Subunit Interactions of Endothelial Nitric-oxide Synthase

COMPARISONS TO THE NEURONAL AND INDUCIBLE NITRIC-OXIDE SYNTHASE ISOFORMS

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Endothelial nitric-oxide synthase (eNOS) is comprised of two identical subunits. Each subunit has a bidomain structure consisting of an N-terminal oxygenase domain containing heme and tetrahydrobiopterin (BH4) and a C-terminal reductase domain containing binding sites for FAD, FMN, and NADPH. Each subunit is also myristoylated and contains a calmodulin (CaM)-binding site located between the oxygenase and reductase domains. In this study, wild-type and mutant forms of eNOS have been expressed in a baculovirus system, and the quaternary structure of the purified enzymes has been analyzed by low temperature SDS-PAGE. eNOS dimer formation requires incorporation of the heme prosthetic group but does not require myristoylation or CaM or BH4 binding. In order to identify domains of eNOS involved in subunit interactions, we have also expressed eNOS oxygenase and reductase domain fusion proteins in a yeast two-hybrid system. Corresponding human neuronal NOS (nNOS) and murine inducible NOS (iNOS) fusion proteins have also been expressed. Comparative analysis of NOS domain interactions shows that subunit association of eNOS and nNOS involves not only head to head interactions of oxygenase domains but also tail to tail interactions of reductase domains and head to tail interactions between oxygenase and reductase domains. In contrast, iNOS subunit association involves only oxygenase domain interactions.

Three distinct isoforms of nitric-oxide synthase (NOS) have been identified in mammalian tissues. Although none of these enzymes has an absolutely tissue-specific pattern of expression, they are commonly referred to as endothelial NOS (eNOS), neuronal NOS (nNOS), and macrophage or inducible NOS (iNOS). eNOS, nNOS, and iNOS catalyze the oxidation of L-arginine to produce NO and L-citrulline via a similar or identical reaction mechanism involving several cofactors or prosthetic groups (1). In accord with their common catalytic function, the NOS enzymes possess many common features including 50–60% amino acid sequence identity and an α2 quaternary structure (2). In addition, all three types of NOS monomers have a bidomain structure consisting of an N-terminal oxygenase domain that contains iron protoporphyrin IX (heme) and tetrahydrobiopterin (BH4) and binds L-arginine and a C-terminal reductase domain that contains binding sites for FAD, FMN, and NADPH (3–6). Each domain can exist and function independently. The isolated oxygenase domain of iNOS can thus form dimers while the isolated reductase domain catalyzes cytochrome c reduction at a rate equivalent to that of native dimeric iNOS (4). The isolated oxygenase and reductase domains of eNOS can be reconstituted in vitro to catalyze production of NO and L-citrulline from L-arginine (6). The three NOS enzymes, therefore, have many similarities in structure and catalytic function. However, because each of the enzymes has a different physiological role and tissue distribution, the molecular mechanisms for regulation of eNOS, nNOS, and iNOS can differ significantly (7).

All three NOS isoforms are dimeric enzymes comprised of two identical subunits. iNOS and nNOS are catalytically active only in dimeric form (8–15). Regulation of NOS subunit interactions could, therefore, provide a mechanism for modulation of enzyme activity in vivo. The cofactors, prosthetic groups, domains, and amino acid residues of iNOS important for subunit interactions have been studied in detail (4, 9, 12–17). Certain features of eNOS and nNOS oligomerization have also been investigated (10, 18–20). Studies of eNOS suggest that this isoform may be different from the other NOS isoforms in terms of its requirements for dimer formation. For example, whereas all of the determinants for iNOS dimerization are thought to be contained in the oxygenase domain of the enzyme (4, 16, 17), eNOS dimer formation has been reported to involve both oxygenase and reductase domains (18). Moreover, while it is now well established that binding of BH4 plays a critical role in formation of the iNOS dimer (8, 9, 12–14), recent evidence suggests that BH4 may not be required for eNOS dimer formation (20).

