Interactions between the Isolated Oxygenase and Reductase Domains of Neuronal Nitric-oxide Synthase

ASSESSING THE ROLE OF CALMODULIN*

Elena A. Rozhkova‡, Norikazu Fujimoto‡, Ikuko Sagami‡, Simon N. Daff¶, and Toru Shimizu‡

From the ‡Institute of Multidisciplinary Research for Advanced Materials, Tohoku University, Sendai 980-8577, Japan and the ¶Department of Chemistry, University of Edinburgh, Edinburgh EH9 3JJ, United Kingdom

Nitric-oxide synthase (NOS) is a fusion protein composed of an oxygenase domain with a heme-active site and a reductase domain with an NADPH binding site and requires Ca²⁺/calmodulin (CaM) for NO formation activity. We studied NO formation activity in reconstituted systems consisting of the isolated oxygenase and reductase domains of neuronal NOS with and without the CaM binding site. Reductase domains with 33-amino acid C-terminal truncations were also examined. These were shown to have faster cytochrome c reduction rates in the absence of CaM. N⁶-hydroxy-l-Arg, an intermediate in the physiological NO synthesis reaction, was found to be a viable substrate. Turnover rates for N⁶-hydroxy-l-Arg in the absence of Ca²⁺/CaM in most of the reconstituted systems were 2.3–3.1 min⁻¹. Surprisingly, the NO formation activities with CaM binding sites on either reductase or oxygenase domains were decreased dramatically on addition of Ca²⁺/CaM. However, NADPH oxidation and cytochrome c reduction rates were increased by the same procedure. Activation of the reductase domains by CaM addition or by C-terminal deletion failed to increase the rate of NO synthesis. Therefore, both mechanisms appear to be less important than the domain-domain interaction, which is controlled by CaM binding in wild-type neuronal NOS, but not in the reconstituted systems.

Nitric oxide (NO) is an important molecule for various biological functions in the cardiovascular, nervous, and immune systems (Refs. 1–8 and references therein). NO is synthesized from l-Arg via formation of N⁶-hydroxy-l-Arg (NHA)¹ as an intermediate by a family of enzymes termed nitric-oxide synthases (NOSs). NOSs are fused proteins composed of an oxygenase domain with a cytochrome P450 (P450)-like heme active site and a reductase domain with FAD, FMN, and NADPH binding sites similar to NADPH-cytochrome P450 reductase (CPR) (Refs. 1–7 and references therein). Electron transfer from NADPH to the heme oxygenase domain is prerequisite for activation of molecular oxygen during the monooxygenation of l-Arg and NHA. The intradomain electron transfer in the reductase domain and the interdomain electron transfer from the reductase domain to the oxygenase domain are facilitated by Ca²⁺/calmodulin (CaM) (1–8). The CaM binding site is located between the two domains (Refs. 1–8 and references therein). The well known fusion protein, cytochrome P450BM3, is composed of a P450 oxygenase domain and a CPR domain and is also driven via intramolecular electron transfer. However, NOS is reported to have a unique intermolecular/intersubunit electron transfer system in which electrons from the reductase domain of one subunit transfer crosswise to the oxygenase domain of the other subunit in the homodimeric enzyme (9–12). The crucial role of (6R)-5,6,7,8-tetrahydro-L-biopterin (H4B) in catalysis with NOS, probably associated with redox function and/or electron transfer, is also noted (13–15).

The oxygenase domain of NOS can be efficiently expressed heterogeneously in Escherichia coli (Refs. 1–15 and references therein). It is stable, easily handled, and purified as a homodimer in the presence of H4B. However, for simplicity, this has not usually included the CaM binding site. Therefore, to further examine the role of CaM binding in catalysis, particularly its effect on the oxygenase domain, we thought necessary to examine the catalytic properties of an oxygenase domain mutant including the CaM binding site. Conversely, most of the isolated reductase domains so far studied include the CaM-binding site to study the effect of CaM on the intramolecular electron transfer from FAD to FMN (16–18). Intriguingly, the C termini of the reductase domains of inducible and constitutive full-length NOSs to attenuate electron flow through the flavine and heme domains (19, 20). The isolated reductase domain without the CaM-binding site and isolated C-terminal truncated reductase domains are also examined in this paper.

* This work was supported in part by Japan Society for the Promotion of Science Fellowship 99324 (to E. A. R.), General Grant 12680624 (to I. S.), and Priority Area Grant 11116201 (to T. S.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; by a Grant-in-Aid for Scientific Research for Advanced Materials, Tohoku University, 2-1-1 Katahira, Aoba-ku, Sendai 980-8577, Japan. Tel.: 81-22-217-5604; Fax: 81-22-217-5604; E-mail: shimizu@tagen.tohoku.ac.jp.

† To whom correspondence should be addressed: Inst. of Multidisciplinary Research for Advanced Materials, Tohoku University, 2-1-1 Katahira, Aoba-ku, Sendai 980-8577, Japan. Tel.: 81-22-217-5604; Fax: 81-22-217-5604; 5664; E-mail: shimizu@tagen.tohoku.ac.jp.

‡ Present address: Dept. of Chemistry, Princeton University, Princeton, NJ 08544.

