Chimeric Enzymes of Cytochrome P450 Oxidoreductase and Neuronal Nitric-oxide Synthase Reductase Domain Reveal Structural and Functional Differences*

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The nitric-oxide synthases (NOSs) are comprised of an oxygenase domain and a reductase domain bisected by a calmodulin (CaM) binding region. The NOS reductase domains share ~60% sequence similarity with the cytochrome P450 oxidoreductase (CYPOR), which transfers electrons to microsomal cytochromes P450. The crystal structure of the neuronal NOS (nNOS) connecting/FAD binding subdomains reveals that the structure of the nNOS-connecting subdomain diverges from that of CYPOR, implying different alignments of the flavins in the two enzymes. We created a series of chimeric enzymes between nNOS and CYPOR in which the FMN binding and the connecting/FAD binding subdomains are swapped. A chimera consisting of the nNOS heme domain and FMN binding subdomain and the CYPOR FAD binding subdomain catalyzed significantly increased rates of cytochrome c reduction in the absence of CaM and of NO synthesis in its presence. Cytochrome c reduction by this chimera was inhibited by CaM. Other chimeras consisting of the nNOS heme domain, the CYPOR FMN binding subdomain, and the nNOS FAD binding subdomain with or without the tail region also catalyzed cytochrome c reduction, were not modulated by CaM, and could not transfer electrons into the heme domain. A chimera consisting of the heme domain of nNOS and the reductase domain of CYPOR reduced cytochrome c and ferricyanide at rates 2-fold higher than that of native CYPOR, suggesting that the presence of the heme domain affected electron transfer through the reductase domain. These data demonstrate that the FMN subdomain of CYPOR cannot effectively substitute for that of nNOS, whereas the FAD subdomains are interchangeable. The differences among these chimeras most likely result from alterations in the alignment of the flavins within each enzyme construct.
binding regions suggests that this tail region bisects the flavin domain between the FMN and FAD and emerges on the other side of the molecule (14), thus impeding the flow of electrons between the flavins. In the presence of CaM, this blockade is partially removed, presumably by repositioning of the tail, facilitating flux between the flavins (12, 13). The tail regions of nNOS and eNOS contain a serine residue that has been demonstrated in eNOS to be phosphorylated as a result of physiological stimuli, resulting in higher rates of both NO production and cytochrome c reduction (15–18). Mutation of this residue in either eNOS or nNOS from serine to aspartate mimics the negative charge of phosphorylation or aspartate mutation results in eNOS to be phosphorylated as a result of physiological stimuli, resulting in higher rates of both NO production and cytochrome c reduction (15–18). Mutation of this residue in either eNOS or nNOS from serine to aspartate mimics the negative charge of phosphorylation and results in an enzyme that is catalytically impaired (12, 13). The tail regions of nNOS and eNOS contain a serine residue that has been demonstrated in eNOS to be phosphorylated as a result of physiological stimuli, resulting in higher rates of both NO production and cytochrome c reduction (15–18). Mutation of this residue in either eNOS or nNOS from serine to aspartate mimics the negative charge of phosphorylation and results in an enzyme that is catalytically impaired (12, 13).

The codons encoding residues 79–238 of CYPOR, encompassing the FMN binding subdomain, were amplified by PCR with the following primers: Upstream, atg cag cat atg gct gaa aac atc gta tct ta; Downstream, aag ctt atg cat atg gga gcc atc gga ggt. The forward primer encodes an NdeI site, and the reverse primer encodes an XhoI site for insertion into the vector. The two PCR products were restriction-digested with NdeI and ligated with NdeI/XhoI-digested pCW vector. This ligation was used to transform XL10-gold cells (Stratagene), and colonies were screened by restriction digest. The correct construct was then used to transform Escherichia coli BL21 cells.

### EXPERIMENTAL PROCEDURES

**Chemicals**

(6R)-5,6,7,8-Tetrahydrobiopterin was from Research Biochemicals International (Natick, MA). All other chemicals were from Sigma and were of the highest grade available.

**Enzymes**

*Pfu* Turbo polymerase was from Stratagene (La Jolla, CA), shrimp alkaline phosphatase was from U. S. Biochemical Corp., and restriction enzymes were from Promega (Madison, WI) or New England Biolabs (Beverly, MA).

**Recombinant DNA Manipulations**

All of the following constructs are summarized in Table I and illustrated in Fig. 1.

