In neuronal nitric-oxide synthase (nNOS), calmodulin (CaM) binding is thought to trigger electron transfer from the reductase domain to the heme domain, which is essential for O2 activation and NO formation. To elucidate the electron-transfer mechanism, we characterized a series of heterodimers consisting of one full-length nNOS subunit and one oxygenase-domain subunit. The results support an inter-subunit electron-transfer mechanism for the wild type nNOS, in that electrons for catalysis transfer in a Ca2+/CaM-dependent way from the reductase domain of one subunit to the heme of the other subunit, as proposed for inducible NOS. This suggests that the different isoforms form similar dimeric complexes. In a series of heterodimers containing a Ca2+/CaM-insensitive mutant (delta40), electrons transferred from the reductase domain to both hemes in a Ca2+/CaM-independent way. Thus, in the delta40 mutant electron transfer from the reductase domains to the heme domains can occur via both inter-subunit and intra-subunit mechanisms. However, NO formation activity was exclusively linked to inter-subunit electron transfer and was observed only in the presence of Ca2+/CaM. This suggests that the mechanism of activation of nNOS by CaM is not solely dependent on the activation of electron transfer to the nNOS hemes but may involve additional structural factors linked to the catalytic action of the heme domain.

An important signaling molecule, nitric oxide (NO), is endogenously produced from L-arginine (L-Arg) by a family of enzymes termed nitric-oxide synthases (NOSs). NOS has a bidomain structure consisting of an N-terminal oxygenase domain that has a cytochrome-P450 (P450)-like heme active site and a C-terminal reductase domain that is similar to NADPH-cytochrome P450 reductase (reviewed in Refs. 1–7). A calmodulin (CaM) binding site is located within the linker region between the two domains. The two constitutive isoforms of NOS, neuronal and endothelial (nNOS and eNOS), are regulated by the reversible binding of Ca2+/CaM, whereas inducible NOS (iNOS) contains CaM as an intrinsic factor and is Ca2+-independent (reviewed in Refs. 1–7).

Many different mechanisms have been proposed for the NO synthesis reaction, but there is still no consensus of opinion. In all the mechanisms, the first step is reduction of the heme of the oxygenase domain by an electron generated by NADPH dehydrogenation in the reductase domain, followed by activation of molecular oxygen. It has also been suggested that the essential cofactor tetrahydrobiopterin (H4B) donates an electron to the ferrous-dioxy intermediate (7–10). How are these electron transfers controlled and what are the electron-transfer routes in NOS? CaM binding regulates electron transfer both within the reductase domain and from the reductase domain to the heme domain (11–14). Siddhanta et al. (15, 16) reported that, in the iNOS dimer, which always binds CaM, electron transfer occurs from the flavins of one subunit to the heme domain of the other subunit during catalytic turnover. They therefore proposed a domain swap model for the structure of native iNOS. This also explains why homodimer formation is a prerequisite for NOS activity. However, it was not certain that electron transfer occurs via the same mechanism in the nNOS and eNOS dimers, both of which are controlled by reversible Ca2+/CaM binding.

Sequence alignment between the three NOS isoforms reveals that both nNOS and eNOS contain an extra 40–46 amino acids within their FMN binding subdomains, which are absent in iNOS. Our previous report and others demonstrated that removal of these amino acids affects the Ca2+/CaM-dependent activation of nNOS and eNOS, suggesting that they are auto-inhibitory elements (14, 17–19). The heme of wild type nNOS was reduced with NADPH only in the presence of Ca2+/CaM, whereas the heme of the 40-amino acid deletion mutant (delta40) was reduced spontaneously upon addition of NADPH in the absence of Ca2+/CaM. The delta40 mutant had 10% of the activity of the wild type enzyme without Ca2+/CaM, which was increased 3-fold upon addition of Ca2+/CaM (14). Our goal is to understand how CaM binding regulates inter-subunit/toothanol; PAGE, polyacrylamide gel electrophoresis; Ni-NTA, nickel-nitrilotriacetic acid; PCR, polymerase chain reaction.
intra-subunit electron transfer to mediate heme reduction and NO formation activity in both the wild type enzyme and the delta40 mutant.

