Biochemical and Biophysical Research Communications **265,** 184–188 (1999)
Article ID bbrc.1999.1643, available online at http://www.idealibrary.com on IDEAL®

Rapid Kinetic Studies of Electron Transfer in the Three Isoforms of Nitric Oxide Synthase[1]

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Received October 7, 1999

The nitric oxide synthases (NOSs) consist of a flavin-containing reductase domain, linked to a heme-containing oxygenase domain, by a calmodulin (CaM) binding sequence. The flavin-containing reductase domains of the NOS isoforms possess close sequence homology to NADPH-cytochrome P450 reductase (CPR). Additionally, the oxygenase domains catalyze monooxygenation of L-arginine through a cytochrome P450-like cysteine thiolate-liganded heme bound in the active site. With these considerations in mind, we conducted studies in an attempt to gain insight into the intermediates involved in flavoprotein-to-heme electron transfer in the NOSs. Static, steady-state, and stopped-flow kinetic studies indicated that nNOS must be reduced to a more than one-electron-reduced intermediate before efficient electron transfer can occur. Therefore, the possibility exists that the oxygenase domains of the NOS isoforms may receive their electrons from the reductase domains by a mechanism resembling the CPR-P450 interaction. Furthermore, the rate-limiting step in electron transfer appears to be the transfer of electrons from the flavoprotein to the oxygenase domain facilitated by the binding of CaM at increased intracellular Ca^{2+} concentrations. Thus, modulation of electron transfer rates appears to be regulated at the level of the flavoprotein domains of the NOS isoforms.

The nitric oxide synthases (NOS) catalyze the NADPH- and oxygen-dependent conversion of L-arginine to L-citrulline plus nitric oxide ($NO^{\bullet}$) (1–4). The NOSs consist of a flavin-containing reductase domain linked to a heme-containing oxygenase domain (5) by a calmodulin (CaM)-binding sequence which is located between the two domains (5–7). The flavin-containing reductase domain, which structurally and functionally resembles cytochrome P450 reductase (CPR) (6), contains FAD and FMN in equimolar amounts (8) and accepts reducing equivalents from NADPH. The oxygenase domain is a cytochrome P450-type hemoprotein containing iron protoporphyrin IX (9–12) and binding sites for the substrate L-arginine and the cofactor tetrahydrobiopterin (BH_4) (13). CaM binding to NOS, triggers electron transfer between the flavins as well as facilitating flavoprotein-to-hemeprotein electron transfer, and thus product formation (14).

Therefore, considering the homology of the nNOS reductase domain with CPR (6) and the functional similarity of the NOS oxygenase domain to the cytochromes P450, we investigated the possibility that electron transfer reactions in the NOS isoforms proceed *via* a mechanism similar to what has been proposed for the CPR-cytochrome P450 interaction (15), i.e., a mechanism whereby the flavin-containing reductase cycles between a 1- and 3-electron-reduced species (16, 17). Furthermore, since the three NOS isoforms differ in the rates of flavin-mediated reactions (cytochrome *c* and DCIP reduction) as well as in rates of heme-mediated reactions ($NO^{\bullet}$ and L-citrulline formation) (18–25), we examined the NADPH-induced reduction kinetics of the three NOS isoforms in an attempt to account for the differing rates of electron transfer. These techniques have allowed us to gain a better understanding of the intermediates involved in flavoprotein-to-hemoprotein electron transfer in the NOSs. Our studies indicate that the three NOS isoforms possess differing rates of flavin and heme reduction and that the rate-limiting step in electron transfer

Abbreviations used: nNOS, rat neuronal nitric oxide synthase; eNOS, bovine endothelial nitric oxide synthase; iNOS, murine inducible nitric oxide synthase; $NO^{\bullet}$, nitric oxide; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; BH_4, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; CaM, calmodulin; CPR, NADPH-cytochrome P450 reductase; Tris, (Tris[hydroxymethyl]aminomethane); K_3FeCN_6, potassium ferricyanide.

