

# The FNR modules contribute to control nitric oxide synthase catalysis revealed by chimera enzymes

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**Abstract.** The reductase domains of neuronal NOS, endothelial NOS and two constitutive nitric oxide synthase (cNOS) share higher sequence similarity (>60%). In order to evaluate the role of ferredoxin-NADP<sup>+</sup> reductase (FNR) module in adjusting NOS catalytic activities, chimeras were by interchanging the FNR-like module between endothelial NOS and neuronal NOS in the present study. The assays of steady-state enzymatic activities for cytochrome *c* and ferricyanide reduction, NO synthesis and NADPH oxidation were performed spectrophotometrically. The two NOS FNR modules transferred their ferricyanide reductase character to the chimera enzymes. Results showed that the FNR module was important in adjusting electrons flow through the reductase domain and out of the FMN module. Results indicated that the FNR module was critical in controlling the electron transfer capacities of the FMN module.

## Introduction

Nitric oxide (NO) is synthesized by the NO synthase (NOS) enzyme. NO is a cytotoxin and a ubiquitous signaling molecule (1,2). Nitric oxide synthases (NOSs) are hemoproteins

and catalyze the oxidation of arginine to nitric oxide (NO) and citrulline. Three genetically and functionally distinct NOS isoforms, endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS) have been identified from endothelial cells, macrophages and neurons, respectively (3,4). NOSs are incorporate three domains in a single polypeptide: i) The N-terminal P450-like oxygenase domain that binds heme, tetrahydrobiopterin (H<sub>4</sub>B), and L-arginine, is the site of oxidation of arginine; ii) a C-terminal cytochrome P450 reductase (CPR)-related reductase domain that contains binding sites for FAD, FMN and NADPH, is responsible for providing electrons to the oxygenase domain; iii) a connecting peptide between the two domains binds calmodulin in a Ca<sup>2+</sup>-dependent manner for constitutive NOS (cNOS), nNOS and eNOS and essentially Ca<sup>2+</sup>-independent manner in iNOS (1,3,5-9). Regulation of NOS activity by CaM has been shown to occur via the control of electron transfer chain in NOS (NADPH<sup>®</sup>FAD<sup>®</sup>FMN<sup>®</sup>Heme) across the domain-domain interaction (10,11). It was shown that CaM can activate NADPH-dependent flavin reduction by suppressing the repressors within reductase domain, such as a C-terminal tail (CT) (12-14) and an autoinhibitory insert (AI) (15-17) within the middle of FMN sub-domain of cNOS.

NOS reductase domain can be dissected into two domains: The N-terminal FMN binding domain is homologous to bacterial flavodoxin, and the C-terminal fragment, containing binding sites for NADPH and FAD is related to the ferredoxin-NADP<sup>+</sup> reductase (FNR) (18). Each flavin nucleotide in NOS plays an obvious role in the electron flow sequence: FAD can transfer NADPH-divided electrons to linked FMN or exogenous electron acceptors such as ferricyanide and 3-acetylpyridine adenine dinucleotide phosphate (AcPyADP<sup>+</sup>), and the FMN can transfer the electrons to attached heme or non-physiological acceptors such as cytochrome *c* (19,20). It has been demonstrated that each of the recombinant flavin-binding domains can be expressed separately and retains the chemical and catalytic properties of their native counterparts (19-21). Recent reported crystal structures of rat nNOS reductase domain and mutation studies suggested that a movement of the FMN module from FNR may be required for its optimal position to transfer electron out of FMN (18). Crystal structure studies of the sulfite

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**Abbreviations:** NO, nitric oxide; NOS, NO synthase; eNOS, endothelial NOS; nNOS, neuronal NOS; cNOS, constitutive NOS (nNOS and eNOS); NOS<sub>red</sub>, reductase domain of NOS; FNR, ferredoxin-NADP<sup>+</sup> reductase; nNOS<sub>eFNR</sub>, nNOS incorporates with eNOS FNR module; eNOS<sub>nFNR</sub>, eNOS incorporates with nNOS FNR module; EPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; CaM, calmodulin; EGTA, ethylene glycol tetraacetic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; PMSF, phenylmethylsulphonyl fluoride.