**Soluble CYPOR**—The coding regions 79–238 of CYPOR, encompassing the FMN binding subdomain, were amplified by PCR with the following primers: Upstream, atg cag cat atg gct gaa aac atc gta tct ta; Downstream, aag ctt atg cat atg gga gcc atc gga ggt. The forward primer encodes an NdeI site, and the reverse primer encodes an XhoI site for insertion into the vector. The two PCR products were restriction-digested with NdeI and ligated with NdeI/XhoI-digested pCW vector. This ligation was used to transform XL10-gold cells (Stratagene), and colonies were screened by restriction digest. The correct construct was then used to transform Escherichia coli BL21 cells along with groEL/S plasmid.

| Chimeric enzyme                  | Parent enzyme | Residue number of parent | Start/end amino acid sequence |
|----------------------------------|---------------|--------------------------|------------------------------|
| Soluble CYPOR (CYPOR)            | CYPOR         | 79–678                   | MANIV/F/LDVWS               |
| nNOS<sub>CFMN</sub>/F<sub>FAD,NADPH</sub> (NCC) | nNOS       | 1–963                    | MAENTF/F/ISNDR               |
| C<sub>FMN</sub>nNOS<sub>FAD,NADPH</sub> (CN) | CYPOR           | 241–678                  | SIRQY/LDVWS                  |
| C<sub>FMN</sub>nNOS<sub>FAD,NADPH</sub> (tr1) (CN-tr1) | CYPOR           | 962–1430                 | DRSWK/EVFSS                  |
| nNOS<sub>CFMN</sub>/nNOS<sub>FAD,NADPH</sub> (CN) | nNOS       | 962–1430                 | DRSWK/HEDIFG                 |
| C<sub>FMN</sub>nNOS<sub>FAD,NADPH</sub> (tr1) (CN-tr1) | nNOS       | 1–754                    | MAENTF/F/AMAKRVR             |
| C<sub>FMN</sub>nNOS<sub>FAD,NADPH</sub> (tr1) | nNOS       | 1–754                    | NIIVF/EVFSS                 |
| C<sub>FMN</sub>nNOS<sub>FAD,NADPH</sub> (tr1) | nNOS       | 79–1397                  | MAENTF/F/AMAKRVR             |
| CYPOR                            | CYPOR         | 79–678                   | NIIVF/LDVWS                  |

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nNOS and CYPOR Chimeras Reveal Functional Differences

*TABLE I*

**Composition of chimeric enzymes**

| Chimeric enzyme                  | Parent enzyme | Residue number of parent | Start/end amino acid sequence |
|----------------------------------|---------------|--------------------------|------------------------------|
| Soluble CYPOR (CYPOR)            | CYPOR         | 79–678                   | MANIV/F/LDVWS               |
| nNOS<sub>CFMN</sub>/F<sub>FAD,NADPH</sub> (NCC) | nNOS       | 1–963                    | MAENTF/F/ISNDR               |
| C<sub>FMN</sub>nNOS<sub>FAD,NADPH</sub> (CN) | CYPOR           | 241–678                  | SIRQY/LDVWS                  |
| C<sub>FMN</sub>nNOS<sub>FAD,NADPH</sub> (tr1) (CN-tr1) | CYPOR           | 962–1430                 | DRSWK/EVFSS                  |
| nNOS<sub>CFMN</sub>/nNOS<sub>FAD,NADPH</sub> (CN) | nNOS       | 962–1430                 | DRSWK/HEDIFG                 |
| C<sub>FMN</sub>nNOS<sub>FAD,NADPH</sub> (tr1) (CN-tr1) | nNOS       | 1–754                    | MAENTF/F/AMAKRVR             |
| C<sub>FMN</sub>nNOS<sub>FAD,NADPH</sub> (tr1) | nNOS       | 1–754                    | NIIVF/EVFSS                 |
| C<sub>FMN</sub>nNOS<sub>FAD,NADPH</sub> (tr1) | nNOS       | 79–1397                  | MAENTF/F/AMAKRVR             |
| CYPOR                            | CYPOR         | 79–678                   | NIIVF/LDVWS                  |

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### EXPERIMENTAL PROCEDURES

**Chemicals**

(6R)-5,6,7,8-Tetrahydrobiopterin was from Research Biochemicals International (Natick, MA). All other chemicals were from Sigma and were of the highest grade available.

**Enzymes**

*Pfu* Turbo polymerase was from Stratagene (La Jolla, CA), shrimp alkaline phosphatase was from U. S. Biochemical Corp., and restriction enzymes were from Promega (Madison, WI) or New England Biolabs (Beverly, MA).

**Recombinant DNA Manipulations**

All of the following constructs are summarized in Table I and illustrated in Fig. 1.