[1] This work was supported by NIH Grants GM52419 and HL30050 and a grant from the Robert A. Welch Foundation AQ1192 to B.S.S.M. R.T.M. was supported by a National Heart, Lung, and Blood Cardiovascular Training Grant HL07350.

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0006-291X/99 $30.00

appears to be the transfer of electrons from the flavoprotein to the oxygenase domain. Thus, modulation of electron transfer rates appears to be regulated at the level of the flavoprotein domains of the NOS isoforms.

EXPERIMENTAL PROCEDURES

Chemicals and enzymes. NADPH, Trizma base, bovine brain calmodulin, L-arginine, and $CaCl_2 \cdot 2H_2O$ were obtained from Sigma Chemical Co. (St. Louis, MO). (6R)-5,6,7,8-Tetrahydro-L-biopterin was from Research Biochemicals International (Natick, MA). All other chemicals and reagents were obtained from commercial sources.

nNOS, eNOS, and iNOS. Rat brain nNOS was prepared as described by Roman *et al.* (21). Wild-type bovine eNOS was prepared as described by Martásek *et al.* (22). iNOS was coexpressed with CaM and worked up by using the nNOS protocol (21), except that it was grown in the absence of the chaperonins GroEL/GroES. The flavoprotein module of nNOS, including amino acids 722–1429, was prepared as previously described (26). All enzymes were prepared fresh before experimentation and were never subjected to freeze-thaw cycles.

Static and steady-state spectroscopy. Absorbance spectra and routine analyses were performed on a Shimadzu UV-2101PC dual beam spectrophotometer at 23°C. Spectral titrations of the flavoprotein module of nNOS were carried out under an aerobic atmosphere. In experiments involving spectral titrations, a two-minute re-equilibration period was allowed between each addition of either NADPH or K_3FeCN_6. All static spectra and spectral titrations were performed at 23°C in 50 mM HEPES containing 100 mM NaCl, pH 7.6.

Stopped-flow spectroscopy. Stopped-flow reactions were performed at 23°C aerobically under turnover conditions in 50 mM Tris containing 100 mM NaCl, pH 7.6. One syringe of the stopped-flow apparatus contained 4 μM NOS, 200 μM L-arginine, 20 μM BH_4, 800 μM $CaCl_2$, and 6 μM CaM (except for experiments involving iNOS). The second syringe contained 200 μM NADPH in buffer. Rapid kinetic reactions were recorded on an Applied Photophysics SX.18MV microvolume stopped-flow reaction analyzer (Applied Photophysics, Leatherhead, UK) which had a dead time of 2 msec. Data were fit to either a single or double exponential equation using the package provided by the instrument manufacturer. Spectral changes associated with the NOS isoforms upon exposure to NADPH were measured at various wavelengths: 397 nm, heme reduction; 485 nm, flavin reduction; 436 nm, ferrous-nitrosyl complex formation. The kinetic traces are presented as averages of a number of individual reactions (5–10) and the residuals throughout the reaction period are displayed below each individual trace.

Protein determination. The protein concentration of the NOS isoforms was determined based on heme content by reduced CO difference spectra using an extinction coefficient of 100 mM^{-1} cm^{-1} for $\Delta\epsilon$ 444–475 (27). CO difference spectra were obtained after reducing the enzyme with a few grains of dithionite and then bubbling with CO. These determinations are probably underestimations of protein concentration since apoprotein (minus heme or flavin) exists in all preparations to some degree. However, the average heme content of NOS preparations from this laboratory is approximately 80%[3]. The concentration of the flavoprotein module of nNOS was determined by total flavin absorbance at 455 nm using an extinction coefficient of 21.6 mM^{-1} cm^{-1}, based on the presence of two flavins per mole.

[3] A.-L. Tsai, P. Martásek, L. J. Roman, and B. S. S. Masters, unpublished observations.

