 mM. EPR conditions were: microwave frequency, 9.61 GHz; power, 1 mW; modulation, 10 G; temperature, 11K; time constant, 0.33 s, and each spectrum is the average of two scans.

**Figure 4.** Stoichiometric titration of eNOS$_{ox}$ by sodium dithionite in the presence of L-arginine. Purified eNOS$_{ox}$, not replenished with exogenous BH$_4$, at 18.0 µM, containing 500 µM L-arginine was titrated by 16.2 mM sodium dithionite anaerobically. A 1.8 mL total volume of reaction mixture in 50 mM HEPES, pH 7.5, containing 0.1 M NaCl and 10 % glycerol was used in the titration. Panel A shows the absorption spectra during the reductive titration. The INSET was the
difference spectra from panel A (against resting). Panel B is the changes of $A_{444}$ minus $A_{388}$ as a function of reducing equivalent per mole eNOS$_{ox}$. The solid straight lines indicate the initial, ending levels and the initial slope of the heme titration.

Figure 5. Molecular weight estimation by gel filtration. eNOS$_{ox}$ (~ 1 mg) was gel filtered through a Sephacryl 200 HR column (1.5 × 50 cm), the ratio of sample elution volume ($V_e$) and column void volume ($V_o$) determined by Blue Dextran was plotted with five molecular weight standards: horse heart cytochrome c (12,400 kDa), bovine erythrocytes carbonic anhydrase (29,000 kDa), bovine serum albumin (66,000 kDa), yeast alcohol dehydrogenase (150,000 kDa) and sweet potato β-amylase (200,000 kDa) (Solid circles). The data for eNOS$_{ox}$ (solid triangle) was interpolated into the standard curve to obtain the estimated molecular weight. There was only one protein peak monitored by $A_{280}$ absorbance detected for eNOS$_{ox}$ in the gel-filtration profile.

Figure 6. Kinetics of chemical modification for thiols by 4,4’-dithiopyridine. 50 µM 4,4’-dithiopyridine was added individually to 4 µM human eNOS$_{ox}$ (solid circles), 4 µM bovine eNOS$_{ox}$ (open circles), 4 µM human eNOS$_{ox}$ pretreated with 5 M urea for 2 hrs (cross) and 9 µM free L-cysteine (open triangles) in 50 mM KPi, pH 7.5. Formation of 4-thiopyridine after reaction with –SH groups of each sample was monitored at 324 nm ($\varepsilon = 19.8 \text{ mM}^{-1}\text{cm}^{-1}$) on HP8453 diode-array spectrophotometer for a period of 2 hrs at room temperature. Solid lines are one or two-exponential fits for each kinetic data.

Figure 7. Transient formation of BH$_4$ radical by eNOS$_{ox}$. 280 µM eNOS$_{ox}$ containing 0.7
equivalent BH$_4$/heme was first mixed with 1 mM L-arginine then reduced by dithionite titration in a tonometer with a side-arm attaching an optical cuvette. This ferrous eNOS$_{ox}$ was reacted with oxygenated buffer at 1:1 ratio on a rapid-freeze quench apparatus and reaction mixture was freeze quenched in isopentane and the ice particles collected at several reaction times from 20 to 200 ms at a ram velocity of 2.5 cm/s at room temperature by our specially designed packing device (42). Liquid helium EPR (A) was recorded for the intermediate trapped at 100 ms (solid) with a parallel control sample of L-arginine treated eNOS$_{ox}$ (dash). EPR conditions were the same as that in Fig. 3. Liquid nitrogen temperature EPR was recorded in the radical region in A as a 100-G scan. The EPR conditions were: microwave frequency: 9.29 GHz; power, 1 mW; modulation, 2 G; time constant, 0.33 s and temperature was 115 K. Spectrum in B (solid) is from a single scan. Dashed lines are computer simulation using the following parameters: $g_x = g_y = g_z = 2.0023$, line width: 12/11/11 G, $A_{xx}/A_{yy}/A_{zz}$ for the nitrogen nucleus is 2/1.5/23 G, and for the two hydrogens are: 4.6/21.6/11.8 G and 12.4/10.9/14.0 G, respectively.

Figure 8. Calmodulin effect on flavin fluorescence. Flavin emission spectra between 450-650 nm of 1.9 $\mu$M eNOS$_{red}$ was recorded before (solid line) and after addition of 250 $\mu$M Ca$^{2+}$ and 3.4 $\mu$M Calmodulin (long dash) to follow the formation of CaM-eNOS$_{red}$ complex and the dissociation of this complex by 3.5 mM EDTA addition (short dash). A negative control excluding eNOS$_{red}$ and CaM was subtracted from the data. INSET: stoichiometric titration of eNOS$_{red}$ by CaM monitored by flavin fluorescence change at 530 nm. The intersection of two straight lines is CaM binding stoichiometry.
Figure 9. Stoichiometric titration of eNOS$_{\text{red}}$ and computer simulations. eNOS$_{\text{red}}$ at 65 $\mu$M in 50 mM HEPES, pH 7.5, containing 0.1 M NaCl and 10% glycerol was titrated with standardized 30.8 mM sodium dithionite anaerobically. Panel A shows the absorption spectra (1-5) up to the addition of 1 reducing equivalent. The spectra of the fully oxidized flavins (dash-dot-dash) and semiquinone form of FMN (solid line) were highlighted. Panel B shows the spectra 5-10 for the titration between 1 and 3 reducing equivalents while the spectra 10-20 shown in panel C represent the titration data for addition of the 3-6 reducing equivalents. The hydroquinone of the second flavin (FADH$_2$) (solid line) and FADH$^-$ semiquinone (dash-dot-dash) were highlighted in panel C.

Panel D is the plot of absorbance changes at 456, 508, and 600 nm against the reducing equivalents consumed per mole of eNOS$_{\text{red}}$. Lines going through data points of each wavelength are the simulation obtained as detailed in the main text. The arrows in Panels A – C indicate the direction of the spectra changes with increasing dithionite. Another two duplications of titration show very similar results.
Fig. 1.
Fig. 2.
Fig. 3.

![Graph showing magnetic field and EPR amplitude](image-url)
Fig. 4.
A

Absorbance

Wavelength /nm

0.0
0.5
1.0
1.5
2.0

0 1 2 3 4 5 6

0.0
0.4
0.8
1.2

ΔA 444 - 388 nm

Reducing Equivalent / eNOS$_{ox}$

B

Δ A$_{444 - 388 \text{ nm}}$

0.0
0.4
0.8
1.2

0 1 2 3 4 5 6

Reducing Equivalent / eNOS$_{ox}$
Fig. 5.
Fig. 6

The graph shows the time (min) on the x-axis and the equivalents of -SH group on the y-axis. The graph compares the treatment of L-Cys, eNOS<sub>ox</sub> + Urea, and eNOS<sub>ox</sub>.
Fig. 7.
EPR Amplitude

Magnetic Field, G

A

EPR Amplitude

Magnetic Field, Gauss

B

45
Fig. 8.
Fig. 9

A

B

C

D

Wavelength /nm

Absorbance

Reducing equivalent /eNOS

600 nm

508 nm

456 nm

0.0

0.5

1.0

1.5

2.0

0.0

0.5

1.0

1.5

2.0

300 400 500 600 700 800

Wavelength /nm

300 400 500 600 700 800

300 400 500 600 700 800

Wavelength /nm

0 1 2 3 4 5 6

Wavelength /nm

0 1 2 3 4 5 6

Reducing equivalent /eNOS

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*J. Biol. Chem. published online December 11, 2002*

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