The Function of the Small Insertion in the Hinge Subdomain in the Control of Constitutive Mammalian Nitric-oxide Synthases*

Received for publication, March 12, 2004, and in revised form, June 14, 2004
Published, JBC Papers in Press, June 21, 2004, DOI 10.1074/jbc.M402808200

Rachel J. Jones, Susan M. E. Smith, Ying Tong Gao, Bradley S. DeMay, Kevin J. Mann, Kathleen M. Salerno, and John C. Salerno‡

From the Department of Biology, Rensselaer Polytechnic Institute, Troy, New York 12180

Control of nitric oxide (NO) synthesis in the constitutive nitric-oxide synthases (NOS) by calcium/calmodulin is exerted through the regulation of electron transfer from NADPH through the reductase domains. This process has been shown previously to involve the calmodulin binding site, the autoinhibitory insertion in the FMN binding domain, and the C-terminal tail. Smaller sequence elements also appear to correlate with control. Although some of these elements appear well positioned to function in control, they are poorly conserved; their role in control is neither well established nor defined by available information. In this study mutations have been induced in the small insertion of the hinge subdomain, which has been shown recently to form a β hairpin in structural studies of the neuronal NOS reductase domains adjacent to the calmodulin site and the autoinhibitory element. Modification of the small insertion in neuronal NOS tends to increase cytochrome c reduction but not NO synthesis activity; some modifications or deletions in the corresponding region in endothelial NOS modestly increase activity under some conditions. Unexpectedly, some minor changes in the sequence introduce a loss in the content of heme relative to flavin cofactors. Taken together, these results suggest that the small insertion protects the calmodulin binding site and that it may be a modulator of NOS activity.

Nitric-oxide synthases (NOS)† are a growing family of modular enzymes, including three mammalian isoforms, as well as a number of related euukaryotic and more distantly related prokaryotic enzymes. Because of the discovery of the function of nitric oxide (NO) as a molecular messenger in a variety of signal transduction pathways (1–4), the endothelial and neuronal nitric-oxide synthases (eNOS and nNOS) have been shown to function as signal generators controlled by calcium/calmodulin (Ca2+/CaM) (5). The other mammalian isoform, iNOS, is induced during immune response (6–9); it is constitutively active and binds CaM at basal Ca2+ levels.

NO synthesis from arginine and molecular oxygen requires 3 mol of electrons supplied by NADPH/mol of NO formed (10). The reductase domains in eukaryotic NOS are homologous to NADPH P450 reductase (11). These domains contain one flavin mononucleotide (FMN) and one flavin adenine dinucleotide (FAD) cofactor (11–18), which function in electron transfer from NADPH to the heme-containing active site (13, 17, 19, 20).

Ca2+/CaM control in NOS is exerted through the regulation of electron transfer between NADPH and heme (5). This regulation is primarily a function of the reductase domains (21). The canonical CaM binding site was recognized in the sequence region connecting the oxygenase and reductase regions (11). A major insertion in the FMN binding region, correlating with Ca2+/CaM control, was identified as a control element with autoinhibitory character (autoinhibitory insertion, or AI) (22, 23). This has been confirmed by a number of groups (e.g. 24, 25).

Recently the C-terminal region has been shown to be necessary for control (26); C-terminal truncated enzymes are uncoupled in the absence of CaM, and the region contains a regulatory phosphorylation site (27). Mutations at this site (Ser-1179 in eNOS) (28) and the nearby FAD stacking residue (Phe-1164) (29) have an altered calcium response. NADPH has been shown to be involved in producing a “conformational lock” in rapid kinetics experiments (30); displacement of NADPH by 2′-AMP and other putative analogs activates eNOS and nNOS.

Two additional regions contain sequence elements that correlate with Ca2+/CaM control. The strap connecting the FAD and FMN binding domains is slightly longer in eNOS, which may provide additional conformational flexibility. Of greater interest is the small insertion (SI) consisting of four to seven residues in the hinge subdomain contained within the FAD binding region. This sequence element has recently been shown to form a β hairpin adjacent to the AI and to the CaM binding site in the first structure of nNOS reductase domains (31). There is, however, no homology at the amino acid level between the SIs of eNOS and nNOS; homology was destroyed by coupled frameshift mutations, which were tolerated despite completely changing the character of the sequence. Most recently, deletion of the SI in eNOS by substitution of the corresponding INOS region produced an enzyme activated at 5-fold lower Ca2+, leading to a proposal that the SI functions as an additional AI element (32). The –SI enzyme is not more active than the wild

† This work was supported by the American Diabetes Association and the American Heart Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Biology, RJ Science Center, Rensselaer Polytechnic Institute, 110 8th St., Troy, NY 12180. Tel.: 518–276–8392; Fax: 518–276–2344; E-mail: salerj@rpi.edu.