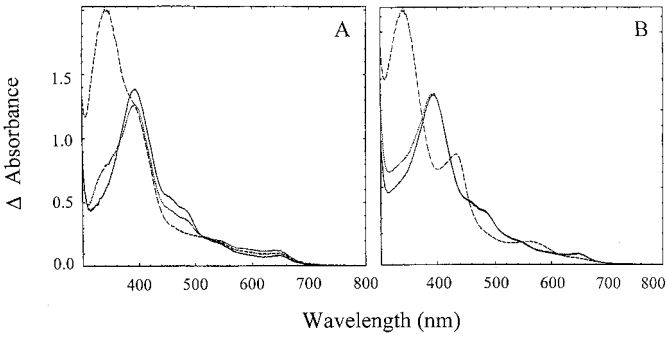

FIG. 1. Spectral changes which occur following NADPH addition to nNOS. Cuvettes contained 10 μM nNOS, 10 μM BH_4, 400 μM $CaCl_2$, 100 μM L-arginine in the absence (A) or presence (B) of 15 μM CaM. In each case, spectral scans were recorded in the following sequence: oxidized enzyme (—), after addition of 300 μM NADPH (---), and, following oxidation of excess NADPH (· · ·) under conditions described in Experimental Procedures.

RESULTS AND DISCUSSION

The spectral changes which occur upon addition of NADPH to CaM-free or CaM-bound nNOS are shown in Figures 1A and 1B. Addition of NADPH to CaM-free nNOS (Figure 1A) causes flavin reduction exclusively as indicated by the decrease in absorbance at 485 nm. Following reduction by excess NADPH, there is an initial decrease in absorbance in the 485 nm region and an absorbance increase in the 585 nm region. Following air oxidation, the 485 nm absorbance returns to an intermediate value which is accompanied by a further increase in absorbance in the 585 nm region, indicative of a one-electron-reduced flavoprotein (16, 28). In experiments in which the isolated reductase domain of nNOS was titrated with NADPH, a decrease in absorbance was noticed along the entire flavin spectrum (from 360–500 nm) with isosbestic points occurring at 363 nm and 502 nm (Figure 2). With the CaM-free nNOS holoenzyme, an isosbestic point occurs at 510 nm between the fully oxidized spectrum and the 1-electron-reduced spectrum (Figure 1A). Spectral changes occurring in the 380 nm region during NADPH reduction suggested that no anionic, red semiquinone (as in P450 BM3) was being formed under our assay conditions (29). The spectral behavior of nNOS and its flavoprotein domain are consistent with the early observations of Iyanagi and Mason (28) and Masters *et al.* (16) in which a one-electron-reduced form of CPR was identified. Furthermore, the 1-electron-reduced form of CPR does not efficiently transfer its lone electron to P450 (30) or to cytochrome *c* (16) without the addition of excess NADPH, and this is also true with nNOS (31). Several studies have suggested that the mechanism of electron transfer from CPR to various electron acceptors involves cycling of the flavins between a 1- and 3-electron-reduced state (32–36).

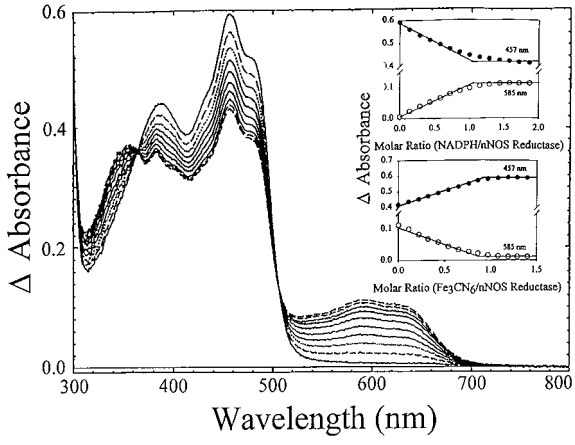

FIG. 2. Titration of the isolated flavoprotein module (amino acids 722–1429) of nNOS, in the absence of CaM, and under aerobic conditions. Titrations of the flavoprotein module (26 μM) were conducted in 500 μL volumes at 23°C as described in Experimental Procedures. The upper panel of the inset shows sequential 2.5 μM additions of NADPH to the flavoprotein module of nNOS until maximal absorbance changes were achieved at 457 nm and 585 nm. The flavoprotein module of nNOS was then reoxidized by back-titrating with sequential 2.5 μM additions of K_3FeCN_6 until the absorbances plateaued at 457 nm and 585 nm (inset; lower panel). Some spectra were omitted for clarity.