**Key words:** chimera enzyme, nitric oxide synthase, electron transfer

reductase indicated that the interaction between the FNR and FMN modules displays lower affinity than in the case of CPR (22). FMN-free mutant studies suggested that removing FMN relieved suppression of FAD reduction within the FNR module and increase ferricyanide reduction (23). These data established the importance of domain-domain interaction and domain movement in flavoprotein catalysis.

The two constitutive NOS, eNOS and nNOS share similarities both in their structure and biochemical behaviors. However, nNOS exhibits much higher overall enzymatic activities than eNOS (24,25). Investigations of chimera enzymes that possess exchanged oxygenase and reductase domain indicated that heme reduction rate in cNOS is controlled mainly by the reductase domains (25). The heme reduction rate is almost independent of oxygenase domain (25). Previous results showed that the lower intrinsic activity of eNOS is caused by a lower ability of its electron transfer rate to the catalytic heme domain or external acceptors (26), but the NADPH-dependent flavin reduction rates of both CaM-bound cNOS are similar (27). Results showed that the electron transfer from full-reduced FMN (FMNH<sub>2</sub>) to heme or cytochrome *c* in cNOS is primarily influenced through a mechanism that does not involve changing the rate of flavins reduction. The details are not clear. It was suspected that the interaction of the FNR and FMN modules may differ between nNOS and eNOS. In this work, we will investigate how electron transfer and catalysis is related to the domain-domain interaction.

In the present study, we have constructed chimeric NOS enzymes by interchanging the FNR module between the two cNOS, and have studied the consequence of FNR module on catalytic activities, electron transfer properties by spectral methods. Our results clearly indicated that FNR module is important in controlling electron flow through the reductase domain to attached heme and outside from FMN module.

## Materials and methods

**Materials.** His-binding resin and CaM-sepharose were products of Amersham Biosciences (Uppsala, Sweden). Cytochrome *c* was purchased from Sigma (St. Louis, MO, USA). Restriction enzymes were purchased from New England Bio-lab (Ipswich, MA, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Generation of chimera DNAs.** The original vector coding was a gift to R.W. from Professor Philip Marsden (University of Toronto, Canada). Briefly, a unique restriction site Nru I was generated at S866 and R877 for nNOS, and S656 and R657 for eNOS by Site-directed mutagenesis using Quick Change polymerase chain reaction (PCR) *in vitro* mutagenesis kit from Stratagene (Agilent Technologies, Inc., Santa Clara, CA, USA).

**Expression and purification of chimera and wild-type enzymes.** Like wild-type rat nNOS and bovine eNOS, the chimera enzymes containing a His-6 tag attached to their N-termini were over-expressed in *E. coli* BL21 and purified by sequential chromatograph on Ni<sup>2+</sup>-NTA and Calmodulin-sepharose resins (28,29). The ferrous-CO adduct absorbing at 444 nm was used to quantitative heme protein content using an extinction

Table I. NADPH oxygenase activities of native and chimeric enzymes.

CaM/Arg or Aga/H <sub>4</sub> B	nNOS	eNOS	nNOS <sub>eFNR</sub>	eNOS <sub>nFNR</sub>
-/Arg/+	8.0±0.5	4.8±0.6	2.4±0.1	22±1.0
+/Aga/+	27±3.0	11±1.5	5.9±0.5	27±2.0
+/Arg/+	117±10	33±2.0	15±1.0	37±2.0

All values are the mean ± standard error for three separate determinations. All reactions were performed at 25°C. Arg, Arginine; Aga, Agatine; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase; FNR, ferredoxin-NADP<sup>+</sup> reductase.

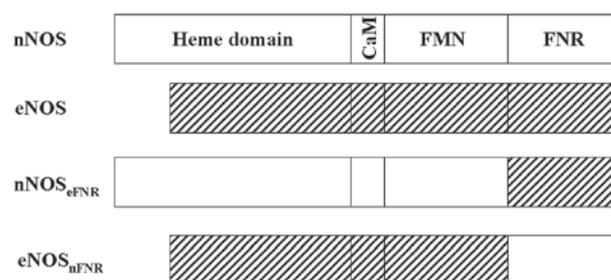


Figure 1. Illustration of constructed nNOS and eNOS chimeras. The open bar represents the nNOS and the black bar represents the eNOS. eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase; FNR, ferredoxin-NADP<sup>+</sup> reductase.

coefficient of 74 mM/cm. The contents of FAD and FMN were measured by HPLC with fluorometric detection.