¶ To whom correspondence should be addressed: Inst. of Multidisciplinary Research for Advanced Materials, Tohoku University, 2-1-1 Katahira, Aoba-ku, Sendai 980-8577, Japan. Tel.: 81-22-217-5604; Fax: 81-22-217-5604; 5664; E-mail: shimizu@tagen.tohoku.ac.jp.

¹ The abbreviations used are: NHA, N⁶-hydroxy-l-Arg; NOS, nitric-oxide synthase; P450, cytochrome P450; CPR, NADPH-cytochrome P450 reductase; CaM, calmodulin; nNOS, neuronal nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; eNOS, endothelial nitric-oxide synthase; H4B, (6R)-5,6,7,8-tetrahydro-L-biopterin; DTT, dithiothreitol; SOD, superoxide dismutase; NAME, N-nitro-l-Arg methyl ester; Ox, neuronal nitric-oxide synthase oxygenase domain not containing the CaM-binding site (residues 1–720); OxCaM, neuronal nitric-oxide synthase oxygenase domain containing the CaM-binding site (residues 1756); Red, neuronal nitric-oxide synthase reductase domain; RedCaM, neuronal nitric-oxide synthase reductase domain containing the CaM-binding site (residues 721–1395); RedD333, neuronal nitric-oxide synthase reductase domain, Red, truncated of 33 amino acids (residues 721–1395); RedCaM, truncated of 33 amino acids (residues 721–1395) at C terminus; full-length nNOS333, full-length neuronal nitric-oxide synthase truncated with 33 amino acids at C terminus.

This paper is available on line at http://www.jbc.org
The interaction between the oxygenase domain and the reductase domain is not clearly understood, despite its importance in controlling intermolecular/intersubunit electron transfer in the NOS enzyme system. The catalytic properties of the oxygenase domains (not including the CaM binding sites) of inducible NOS (iNOS) and endothelial NOS (eNOS) in reconstituted systems with the isolated recombiant reductase domain (including the CaM-binding site) have been reported (21, 22). Hitherto, no study of the neuronal NOS (nNOS) oxygenase domain in a similar reconstituted system has been reported. In addition, it is worthwhile examining how the CaM-binding site, CaM binding itself, and the C-terminal truncation of the isolated reductase domain affect the reconstituted system. Another interesting issue is whether or not native CPR purified from rat liver microsomes is able to support NO synthesis in the reconstituted system containing oxygenase domain of NOS.

In the present study, we examined the interaction between the oxygenase domain of rat nNOS with and without the CaM binding site and the reductase domain with and without the CaM binding site. Reductase domains truncated by 33 amino acids at the C terminus (18, 19) and native CPR purified from rat liver microsomes were also examined in conjunction with the nNOS oxygenase domains. NO formation activity was observed with the substrate NHA in a reconstituted system composed of the oxygenase and reductase domains with and without the CaM binding sites. Surprisingly, addition of Ca2+/CaM to the system markedly inhibited the NO formation activity of the reconstituted systems in direct contrast to its effect on the wild-type enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**

The complete cDNA for rat nNOS (GenBank accession no. X59949) in pBluescript SK+ was kindly provided by Dr. S. Snyder (The Johns Hopkins University School of Medicine, Baltimore, MD). The complete cDNA of GroESL was kindly provided by Dr. A. A. Gatenby (DuPont Central Research and Development, Wilmington, DE). An E. coli expression vector, pCWoRI, was provided by Dr. M. R. Waterman (Vanderbilt University School of Medicine, Nashville). E. coli strains JM109 and BL21 were used in this study were purchased from Takara Shuzo Co. (Kyoto, Japan). Restriction enzymes and other DNA modifying enzymes were purchased from Takara Shuzo Co., Roche Molecular Biochemicals (Mannheim, Germany), and New England Biolabs (Beverly, MA), Toyobo Co. (Osaka, Japan), and Invitrogen Oriental (Tokyo, Japan). H4B was purchased from Schircks Laboratories (Jona, Switzerland). Oligonucleotides for mutagenesis were purchased from Sawady Technology (Tokyo, Japan). PCR kits were obtained from Takara Shuzo, 2',5'-ADP-Sepharose and CaM-Sepharose were products of Amersham Biosciences AB (Uppsala, Sweden); Nickel-nitrioltriacetic acid-agarose was a product of Qiagen Inc. (Valencia, CA). Bio-Spin 30 Tris column was from Bio-Rad. Other reagents of the highest grade available were obtained from Sigma or Wako Pure Chemicals (Osaka, Japan).

**Construction of the nNOS Oxygenase Domain Plasmids and the nNOS Reductase Domain Plasmids**

Expression plasmids for wild-type nNOS, the nNOS oxygenase domain (including the CaM-binding site) have been reported (21, 22). Hitherto, no study of the neuronal NOS (nNOS) oxygenase domain with wild-type nNOS cDNA as a template and 5'-GCCGTCCTCGCTCCTTT-3' as a backward primer were used. The products were isolated in an agarose gel electrophoresis and recovered by band excision after digestion, with SphI and EcoRI enzymes, and ligated to pUC19 vector. The desired products were confirmed by sequencing using an internal primer (DSQ-2000L, Shimadzu Co., Kyoto, Japan). The NdeI/SphI fragment from the wild-type nNOS cDNA were ligated into NdeI/SphI sites of pCWoRI'.