Addition of NADPH to CaM-bound nNOS (in the presence of L-arginine and cofactors; Figure 1B) causes flavin reduction (485 nm), a slight increase in absorbance at 585 nm (heme reduction; α/β Soret bands), a decrease in absorbance at 397 nm (heme reduction), and formation of a ferrous-nitrosyl species (436 nm). The ferrous-nitrosyl species is formed by enzymatically-produced $NO^{\bullet}$, which binds to the heme iron of nNOS and slowly inactivates the enzyme (37). Following consumption of the excess NADPH, a slight residual absorbance in the 585 nm region remained, indicative of the fleeting presence of a one-electron-reduced flavin species (Figure 1B). However, in the holoenzyme, interpretation of the spectral changes occurring at these longer wavelengths, which are diagnostic for the presence of one-electron-reduced flavin, is difficult due to concurrent spectral contributions due to heme reduction. The inability of CaM-bound nNOS to remain in the one-electron-reduced state for prolonged periods of time is due to a destabilizing effect of CaM on the semiquinone.[4] To investigate whether the lone electron in CaM-bound nNOS was capable of reducing the heme within the oxygenase domain or, alternatively, of being lost to molecular oxygen, we reduced nNOS with excess NADPH and then allowed the enzyme to air-oxidize to the 1-electron-reduced semiquinone state. Addition of CaM at that point did not trigger electron transfer to the heme. This can be clearly seen in Figure 3 where a spectrum was collected immediately following the addition of CaM to the 1-electron-reduced form of nNOS. No significant spectral changes in either the flavin region or the heme region were evident following the addition of CaM. These data suggest the loss of the single electron is from flavin-to-oxygen and not from flavin-to-heme. Furthermore, this electron loss occurs at a faster rate with CaM-bound nNOS than with CaM-free nNOS[3]. However, irrespective of their individual rates of decay, the main finding in this set of studies is that both CaM-free, as well as CaM-bound, nNOS can stabilize the 1-electron-reduced species to some extent.

In order to investigate further and to confirm that the spectral changes we observed were being caused by changes occurring within the flavoprotein domain of nNOS, we titrated the isolated flavoprotein module (amino acids 722–1429) of nNOS with NADPH under aerobic conditions (Figure 2). Isosbestic points occurred at 363 nm and 502 nm during titration with NADPH, consistent with the conversion of a fully oxidized flavoprotein to a one-electron-reduced species. The peak-splitting in the region of 380 nm and the absorbance increase in the 585 nm region are also consistent with the formation of a 1-electron-reduced flavin species (38). The insets of Figure 2 show that 1 mole of NADPH (2 electrons)/mole NOS flavoprotein are required to produce the maximum amount of semiquinone (upper panel). One electron is lost to oxygen during this process. When the 1-electron-reduced flavoprotein was back-titrated with K_3FeCN_6, it can be seen that only one electron was contained within the enzyme (lower panel).