**Soluble CYPOR**—The codons encoding residues 79–678 of cytochrome P450 reductase were amplified by PCR, using the original CYPOR plasmid kindly provided by Dr. Charles Kasper as template, with the following primers: Upstream, atg cag cat atg gct gaa aac atc gta tct ta; Downstream, aag ctt atg cat atg gga gcc atc gga ggt. The forward primer encodes an NdeI site, and the reverse primer encodes an XhoI site for insertion into the vector. The two PCR products were restriction-digested with NdeI and ligated with NdeI/XhoI-digested pCW vector. This ligation was used to transform XL10-gold cells (Stratagene), and colonies were screened by restriction digest. The correct construct was then used to transform Escherichia coli BL21 cells.
open boxes represent nNOS sequences, and diagonally striped boxes represent CYPOR sequences.

**Protein Expression and Purification**

nNOS, CYPOR, and chimeric proteins were expressed and purified as previously described (24) except that cultures were grown in 500 ml of medium in Fernbach flasks, all of the column equilibration and wash buffers contained 100 mM NaCl, and the protein was eluted with buffer containing 500 mM NaCl and 15 mM 2,3′-AMP. The reductase proteins (CYPOR, C_FMN/nNOSPAD, and C_FMN/nNOSPAD(tr1)) were fully oxidized by ferricyanide titration before gel filtration chromatography. Calmodulin was prepared by the method of Zhang and Vogel (25). Based on the gel filtration chromatography step, all of the heme domain-containing chimeras were primarily dimeric, including NCC, which contains no nNOS-derived reductase domain modules, indicating that all the determinants necessary for dimerization are contained within the heme domain of nNOS.

**Spectrophotometric Methods**

CO difference spectra were performed as described (24). The molar protein concentrations for heme-containing chimeras were determined based on heme content via reduced CO difference spectra, where $\epsilon = 100 \text{ mM}^{-1} \text{cm}^{-1}$ for $\Delta A440-470$. The molar protein concentrations for non-heme-containing chimeras were determined based on flavin content via absolute absorbance, where $\epsilon = 21 \text{ mM}^{-1} \text{cm}^{-1}$ (10.5 mM$^{-1}$ flavin) at 454 nm. All spectral analyses were performed using a Shimadzu model 2401PC UV-visible dual-beam spectrophotometer.

**Stopped-flow Spectroscopy**

Stopped-flow reactions were performed aerobically under turnover conditions at 23 °C, as described in Miller et al. (26) and Roman et al. (12, 13), using an Applied Photophysics SX.18MV diode array stopped-flow spectrophotometer, with a dead time of 2 ms. Reactions contained 3 mM enzyme and 100 mM NADPH in 50 mM Tris-HCl, pH 7.4, and 100 mM NaCl. Where indicated, 10 mM CaM was also added. Flavin reduction was monitored at 485 nm.

**Measurement of Activity**

Nitric oxide formation (hemoglobin capture assay) and cytochrome c reduction were measured at 23 °C as described (27, 28), with the exception that both assays were performed in a buffer containing 50 mM Tris-HCl, pH 7.4, and 100 mM NaCl. Rates of NO synthesis and cytochrome c reduction were determined using extinction coefficients of 60 mM$^{-1}$ cm$^{-1}$ at 401 nm and 21 mM$^{-1}$ cm$^{-1}$ at 550 nm, respectively. Ferricyanide reduction was performed in the same manner as cytochrome c reduction, except that the ferricyanide concentration was varied, and the extinction coefficient was 1.02 mM$^{-1}$ cm$^{-1}$ at 420 nm. The reoxidation of reduced flavins was monitored at 485 nm for all enzymes in the presence of 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 20 mM NADPH, and 2 mM enzyme at 23 °C.
RESULTS

Expression and Spectral Characterization of Chimeric Enzymes—All of the chimeric enzymes expressed very well in E. coli, with yields comparable with those of wild-type nNOS and CYPOR (i.e. 100–150 nmol/liter cells), and all were of the correct mass as visualized by SDS-PAGE. All of the chimeric enzymes looked spectrally healthy. All chimeric holoenzymes contained both heme and flavins, as determined by absolute spectra, and were isolated in a mixed low spin/high spin form that shifted completely to low spin with the addition of 1 mM imidazole and completely to high spin upon the addition of 100 mM arginine (Fig. 2A). The absorbance peak shifted an additional 4 nm lower upon the addition of H$_4$B (not shown), suggesting that the binding site for this cofactor is intact. All formed the characteristic peak at ~$\lambda_{max}$ nm in the reduced, CO-bound form, indicating proper insertion of the heme moiety in the chimeras. Spectral measurement of the binding constant for arginine ($K_a$) yielded values comparable with that of wild-type NOS (717 nm; Ref. 24). Shown in Fig. 2B are the substrate difference spectra for titration of nNOS ox/CFMN,FAD,NADPH by arginine, which yielded $K_a = \sim 300$ mM. The similarity of these values to the wild type suggests that the binding site for arginine is unimpaired in the chimeras. All of these characteristics are similar in both the wild-type and chimeric proteins, suggesting an intact and potentially functional heme domain.