§ The abbreviations used are: NOS, nitric-oxide synthase; AI, autoinhibitory insertion; CaM, calmodulin; NO, nitric oxide; eNOS, endothelial nitric-oxide synthase (NOSIII); iNOS, inducible nitric-oxide synthase; nNOS; 4-morpholinepropanesulfonic acid; nNOS, neuronal nitric-oxide synthase (NOSI); SI, small insertion; CNOS, constitutive nitric-oxide synthase.

2 Y. T. Gao, S. M. E. Smith, R. Jones, and J. C. Salerno, unpublished data,
type enzyme under all conditions, however, and does not follow the pattern observed on AI deletion/substitution. These conflicting indications concerning the importance of the region are addressed here by a series of mutagenesis experiments.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals used for purification were obtained from Sigma. The genes for eNOS and nNOS were gifts from Professor B. S. Masters (University of Texas Health Science Center at San Antonio). The iNOS expression system was the gift of Professor Dipak Ghosh (Duke University and VA Medical Center). GroE(S) plasmid was provided by Dr. Anthony Gatby (PerkinElmer Life Sciences).

**Expression and Purification of Wild Type and Mutant Bovine eNOS and Rat nNOS**—Expression and purification of bovine eNOS and rat nNOS were performed using procedures similar to those described previously (33). Transformed cells were broken with a French press, and after centrifugation to remove cell debris the supernatant was loaded on a 2′-5′-ADD affinity column, washed, and eluted with 2′-AMP as in the previous paper (33). High purity preparations can be obtained with a size exclusion step; we used a Superose 6 HR 10/30 column (Amersham Biosciences); flow rate, 0.4 ml/min; buffer composition, 50 mM Na-Hepes, pH 7.5, 0.1 mM EDTA, 1 mM D-mercaptoethanol, 100 mM NaCl, 10% glycerol (v/v). During the purification procedure tetrahydrobiopterin or L-arginine was added after elution from the affinity column. Enzyme sample concentrations were determined on the basis of heme concentration, except in heme-free preparations, where the flavin concentration was measured. UV-visible absorbance spectra were recorded on an Aminco DW-2000 spectrophotometer.

NO production was assayed using the Griess assay as adapted for microtiter plates (34); calcium dependence was measured using EDTA as a Ca<sup>2+</sup> buffer system. NO production by NO isoforms was routinely assayed with different buffering systems because eNOS is much more active in MOPS, whereas iNOS and nNOS work well in Tris. NADPH-reduction was measured spectrophotometrically as described by McMillan et al. (17), adapted to 96-well plate mixtures. In each well, the 500-μl reaction mixture contained 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 50 μM NADPH, 50 μM horse heart cytochrome c, and ~10 nm nNOS. Cytochrome c reduction was monitored at 550 nm (ε = 2.1 × 10<sup>3</sup> M<sup>−1</sup> cm<sup>−1</sup>). In Ca<sup>2+</sup>-/CaM dependence studies, 0.75 μM CaCl<sub>2</sub> and 10 pg/ml CaM were added to reaction mixtures. Assays were performed on a SpectraMAX plate reader (Molecular Devices).

Protein concentrations were estimated by several methods. The Bio-Rad DC protein kit, an adaptation of the method of Lowry et al. (35), provided the primary measurements; a correction for tyrosine and tryptophan content was applied. Holoenzyme and reductase domain concentrations were measured using UV-visible absorbance spectra recorded on an Aminco DW-2000 spectrophotometer.

**Generation of NOS Mutants**—NOS genes in pCwori+ were mutated using a method we devised employing the Stratagene QuikChange mutagenesis kit. As suggested by Wang and Malcolm (36), we began by separating the forward and reverse primers, but instead of a single preliminary step with separate primers followed by reversion by the Stratagene protocol, we employ 25 cycles of linear amplification with no additional steps other than to combine and anneal the samples. The removal of the “PCR” steps, which are unproductive and which actually destroy mutant strands by extension, removes the limitations on the separate linear amplification steps and greatly improves the performance of the procedure. In particular, we have obtained a consistent high yield of mutants and a very low background (<5%) of parentals. A more detailed evaluation of the method will be published elsewhere.

**Results**

An alignment of the SI region in eNOS, nNOS, iNOS, and NADPH P450 oxidoreductase is shown in Fig. 1. These sequences are part of the hinge subdomain within the FAD binding domain. Alignments of two dozen eukaryotic NO sequences (not shown for reasons of space) indicate that corresponding SIs are a feature of other NO sequences that contain AI cognates in the FMN binding domain; iNOSs lack this feature. The SI regions of constitutive NO isoforms of primitive vertebrates and invertebrates resemble nNOS more closely than eNOS. The SI in mammalian nNOS is characterized by its hydrophobic character and by a glycine residue that marks a turning point in the path of the backbone. It can be argued that a centrally located glycine (or glycines) allowing a sharp turn is the only common SI sequence element. To examine the significance of this residue we constructed several mutants. We reasoned that the standard alanine mutant would not be particularly informative in this case because Gly and Ala both have small side chains associated with sharp turns. Tyr and Asp were selected as residues that would introduce strongly different character into the region, Tyr by virtue of its large side chain volume and Asp by introducing a negative charge into a hydrophobic region. We preferred the relatively short side chain of acidic residues to the very long positively charged side chains of Arg and Lys, both of which are capable of interacting over a considerable distance.