In the present study, we have generated the following nNOS mutants: full-length E592A (a point mutation in the heme domain removing substrate binding capability), full-length E592A delta40 mutant (combining the E592A mutation with deletion of 40-amino acids in the FMN binding subdomain), and the oxygenase domains of the wild type enzyme (Wildox) and the E592A mutant (E592Aox). To elucidate the route of electron transfer between the heme domain and the reductase domain in nNOS (i.e. intra- or inter-subunit), we prepared a series of heterodimers, each composed of a full-length subunit and an oxygenase domain subunit. We discuss the electron transfer routes from the reductase domain to the heme active sites in the heterodimers and the regulation of catalytic activities by Ca²⁺/CaM.

**EXPERIMENTAL PROCEDURES**

**Materials**—pGroESL for expression of *Escherichia coli* groEL and groES proteins was kindly provided by Dr. A. A. Gatenby (DuPont Central Research and Development, Wilmington, DE). An *E. coli* expression vector, pCWori, was gifted from Dr. M. R. Waterman (Vanderbilt University). ODA-ALA PCR kit for site-directed mutagenesis was obtained from Takara Shuzo (Tokyo, Japan). H₄B was purchased from Schircks Laboratories (Jona, Switzerland). 2',5'-ADP-Sepharose and CaM-Sepharose were products of Amersham Pharmacia Biotech. Other reagents were obtained from Sigma Chemical Co. or Wako Pure Chemicals (Osaka, Japan).

**Construction of the nNOS Mutants Expressing Plasmids**—The cDNA for rat nNOS was kindly given by Dr. S. H. Snyder (Johns Hopkins University, School of Medicine). The expression plasmids for full-length nNOS of wild type and delta40 mutant were constructed as described previously (14, 20). For E592A, site-directed mutagenesis was performed with the polymerase chain reaction-based strategy using the ODA-ALA PCR kit from Takara Shuzo and a primer, 5'-ATG GGC ACA GGC ATC GGC GTC-3'. To clone Wildox containing the oxygenase domain (residues 1–720), the downstream primer used for PCR was 5'-GGGCTAGATTTAGTGTTGGGCCTTTCCACACG-3', which corresponds to the sequence in the pKF19k plasmid. The desired PCR products were sequenced in the heterodimers and the regulation of catalytic activities by Ca²⁺/CaM.

**Purification of Full-length nNOSs and the Oxygenase Domains—**In the present study, we have generated the following nNOS mutants: full-length E592A (a point mutation in the heme domain removing substrate binding capability), full-length E592A delta40 mutant (combining the E592A mutation with deletion of 40-amino acids in the FMN binding subdomain), and the oxygenase domains of the wild type enzyme (Wildox) and the E592A mutant (E592Aox). To elucidate the route of electron transfer between the heme domain and the reductase domain in nNOS (i.e. intra- or inter-subunit), we prepared a series of heterodimers, each composed of a full-length subunit and an oxygenase domain subunit. We discuss the electron transfer routes from the reductase domain to the heme active sites in the heterodimers and the regulation of catalytic activities by Ca²⁺/CaM.

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**Purification of Full-length nNOSs and the Oxygenase Domains—**Intra-subunit Electron Transfer in Neuronal Nitric-oxide Synthase

**EXPERIMENTAL PROCEDURES**

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change the H₄B binding in terms of the absorption spectral character.

Under anaerobic conditions, the addition of NADPH to E592A caused a broad decrease in absorbance between 400 and 500 nm, indicating the reduction of the bound flavins of the enzyme. In the absence of Ca²⁺/CaM, no further change was observed over a period of 30-min incubation (Fig. 1A). Addition of Ca²⁺/CaM, however, initiated heme reduction as observed for wild type full-length nNOS and resulted in the appearance of a peak at 444 nm in the presence of CO. On the other hand, the E592A/delta40 mutant showed clear heme reduction with NADPH in the absence of Ca²⁺/CaM under anaerobic conditions (Fig. 1B). The Fe(II) heme-CO complexes of both the mutants had a peak at 444 nm as observed for wild type full-length nNOS.

To elucidate how much heme reduction with NADPH occurred in the absence or presence of Ca²⁺/CaM, we measured the absorbance change of the mutants at 444 nm with respect to time in a saturated solution of CO under anaerobic conditions. As summarized in Table I, after 30-min incubation with NADPH, heme reduction was about 30% complete in wild type and the wild type enzyme. These results confirm that the mutants are insensitive to the substrate.