We next employed rapid-reaction stopped-flow spectrophotometry in an attempt to gain insight into the reason(s) for the differing rates of turnover between the NOS isoforms. In Figure 4, the data obtained with

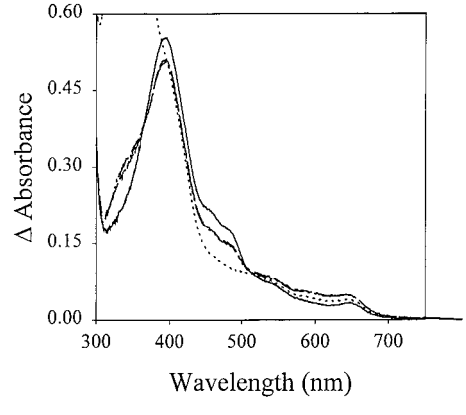

FIG. 3. Effect of adding CaM to the one-electron-reduced form of nNOS. Oxidized nNOS 5 μM (—) was reduced with excess NADPH (· · ·). The enzyme was then allowed to air-oxidize to the stable one-electron-reduced form (- - -), at which time excess CaM was added and a spectrum recorded (· · –). Note how the stable one-electron-reduced spectrum of nNOS perfectly overlays the spectrum recorded following CaM addition.

[4] R. T. Miller and B. S. S. Masters, unpublished observations.