**Mutants of nNOS**—The structure of the nNOS region can be seen in Fig. 2, in which the structure of cytochrome P450 oxidoreductase (38) is overlaid with the structure of a two-domain nNOS construct, including the FAD and NADPH binding domains and the hinge subdomain (31). The SI is shown in white; it is adjacent to the edge of the FMN binding domain opposite the FMN binding site and is directly adjacent to bound CaM and the AI. In nNOS the sequence immediately preceding the SI contains the tripeptide LEE, corresponding to LDE in iNOS and NADPH P450 oxidoreductase and LEK in eNOS. The second acidic residue in the figure, corresponding to Glu-352 in NADPH P450 oxidoreductase and Glu-1068 in rat nNOS, is marked in deep red in both cases. Immediately after the SI is a tripeptide KKK in NADPH P450 reductase and NWK in nNOS. The serine residues, Ser-354 and Ser-1077 in NADPH P450 oxidoreductase and Glu-1068 in rat nNOS, is marked NCPR in both cases. The serine residues, Ser-354 and Ser-1077 in NADPH P450 oxidoreductase and nNOS respectively, are marked in yellow. This makes the relative positions of cognate structures (e.g. the AI and CaM binding site) surrounding the SI region in the two proteins clear.

In designing deletion mutants we were influenced by the pattern observed in large multiple sequence alignments of NO isoforms, by the structures of P450 reductase and NOS reductase domains shown in Fig. 2, and by structural models of the NOS reductase domains we have constructed over the past 10 years using information from these and other solved homologous proteins (e.g. 22). In P450 oxidoreductase the motif LDEES (see Fig. 1) forms the turn at the end of a series of β

**FIG. 1. Alignment of the gene and amino acid sequences of rat nNOS, bovine eNOS, and mouse iNOS in the SI region showing the position and extent of the insertion in the constitutive isoforms relative to iNOS.** Apparent frameshifts have destroyed all similarity at the amino acid level between eNOS and nNOS; several slightly different equivalent alignments are possible. Although the presence of the SI correlates well with Ca<sup>2+</sup>/CaM control and the presence of an AI elsewhere in the sequence, there is no homology at the amino acid level. The amino acid sequence of human NADPH P450 oxidoreductase (NCPR) is shown for comparison.

**FIG. 2. Detailed evaluation of the method will be published elsewhere.**
pair structures; this is probably approximated in iNOS by the
cognate sequence LDESGS. The insertion of six or seven resi-
dues relative to iNOS or P450 reductase causes the correspond-
ning sequence element to assume a different position; it now
forms one side of a terminal β hairpin structure. Removal of the
entire insertion (corresponding to within one residue to TAL-
GVIS in nNOS and PGGPPP in eNOS) should cause a rever-
sion to the P450 reductase-like structure if this is consistent
with the structural context. Partial deletions should cause
shortening of the β hairpin with potential disruption of the
local geometry because the shorter sequence cannot make the
same turn as the wild type parent. Note the large difference in
structure between the flanking homologous regions in the two
structures overlaid in Fig. 2; this might have less to do with the
insertion than with the differences in structurally adjacent
regions in the two enzymes shown and with the absence of the
FMN binding domain in the nNOS structure.

Fig. 3A shows optical spectra of preparations (partially pu-
rified by 2.5'-ADP affinity chromatography) of the G1074Y
and G1074D rat nNOS mutants as well as wild type enzyme.
G1074Y has an abnormal absorbance spectrum reflecting a
heme:flavin ratio of 1:4 rather than the 1:2 ratio of wild type
enzyme, whereas the spectrum of G1074D closely resembles
that of wild type nNOS. This is most obvious from the relative
size of the heme Soret band near 410 nm, indicative of a
mixture of high spin and low spin ferriheme, and the bands
near 480 nm from FAD and FMN. We have confirmed the loss
of heme by decomposition of the spectra into heme and flavin
components. This unusual and somewhat counterintuitive re-
sult implies that a mutation in the reductase domains reduced
the heme content while leaving the flavin content relatively
unchanged. CO difference spectra (not shown) indicate that
the ferrous CO complex of the remaining heme has its Soret max-
imum at 447 nm, indicating that the heme still has its native
axial thiolate ligand; little conversion to the denatured P420
form has occurred. This indicates that the remaining heme
is located in correctly folded oxygenase domains.