In the presence of l-Arg and Ca²⁺/CaM, NADPH consumption and NO formation are well coupled in the wild type enzyme.

For the delta40 mutant, significant NO formation was observed in the absence of Ca²⁺/CaM but not as much as expected from the heme reduction rate and ratio. Addition of Ca²⁺/CaM increased the NO formation activity and rate of NADPH oxidation in the presence of l-Arg. Coupling between NO formation and NADPH consumption was not as efficient in the mutant even in the presence of l-Arg and Ca²⁺/CaM as described previously for the delta40 mutant expressed in yeast (14). Addition of l-Arg did not lower the NADPH consumption rates for either the wild type enzyme or the delta40 mutant in the absence of Ca²⁺/CaM.

As expected, full-length E592A and E592A/delta40 mutants had no NO formation activities. The NADPH oxidation rates of these mutants were increased on addition of Ca²⁺/CaM, but were not subsequently lowered by addition of l-Arg, unlike the wild type enzyme. These results confirm that the mutants are insensitive to the substrate.

We also analyzed the dimer formation ability of these mutants using fast-protein liquid chromatography gel filtration. More than 75% of each of the delta40, E592A, and E592A/delta40 mutants were eluted as dimers after incubation with 1 mM l-Arg and 10 μM H₂B (data not shown).

Preparation of Heterodimers—To prepare heterodimers consisting of a full-length nNOS subunit and an oxygenase domain subunit with a six-histidine tag at the C terminus, we first treated the full-length dimers and oxygenase domain dimers with 3 M urea. Fig. 2 shows typical gel-filtration profiles for the E592A/delta40 mutant, the Wildox mutant, and E592A/delta40/Wildox heterodimer. After urea treatment, about 30% of the E592A/delta40 was eluted as monomer, whereas only 10% of the Wildox was monomeric. Similarly, the monomeric forms of the other full-length nNOSs (wild type, E592A, and delta40) were obtained more easily than those of the corresponding oxygenase domain proteins. The monomer proportions were not increased even when the dimers were treated with higher concentrations of urea for longer time periods. More severe conditions destroyed the NOS proteins. After renaturation by 10 × dilution and incubation with buffer containing H₂B and l-Arg, each heterodimer was separated and purified from the homodimers and the monomers by sequential chromatographies. Namely, at first, we isolated the enzyme fractions containing histidine tags (i.e. containing oxygenase domain subunits) using Ni-NTA column chromatography. We then isolated the enzymes containing NADPH binding sites (i.e. containing full-length subunits) using a 2′,5′-ADP-Sepharose column chromatography. Consequently, only heterodimers containing a full-length nNOS subunit and an oxygenase domain subunit are retained. The Fe(III), Fe(II), and Fe(II)-CO complexes of the heterodimers had similar spectra to those of the wild type enzyme. In the spectra of the Fe(II)-CO complexes, no absorption band at around 420 nm ascribed to the denatured complex, P420, was observed (as discussed later in Fig. 5), confirming that the heme domains were not altered by these treatments.

To determine the molecular size, we analyzed the isolated heterodimer fraction using gel filtration chromatography (Fig. 2). The E592A/delta40/Wildox heterodimer fraction was eluted as one peak at about 245 kDa, i.e. between the E592A/delta40 homodimer (320 kDa) and the Wildox homodimer (160 kDa). The other heterodimers, E592A/Wildx, Wildx/E592Aox, delta40/Wildx, and delta40/E592Aox had similar gel filtration profiles. We also examined the components of the heterodimer fractions using SDS-PAGE after denaturation by heating with
Wildox, and delta40/E592Aox heterodimers were reduced by NADPH in the absence of Ca\(^{2+}\)/CaM under anaerobic conditions unlike in the presence of Ca\(^{2+}\)/CaM, which were similar to that of the full-length monomers (FL) and oxygenase domain monomers (OX).