To date, a comprehensive investigation of the cofactors, prosthetic groups, domains, and amino acid residues of eNOS involved in oligomerization has not been carried out. Furthermore, conclusions about the differences and similarities in dimerization requirements of NOS isoforms can currently be made based only on individual studies of a single isoform. Comparative analysis of all three isoforms in the same experimental model system has not been performed. In this study, therefore, we have investigated eNOS dimerization requirements by expression and purification of wild-type and mutant forms of the bovine enzyme in a baculovirus/Sf9 insect cell system. In addition, we have investigated interactions of eNOS oxygenase and reductase domains in a yeast two-hybrid system. Human nNOS and murine iNOS have been expressed in the same two systems allowing direct comparisons to be made of all three NOS isoforms.

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1 The abbreviations used are: NOS, nitric-oxide synthase; NO, nitric oxide; eNOS, endothelial NOS; nNOS, neuronal NOS; iNOS, inducible NOS; BH4, tetrahydrobiopterin; CaM, calmodulin; DAHP, 2,4-diaminoptereic acid; L-arginine; L-citrulline; NOS-catalyzed pyridoxal-5'-phosphate aminolysis; LT-PAGE, low temperature SDS-polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; BAEC, bovine aortic endothelial cell(s).
Materials—S9 insect cells, baculovirus transfer vector pVL1393, and Baculogold™ viral DNA were obtained from Pharmingen (San Diego, CA). Bovine aortic endothelial cells (BAEC) were purchased from Cell Systems Inc. (Kirkland, WA). Monoclonal antibodies to eNOS and iNOS were purchased from Transduction Laboratories (Lexington, KY). AG-1-XW-XS cation exchange resin was obtained from Bio-Rad, and L-[1-14C]arginine and ECL reagents were purchased from Amersham. Corp. 2'-5'-ADP-Sepharose was from Pharmacia Biotech Inc. pGBK7 (DNA binding domain hybrid cloning vector), and pGAD424 (activation domain hybrid cloning vector), and Saccharomyces cerevisiae SFY526 were purchased from Clontech (Palo Alto, CA). Chlorophenol red-D-galactopyranoside came from Boehringer Mannheim and (6R)-5,6,7,8-tetrahydro-1-biotin came from Research Biochemicals International. Oligonucleotide primers for PCR, Protein A and G agarose and immunoprecipitant were purchased from Life Technologies, Inc.

Plasmid Construction—Baculovirus pVL1393 plasmid transfer vectors encoding wild-type bovine eNOS, wild-type murine iNOS, wild-type human nNOS, (−myr) eNOS, and eCam eNOS have been described previously (21, 22). The (−hem) eNOS mutant in pVL1393 was created by mutating the cysteine 186 codon (TGC) in the wild-type bovine eNOS cDNA to an alanine codon (GCC) using the PCR-based splicing by overlap extension technique (23). cDNA constructs encoding hybrids of the NOS oxygenase and reductase domains and the GAL4 DNA binding and activation domains were created by subcloning into the shuttle/ expression vectors, pGBK7 and pGAD424 (Clontech). DNA sequences encoding bovine eNOS residues 1–505 and 506–1205, murine iNOS residues 1–1498 and 499–1144, and human nNOS residues 1–744, 239–749, 240–749, and 15–749 were generated by PCR amplification using primers encoding eNOS, wild-type iNOS, and wild-type nNOS full-length cDNAs, respectively. PCR primers were designed to incorporate 5′ EcoRI and SfI restriction sites into amplified products for subcloning. G442A and A445I point mutations in eNOS fusion constructs were created using the overlap extension technique to replace the glycine 442 (GCC) codon and the alanine 445 (GCC) codon with alanine (GCC) and isoleucine (ATC) codons, respectively. G450A and A453I mutants of iNOS fusion constructs replaced glycine 450 (GGC) with alanine (GCC) and alanine 453 (ATC) with isoleucine (ATA). All DNA constructs were sequenced in the Molecular Biology Core Facility of the Institute for Molecular Medicine and Genetics, Medical College of Georgia, using a Perkin-Elmer ABI 377 automated DNA sequencer. Sequence analysis confirmed that the overlap extension technique to replace the glycine 442 (GCC) codon and the alanine 445 (GCC) codon with alanine (GCC) and isoleucine (ATC) codons, respectively. G450A and A453I mutants of iNOS fusion constructs replaced glycine 450 (GGC) with alanine (GCC) and alanine 453 (ATC) with isoleucine (ATA). All DNA constructs were sequenced in the Molecular Biology Core Facility of the Institute for Molecular Medicine and Genetics, Medical College of Georgia, using a Perkin-Elmer ABI 377 automated DNA sequencer. Sequence analysis confirmed that only the desired mutations were incorporated into the various constructs and that in-frame fusions were created by sequences encoding the hybrid proteins.