All of the non-heme domain-containing flavoprotein constructs were isolated as greenish in color, indicating the formation of an air-stable flavin semiquinone, which was confirmed spectrophotometrically (not shown), as is typically seen with CYPOR. This semiquinone form persisted until oxidized by the addition of ferricyanide. None of the chimeras were as stable as nNOS or CYPOR, as judged by loss of activity over a period of a week on ice, but all were stable (less than 10% drop in activity) for at least 2 days. Therefore, all activity measurements were performed on freshly prepared enzyme within 2 days of isolation.

Cytochrome c Reduction—To determine the functionality of the reductase domains, cytochrome c reduction was assayed. Reduction of cytochrome c involves passage of electrons from NADPH to FAD to FMN to cytochrome c and, thus, requires an intact reductase domain. Fig. 3 shows that all of the chimeric holoenzymes were able to reduce cytochrome c. Wild-type nNOS, nNOS (tr1), and CYPOR are shown for comparison. As reported previously, the rates of reduction by nNOS and nNOS (tr1) in the presence of CaM are very similar to that of CYPOR, 3000–4500 min$^{-1}$. The $K_m$ values for cytochrome c were determined for all of the enzymes shown, and all are between 3–6 $\mu$M (not shown), so any differences observed among the chimeras are not due to altered interaction with cytochrome c.

The profile for NNC resembles that of nNOS (tr1) in that in the absence of CaM, cytochrome c reduction is very high, 2–2.5-fold higher than either CYPOR or nNOS in the presence of CaM. In the presence of CaM, cytochrome c activity decreases by 75%, to a level half that of CYPOR. NNC retains the auto-

![Fig. 2. Spectra of chimera NCC. A, absolute spectra showing 0.85 $\mu$M enzyme as isolated (solid line), with 1 mM imidazole (dotted line), and with 200 $\mu$M arginine (dashed line); B, substrate difference spectra with 2 $\mu$M enzyme showing the addition of 0, 0.2, 0.6, 1.2, and 2.0 $\mu$M arginine, with the higher concentrations of arginine causing the greater deviations from the base line (other concentrations were also measured but are not shown for clarity). The buffer was 50 mM Tris HCl, pH 7.4, 100 mM NaCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 10% glycerol, and all spectra were recorded at room temperature.](http://www.jbc.org/Downloaded from)
Diagonally striped bars, no CaM added; filled bars, 1 
μM CaM added. Reactions were performed as described under Experimental Procedures using 1 μM enzyme in 1-ml final volume of 50 mM Tris HCl, pH 7.4, and 100 mM NaCl. Reactions also contained 80 μM cytochrome c and 100 μM NADPH and were begun with the addition of enzyme.

In the presence of CaM, the turnover number for wild-type nNOS ferricyanide reduction does not change, but the $K_m$ value decreases by 50% (to 780 μM). The activity of the chimeras is essentially unchanged by CaM (not shown).

**NO Synthesis**—Only one of the chimeras was competent to synthesize NO. NNC catalyzed NO production in the presence of CaM at a rate of 88 ± 1 min$^{-1}$, as compared with wild-type nNOS, which has a turnover number of 71 ± 6 min$^{-1}$ under the same conditions (Fig. 4). Thus, despite the chimeric nature of its reductase domain, NNC is at least as good as, and perhaps better than, the wild-type enzyme at synthesizing NO. NNC also synthesizes NO at almost 3× the rate of nNOS (tr1) in the presence of CaM but, unlike nNOS (tr1), forms no NO in the absence of CaM. NNC and nNOS (tr1) have identical heme domains and FMN subdomains. They differ only in the connecting/FAD subdomain, and that of nNOS (tr1) was initially designed to mimic that of CYPOR, which is the parent of the NNC connecting/FAD subdomain.

To determine whether the other chimeric holoproteins were able to transfer electrons to their heme domains even though NO is not formed, heme reduction by electrons donated by NADPH was measured in the presence of CO, superoxide dismutase, and catalase. Heme reduction was monitored by an absorbance increase at 445 nm, the characteristic peak observed for the reduced, CO-bound form of the enzyme. None of the chimeric enzymes NNC, NCC (tr1), or NCC formed the reduced, CO-bound heme, indicating that electrons are not passed from the reductase domain to the heme in these proteins.