Further purification by gel filtration of nNOS constructs
which express significantly as holoenzyme resulted in prepa-
trations with UV-visible spectra very similar to wild type nNOS,
yields were comparable for G1074D and low (about one-quarter
normal) for G1074Y. Protein concentrations were measured by
several methods. The mM \(^{-1}\) extinction coefficients at 280 nm
were 175 for nNOS holoenzyme and 70 for the reductase do-
main from assays for tetrahydrobiopterin-free preparations or
for spectra from which the biopterin contribution had been
subtracted, which agreed surprisingly well with calculations
based on the content of aromatics. As shown in Table I, mu-
tants that express significantly as holoenzyme have close to one
heme/monomer. G1074Y has slightly lower heme content even
in preparations that have been further purified by gel filtra-
tion. Because all of the nNOS mutants have nearly a full
complement of flavin cofactors, very few oxygenase sites are
associated with flavin-deficient reductase units, and the meas-
ured activities do not reflect trivial effects based on flavin
content.

The effects on enzyme activity and Ca\(^{2+}\)/CaM control of
G1074Y and G1074D nNOS mutations were similar. Although
the SI appears well placed to interact with the CaM binding
region, the calcium dependence of activation is unchanged.
However, the NO synthase activity of both mutants, based on
heme content as measured at 447 nm in the CO difference
spectrum, is only about two-thirds that of the wild type enzyme
on a per heme basis. The activities of the mutants are summa-
ized in Table I.

Deletion of half of the SI (TAL corresponding to 1071–1073 in
nNOS) produced an effect on the spectral properties of the
expressed protein analogous to the substitution of Tyr for Gly;
in this case the heme:flavin ratio is reduced only to about 1:2.4
(Table I) (spectrum not shown). The calcium dependence was
unaltered. Unlike the Tyr mutant, this mutant had wild type
activity on a per heme basis. Deletion of the entire SI produced
a spectrally similar nNOS mutant with low activity on a per
heme basis.

Cytochrome c reduction by NOS isoforms has been assumed
to occur primarily through FMN; recently conducted experi-
ments with an nNOS shielding residue mutant lacking FMN
support this view.\(^2\) Cytochrome c reduction is therefore a mea-
sure of electron transfer through the reductase domains, which
does not require the oxygenase component. It is activated by

---

**Small Insertion Function in the Control of NO Synthases**

Fig. 2 Overlay of structures of NADPH P450 oxido-reductase (dark blue trace with selected β strands in light blue with ribbon) and a two-domain structure consisting of the FAD and NADPH binding domains of nNOS (cyan trace with selected β strands in red-brown). The SI is in white in the upper right. To emphasize the relative positions of the structure containing the SI in nNOS and its cognate in NADPH P450 oxido-reductase and Glu-1068 in nNOS are marked in deep red in both cases and following residues Ser-354 in yellow.
CaM binding but does not strictly correlate with NO production. The cytochrome c reduction experiments presented here correspond to the low salt experiments of Knudsen et al. (32).

The effects of SI mutations on cytochrome c reduction rates in nNOS are shown in Fig. 4. The G1074Y mutant has a slightly reduced rate of cytochrome c reduction consistent with its lower rate of NO production. This suggests that the low rate of NO production is the result of slow electron delivery. The enhancement of cytochrome c reduction by CaM is comparable (about 5-fold) in wild type nNOS and all mutants except the full SI deletion, which has very low cytochrome c reduction activity. This is in sharp contrast to AI mutants, which tend to lose CaM sensitivity (e.g. 23).

Mutants of eNOS—In contrast to nNOS, the SI in eNOS consists entirely of proline and glycine residues unless the flanking residue serine 838 is considered. The eNOS SI was less tolerant of mutations than the nNOS SI, probably because of rigidity imposed by the prolines. In general, 20% of wild type expression levels would produce more than enough enzyme for the purposes of this paper, but most of the mutants produced only about 10% heme-containing protein as judged by the small peak in the Soret region; we could detect no CO difference spectra in these preparations at 450 nm (cysteinyl ligand intact) or 420 nm (denatured form).

Neither the G841Y nor G841E mutant of bovine eNOS was significantly expressed as a heme protein, in contrast to their nNOS cognates. The lower heme:flavin ratio observed in G1074Y rat nNOS was reflected in an exaggerated form in the eNOS mutants, which could be partially purified as flavoproteins on a 2',5'-ADP column. The optical spectra of these preparations are compared with wild type eNOS in Fig. 3B. The reductase domains of these mutants are obviously inactive in NO production, but they exhibit uncoupled NADPH oxidation (based on NADPH oxidation by O_2 during Griess assays; data

![nNOS Mutant Spectra](image1)

![eNOS Mutant Spectra](image2)