Expression plasmids for the C-terminal truncated reductase domain with (designated RedCaM33) and without (designated Red33) CaM-binding site were also constructed in the present study. The synthetic expression plasmid 5'-GGGATCCATATGGGGACCCCCACGAAG-3', was used as a forward primer. nNOS cDNA and 5'-GGTCTAGATAAAAA-GAGTGCCTCAG-3' were used as a template and backward primer for PCR. Amplified product was isolated in an agarose gel electrophoresis, recovered by band excision after digestion with XbaI and EcoRI enzymes, and ligated to pBSK II vector. The product was confirmed by sequencing. The XbaI/Xbal fragment of the plasmid was ligated to SphI/HindIII fragments of plasmids of RedCaM and Red to construct the expression plasmids for RedCaM33 and Red33.

**Expression of nNOS Proteins**

Full-length nNOS of wild type and the nNOS oxygenase domains, OxCaM and Ox, were expressed in E. coli cell line, BL21, which contains another plasmid, pGroESL, for expression of chaperone proteins as described previously (12, 23, 24, 26).

For the reductase domains, the BL21 E. coli cells double-transformed with two kinds of plasmids (RedCaM, Red, RedCaM, and pGroESL) were cultured in a Terrific Broth medium containing 50 μg/ml ampicillin, 35 μg/ml chloramphenicol, 3 μg riboflavin, and 1 mM ATP at 25 °C. The protein expression was induced at A600nm = 0.7 with 0.5 mM isopropyl-β-D-thiogalactopyranoside. Cells were further incubated for 36–40 h after isopropyl-β-D-thiogalactopyranoside addition.

**Purification of nNOS Oxygenase Domains and nNOS Reductase Domains**

OxCaM and Ox protein were purified as described previously (12). RedCaM, RedCaM33, and Red33—The E. coli cells expressing RedCaM, RedCaM33, or Red33 were suspended in buffer A (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 10% glycerol, 10 μM H4B, 0.1 mM DTT, 1 mM phenylmethylsulfonfluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μg/ml pepstatin). The cells were crushed by pulsed sonication for 2 min (three times with 2-min interval) in ice using the Ultrasonic Disruptor UD-201 (Toho Seiko, Tokyo, Japan) and centrifuged at 35,000 rpm for 35 min at 4 °C. The supernatant was applied to a DEAE-Toyopearl 650M (Toho Co., Tokyo, Japan) column (3.5 × 20 cm) pre-equilibrated with 0.1 mM NaCl in buffer A. The flow-through fractions were pooled and applied to a 2',5'-ADP-Sepharose 4B (2 × 5 cm) pre-equilibrated with buffer A containing 0.1 mM NaCl. After loading the column was washed sequentially with 100 mM of buffer A containing 0.1 mM NaCl, 50 mM of buffer A containing 0.4 mM NaCl, and 50 mM of buffer B (50 mM Tris (pH 7.5), 0.1 mM EDTA, 0.1 mM EGTA, 10% glycerol, 10 μM H4B, and 1 mM DTT) containing 0.1 mM KCl. The desired protein was eluted with buffer B containing 0.1 mM KCl and 10 mM NADPH. Fractions containing the RedCaM protein were pooled and concentrated with an Ultrafree-15 centrifugal filter device (Millipore Japan, Tokyo, Japan).

Red—The E. coli cells were resuspended in buffer A. The cells were crushed on ice with sonication. After centrifugation at 35,000 rpm for 35 min at 4 °C, ammonium sulfate was added to the resulting supernatant up to 40% saturation. The precipitate was collected and then dissolved in buffer C (50 mM sodium phosphate buffer (pH 7.8), 5 μM H4B, 1 mM β-mercaptoethanol, 0.1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonfluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin) containing 10 mM imidazole. The solution was passed through a Sephadex G-25 column (4 × 20 cm) pre-equilibrated with the same buffer. The eluted solution was applied to a nickel-nitrioltriacetic acid-agarose column pre-equilibrated with buffer C containing 10 mM imidazole. The column was washed sequentially with buffer C containing 10 mM imidazole. The protein was eluted with buffer C containing 10 mM imidazole. The protein fractions were pooled and concentrated. After concentration the reductase domains were quickly frozen in liquid nitrogen and stored at −80 °C.

Before analysis, the buffer of protein was changed to a Tris buffer (50 mM Tris-HCl (pH 7.5), 0.1 mM KCl, 0.1 mM EDTA, 10% glycerol, 5 μM H4B, and 20 μM DTT) using a Sephadex G-25 column. All purified
nNOS enzymes were more than 95% pure, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis stained with Coomassie Blue R-250. The concentrations of the nNOS oxygenase domains were determined optically from the [CO-Fe(II) – [Fe(II)] difference spectrum using $\Delta A_{570-523} = 55 \text{ mm}^{-1} \cdot \text{cm}^{-1}$. The $\Delta A$ value was estimated by the tyrosine hemochromogen method (25), assuming that one heme is bound to one subunit of the protein. Concentrations of the reductase domains were determined from the absorption spectrum, $\Delta A_{457} = 22.9 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ for both domains with or without CaM-binding site.