We next analyzed a series of heterodimers formed with the delta40 mutant, in which 40 amino acids have been deleted. Interestingly, the heterodimers containing the wild type reductase domain. The heme reduction rates were similar to those of the other heterodimers and slower than those of the wild type and delta40 homodimers (Table I). Two of the three heterodimers, delta40/Wildox and E592Adelta40/Wildox, had NO formation activities in the presence of Ca\(^{2+}\)/CaM, which were similar to that of the Wild/Wildox heterodimer. Like the Wild/E592Aox heterodimer,
erodimer caused by addition of NADPH in the presence of L-Arg and CO under anaerobic conditions. The heme reduction was started by addition of 0.1 mM NADPH to the solution composed of nNOS (20 μM), 50 mM Tris-HCl (pH 7.5), 10 μM HμB, 50 μM DTT, 0.1 mM NADPH, 1 mM L-Arg, and 1.2 mM CO. A, the absorption spectra were monitored at 5, 10, and 20 min after addition of NADPH, 20 min after addition of 10 μg/ml CaμM and 1 mM CaClμ2 (solid line), and after addition of sodium dithionite (dotted line). An arrow indicates the increase of delta absorbance (A, 444-457 nm) with time.

The heme reduction was monitored at 5, 10, and 20 min after addition of NADPH, 20 min after addition of 10 μg/ml CaμM and 1 mM CaClμ2 under anaerobic conditions. Also, the absorption spectra were monitored at 5, 10, and 20 min after addition of CO and NADPH in the presence of Caμ2/CaμM, indicating a critical role for Caμ2/CaμM in NOS activity. Although FMN to heme electron transfer is triggered by Caμ2/CaμM binding, 38% of the heme of the wild type enzyme was reduced by NADPH in the absence of Caμ2/CaμM under anaerobic conditions, albeit at a slow rate, as shown in Table I. Addition of Caμ2/CaμM to the system resulted in the appearance of NO formation activity concomitant with a dramatic increase in the heme reduction rate. The E592A mutant also showed similar results for heme reduction by NADPH, except it could not support NO formation from L-Arg due to its inherent defective substrate binding ability, as mentioned above. It appears, therefore, that Caμ2/CaμM and substrate binding are not absolutely essential for electron transfer from the reductase domain to the heme in nNOS. These results are also consistent with recent reports that CaμM has very little influence on the flavin reduction potentials of nNOS (30) and that the reduction potential of the nNOS heme was not affected by substrate binding and is thermodynamically accessible to reduction by the flavins (31). On the other hand, the wild type enzyme absolutely required Caμ2/CaμM binding for NO formation activity. CaμM binding is likely to induce a conformational change for efficient electron transfer between the domains. It is thus suggested that there might be differences in active site conformation and/or the electron transfer route for the first electron required for initial heme reduction and subsequent electrons required for catalytic turnover.

**Inter-subunit Electron Transfer in Wild type nNOS**—Analysis of a heterodimer consisting of a full-length wild type subunit and a wild type oxygenase domain subunit revealed that only one heme in the dimer was reduced by NADPH in the presence of Caμ2/CaμM (Table II). This is similar to the results obtained by Siddhanta et al. using iNOS heterodimers (15, 16). They demonstrated that a single reductase domain is sufficient to support heme reduction and NO formation activity at one heme site. They also proposed a domain-swapping model for iNOS, in which electron transfer occurs only between the flavins and hemes located on adjacent subunits (16). iNOS irreversibly binds CaμM and its activity is insensitive to Caμ2 concentration. Therefore, it is very difficult to elucidate how CaμM binding regulates NO synthesis and the individual electron transfer steps using iNOS as a model. In the case of nNOS, the heterodimers E592A/Wildox and Wild/E592Aox showed that only one of two hemes was reduced by NADPH in the presence of Caμ2/CaμM as in the corresponding heterodimers of iNOS. Also, NO formation activity was observed only in the presence of Caμ2/CaμM for the E592A/Wildox heterodimer (this has a deactivated full-length subunit and a wild type oxygenase domain subunit), whereas the Wild/E592Aox heterodimer did not show any activity despite containing a full-length wild type subunit (Table II). These results support the model proposed for iNOS, in which electrons for heme reduction flow from the flavins of