**Spectrophotometric methods.** The assays of steady-state enzymatic activities for ferricyanide and cytochrome *c* reduction, NO synthesis, and NADPH oxidation were carried out spectrophotometrically. The rates of ferricyanide reduction were obtained by determining the absorbance decrease at 420 nm using an extinction coefficient of 1.02 mM/cm. The concentration of ferricyanide and NADPH was 1.0 mM and 0.3 mM, respectively. Cytochrome *c* reduction was determined by monitoring the absorbance increase at 550 nm using a difference extinction coefficient of 21 mM/cm between reduced and oxidized forms, with 0.3 mM NADPH and 0.1 mM cytochrome *c*. The rate of NADPH oxidation was measured by monitoring the absorbance decrease at 340 nm using an extinction coefficient of 6.22 mM/cm with 0.1 μM NADPH and in the presence or absence of 5 μM CaM, 300 μM Ca<sup>2+</sup>, 10 μM H<sub>4</sub>B, 250 μM Arginine or Agatine. NO synthesis rate was monitored from the conversion of oxyhemoglobin to methemoglobin mediated by NO. The rate was obtained by using a difference extinction coefficient of 38 mM/cm between oxyhemoglobin and methemoglobin at 401 nm, with 0.3 mM NADPH, 4 μM H<sub>4</sub>B, 10 μM oxyhemoglobin and 1 mM L-arginine. All results were obtained at 25°C in EPPS buffer (40 mM, pH 7.6) containing 0.25 M NaCl, 10% glycerol, 10 units/ml superoxide dismutase (SOD), 0.5 μM FAD/FMN, 100 units/ml catalase, and 50 μM EDTA (for minus CaM assays) using a U-3110 spectrophotometer (HITACHI Ltd,

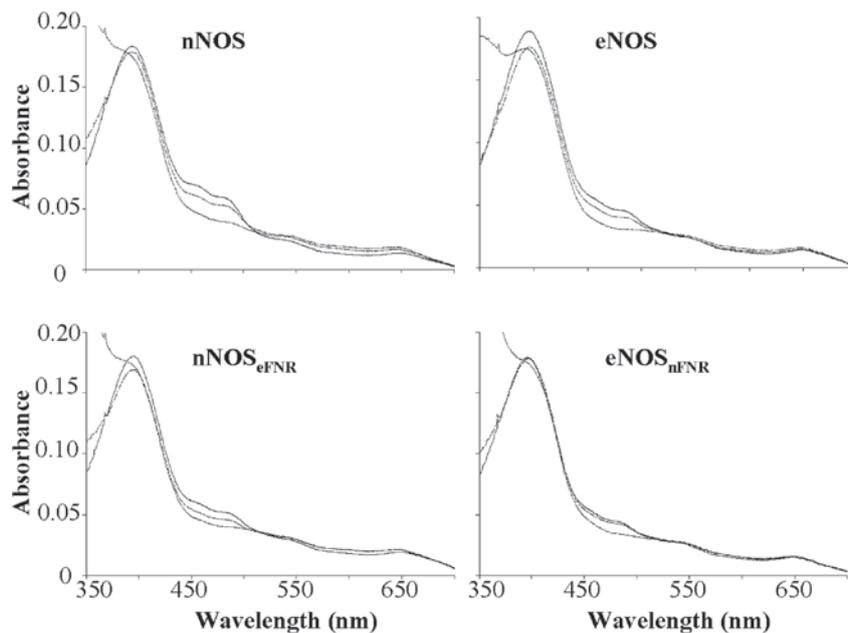


Figure 2. Absorbance spectra record during oxidation-reduction reactions of wild-type and chimera enzymes. (—) The oxidized enzymes. (---) The spectra were recorded immediately after mixing excess NADPH with enzyme. (----) The spectra were recorded at 30 min after addition of excess NADPH to enzyme solution. Final concentrations: Enzyme, 1.8  $\mu\text{M}$ ; NADPH, 100  $\mu\text{M}$ ; CaM, 5  $\mu\text{M}$ ;  $\text{Ca}^{2+}$ , 300  $\mu\text{M}$ ;  $\text{H}_4\text{B}$ , 10  $\mu\text{M}$ ; Agatine, 250  $\mu\text{M}$ . Results are representative of three similar individual experiments. nNOS, neuronal nitric oxide synthase;  $\text{H}_4\text{B}$ , tetrahydrobiopterin; eNOS, endothelial nitric oxide synthase; FNR, ferredoxin-NADP<sup>+</sup> reductase.