**Fig. 3.** UV-visible absorbance spectra of wild type NOS enzymes and mutants, showing contributions from the heme and flavin cofactors. A, spectra isolated from wild type nNOS and the G1074Y and G1074D mutants; compare the heme Soret band near 410 nm with the FAD and FMN features in the 450–500 nm region. B, spectra isolated from wild type eNOS and the corresponding G841Y and G841D mutants; both mutants are almost completely heme-free.

| Enzyme                  | Heme:flavin ratio | Nitrite production | Heme content/monomer |
|-------------------------|-------------------|--------------------|----------------------|
| Wild type nNOS          | 1:1.9             | 292.2              | 0.9                  |
| G1074D mutant           | 1:2.1             | 179.8              | 0.9                  |
| G1074Y mutant           | 1:4.0(a)          | 186.2              | 0.85(c)              |
| TAL deletion mutant     | 1:3.2(b)          |                    |                      |
|                         | 1:2.4(a)          |                    |                      |
|                         | 1:2.1(b)          |                    |                      |

**Table I**

Some properties of nNOS mutants expressed in ER2566 cells and partially purified using 2',5'-ADP affinity chromatography

Nitrite production was measured with a Griess assay on the basis of heme content; NOS concentration in mg/ml was obtained by multiplying the spectrophotometrically determined heme content by the molecular mass of full-length nNOS. Heme contents were determined to an error of 0.05 assuming 100% purity of holoenzyme preparations. (a), affinity column only; (b), additional sizing column; (c), estimated from 280 and Soret bands.
not shown) at two to three times the rate of wild type eNOS. When further purified by gel filtration, the spectra of the Gly-841 deletion mutant closely resembled wild type enzyme. We calculated a minimum heme:monomer ratio of 0.5–0.6 assuming 100% purity; the extinction coefficients of 150 and 75 for eNOS holoenzyme and reductase domain agree well with expected values based on aromatic contents, subject to interference by tetrahydrobiopterin. The flavin content of affinity-purified preparations was about 1.7 flavins/heme. Mutants that did not express significantly as holoenzyme could be partially purified by affinity chromatography followed by gel exclusion. Total flavin:monomer ratio ranged from 0.5 to 1 assuming 100% purity. The activities and spectra of these constructs indicate that most of the flavin is present in molecules with one FMN and one FAD.

As mentioned previously, Knudsen et al. (32) reported the effects of SI deletion in eNOS; calcium dependence was affected, but neither the cytochrome c nor the NO production activities were significantly affected, apart from a modest change in salt dependence. The eNOS mutants produced here (except for the Gly-841 deletion, which is similar to wild type) are CaM-insensitive in all assays, but this may result from exposure of the CaM binding site to proteolysis rather than loss of control because of the removal or mutation of the SI. The cytochrome c reduction data for selected eNOS mutants is summarized in Fig. 5. The pattern of activity is otherwise similar to that observed in nNOS mutants; the tyrosine mutant has low activity, whereas the G841D mutant and the Gly-841 deletion have slightly higher than wild type rates of cytochrome c reduction. It is unlikely that G841Y has a different cofactor distribution than the other mutants because it is spectrally similar, and the flavins are reducible with NADPH. Since the original submission of the manuscript, we have produced an eNOS SI deletion mutant that differs slightly from the Knudsen et al. (32) construct in that the flanking regions of our deletion are not iNOS-derived. We have thus far obtained only the reductase domains, which are highly controlled by CaM and, unlike the Knudsen et al. (32) construct, hyperactive in the reduction of cytochrome c. Clearly, small details can have large effects.

The G841D mutant was essentially the same as the Gly-841 deletion on a per flavin basis, although lack of heme suggested that it was expressed as a reductase domain preparation, and the Gly-841 deletion is expressed as holoenzyme. The absolute rates are therefore not directly comparable. The G841Y mutant, also hemeless, had low activity and was CaM-insensitive, in contrast to the corresponding nNOS construct.

**Proteolysis of Mutants and iNOS**—The low heme content in some mutants suggested that the enzyme was susceptible to proteolysis; in purified eNOS and nNOS the exposed CaM binding site is the most sensitive to proteolytic cleavage. Because the 2',5'-ADP affinity site is associated with the reductase domains, in vivo cleavage at the CaM site would produce flavin-bearing reductase domains without heme.

The iNOS holoenzyme, which lacks the SI, cannot be purified without CaM coexpression (39). When we expressed iNOS without CaM coexpression and fractionated the extract on a 2',5'-ADP affinity column, we obtained a yellow flavoprotein preparation spectroscopically indistinguishable from the heme free eNOS mutants. The dominant proteins in 2',5'-ADP affin-
Discussion

Previously identified regions involved in the control of NO synthesis by Ca\(^{2+}\)/CaM have either been CaM binding sites or elements involved in suppressing activity in the absence of Ca\(^{2+}\)/CaM. The SI is clearly correlated with control in the evolution of NOS and is structurally positioned to interact with established control elements. The authors of the previous mutagenic study suggested that the SI functions as a secondary autoinhibitory element, but the information obtained in the studies presented here indicate that the role of the SI is more complex and may even differ among isoforms. Unlike the AI and the C-terminal tail, modification, truncation, or deletion of the SI does not consistently result in activation of the enzyme. Although AI deletion results in increased activity and reduction of calcium sensitivity, SI modifications result in less straightforward changes in the activity of NO synthesis.