**NADPH-Cytochrome P450 Reductase**

The NADPH-cytochrome P450 reductase was purified from phenobarbital-induced rat liver microsomes with DEAR-Toyopearl and 2', 5'-ADP-Sepharose 4B column chromatographies as previously described (29, 30). Concentration of the reductase was determined from the absorption spectrum of the oxidized form using $\Delta A_{457} = 10.7 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ (29, 30).

**Catalytic Activities**

NO concentrations generated in the reconstituted system were fluorometrically determined as NO by using the NO/NO$_3$ Assay Kit (Griess method) of Cayman Chemical Co. (Cayman, MI). Unless otherwise indicated, catalytic assays were carried out at 25°C in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 0.1 mM heme domain, 0.5 mM reductase domain or CPR, 0.1 mM NADPH, 0.5 mM NHA, 5 mM H4B, 20 mM DTT, 10 units/ml SOD, and 100 units/ml catalase in the presence or absence of 1 mM CaCl$_2$ and 10 $\mu$g/ml CaM. The reaction mixture without the heme domain was preincubated at 25°C for 5 min, after which 0.1 mM heme domain was added to start the reaction. The reaction mixture was incubated at 25°C for 30 min. The reaction was terminated by removing the enzyme with a membrane filter, Ultrafase-MC UFC35LCG (Millipore Japan Ltd., Tokyo, Japan), with centrifugation at 7,000 rpm for 10 min.

NO concentrations generated in the H$_2$O$_2$-dependent system were spectrophotometrically determined as NO$_2$ by using the NO/NO$_3$ Assay Kit (Griess method) of Cayman Chemical Co. (Cayman, MI). Unless otherwise indicated, catalytic assays were carried out at 25°C in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 0.1 mM NOS, 1 mM NHA, 5 mM H4B, 20 mM DTT, and 10 units/ml SOD in the presence or absence of 1 mM CaCl$_2$ and 10 $\mu$g/ml CaM. The reaction mixture without H$_2$O$_2$ was preincubated at 25°C for 5 min, and then 30 mM H$_2$O$_2$ was added to start the reaction. The reaction mixture was incubated at 25°C for 10 min. The reaction was terminated by adding 13,000 units/ml catalase.

The NADPH oxidation rate was determined spectrophotometrically as an absorbance decrease at 340 nm, using the extinction coefficient of 6.22 mm$^{-1} \cdot$ cm$^{-1}$ (25–28). Cytochrome c reductase activity was determined by monitoring the absorbance at 550 nm using a $\Delta A_{457} = 21$ mm$^{-1} \cdot$ cm$^{-1}$.

**Optical Absorption and Fluorescence Spectra**

Spectral experiments under aerobic conditions were carried out on a Shimadzu UV-2500 spectrophotometer maintained at 25°C by a temperature controller (25–28). Anaerobic spectral experiments were conducted on a Shimadzu UV-160A spectrophotometer maintained at 15°C in a glove box under a nitrogen atmosphere with an O$_2$ concentration of less than 50 ppm (25, 28). Fluorescence spectra were obtained with Shimadzu RF-500 and RF-5300PC spectrofluorophotometers. To ensure that the temperature of the solution was appropriate, the cell was incubated prior to spectroscopic measurements. Titrations were repeated at least three times for each complex. Regression analyses were performed, and lines giving an optimum correlation coefficient were selected (31). Experimental errors were less than 20%.

**RESULTS**

The OxCaM-Fe(III) complex had a Soret peak around 405 nm and the addition of L-Arg moved the peak to 395 nm, indicating that the substrate binding site is not altered in this domain on elimination of the reductase domain. The Ox-Fe(III) complex had a peak at around 415 nm ascribed to a low spin complex, suggesting that in the absence of the CaM binding site, the properties of the heme are altered. However, the Soret absorption band was moved to 400 nm on addition of L-Arg, confirming that the substrate binding site was preserved in this mutant. To confirm that the substrate and H4B binding sites were preserved, we observed spectral changes with the reconstituted system composed of Ox and RedCaM (Fig. 1). On addition of the substrate, NHA, to the solution, the Soret spectrum altered so as to indicate an increase in the high spin content, but the spectra were still a mixture of the low and high spin complexes. Further addition of H4B resulted in the formation of the high spin complex. Therefore, it is suggested that the NHA and H4B binding sites of the oxygenase domain in the reconstituted system were not altered by isolating the domain from the full-length wild-type protein. The Fe(II)-CO complex did not show any absorption at ~420 nm (shown later), suggesting that the thiol coordination to the heme was preserved and no denatured form was detected in the reconstituted systems. These spectral findings are very similar to those observed for the reconstituted system composed of OxCaM and Red, and also to the isolated oxygenase domains per se (1–10).

Red and RedCaM had absorption spectral peaks at ~387 and 457 nm with a shoulder at ~479 nm and a broad absorption centered at ~590 nm (data not shown). These spectral features are very similar to those reported previously (16–18). As summarized in Table I, the cytochrome c reduction rate with Red was 480 min$^{-1}$, which is similar to that (380 min$^{-1}$) reported previously (16–18). The rate with RedCaM in the absence of Ca$^{2+}$/CaM was 510 min$^{-1}$, and addition of Ca$^{2+}$/CaM increased the rate up to 2,550 min$^{-1}$, as reported previously (16–18). The rate of cytochrome c reduction with RedCaM was higher than that in its absence, unlike full-length nNOSA33, which has been shown to be decreased slightly in the presence of Ca$^{2+}$/CaM. However, overall, the reductase domains behave as expected.