**Flavin Reduction**—Initial flavin reduction in the presence of oxygen and NADPH was measured via stopped-flow spectrophotometry, and the results are shown in Table III. All enzymes showed the biphasic flavin reduction characteristic of the wild-type nNOS (26). There is clearly no effect of CaM on the rate of either the fast or slow phase of flavin reduction in any of the chimeric holoproteins. Furthermore, the rates of flavin reduction in the chimeras are similar to that of wild-type nNOS in the presence of CaM, indicating that whatever elements or conformation that the binding of CaM alters to relieve inhibition of electron flow through the flavin domain are no longer present or effective in the chimeras.
globin, 100/H9262
actions also contained 8/H9262
the stable semiquinone state.

data are representative of all the chimeras; they all reoxidize to
shows that NCN (tr1) forms a stable semiquinone, and these
absorbance change, the semiquinone form is present. Fig. 5
flavins have been fully reoxidized. If it regains only half the
absorbance plateaus at the same absorbance as it started, the
NADPH is exhausted, the flavin absorbance returns to a pla-
tau whose absorbance represents the air-stable form. If the
NCN (tr1), and NNC.
with flavins fully oxidized. NADPH
ras, flavin reoxidation was followed. In Fig. 5, the enzyme is
the air-stable semiquinone form was generated in the chime-
tail region removed to resemble CYPOR. To determine whether
does not have the tail region, and nNOS (tr1), which had the
exposure of the flavins to solvent between CYPOR, which
fully reoxidized (12, 13), suggesting significant differences in
zymes no longer formed the semiquinone but quickly became
fully reoxidized (12, 13), suggesting significant differences in
the exposure of the flavins to solvent between CYPOR, which
do not have the tail region, and nNOS (tr1), which had the
tail region removed to resemble CYPOR. To determine whether
the air-stable semiquinone form was generated in the chime-
as, flavin reoxidation was followed. In Fig. 5, the enzyme is
initially in its resting state, with flavins fully oxidized. NADPH
is added, the flavins reduced, and catalysis is begun. When the
NADPH is exhausted, the flavin absorbance returns to a pla-
tau whose absorbance represents the air-stable form. If the
absorbance plateaus at the same absorbance as it started, the
flavins have been fully reoxidized. If it regains only half the
absorbance change, the semiquinone form is present. Fig. 5
shows that NCN (tr1) forms a stable semiquinone, and these
data are representative of all the chimeras; they all reoxidize to
the stable semiquinone state.

DISCUSSION
We have produced a series of chimeric enzymes by swapping
the FMN binding and FAD/NADPH binding subdomains be-
tween nNOS and CYPOR. The successful production of en-
zymes able to reduce cytochrome c indicates that the FN
binding and FAD/NADPH binding subdomains have discrete
domain structures that are appropriately folded and similar
enough in structure that swapping domains still yields proteins
that in each case support electron transfer through the two
flavins.

The heme domain-containing constructs are more active
than their corresponding reductase domain constructs (NCN
versus CN; NCN (tr1) versus CN (tr1)), indicating an influence
of the heme domain on folding/stability of the flavin domain. In
fact, NCC, in which the entire reductase domain is donated by
CYPOR, exhibits a 2-fold increase in both cytochrome
c and
ferricyanide reduction over that of CYPOR. Such behavior is
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CYPOR, exhibits a 2-fold increase in both cytochrome

\begin{table}
\centering
\caption{Ferricyanide reduction}
\begin{tabular}{lcc}
\hline
Construct & $k_{cat}$ & $K_m$ \\
\hline
nNOS & 34400 & 1840 \\
nNOS + CaM & 31200 & 280 \\
CYPOR & 24 11 & 23 19 \\
nNOS$_{FMN}$/FAD/NADPH (NCC) & 21 6 & 23 7 \\
nNOS$_{FMN}$/nNOS$_{FAD}$/NADPH (NCN) & 13 2 & 23 3 \\
nNOS$_{FMN}$/nNOS$_{FAD}$/NADPH (tr1) (NCN-tr1) & 10 3 & 23 4 \\
\hline
\end{tabular}
\end{table}