**FIG. 4.** Absorption spectral changes at the Soret region (A) and time course of heme reduction (B) of the delta40/Wildox heterodimer caused by addition of NADPH in the presence of L-Arg and CO under anaerobic conditions. The heme reduction was started by addition of 0.1 mM NADPH to the solution composed of nNOS (20 μM), 50 mM Tris-HCl (pH 7.5), 10 μM HμB, 50 μM DTT, 0.1 mM NADPH, 1 mM L-Arg, and 1.2 mM CO. A, the absorption spectra were monitored at 5, 10, and 20 min after addition of NADPH, 20 min after addition of 10 μg/ml CaμM and 1 mM CaClμ2 (solid line), and after addition of sodium dithionite (dotted line). An arrow indicates the increase of delta absorbance (A, 444-457 nm) with time.

**FIG. 5.** A, absorption spectral changes of the delta40/E592Aox heterodimer. Resting form of the delta40/E592Aox heterodimer (solid line), 30 min after addition of CO and NADPH in the absence of Caμ2/CaμM (broken line), and 10 min after addition of 10 μg/ml CaμM and 1 mM CaClμ2 (dotted line). B, the heme reduction of the delta40/E592Aox (triangle) and E592A/delta40/Wildox (circle) caused by addition of NADPH. 10 μg/ml CaμM and 1 mM CaClμ2 were added to the mixture 30 min after addition of NADPH as indicated with an arrow.

**Effect of the E592A Mutation on Electron Transfer and Dimer Formation**—The crystal structures of the eNOS and iNOS heme domain dimers revealed that L-Arg binds to the active site of the heme domain with hydrogen bonding to Glu-371 of mouse iNOS (Glu-361 in human eNOS, corresponding to Glu-592 in rat nNOS) (23–26). The E592A mutation resulted in a loss of sensitivity to L-Arg, characterized by a lack of spectroscopic perturbation on its addition (Fig. 1). This was corroborated by results from competitive imidazole binding experiments. Addition of L-Arg to the mutant in the presence of bound imidazole did not cause ligand displacement, or any spectral perturbation, unlike for the wild type enzyme (data not shown). Addition of HμB to the mutant caused a shift in the Soret peak position from 400 to 396 nm, suggesting that the mutation was not likely to affect HμB binding. As expected, the E592A mutant did not show any NO formation activity like the corresponding mutants of iNOS and eNOS (16, 28). In the wild type enzyme, the NADPH oxidation rate was lowered on addition of L-Arg due to coupling with NO formation. In contrast, the rate of NADPH oxidation by the E592A mutant was not affected by L-Arg, consistent with its binding site being defective. For NO formation activity, NOS dimer formation is prerequisite. Gel filtration analysis indicated that the mutation did not affect either the percentage of homodimer formed by the full-length enzyme, or the percentage of heterodimer formed with the isolated oxygenase domain mutants. These results are consistent with the previous reports, which demonstrated that dimer formation does not require L-Arg in any of the NOS isoforms (27–29).

**CaM Binding Is Essential for NO Formation but Not for Heme Reduction**—It has been demonstrated that the binding of Caμ2/CaμM is a trigger for electron transfer from the reductase domain to the heme in nNOS and eNOS (11, 12). CaμM binding also facilitates flavin reduction by NADPH and electron transfer from the reductase domain to exogenous electron acceptors such as cytochrome c or ferricyanide (13, 14). Like the eNOS isoform, wild type nNOS has NO formation activity only in the presence of Caμ2/CaμM, indicating a critical role for Caμ2/CaμM in NOS activity. Although FMN to heme electron transfer is triggered by Caμ2/CaμM binding, 38% of the heme of the wild type enzyme was reduced by NADPH in the absence of Caμ2/CaμM under anaerobic conditions, albeit at a slow rate, as shown in Table I. Addition of Caμ2/CaμM to the system resulted in the appearance of NO formation activity concomitant with a dramatic increase in the heme reduction rate. The E592A mutant also showed similar results for heme reduction by NADPH, except it could not support NO formation from L-Arg due to its inherent defective substrate binding ability, as mentioned above. It appears, therefore, that Caμ2/CaμM and substrate binding are not absolutely essential for electron transfer from the reductase domain to the heme in nNOS. These results are also consistent with recent reports that CaμM has very little influence on the flavin reduction potentials of nNOS (30) and that the reduction potential of the nNOS heme was not affected by substrate binding and is thermodynamically accessible to reduction by the flavins (31). On the other hand, the wild type enzyme absolutely required Caμ2/CaμM binding for NO formation activity. CaμM binding is likely to induce a conformational change for efficient electron transfer between the domains. It is thus suggested that there might be differences in active site conformation and/or the electron transfer route for the first electron required for initial heme reduction and subsequent electrons required for catalytic turnover.
In the absence of Ca²⁺/CaM, both hemes in the delta40 homodimer were reduced under anaerobic conditions with a similar rate to that of the wild type enzyme in the presence of Ca²⁺/CaM (Table I). The deletion seems to activate both heme reduction and NO synthesis in a way similar to that of Ca²⁺/CaM binding, which activates the wild type enzyme. In the homodimer of the E592A delta40 mutant, 60% of the heme was reduced with NADPH in the absence of Ca²⁺/CaM. The addition of Ca²⁺/CaM resulted in further heme reduction up to 85%, as in the delta40 homodimer mutant (Table I). Loss of substrate binding to the heme site did not appear to unduly affect heme reduction.