Tokyo, Japan). For assays of calmodulin activation, 5  $\mu\text{M}$  CaM and 0.5 mM  $\text{Ca}^{2+}$  were added to the reaction mixture, respectively.

**Kinetics of NADPH-dependent flavin and heme reduction.** Rapid mixing stopped-flow reaction were performed using a stopped-flow spectrophotometer. The stopped-flow apparatus had a dead time of 2 ms and was equipped with an anaerobic workbox. Stopped-flow experiments were obtained in 0.25 M NaCl, 40 mM pH 7.6 EPPS buffer. The constant temperature is 10°C. Flavin reduction was measured by monitoring the decrease of absorbance (485 nm) after mixing 50  $\mu\text{M}$  NADPH with 2  $\mu\text{M}$  enzyme in the presence and absence of  $\text{Ca}^{2+}$ /CaM under anaerobic conditions. Anaerobic heme reduction rate was obtained by monitoring the increase of absorbance at 444 nm, and this rate represents the formation of ferrous-CO complex. Reaction was initiated by rapid mixing 50  $\mu\text{M}$  NADPH with 2  $\mu\text{M}$  chimera or wild-type in 40  $\mu\text{M}$  EPPS buffer, pH 7.6, 10  $\mu\text{M}$   $\text{H}_4\text{B}$ , 1 mM L-arginine, 10  $\mu\text{M}$  CaM, and 1 mM  $\text{Ca}^{2+}$ . In these experiments, NADPH, chimera and wild-type were prepared in an anaerobic CO-saturated solutions.

## Results

**Expression and purification of chimera enzymes.** FNR swapped chimera enzymes, nNOS<sub>eFNR</sub> and eNOS<sub>nFNR</sub> were expressed in *E. coli* BL21. Their yields were similar to those of wild-type nNOS and eNOS, respectively. The chimera enzymes were purified in the same way as wild-type enzymes as described in experiment procedures. Both the chimera enzymes showed correct molecular mass and over 90% purity as judged by SDS-PAGE. The chimera enzymes (Fig. 1)

contained 1 FMN and 1 FAD per NOS heme measured by HPLC.

**Optical spectra properties of chimera enzymes.** Spectroscopic analysis showed that the chimera enzymes were consistent with the flavins-bound wild-type proteins as shown in Fig. 2 spectrum a. Dithionite reduction of the chimera enzymes produced the expected absorbance peak at 444 nm for the ferrous-CO complex in all cases in the presence of Arg,  $\text{H}_4\text{B}$  and CO. Results indicated that swapped FNR sub-domain between nNOS and eNOS did not alter proteins folding, expression and physical properties.

**NADPH oxidation.** The steady-state oxidation of NADPH was measured in the presence or absence of CaM, Arginine or Agatine,  $\text{H}_4\text{B}$  as shown in Fig. 3 and Table I. Compared with wild-type nNOS, the chimera enzyme, nNOS<sub>eFNR</sub> showed a lower (approximately 12-30 %) NADPH oxygenase activity both in the presence and absence of CaM. In contrast, the chimera enzyme, eNOS<sub>nFNR</sub> showed higher (~4.5-fold) NADPH oxygenase activity than that of wild-type eNOS without CaM. However, unlike nNOS<sub>eFNR</sub>, it did not increase upon CaM.

**NO synthesis.** The binding of CaM is essential for electron transfer from FMN to heme thereby initiated NO synthesis. Wild-type nNOS showed higher ability of NO synthesis (~5-fold) than that of wild-type eNOS. We measured the steady-state NO synthesis of chimera enzymes as shown in Fig. 4 and Table II. As the wild-type enzymes, both chimera enzymes are required CaM binding for NO synthesis activity. The nNOS chimera (nNOS<sub>eFNR</sub>) incorporates eNOS FNR module and showed NO synthesis activity that was about 12%

Table II. Steady-state activities for NO synthesis, cyt. *c* reduction and ferricyanide reduction.