\begin{table}
\centering
\caption{Stopped-flow analysis of flavin reduction}
\begin{tabular}{lcccc}
\hline
Chimeric enzyme & Minus CaM & Plus CaM \\
& Fast & Slow & Fast & Slow \\
\hline
nNOS* & 53 & 11 & 53 & 22 \\
nNOS (tr1)* & 367 & 380 & 367 & 380 \\
CYPOR & 187 & 157 & 187 & 157 \\
nNOS$_{FMN}$/FAD/NADPH (NCC) & 276 & 237 & 280 & 246 \\
CYPOR$_{FMN}$/nNOS$_{FAD}$/NADPH (CN) & 252 & 12 & 473 & 61.8 \\
CYPOR$_{FMN}$/nNOS$_{FAD}$/NADPH (tr1) (CN-tr1) & 330 & 20 & ND & ND \\
nNOS$_{CMN}$/nNOS$_{FAD}$/NADPH (NNC) & 402 & 45.2 & 473 & 61.8 \\
nNOS$_{CMN}$/nNOS$_{FAD}$/NADPH (tr1) (NCN-tr1) & 418 & 8.49 & 558 & 82 \\
nNOS$_{CMN}$/FAD/NADPH (NCC) & 446 & 1.52 & 474 & 14.6 \\
\hline
\end{tabular}
\begin{tablenotes}
\item [*] Data from Roman et al. (13).
\item [a] ND, not determined.
\end{tablenotes}
\end{table}
Interestingly, CaM regulation is completely gone in all chimeras except for NNC, which contains the nNOS FMN binding subdomain and, thus, the autoregulatory insert. A nNOS construct in which the autoregulatory insert has been removed still responds to CaM (9, 33), indicating that something more than the autoregulatory insert was involved. The chimera NCN could, thus, potentially still retain modulation by CaM, but it clearly does not. The cytochrome c reduction activities of these chimeras, however, are closer to those observed with wild-type enzyme in the presence of CaM, so the chimeras must be missing those inhibitory elements that CaM relieves to allow electron transfer through the flavin domain. Experiments by Matsuda and Iyanagi (34) demonstrate that CaM activates the transfer of electrons between the two flavin subdomains. This is consistent with our data showing that CaM has no effect on the $K_{cat}$ value of ferricyanide reduction, but a significant effect on the $K_{cat}$ value of cytochrome C reduction by nNOS. Because CaM appears to have no effect on either the fast or slow phases of flavin reduction or on cytochrome c activity in any of the chimeric constructs, it is obvious that the block CaM relieves in the wild-type enzyme reductase domain is not present in the chimeras. Craig et al. (35) propose that this block, which is relieved by CaM binding, is a conformational block imposed by NADPH binding, which restricts the access of external electron acceptors to the FMN. In NNC, which retains the nNOS FMN subdomain, or in NCN and NCN (tr1), which retain the nNOS FAD subdomain and NADPH binding site, or even in nNOS (tr1), which retains all FMN, FAD, and NADPH binding sites from nNOS but lacks the C-terminal tail, no such block appears to exist, indicating that both nNOS subdomains must be intact and present for the NADPH/CaM mediation to occur.

Electron transfer to the heme domain, however, does not occur in NCN, NCN (tr1), or NCC. This may be because of the inability of the FMN subdomain of CYPOR to interact properly with the heme domain of nNOS, as discussed below, but may also be related to the lack of CaM modulation. Sagami et al. (36) propose that the mechanism of activation of nNOS by CaM was not merely dependent on the activation of electron transfer to the heme domain. Because nNOS mutants in which the calmodulin site had been deleted still show heme reduction but not NO production, they suggest that additional structural features in the heme domain must be affected by CaM binding. These structural features are still present, because the heme domains of the chimeras are intact and respond appropriately to substrate. One possibility suggested by these non-functional chimeric proteins is an interaction between the autoinhibitory region, which is not present in the chimeras, and sites on the heme domain.

In addition, little difference exists between the chimeras whether the tail region is present or not (NCN versus NCN (tr1)), even in the flavin reoxidation state. All of the chimeras are able to form a stable semiquinone, unlike nNOS (tr1), whether the FAD binding subdomain is donated by CYPOR or by nNOS, indicating that either the nNOS or the CYPOR FMN domains has the capacity to stabilize the semiquinone form in these chimeras. Thus, in the chimeras, the tail does not obstruct or limit electron flow through the reductase domain even in the absence of CaM, due to either loss of a direct interaction between the tail region and the autoregulatory insert, as proposed by Lane and Gross (21), or an alternate alignment of FMN binding and FAD binding subdomains. Because nNOS still responds to CaM in the absence of its autoregulatory insert, it is most likely that the spatial apposition of the flavins in the chimeric proteins is such that the tail does not obstruct electron flow.