The deletion mutation appears to cause both hemes of the heterodimers to be reduced by NADPH in the absence or presence of Ca²⁺/CaM. These results indicate that, in the delta40 mutants, electrons can be transferred to the heme domains from the flavin located on either the same subunit (intra-subunit) or the adjacent subunit (inter-subunit), as shown in Fig. 6, E–H. It is unlikely that inter-heme electron transfer occurs in these heterodimers, because it would also be expected to occur in the other heterodimers (such as Wild/Wildox (B), E592A/Wildox (C), and Wild/E592Aox (D) in Fig. 6) in the presence of Ca²⁺/CaM.

The delta40 heterodimers were found to require Ca²⁺/CaM binding for NO formation activity, just like the wild type enzyme (Table I). The delta40 homodimer, however, retained about 30% of its activity in the absence of Ca²⁺/CaM (14). These results again suggest that there is a conformational difference between the homodimers and heterodimers. In this case, the effect of heterodimer formation is to prevent catalytically viable electron transfer from the delta40 reductase to the hemes in the absence of Ca²⁺/CaM (Fig. 6E). It is interesting that the reductase domain of the delta40 heterodimers is able to reduce both hemes in the absence of Ca²⁺/CaM but is unable to drive NO synthesis (Fig. 6, F–H). An optimal structure/conformation necessary for NO formation activity may not be retained in the delta40 heterodimer in the absence of Ca²⁺/CaM, whereas, in the delta40 homodimer, that would be well preserved, because the homodimer has two reductase domains. These results support the idea that CaM binding and/or the presence of the two reductase domains are required for the formation of catalytically active dimer as proposed previously (32, 33). Under assay conditions, it may be that electron transfer is too slow to drive NO synthesis in the delta40 heterodimers in the absence of Ca²⁺/CaM. The deletion mutation also had the effect of activating the reductase domain in the absence of Ca²⁺/CaM. It would not, therefore, be surprising if heterodimers containing this domain were more likely to undergo inter-dimer heme reduction.

It is possible that the route of the first electron transfer to the heme is different from the route of the second electron transfer, which is required for activation of the ferrous-dioxy intermediate during catalysis. Recent studies suggest that the second electron might be transferred from H₄B, which is then regenerated by the reductase domain (7–10). Ca²⁺/CaM binding may be required for this process to occur (in all but the delta40 homodimers). From the analysis of nNOS-NO complex formation, we found that the heme-NO complex of nNOS is easily reduced by NADPH in the presence of both l-Arg and H₂B even in the absence of Ca²⁺/CaM (34). These results support the idea of different transfer routes and regulation mechanisms for the first and second electrons. To confirm this and understand the precise role of CaM binding, further experiments remain to be carried out.