We detected a small amount of NO formation activity with L-Arg (less than 0.22 min$^{-1}$) in the reconstituted systems composed of nNOS oxygenase and reductase domains. Ca$^{2+}$/CaM addition had little effect on the activity. With CPR, on the other hand, we did not detect any NO formation activity using either the fluorometric method or the Griess method. The H$_2$O$_2$-supported system was unable to support NO formation activity from L-Arg.

NO formation from NHA was observed for all reconstituted systems composed of the oxygenase domains and the reductase domains or CPR (Table II). By increasing the RedCaM concentration, the activity of OxCaM increased linearly with up to a ratio of 1:5 heme-reductase (Fig. 2). This optimal ratio of the oxygenase to the reductase domain was similar to that ob-
Domain-Domain Interactions of Nitric-oxide Synthase

Table I

| Ca\(^{2+}\)/CaM | Red | RedCaM | RedΔ33 | RedCaMΔ33 | Full-length nNOSΔ33 | CPR\(^a\) | nNOS wild type |
|-----------------|-----|--------|--------|------------|---------------------|---------|---------------|
|                 | 480 ± 40 | 450 ± 40 | 510 ± 10 | 2,550 ± 20 | 5,140 ± 130 | 3,240 ± 570 | 1,400 ± 70 | 2,180 ± 300 |
|                 | 1,000 ± 77 | 6,440 ± 140 |        |            | 3,550 ± 70 | 2,280 ± 110 | 2,440 ± 70 | 1,130 ± 300 |

\(^a\) Data from Ref. 20.

Table II

| Ca\(^{2+}\)/CaM | OxRed | OxRedCaM | OxCaM/Red | OxCaM/RedCaM | OxRedΔ33 | OxRedCaMΔ33 | OxCaM/RedΔ33 | OxCaM/RedCaMΔ33 | OxCaM/CPR\(^b\) | Ox/CPR\(^b\) | nNOS wild type |
|-----------------|-------|----------|------------|--------------|----------|--------------|---------------|-----------------|-----------------|--------------|---------------|
|                 | 2,430 ± 200 | 1,400 ± 270 | <10 | <10 | 640 ± 40 | 3,100 ± 800 | 1,840 ± 340 | 2,890 ± 220 | 720 ± 50 | 90 ± 10 | <10 | 12,000 ± 330 |
| Ratio (--) | 1.7 | >200 | >200 | >3 | 2.1 | 3.9 | 1.6 | 5.8 | 3.8 | 1.3 | 1.3 | 1.3 |

\(^a\) The solution contained 0.1 \(\mu\)M nNOS heme, 0.5 \(\mu\)M NHA, 0.5 \(\mu\)M nNOS reductase or CPR, 100 \(\mu\)M NADPH, 50 \(\mu\)M Tris-HCl (pH 7.5), 5 \(\mu\)M H4B, 20 \(\mu\)M DTT, 10 units/ml SOD, and 100 units/ml catalase with and without 1 mM CaCl\(_2\), 10 \(\mu\)M CaM.

Figure 2. Relation of the ratio of purified reductase to oxygenase domains to NO formation. The formation of NO was monitored with a fixed concentration of 0.1 \(\mu\)M OxCaM and various concentrations of RedCaM (0–1 \(\mu\)M).

The formation of NO was monitored with a fixed concentration of 0.1 \(\mu\)M OxCaM and various concentrations of RedCaM (0–1 \(\mu\)M).
Red

6.22 m M

or RedCaM, indicating severe uncoupling in the reconstituted system (21). If this is the root cause, the interaction must be facilitating electron transfer even in the absence of calcium. Very similar spectral changes were observed for OxRedCaM, Ox/Red, and OxCaM/Red as well.

Addition of sodium dithio-

 effect on cytochrome 

c. It has been reported that dimer formation is required for NO formation in full-length nNOS (1–7). Both oxygenase domains, OxCaM and Ox, studied here were mainly dimeric both in the presence and absence of H4B in terms of gel-filtration column chromatography (data not shown).

TABLE III

NADPH oxidation rates (min⁻¹) in the reconstituted systems in the absence and presence of Ca²⁺/CaM

| System                  | -       | +       |
|-------------------------|---------|---------|
| OxCaM/Red               | 104 ± 4 | 82 ± 2  |
| OxRedCaM                | 40 ± 5  | 107 ± 10|
| OxCaM/Red               | 113 ± 2 | 137 ± 4 |
| OxCaM/RedCaM            | 56 ± 2  | 101 ± 1 |
| OxRedΔ33                | 19 ± 2  | 26 ± 2  |
| OxRedCaMΔ33             | 24 ± 2  | 140 ± 8 |
| OxCaM/RedΔ33            | 28 ± 1  | 26 ± 1  |
| OxCaM/RedCaMΔ33         | 20 ± 1  | 86 ± 9  |
| OxCPR                   | <10     | <10     |
| OxCaM/CPR               | 24 ± 0.4| 14 ± 0.3|
| nNOS wild type          | 35 ± 1  | 47 ± 1  |

The NADPH oxidation rate was determined spectrophotometrically as an absorbance decrease at 340 nm, using an extinction coefficient of 6.22 mM⁻¹ cm⁻¹ (25–28). Experimental conditions were the same as those in Table II.