Typically, –AI mutants synthesize NO at low Ca\(^{2+}\) concentrations and have high levels of cytochrome c reductase activity in the absence of CaM. In contrast, –SI mutants require CaM for optimal cytochrome c reductase activity as well as NO synthesis, except in cases in which the CaM binding site has been exposed to cleavage, producing flavoprotein expression. We point out that changes in the calcium dependence of mutants in positions adjacent to the CaM binding site can result from interactions with control elements that function as activators as well as displacement of inhibitors because these changes merely reflect the necessity of doing work on a protein structural element during CaM binding.

Intentional disruption of the structure of the SI with incompatible substitutions causes significant loss of NO synthesis activity in NOS, whereas deletion of a major portion of the SI had a much smaller effect. This suggests that the functions of the SI are ancillary rather than essential for activity; eNOS with a SI deletion or nNOS with a reduced SI can still function, but a disrupted SI can interfere with activation. It is certainly possible that a slightly different full SI deletion in nNOS would also be fully active.

In this context it is worth considering that wild type NOS enzymes lacking a SI are active as long as they also lack an AI. When we designed these mutants we thought it possible that the AI and SI acted cooperatively and that the SI might be needed for AI-mediated inhibition (e.g., as a lock and clasp). The data presented here strongly argue against this view and suggest that instead the SI may function as an accessory element because SI mutants may either positively or negatively affect activity.

Several hypotheses can be constructed for an ancillary role in control. In a direct activation hypothesis, the SI interacts directly with CaM to shift the domain alignment equilibrium, allowing the conformational changes needed for electron transfer to the active site. The SI might also act to destabilize an inactive, conformationally restricted state. It is clear that any such function must represent an enhancement of a mechanism that is operational without the SI because eNOS and nNOS tolerate its removal and/or truncation.

Our recent proposal of a tethered shuttle mechanism for NOS electron transfer and control (40) provides a context for these studies. In this model the FMN binding domain shuttles between FAD and heme facing states, both of which bind CaM.CaM facilitates the release of the FMN domain from the reductase complex, where it is in close association with the FAD and NADPH binding domains. The release of the reduced FMN binding domain allows cytochrome c reduction but is not sufficient to allow NO production. In holoenzyme, realignment of the FMN binding domain, also CaM-facilitated, is necessary for subsequent electron transfer into the oxygenase domain to support catalysis. Since the original submission of this manuscript, a related paper has been published describing differential activation of eNOSs and iNOS by CaM chimera, which supports this interpretation of CaM activation (41).

The evolutionary ancestors of the NOS reductase domains existed as separate proteins closely related to ferredoxin NADPH reductase and flavodoxin, and in these ancestral electron transfer systems ferredoxin/flavodoxin functioned as a shuttle. The FMN binding domain is essentially a ferredoxin tethered to the two-domain reductase unit. In reductase systems in which one component acts as a shuttle, it is common to observe maximum activity at a salt concentration that allows formation of binary complexes for electron transfer (usually optimized at low salt) but does not produce complexes with
such slow dissociation rates (optimized at high salt) that the dissociation rate limits the shuttle (42). Salt-inhibited shuttles (iNOS) are characterized by relatively weak interactions, whereas salt-stimulated shuttles (eNOS) are characterized by stronger interactions.

The eNOS–SI mutants studied by Knudsen et al. (32) were described in terms of SI inhibition which was “masked” by the AI and salt. Cytochrome c reduction by these mutants at low KCl is not significantly different from the corresponding activity of the parents; at high salt the –SI mutant has somewhat lower cytochrome c reductase activity, and the –SI–AI mutant somewhat higher cytochrome c reductase activity, than their parents. Under the conditions that produce enhanced cytochrome c reduction, NO production is lower than or at best equal to that of their parents. Enhanced NO production in –SI and –SI–AI mutants with respect to the parent wild type and –AI mutant eNOS enzymes is only observed at low salt, which must be unrelated to enhanced cytochrome c reduction at high salt. This suggests that steady-state cytochrome c reduction by eNOS and all –AI and –SI–AI mutants is limited at low salt by the dissociation of a tight complex which is stabilized by the AI but not the SI and at high salt by interactions with cytochrome c which can be modestly enhanced by SI removal only in the –AI construct.