	NO synthesis		Cyt. <i>c</i> reduction		Ferricyanide reduction	
	-	+	-	+	-	+
nNOS	ND	51±4.4	554±13	4,798±148	7,800±508	11,983±745
eNOS	ND	11±0.9	63±4	340±10	6,012±165	7,168±248
nNOS <sub>eFNR</sub>	ND	6.3±0.3	59±4.0	151±14	6,349±312	6,665±100
eNOS <sub>nFNR</sub>	ND	13±1.0	787±42	562±32	8,366±194	8,627±131

NO, nitric oxide; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase; FNR, ferredoxin-NADP<sup>+</sup> reductase.

Table III. Observed rate constants for NADPH-dependent flavin and heme reduction in the presence and absence of Ca<sup>2+</sup>/CaM (10°C).

	Flavin reduction		Heme reduction
	k1 (%)	k2	k
nNOS	-4.3±0.2 (63) +48±2.1 (61)	0.4±0.02 5.1±0.004	ND 3.6±0.2
eNOS	-0.3±0.03 (59) +59±4.4 (77)	0.02±0.002 4.3±0.9	ND 0.005±0.0004
nNOS <sub>eFNR</sub>	-0.17±0.003 (40) +19±3.5 (43)	0.019±0.0007 0.19±0.006	ND 0.0018±0.0002
eNOS <sub>nFNR</sub>	-18±1.0 (62) +32±1.7 (64)	1.0±0.02 1.1±0.03	ND 0.0024±0.0002

NOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; FNR, ferredoxin-NADP<sup>+</sup> reductase.

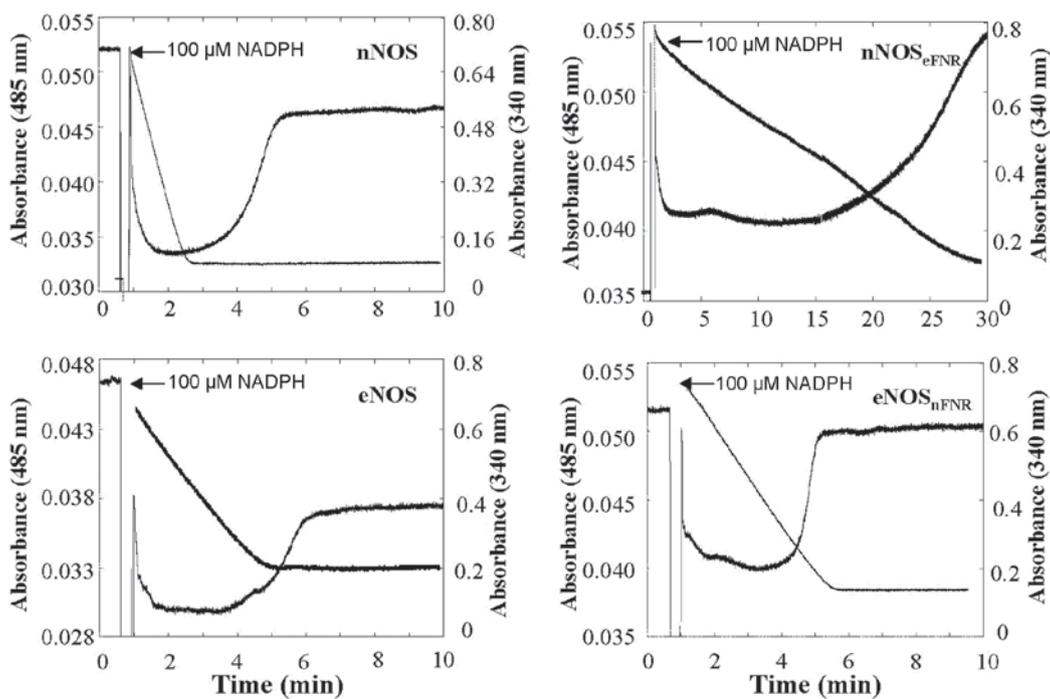


Figure 3. Time course of absorbance change at 485 nm represent flavin oxidation-reduction, and at 340 nm represents NADPH oxidation, respectively. The enzyme solution contain: Enzyme, 1.8 μM; CaM, 5 μM; Ca<sup>2+</sup>, 300 μM; H<sub>4</sub>B, 10 μM; Aratine, 250 μM. The narrows indicate the point of addition of 100 μM NADPH. Results are representative of three similar individual experiments. H4B, tetrahydrobiopterin; eNOS, endothelial nitric oxide synthase; FNR, ferredoxin-NADP<sup>+</sup> reductase.