RedΔ33, and OxCaM/CPR systems. There is no correlation between NO formation and NADPH consumption. NADPH consumption is much faster than NO formation in all cases (>10-fold), indicating severe uncoupling in the reconstituted systems. This may originate primarily from the reaction of O₂ with the reductase domains alone (which are present in excess).

The reaction of NHA with H₂O₂ or superoxide anion to form NO and L-citrulline is catalyzed by the NOS oxygenase domain (Ref. 4 and references therein). We examined the H₂O₂-dependent (shunt reaction) NO formation activity using NHA as the substrate. Both OxCaM and Ox provided high NO formation activities (4.0–6.0 min⁻¹) similar to those observed for the full-length nNOS. The NO formation activities observed for OxCaM and full-length nNOS were only slightly (10%) enhanced by Ca²⁺/CaM. It appears, therefore, that the mechanism occurring in the shunt reaction is fairly different from that observed in the reconstituted system.

It has been reported that dimer formation is required for NO formation in full-length nNOS (1–7). Both oxygenase domains, OxCaM and Ox, studied here were mainly dimeric both in the presence and absence of H4B in terms of gel-filtration column chromatography (data not shown).

DISCUSSION

Previous studies on the constitutive NOS enzymes have yielded a number of interesting facts. 1) The intramolecular electron transfer from FAD to FMN in the reductase domain is facilitated by CaM binding (1–8, 32, 33). 2) The interdomain electron transfer from the reductase domain to the heme domain is facilitated by CaM binding (1–8, 32, 33). 3) Interdomain electron transfer is conducted in a cross-wise manner from the reductase domain of a subunit to the oxygenase domain of the other subunit (5, 9, 10). 4) There is an autoinhibitory loop in the FMN-binding subdomain of the reductase domain in nNOS and eNOS not present in iNOS (18, 27, 34–36). 5) The C terminus of the reductase domain inhibits reduction of FAD (19, 20). The present paper explores additional interesting characteristics of CaM-binding and the CaM-binding site.

Substrate Specificities—Optical absorption spectral changes caused by adding l-Arg suggest that the substrate binding site is well conserved in the both oxygenase domains, Ox and OxCaM. It is interesting to note, however, that L-Arg is not a viable substrate for the reconstituted system, despite its being the physiological substrate for the holoenzyme. A certain important conformation/configuration appears to be required for the monooxygenation of L-Arg. This important structural feature is a characteristic found only in the dimeric full-length nNOS protein, whereas the oxygenase domains, both OxCaM and Ox, must lack this feature. A previous report has suggested that a specific interaction between the oxygenase domain and the reductase domain may be lacking in the iNOS reconstituted system (21). If this is the root cause, the interaction must be influencing the conformation/configuration of the heme active site, which is responsible for catalytic monooxygenation of L-Arg.

The Isolated Reductase Domains—The cytochrome c reduction rates of RedCaM and RedCaMΔ33 were markedly increased by addition of Ca²⁺/CaM (Table I), suggesting that the function of the CaM-binding site in the reductase domain is well preserved in these domains. The cytochrome c reduction rates of full-length nNOSΔ33 in the absence of Ca²⁺/CaM are much higher than the wild-type nNOS under the same conditions (Table I) (20). Both RedΔ33 and RedCaMΔ33 in the absence of Ca²⁺/CaM also have higher rates than those of the corresponding domains, Red and RedCaM, in the absence of Ca²⁺/CaM, confirming that the effect of the Δ33 mutation is preserved in the isolated domain.

The NADPH oxidation rates for systems containing RedCaM or RedCaMΔ33 in the presence of Ca²⁺/CaM were always more than 2-fold higher than in its absence (Table III). Therefore, it appears that the CaM-binding site of the isolated reductase domain binds CaM and facilitates electron transfer even in the reconstituted system. This behavior is also consistent with the effect on cytochrome c reduction, which is similar in the reductase domain of full-length nNOS (25–28).
In Either Domain, the Presence of the CaM-binding Site Appears to Assist NO Formation—The OxCaM systems were found to have slightly higher NO formation activity than the Ox systems in the absence of Ca$^{2+}$/CaM (Table II). In particular, those of OxCaM/RedΔ33 and OxCaM/RedCaMΔ33 were 3 times higher than those of Ox/RedΔ33 and Ox/RedCaMΔ33, respectively. The CaM-binding site may itself be an important component of the heme domain, required for optimal NO formation catalysis. The position of the Soret peak of the Ox protein was at a higher wavelength than for the OxCaM protein after purification, suggesting that the heme is in some way different, even though the substrate binding sites are retained. The CaM binding site may therefore have a subtle effect on the enzyme’s active site.

The highest NO formation activity was observed for the reconstituted system in which both domains contained the CaM-binding site in the absence of Ca$^{2+}$/CaM (Table II). The CaM-binding site of RedCaM or RedCaMΔ33 may be important for the domain-domain interaction and must lie in the proximity of the domain-domain interface.