Salt effects on the activity of NOS isoforms were studied independently by two groups (43, 44); the observations were generally similar in that cNOSs were found to be stimulated by moderate salt concentrations comparable with those used by Knudsen et al. (32) and inhibited as the salt concentration was further increased. Schrammel et al. (43) provided data for eNOS and iNOS as well as nNOS and reported that for iNOS cytochrome c reduction and NO synthesis were monotonically inhibited by salt. At least part of the salt inhibition of cytochrome c reduction was attributed to their inability to saturate with cytochrome c at high salt. Schrammel et al. (43) and Nishimura et al. (44) differ with respect to nNOS cytochrome c reduction, which the former report to be monotonically inhibited and the latter initially stimulated by salt. It is likely that the modest gain in cytochrome c reductase activity in the –SI–AI eNOS mutant represents a $K_m$ effect with respect to cytochrome c.

NO formation is not rate-limited by the process that limits cytochrome c reduction. In wild type eNOS it is slightly enhanced, but in the –SI and –AI–SI mutants NO formation is significantly slower at 0.2 mM KCl. This suggests the participation of a second complex characterized by weaker interactions in the –SI mutants; clearly, this must be an internal complex involving associations between NO domains.

Interpretation of salt effects is complicated further by the effects on substrate binding and NO dissociation (44). It is of interest that a slightly different SI deletion produces a mutant hyperactive in cytochrome c reduction but, unlike AI deletions, strongly CaM-dependent.

The SI is located in the hinge subdomain, which interacts with all three reductase domains (FMN, FAD, and NADPH binding). The position of the SI indicates that it is in direct contact with bound CaM, and at the same time other residues in the subdomain are hydrogen-bonded both to residues in the other domains and directly to NADP. Although we are not confident enough in the details of models based on incomplete domain structures to assign specific interactions on the FMN binding domain or bound CaM to the SI, its displacement by CaM will clearly affect FMN domain mobility because it forms the terminus of a β hairpin, which forms a three-stranded β structure with the polypeptide strap linking the FMN and FAD binding domains. In this regard it may serve as an amplifier of CaM-driven conformational effects. At the same time, CaM-driven conformational effects on the hinge subdomain are likely to be transmitted to the NADPH binding site through this strap, linking CaM binding, conformation, and nucleotide binding.

The SI has at least one function indirectly related to control; eNOS and nNOS are resistant to proteolysis in cells even without bound CaM, whereas iNOS cannot survive in proteolytically deficient E. coli without CaM coexpression. The increased sensitivity of the CaM binding region in eNOS and nNOS SI mutants implies that the proximity of the SI to the CaM binding region provides some protection to the enzyme from degradation by proteases. It is possible that this is a major function of the SI in cNOS, although it will be necessary to express these mutants in mammalian cells to determine whether the compartmentalization of activities is sufficient to protect the CaM site in SI-deficient enzymes.

It is obvious that the protective effect of CaM on iNOS is exerted largely by protecting the protease-sensitive CaM binding site. It should be possible to produce intact versions of some mutants which are otherwise produced as reductase fragments by coexpression with CaM, much as iNOS is produced in recombinant systems. In addition, the results presented here suggest the possibility of producing full-length iNOS holoenzyme without CaM coexpression by introducing an SI from eNOS or nNOS. Studies are under way to test these hypotheses.