The reductase domain of nNOS shares 58% amino acid sequence similarity with CYPOR, and both CYPOR and the NOS reductase domains can be divided into four subdomains: FMN binding, connecting, FAD binding, and NADPH binding subdomains. In addition to these similarities, significant differences exist between the two. The two most obvious regions of dissimilarity are the autoregulatory insert and the tail region of nNOS, both of which are completely absent in CYPOR. The complete structure of CYPOR has been published (23) but, because the complete structure of the nNOS reductase domain has not yet been reported, no direct comparison can be made. However, Zhang et al. (14) published the structure of a partial nNOS reductase protein consisting of the connecting, FAD binding, and NADPH binding subdomains. They found that the overall polypeptide fold of this protein closely resembled the corresponding regions in CYPOR and that the FAD binding site, the NADPH binding site, and the residues implicated in hydride transfer were strictly conserved between CYPOR and all the NOSs. Given that electron transfer between the FMN and FAD/NADPH binding subdomains occurs readily in the chimeras, particularly when the FAD/NADPH binding subdomain is from CYPOR and the FMN binding subdomain is from nNOS (NCC), this suggests that the electron transfer mechanism of the NOSs is very similar to that of CYPOR.

In the chimeric enzymes, the connecting region between the FMN and FAD binding subdomains is donated by the parent of the FAD binding subdomain. This connecting region shows the

**FIG. 5.** Flavin reoxidation by NCN (tr1) and wild-type nNOS. Solid line, NCN (tr1); dashed line, nNOS. Reactions contained 2 μM enzyme and 20 μM NADPH in 50 mM Tris HCl, pH 7.4, and 100 mM NaCl. The experiments were begun with the addition of NADPH and were performed as described under “Experimental Procedures” at room temperature.
least sequence and structural similarity between CYPOR and nNOS (14). The N-terminal 27 residues of the nNOS-connecting region align with 39 residues in CYPOR, making this region 12 residues shorter in nNOS than in CYPOR, and form an α-helical structure; in CYPOR, these residues form a loop. Also, amino acids 1077–1091 in nNOS form a β-strand finger that may interact with the FMN binding subdomain. They align with a region in CYPOR that is eight residues shorter, and in CYPOR, this region is a short loop.

Zhang et al. (14) also identify a putative FMN domain binding site on the connecting region. In CYPOR, the interaction between the FMN and connecting/FAD binding subdomains is highly electrostatic, with the FMN binding subdomain negatively charged and the connecting/FAD binding subdomain positively charged. In nNOS, the corresponding residues of the connecting region are more neutral, suggesting that the FMN binding subdomain interaction site must also be less charged than in CYPOR (14). The chimeras indicate that the negatively charged FMN binding subdomain is tolerated for interaction with the connecting/FAD binding subdomain but is not tolerated for interaction with the heme domain. Also, the positively charged connecting/FAD binding subdomain of CYPOR is tolerated and in fact performs better than the more neutral nNOS connecting/FAD binding subdomain.

In summary, we have constructed functional chimeric enzymes between nNOS and CYPOR, suggesting that 1) the FMN binding and FAD/NADPH binding subdomains of nNOS and CYPOR are discrete structures that are similar enough in structure to reconstitute electron flow; 2) the electron transfer mechanism of the NOSs is very similar to that of CYPOR; 3) the presence of the nNOS heme domain significantly affects the CYPOR reductase domain; 4) the chimeras are missing the inhibitory elements that CaM relieves; and 5) the tail region of the chimeras does not obstruct electron flow as in wild-type nNOS.

These experiments suggest that regions or subdomains of the nNOS flavoprotein could be uniquely targeted to control electron flow through this NOS isoform. Such experiments also provide insight into the functional relationships among the various interactive subdomains that lead to inhibition or activation of these signaling molecule-producing enzymes.