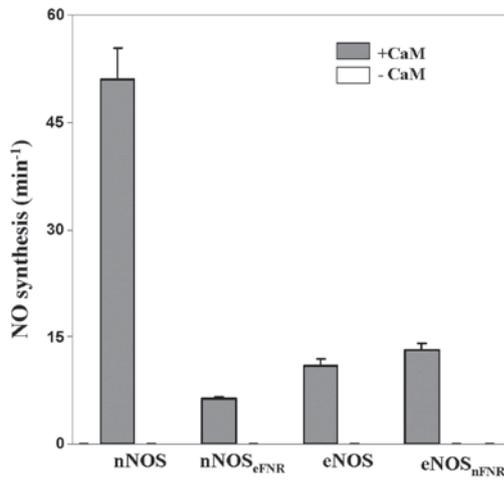


Figure 4. Steady-state NO formation of wild-type and chimera enzymes in the absence and presence of CaM/Ca<sup>2+</sup> as described in Materials and methods. Each bar represents the mean  $\pm$  standard error of three independent measurements. NO, nitric oxide; nNOS, neuronal NO synthase; eNOS, endothelial NO synthase; FNR, ferredoxin-NADP<sup>+</sup> reductase.

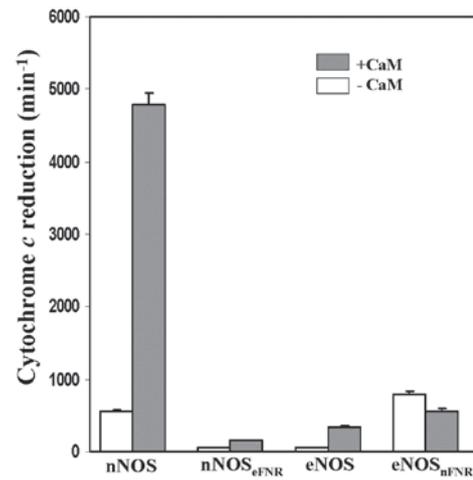


Figure 5. Steady-state cytochrome *c* reduction of wild-type and chimera enzymes. The activities of cytochrome *c* were measured in the absence and presence of CaM/Ca<sup>2+</sup> as described in Materials and methods. Each bar represents the mean  $\pm$  standard error of three independent measurements. nNOS, neuronal NO synthase; eNOS, endothelial NO synthase; FNR, ferredoxin-NADP<sup>+</sup> reductase.

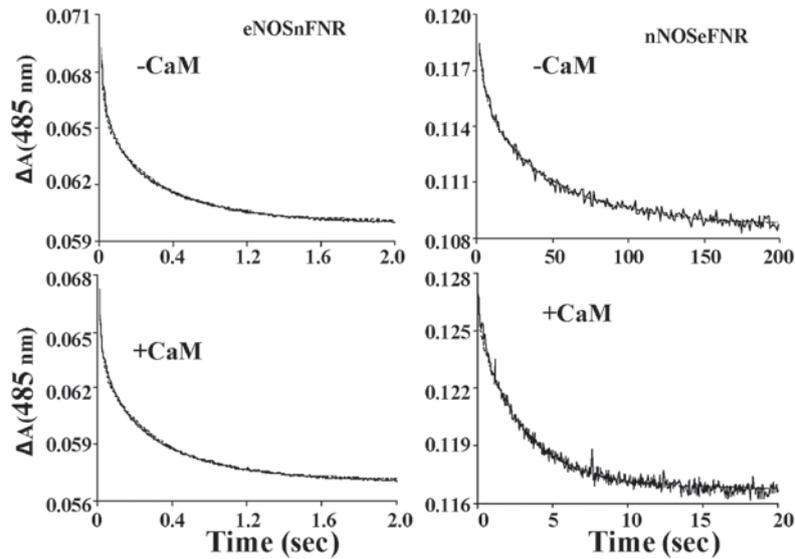


Figure 6. Kinetics of NADPH-dependent flavin reduction in chimera enzymes, eNOS<sub>nFNR</sub> and nNOS<sub>eFNR</sub> in the absence and presence of CaM/Ca<sup>2+</sup>. Spectral changes were recorded at 485 nm represent the flavin reduction in a stopped-flow spectrophotometer after rapid mixing 2  $\mu$ M enzyme and 50  $\mu$ M NADPH in 40 mM EPPS buffer containing 0.25 M NaCl, pH 7.6, under anaerobic conditions at 10°C. Traces shown are the average of 8-10 individual scans. nNOS, neuronal NO synthase; eNOS, endothelial NO synthase; FNR, ferredoxin-NADP<sup>+</sup> reductase.