Suppression of Activities by Ca$^{2+}$/CaM Binding—The NO formation rate in the reconstituted system was substantially suppressed on addition of Ca$^{2+}$/CaM. This is the opposite effect of CaM binding on the full-length wild-type nNOS, with which CaM binding facilitates electron transfer and activates NO formation activity (1–8, 11). The bound CaM could easily impede the catalytically useful collisions between the domains in the reconstituted system. It is suggested that CaM binding activates full-length wild-type nNOS by causing a reorientation between the oxygenase and reductase domains, such that electron transfer becomes viable. When the domains are separate, this effect would be expected to have disappeared. CaM binding does not appear to active nNOS directly at the mechanistic active site.

Previous studies of reconstituted systems consisting of the isolated domains of iNOS (21) and of eNOS (22) did not report that CaM suppresses catalysis. Why are there differences between the present study and previous studies? In iNOS, CaM binds very tightly even in the absence of Ca$^{2+}$ (1–10). It would be difficult, therefore, to observe CaM dependence. It is unclear why the study on eNOS indicated that the reconstituted system was deactivated in the absence of CaM, but this may be because of the lower activity of the eNOS reductase domain, which may limit electron transfer more severely in the absence of CaM. This effect also appears to limit overall turnover in full-length eNOS.

Heme Reduction Rates, NADPH Oxidation Rates, and Catalytic Activities—Differences in heme reduction rates between the systems were marginal in the presence and absence of Ca$^{2+}$/CaM (Fig. 4) in comparison with the marked difference observed for the NO formation activities (Table I). Note that many of the NADPH-derived electrons are not well coupled with catalysis in the reconstituted system and serve to reduce molecular oxygen and produce superoxide anion and/or H$_2$O$_2$. Nevertheless, NADPH oxidation rates in the reconstituted systems containing RedCaM or RedCaMΔ33 were markedly increased by adding Ca$^{2+}$/CaM (Table III). These are not in accordance with the catalytic findings in that addition of Ca$^{2+}$/CaM suppressed the NO formation activities (Table I) in our reconstituted system. The fact that NADPH consumption is not coupled to NO synthesis is unsurprising, given the stoichiometry of 5 reductase domains to 1 oxygenase domain. Electron transfer to the oxygenase domain is clearly hampered on domain separation, but NADPH/oxygen consumption at the flavin sites of the reductase domain will be unaffected. This portion of the NADPH consumption will remain Ca$^{2+}$/CaM-dependent and unaffected by the presence of the oxygenase domain.

The dependence of NO synthesis on electron transfer is complicated in nNOS; as explained by recent catalytic models, faster heme reduction does not necessarily lead to faster NO synthesis. The rate of turnover in these reconstituted systems may be affected by changes at any point in such catalytic models. The rate of NO synthesis must be balanced against the rate of superoxide/peroxide formation, the rate of formation/decomposition of dead-end complexes such as the ferrous heme-NO complex, etc. However, several firm conclusions can be made. 1) The presence of the CaM binding site improves the rate of catalytic turnover and is therefore likely to be an important structural feature of both reductase and oxygenase domains. 2) The effect of CaM-binding on nNOS depends crucially on whether the two domains are linked. CaM binding to both the oxygenase and reductase fragments is detrimental to overall turnover. This reinforces the view that the primary role of CaM binding is to rearrange the two domains with respect to each other. This factor overrides any improvements in reduction activity observed on CaM binding. 3) The Δ33 mutation, although activating the reductase domain, had little effect on NO synthesis. This correlates with the lack of CaM dependence observed, suggesting that NO synthesis is not controlled by factors within the reductase domain, but by interactions between the reductase and oxygenase domain.

Heme reduction in the reconstituted system was not affected by CaM, unlike the NO formation rate. For one cycle of NO formation, three electrons are required to be transferred from the reductase domain to the heme domain. Recent studies suggested that H4B might provide the second electron required for activation of the ferrous-dioxy intermediate during catalysis (13–15). In the absence of H4B, NO formation activity was observed in the reconstituted system (data not shown), suggesting that it plays a critical catalytic role in these systems as well as in native NOS. It is possible that there is a successive or alternative electron transfer pathway via H4B, as has been suggested for the full-length NOS system (13–15). This electron transfer may be hampered by adding Ca$^{2+}$/CaM, rather than electron transfer to the heme, explaining why NO synthesis, but not heme reduction, was impeded by Ca$^{2+}$/CaM binding. Nevertheless, the heme remains the center of catalysis, because heme active site inhibitors, NAME and KCN, markedly inhibited catalysis.

A recently isolated bacterial NOS consisting only of the oxygenase domain appears to function similarly with only NHA acting as a viable substrate (37). A comparative study, therefore, may serve as a useful working hypothesis for the further investigation of NOS function.