REFERENCES

1. Masters, B. S. S. (2000) in Nitric Oxide, Biology, and Pathobiology (Ignarro, L. J., ed) pp. 91–104, Academic Press, Inc., New York
2. Roman, L. J., Martasek, P., and Masters, B. S. S. (2002) Chem. Rev. 102, 1179–1189
3. Michel, T., and Feron, O. (1997) J. Clin. Invest. 100, 2146–2152
4. Crane, B. R., Arvai, A. S., Ghosh, D. K., Wu, C., Getzoff, E. D., Stuehr, D. J., and Tainer, J. A. (1998) Science 279, 2121–2126
5. Raman, C. S., Li, H., Martasek, P., Krai, V., Masters, B. S. S., and Poulos, T. L. (1998) Cell 95, 939–950
6. Pischmann, T. O., Bruza, A., Niu, X. D., Fossetta, J. D., Lunn, C. A., Dolphin, E., Prongay, A. J., Reichert, P., Lundell, D. J., Nurula, S. K., and Weber, P. C. (1999) Nat. Struct. Biol. 6, 233–242
7. Nishida, C. R., and Ortiz de Montellano, P. R. (1998) J. Biol. Chem. 273, 5566–5571
8. Salerno, J. C., Harris, D. E., Irizarry, R., Patel, B., Morales, A. J., Smith, S. M. E., Martasek, 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
9. Daff, S., Sagami, I., and Shimizu, T. (1999) J. Biol. Chem. 274, 30589–30595
10. Nishida, C. R., and Ortiz de Montellano, P. R. (1999) J. Biol. Chem. 274, 14692–14698
11. Chen, P.-F., and Wu, K. (2000) J. Biol. Chem. 275, 13155–13163
12. Roman, L. J., Miller, R. T., de la Garza, M. A., Kim, J.-J., and Masters, B. S. S. (2000) J. Biol. Chem. 275, 21914–21919
13. Roman, L. J., Martasek, P., Miller, R. T., Harris, D. E., de la Garza, M. A., Shea, T. M., Kim, J.-J., and Masters, B. S. S. (2000) J. Biol. Chem. 275, 29225–29232
14. Zhang, J., Paschke, R., Martasek, P., Shea, T., Masters, B. S. S., and Kim, J.-J. P. (2001) J. Biol. Chem. 276, 37506–37513
15. Fulton, D., Gratton, J.-P., McCabe, T. J., Fontana, J., Fugio, Y., Walsh, K., Franke, T. F., Papapetropoulos, A., and Sessa, W. C. (1999) Nature 399, 597–601
16. Dimmeler, S., Fleming, I., Fasliathaler, B., Hermann, C., Busse, R., and Zeiher, A. M. (1999) Nature 399, 601–605
17. Chen, Z.-P., Mitchell, K. I., Michell, B. J., Stapleton, D., Rodriguez-Crespo, I., Witters, L. A., Power, D. A., Ortiz de Montellano, P. R., and Kemp, B. E. (1999) FEBS Lett. 443, 285–289
18. Butt, E., Bernhardt, M., Smolenski, A., Kotsouinis, P., Frohlich, B. G., Sickmann, A., Meyer, H. E., Lohmann, S. M., and Schmidt, H. H. W. (2000) J. Biol. Chem. 275, 5179–5187
19. McCabe, T. J., Fulton, D., Roman, L. J., and Sessa, W. C. (2000) J. Biol. Chem. 275, 6123–6128
20. Adak, S., Santolini, J., Tikunova, S., Wang, Q., Johnson, J. D., and Stuehr, D. J. (2001) J. Biol. Chem. 276, 1244–1252
21. Lane, P., and Gross, S. S. (2002) J. Biol. Chem. 277, 19087–19094
22. Breit, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R., and Snyder, S. H. (1991) Nature 351, 714–718
23. Wang, M., Roberts, D. L., Paschke, R., Shea, T. M., Masters, B. S. S., and Kim, J.-J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8411–8416
24. Roman, L. J., Shei, E. A., Martasek, P., Gross, S. R., Liu, Q., and Masters, B. S. S. (1999) Proc. Natl. Acad. Sci. U. S. A. 92, 8428–8432
25. Zhang, M., and Vogel, H. J. (1994) Biochemistry 33, 1163–1171
26. Miller, S. T., Martasek, P., Osurow, T., and Masters, B. S. S. (1999) Biochem. Biophys. Res. Commun. 265, 184–188
27. Sheta, E., McMillan, K., and Masters, B. S. S. (1994) J. Biol. Chem. 269, 15147–15153
28. Martasek, P., Miller, R. T., Roman, L. J., Shea, T. M., and Masters, B. S. S. (1999) Methods Enzymol. 301, 70–78
29. Masters, B. S. S. (1994) Annu. Rev. Nutr. 14, 131–145
30. Abu-Soud, H. M., and Stuehr, D. J. (1995) Proc. Natl. Acad. Sci. U. S. A. 90, 10769–10772
31. Pou, S., Keston, L., Surichamorn, W., and Rosen, G. M. (1999) J. Biol. Chem. 274, 8073–8080
32. Noble, M. A., Munro, A. W., Rivers, S. L., Robledo, L., Daff, S. N., Yellowlees, I., Witters, L. A., Power, D. A., Ortiz de Montellano, P. R., and Kemp, B. E. (1999) Biochemistry 38, 16413–16418
33. Montgomery, H. J., Romanov, V., and Guillemette, G. (2002) J. Biol. Chem. 277, 5058–5058
34. Matsuda, H., and Iyana, T. (1999) Biochem. Biophys. Res. Acta 1473, 345–355
35. Craig, D. H., Chapman, S. K., and Daff, S. (2002) J. Biol. Chem. 277, 33987–33994
36. Sagami, I., Daff, S., and Shimizu, T. (2001) J. Biol. Chem. 276, 30036–30042