of native nNOS. However, the eNOS chimera (eNOS<sub>nFNR</sub>) incorporates nNOS FNR module and showed similar NO synthesis activity with native eNOS.

**Cytochrome *c* and ferricyanide reductions.** Both the chimera enzymes did not alter their ferricyanide reductase activities, however, they dramatically changed their cytochrome *c* reductase activities comparing with the wild-type enzymes (Fig. 5, Table II). The CaM-free nNOS<sub>eFNR</sub> chimera showed a lower (~4%) cytochrome *c* reductase activity than that of wild-type nNOS. On the contrary, cytochrome *c* reductase activity of eNOS<sub>nFNR</sub> chimera was 14-fold higher than that of wild-type eNOS in the absence of CaM. However, its activity did not increased up on CaM binding.

**Kinetics of flavin and heme reduction.** Under anaerobic conditions, the rates of NADPH-dependent flavin and heme reduction were measured by the aid of stopped-flow spectroscopy. Fig. 6 showed the spectra traces (485 nm) of flavin reduction of chimera enzymes in the absence and presence CaM as an absorbance decrease vs. time. The traces of flavin reduction were biphasic for both chimera enzymes. The flavin reduction rate was slower in nNOS<sub>eFNR</sub> chimera than that of native nNOS both in the presence and absence of CaM/Ca<sup>2+</sup>. However, in contrast, eNOS<sub>nFNR</sub> chimera showed a higher flavin reduction rate without CaM/Ca<sup>2+</sup>, and it weakly increased upon CaM binding (Table III). These results consistent with the results acquired from steady-state cytochrome *c* reduction. Like wild-type enzymes, both chimeras are required CaM binding

for heme reduction. However, the nNOS<sub>eFNR</sub> chimera showed a lower heme reduction rate comparing with that of wild-type nNOS, and no significant change was found between eNOS<sub>nFNR</sub> and wild-type eNOS.

**Clinical implication.** Experimental and clinical results show that NO is involved in the genesis of depression as well as in antidepressant drug effects (30). NOS catalyze a two-step oxidation of L-arginine to form NO. NOS is also the molecular target of anti-inflammatory compounds (30). Inhibition of NOS activity will exert antidepressant-like effect in animal models (31). NOS chimeras have been successfully created in this work. Investigations provide a better understanding of how distinct protein structures or structural features influence the activities of NOS enzymes. Our results provide general fundamental understanding for rational design of NOS enzymes in order to regulate their functions in various biological settings (32).

The reductase domains of eNOS, nNOS and the two constitutive nitric oxide synthase (cNOS) share higher sequence similarity (over 60%). These domains contain same regulatory peptide (a C-terminal tail and an AI) and same cofactor binding sites. Nevertheless, they differ observably in the ability for transferring their electrons to heme or heme protein acceptors. In order to evaluate the role of FNR module in controlling NOS catalytic activities, chimeras were created by interchanging the FNR-like module between eNOS and nNOS in this work. The eNOS chimera (eNOS<sub>nFNR</sub>) showed a higher cytochrome *c* reductase activity without calmodulin (CaM), and the activity was over 10-fold higher than that of native eNOS. Meanwhile, the activity did not increase upon CaM binding. The NO synthesis rate was similar to that of native eNOS. In contrast, the nNOS chimera (nNOS<sub>eFNR</sub>) had a ~10% cytochrome *c* reductase activity of native nNOS without CaM. The NO synthesis activity for nNOS chimera (nNOS<sub>eFNR</sub>) was ~12% activity of native nNOS. The NOS FNR modules transferred their ferricyanide reductase character to the chimera enzymes. In conclusion, the FNR module is important in adjusting electrons flow through the reductase domain and out of the FMN module. Results indicated that the FNR module plays a critical role in controlling electron transfer capacities of the FMN module.

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