REFERENCES

1. Pfeiffer, S., Mayer, B., and Hemmens, B. (1999) Angew. Chem. Int. Ed. 38, 1714–1741
2. Mayer, B. (ed) (2000) Nitric Oxide, Springer-Verlag, Berlin
3. Ignarro, L. J. (ed) (2000) Nitric Oxide: Biology and Pathobiology, Academic Press, San Diego
4. Ludwig, M. L., and Marletta, M. A. (1999) Structure 7, R73–R79
5. Stuehr, D., and Ghosh, S. (2000) in Nitric Oxide: Biology and Pathobiology (Ignarro, L. J., ed) pp. 91–104, Academic Press, San Diego
6. Masters, B. S. (2000) in Nitric Oxide: Biology and Pathobiology (Ignarro, L. J., ed) pp. 33–91, Springer-Verlag, Berlin
7. Raman, C. S., Martásék, P., and Masters, B. S. S. (2000) Porphyrin Handbook, 4, 293–339
8. Matsuoka, A., Stuehr, D. J., Olson, J. S., Clark, P., and Ikeda-Saito, M. (1994) J. Biol. Chem. 269, 20335–20339
9. Siddhanta, U., Presta, A., Fan, B., Wolan, D., Rousseau, D. L., and Stuehr, D. J. (1998) J. Biol. Chem. 273, 18950–18958
10. Stuehr, D. J. (1999) Biochem. Biophys. Acta 1411, 217–230
11. Panda, K., Ghosh, S., and Stuehr, D. J. (2001) J. Biol. Chem. 276, 23545–23556
12. Sagami, I., Daff, S., and Shimiizu, T. (2001) J. Biol. Chem. 276, 30036–30042
13. Bec, N., Gorren, A. C. F., Voelker, C., Mayers, B., and Lange, R. (1998) J. Biol. Chem. 273, 13502–13508
Domain-Domain Interactions of Nitric-oxide Synthase

14. Hurshman, A. R., Krebs, C., Edmondson, D. E., Huynh, B. H., and Marletta, M. A. (1999) Biochemistry 38, 15689–15696
15. Raman, C. S., Li, H., Martásek, P., Krul, V., Masters, B. S. S., and Poulos, T. L. (1998) Cell 95, 927–937
16. Newton, D. C., Montgomery, H. J., and Guillemette, J. G. (1998) Arch. Biochem. Biophys. 359, 249–257
17. Matsuda, H., and Iyanagi, T. (1999) Biochem. Biophys. Acta 1473, 345–355
18. Montgomery, H. J., Romanov, V., and Guillemette, J. G. (2000) J. Biol. Chem. 275, 5652–5658
19. Roman, L. J., Miller, R. T., de la Garza, M. A., Kim, J.-J. P., and Masters, B. S. S. (2000) J. Biol. Chem. 275, 21914–21919
20. Roman, L. J., Martásek, P., Miller, R. T., Harris, D. E., de la Garza, M. A., Shea, T. M., Kim, J.-J. P., and Masters, B. S. S. (2000) J. Biol. Chem. 275, 29225–29232
21. Ghosh, D. K., Abu-Soud, H. M., and Stuehr, D. J. (1995) Biochemistry 34, 11316–11320
22. Chen, P.-F., Tsai, A.-L., Berka, V., and Wu, K. K. (1996) J. Biol. Chem. 271, 14631–14635
23. McMillian, K., and Masters, B. S. S. (1995) Biochemistry 34, 3686–3693
24. Roman, L. J., Sheta, E., Martásek, P., Gross, S. S., Liu, Q., and Masters, B. S. S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8428–8432
25. Sagami, I., and Shimizu, T. (1998) J. Biol. Chem. 273, 2105–2108
26. Shimanuki, T., Sato, H., Daff, S., Sagami, I., and Shimizu, T. (1999) J. Biol. Chem. 274, 26956–26961
27. Daff, S., Sagami, I., and Shimizu, T. (1999) J. Biol. Chem. 274, 30589–30595
28. Sagami, I., Sato, Y., Daff, S., and Shimizu, T. (2000) J. Biol. Chem. 275, 26150–26157
29. Yasukochi, Y., and Masters, B. S. S. (1976) J. Biol. Chem. 251, 5337–5344
30. Iyanagi, T., Makino, R., and Anan, F. K. (1981) Biochemistry 20, 1722–1730
31. Nakan, R., Sato, H., Watanabe, A., Ito, O., and Shimizu, T. (1996) J. Biol. Chem. 271, 8570–8574
32. Chen, P.-F., and Wu, K. K. (2000) J. Biol. Chem. 275, 13155–13168
33. Lee, S.-J., Beckingham, K., and Stull, J. T. (2000) J. Biol. Chem. 275, 36067–36072
34. Salerno, J. C., Harris, D. E., Iriazary, K., Patel, B., Morales, A. J., Smith, S. M., Martásek, P., Roman, L. J., Masters, B. S. S., Jones, C. L., Weissman, B. A., Lane, P., Liu, Q., and Gross, S. S. (1997) J. Biol. Chem. 272, 29769–29777
35. Nishida, C. R., and Ortiz de Montellano, P. R. (1999) J. Biol. Chem. 274, 14692–14698
36. Nishida, C. R., and Ortiz de Montellano, P. R. (2001) J. Biol. Chem. 276, 20116–20124
37. Adak, S., Bilwes, A. M., Panda, K., Hosfield, D., Aulak, K. S., McDonald, J. F., Tainer, J. A., Getzoff, E. D., Crane, B. R., and Stuehr, D. J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 107–112