REFERENCES

1. Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E., and Chaudhuri, B. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 9265–9269.
2. Puchgott, H. F. (1988) in Vasodilatation: Vascular Smooth Muscle, Peptides, Autonomic Nerves, and Endothelium (Vanhoutte, P.M., ed) pp. 401–414, Raven Press, New York.
3. Palmer, R. M. J., Ferrige, D. G., and Moncada, S. (1987) Nature 327, 542–546.
4. Garthwaite, J., Charles, S. L., and Chess-Williams, R. (1988) Nature 336, 385–388.
5. Abu-Soud, H. M., and Stuehr, D. J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10769–10772.
6. Hauschult, S., Lucknoff, A., Mulech, A., Kohler, J., Besenov, L., and Buse, R. (1990) Biochem. J. 270, 351–356.
7. Knowles, R. G., Palacios, M., Palmer, R. M. J., and Moncada, S. (1990) Biochem. J. 269, 207–210.
8. Curran, R. D., Billiar, T. R., Stuehr, D. J., Hofmann, K., and Simmons, R. L. (1989) J. Exp. Med. 168, 1769–1774.
9. Nishimura, A., and Curran, R. D. (1989) Biochem. J. 262, 293–296.
10. Masters, B. S. (1994) Annu. Rev. Nutr. 14, 131–145.
11. Breit, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. S., and Snyder, S. H. (1991) Nature 351, 714–718.
12. Breit, D. S., Ferris, C. D., and Snyder, S. H. (1992) J. Biol. Chem. 267, 10976–10981.
13. Mayer, B., John, M., Heimel, B., Werner, E. R., and Wachtler, H. (1991) FEBS Lett. 288, 187–191.
14. Jassoum, S. P., Shimouchi, A., Quintermos, T., Blech, D. B., and Blech, D. K. (1992) J. Biol. Chem. 267, 6370–6374.
15. Lamas, S., Marsden, P. A., Li, G. K., Tempest, P., and Michel, T. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6348–6352.
16. Lyons, C. R., Orloff, G. J., and Cunningham, J. M. (1992) J. Biol. Chem. 267, 6370–6374.
17. McMillan, K., Breit, D. S., Hirsch, D. J., Snyder, S. H., Clark, J. E., and Masters, B. S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11141–11145.
18. Xu, Q., Cho, H. J., Calacyk, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., Ding, A., Truo, T., and Nathan, C. C. (1992) Science 256, 225–228.
19. Stuehr, D. J., and Ikeda-Saito, M. (1992) J. Biol. Chem. 267, 20547–20550.
20. White, K. A., and Marletta, M. A. (1992) Biochemistry 31, 6627–6631.
21. Sheta, E. A., McMillan, K., and Masters, B. S. (1994) J. Biol. Chem. 269, 15147–15153.
22. Salerno, J. C., Harris, D. E., Irizarry, K., Patel, B., Morales, A. J., Smith, S. M., Martasek, P., Roman, L. J., Masters, B. S., Jones, C. L., Weissman, B. A., Lane, P., Liu, Q., and Gross, S. S. (1997) J. Biol. Chem. 272, 29769–29777.
23. Salerno, J. C. (July 12, 1996) U.S. Patent 6,150,500.
24. Nishida C. R., and Ortiz de Montellano, P. R. (1999) J. Biol. Chem. 274, 14692–14699.
25. Chen, P. F., and Wu, K. K. (2000) J. Biol. Chem. 275, 13155–13163.
26. Roman, L. J., Martasek, P., Miller, B., Harris, D. E., de la Garza, M. A., Shea, T. M., Kin, J. J., and Masters, B. S. (2000) J. Biol. Chem. 275, 29225–29232.
27. Fulton D., Gratton, J. P., McCabe, T. J., Fontana, J., Fuyio, Y., Walsh, K., Franke, T. F., Papapetroupooulos, A., and Sessa, W. C. (1999) Nature 399, 597–601.
28. Lane, P., and Gross, S. S. (2002) J. Biol. Chem. 277, 19087–19094.
29. Adak, S., Sharma, M., Meade, A. L., and Stuehr, D. J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 13516–13521
30. Craig, D. H., Chapman, S. K., and Daff, S. (2002) J. Biol. Chem. 277, 33987–33994
31. Zhang, J., Martasek, P., Paschke, R., Shea, T., Masters, B. S. S., and Kim, J. J. P. (2001) J. Biol. Chem. 276, 37506–37513
32. Knudsen, G. M., Nishida, C. R., Mooney, S. D., and Ortiz de Montellano, P. R. (2003) J. Biol. Chem. 278, 31814–31824
33. Martasek, P., Liu, Q., Liu, J., Roman, L. J., Gross, S. S., Sessa, W. C., and Masters, B. S. (1996) Biochem. Biophys. Res. Commun. 219, 359–365
34. Gross, S. S. (1996) Methods Enzymol. 268, 159–168
35. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, A. J. (1951) J. Biol. Chem. 193, 265–275
36. Wang, W. Y., and Malcolm, B. A. (1999) BioTechniques 26, 680–682
37. Hainu, M., McManus, M. E., Birkett, D. J., Lee, T. D., and Shively, J. E. (1989) Biochemistry 28, 8639–8645
38. Wang, M., Roberts, D. L., Paschke, R., Shea, T. M., Masters, B. S., and Kim, J. J. P. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8411–8416
39. Fusetta, J. D., Niu, X. D., Lunn, C. A., Zavadny, P. J., Narula, S. K., and Lundell, D. (1996) FEBS Lett. 376, 135–138
40. Ghosh, D. K., and Salerno, J. C. (2003) Frontiers Biosci. 8, D193–D209
41. Spratt, D. E., Moshier, J., Cheyne, B., Montgomery, H. J., Wilson, D. L., Weinberg, J. B., Smith, S. M. E., Salerno, J. C., Ghosh, D. K., and Guillemette, J. G. (2004) J. Biol. Chem. 279, 33547–33557
42. Lambeth, J. D., Seybert, D. W., Lancaster, J. R., Salerno, J. C., and Kamin, H. (1982) Mol. Cell. Biochem. 45, 13–31
43. Schrammel, A., Gorren, A. C., Stuehr, D. J., Schmidt, K., and Mayer, B. (1998) Biochim. Biophys. Acta 1387, 257–263
44. Nishimura, J. S., Narayanasami, R., Miller, R. T., Roman, L. J., Panda, S., and Masters, B. S. (1999) J. Biol. Chem. 274, 5399–5406