Intra-subunit Electron Transfer in Neuronal Nitric-oxide Synthase

FIG. 6. Hypothetical models of intra- and inter-molecular electron transfers in wild type nNOS and mutants. In the wild type homodimer (A), electrons cross over from one subunit to the other subunit for both heme reduction and NO formation catalysis. Similarly, electrons for heme reduction and for molecular oxygen activation cross over from one subunit to the other in a Ca²⁺/CaM-dependent way in the heterodimers (B, C, and D), which are composed of wild type and the E592A mutant subunits. In the delta40 heterodimers (F, G, and H), the first electron to reduce the heme transfers within the same subunit and/or between the subunits in a Ca²⁺/CaM-independent way, but the second electron, required to activate molecular oxygen, crosses over from one subunit to the other in a Ca²⁺/CaM-dependent way. In the delta40 homodimer (E), however, electrons transfer in a Ca²⁺/CaM-independent way. Black and white arrows indicate inter-molecular and intra-molecular electron transfer, respectively. The reductase domain containing the 40-amino acid deletion in the heterodimers (F, G, and H) might have a slightly different orientation with respect to the oxygenase domain from that of the reductase in the homodimer (E) in the absence of Ca²⁺/CaM. Filled squares indicate the extra 40-amino acid sequences in the full-length subunits.

the other subunit in the dimer (A–D in Fig. 6). It should also be noticed that heme reduction in the heterodimers occurred in the presence of Cu²⁺/CaM, but not in its absence, unlike in the case of the wild type and E592A full-length homodimers. There is clearly a difference between the heterodimers and homodimers in terms of the abilities of their respective reductase domains to transfer electrons to the heme domains and to drive NO synthesis. The wild type heterodimer also has a much lower catalytic rate than the corresponding homodimer. This suggests that the conformation of the heterodimer is not identical to that of the homodimer, which may explain why there appears to be no heme reduction without Cu²⁺/CaM in the heterodimers. If the two reductase domains of the homodimer are in close proximity, it is likely that loss of one will affect the conformation of the other. The location and/or position of the NOS reductase domains with respect to the oxygenase domains are, however, uncertain.

Inter- and Intra-subunit Electron Transfer in the delta40 Mutants—In our previous paper, it was shown that the nNOS delta40 mutant, which has a 40-amino acid deletion in the FMN subdomain, binds CaM at lower Ca²⁺ concentrations and retains NO formation activity in the absence of Ca²⁺/CaM, supporting the idea that the insert is an autoinhibitory element
described in the tables are low compared with the relevant NO formation rates and the NADPH oxidation rates as reported for the wild type homodimer in our previous studies (20, 22). The heme reduction described here was determined at 15 °C under anaerobic conditions, whereas NADPH consumption and NO formation rates were determined at 25 °C under aerobic conditions. In addition, note that the heme reduction described here reflects the introduction of the first electron to the heme iron and the slow equilibration between NADPH and heme during incubation. Although it is certain that the involvement of heme is essential for NO formation activity, it is not clear how subsequent electrons are delivered and circulated for the activation of O2 during catalysis. For example, electron donation from H2B to the O2-bound heme has been implied (8–10). Involvement of H2B in reduction of the NO-heme complex (a proposed dead-end complex) has been suggested (34), and electrons from the substrate itself may also be used to active O2 (9, 24). Further studies remain to be carried out to address these questions.

**Summary**—The following were suggested from the present study: 1) Electrons transfer from the reductase domain of one subunit to the oxygenase domain of the other subunit in wild type nNOS homodimers and heterodimers in a Ca2+/CaM-dependent way, as has been suggested for iNOS; 2) In the delta40 mutant heterodimer electrons transfer from the reductase domain of one subunit to both of the oxygenase domains in a Ca2+/CaM-independent way; 3) In the delta40 mutant heterodimers, electrons required for the NO formation activity transfer exclusively from the reductase domain of the delta40 mutant to the oxygenase domain of the other subunit in a Ca2+/CaM-dependent way. It is clear, therefore, that catalytically relevant electron transfer events occur in an inter-subunit manner in both the wild type and delta40 mutant forms of nNOS. This study has also showed that intra-subunit electron transfer is possible in the delta40 mutant, in contrast to the wild type enzyme. The 40 amino acids in the FMN binding subdomain appear to be very important in regulating electron transfer between the domains in nNOS and particularly in controlling the conformational integrity of the interdomain complex in the presence and absence of CaM.

**Note Added in Proof**—During the review of this paper, we became aware of a report describing inter-subunit electron transfer in wild-type nNOS by Dr. D. Stuehr’s group (35). Their results are consistent with those reported in this paper.

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