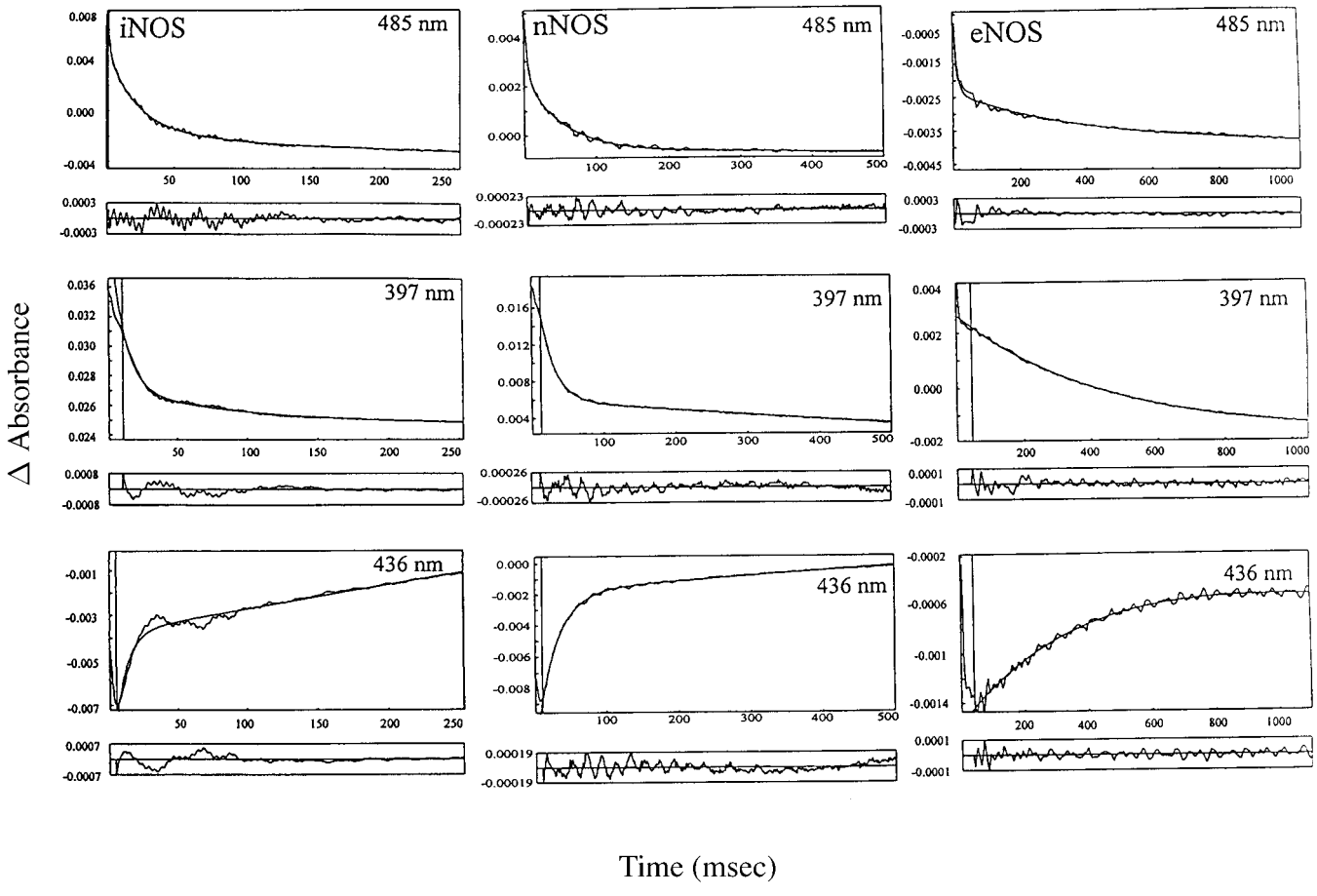

FIG. 4. Stopped-flow kinetic traces obtained following the rapid mixing of NOS with NADPH under turnover conditions. Left column, iNOS; middle column, nNOS; right column, eNOS. Spectral changes were followed at 485 nm for flavin reduction (upper panels), 397 nm for heme reduction (middle panels), and 436 nm for ferrous-nitrosyl complex formation (lower panels). The experimental conditions are described in Experimental Procedures and the reaction rates are summarized in Table 1. The experimental fits for heme reduction and ferrous-nitrosyl complex formation excluded the initial, rapid (10–20 msec) decrease in absorbance, which is attributable to spectral contributions of the flavins at these wavelengths.

iNOS (left column), nNOS (middle column), and eNOS (right column) show stopped-flow traces gathered at various wavelengths to monitor the spectral changes occurring following rapid mixing of NOS with NADPH under turnover conditions. The actual rate constants for these reactions are summarized in Table 1. Again, 485 nm was used to monitor flavin reduction, 397 nm for heme reduction, and 436 nm for monitoring ferrous-nitrosyl complex formation. In all instances, the rank order for flavin reduction, heme reduction, and ferrous-nitrosyl formation was iNOS > nNOS > eNOS. These data clearly demonstrate that the rate of flavin reduction correlates in a positive manner with the rate of heme reduction. Most importantly, the rate of heme reduction is much slower than flavin reduction, most dramatically with eNOS. This can best be seen from the ratios of the rate of flavin reduction to the rate of heme reduction: iNOS, 689/101 = 6.8; nNOS, 242/49 = 4.9; eNOS, 107/2.3 = 46. These ratios suggest that some impediment exists which may be modulating

TABLE 1

Rates of NADPH-Induced Electron Transfer in the NOS Isoforms

	485 nm (flavin reduction)	397 nm (heme reduction)	436 nm (ferrous nitrosyl)
eNOS			
fast phase	107	2.3	2.5
slow phase	3	—	—
nNOS			
fast phase	242	49	36
slow phase	18	—	—
iNOS			
fast phase	689	101	116
slow phase	50	10	—

Note. All rates are expressed as turnover numbers second^{-1}. Stopped-flow reactions were performed at 23°C under turnover conditions. The designations "fast" and "slow" refer to instances where reactions were biphasic, and consisted of an initial "fast phase" followed by a secondary "slow phase."

electron transfer from the reductase to the heme in the constitutive NOS isoforms, even in the presence of bound CaM (Figure 4; Table 1).

Collectively, these studies demonstrate that nNOS stabilizes a 1-electron-reduced form of flavin, presumably [FAD/FMNH$^{\bullet}$], which is more stable in the absence of bound CaM. The 1-electron-reduced form of nNOS is unable to transfer its electron efficiently to the heme iron of its oxygenase domain or to cytochrome *c* (31), and, thus, only a two (or more)-electron-reduced form of nNOS is capable of these electron transfer reactions. Furthermore, the flavin-containing reductase domains of the constitutive NOS isoforms appear to play major role in the regulation of flavoprotein-to-heme electron transfer.

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