Expression and Purification of Wild-type and Mutant NOS Proteins—Expression of NOS proteins in the baculovirus/S9 insect cell system and purification of the proteins by 2′,5′-ADP-Sepharose was carried out as described previously (21, 22). Enzyme activity was determined by monitoring the rate of formation of L-[14C]citrulline from L-[14C]arginine under the conditions described previously (21).

RESULTS AND DISCUSSION
Quantary Structure of Wild-type eNOS, nNOS, and iNOS Expressed and Purified from a Baculovirus System—The eNOS protein has been shown previously to elute anomalously from gel filtration columns (26), a property that precludes analysis of eNOS quaternary structure by conventional means. Like nNOS and iNOS, however, eNOS has the capacity to oligomerize (18). Moreover, the eNOS oligomer is a dimer rather than a higher order oligomer as shown recently by Rodriguez-Crespo et al. (20) using LT-PAGE. In this previous study (20), bovine eNOS was overexpressed in Escherichia coli where it was found in a 50:50 monomer-dimer equilibrium. It is not clear, however, whether such an equilibrium exists when the enzyme is expressed in a eukaryotic expression system or when naturally expressed in endothelial cells. To address this question, we have expressed wild-type bovine eNOS in a eukaryotic baculovirus/S9 insect cell expression system and purified the enzyme to >95% homogeneity by affinity chromatography on 2′,5′-ADP-Sepharose. Purified protein was suspended in SDS sample buffer (to a final concentration of 3% SDS and 7% 2-mercaptoethanol) and incubated for 30 min at 0, 20, 30, 40, 50, and 60 °C. Dimeric and monomeric forms of the enzyme were then separated by LT-PAGE and visualized on gels by Coomassie staining. As shown in Fig. 1A, eNOS migrated on LT-PAGE almost entirely as a dimer if samples were not heated before running the gel. The critical temperature for subunit dissociation under these denaturing conditions was 30–40 °C. In contrast to the prokaryotic expression system (20), therefore, expression of eNOS in eukaryotic cells results in close to 100% of the enzyme forming stable, SDS-resistant homodimers. Furthermore, eNOS is found entirely in dimeric form, not only when expressed and purified from the baculovirus system but also when naturally expressed in endothelial cells. BAEC lysates were also analyzed by LT-PAGE. Monomeric and dimeric eNOS expressed in BAEC were visualized on LT-PAGE by immunoblotting with anti-eNOS antibody. As shown in Fig. 1B, eNOS in BAEC exists under basal conditions exclusively in dimeric form. It is unlikely, therefore, that cofactor or substrate induction of dimer formation functions as a regulatory mechanism for increasing eNOS activity in endothelial cells. In this respect, eNOS differs from iNOS, which is 50–75% monomeric in macrophages (9, 14) and is thus thought to be regulated in part by factors that promote dimer assembly (9). A recent report by Kiltti et al. (10) has shown that BH₄ and L-arginine synergistically convert purified porcine nNOS from a monomeric to dimeric form. In addition, cofactor and amino acid substrate binding together increase the stability of the dimer in a cooperative manner. To determine whether dimeric eNOS is similarly stabilized by these two effectors, purified eNOS enzyme was preincubated with either BH₄ (0.1 mM) or L-arginine (1 mM) alone or the combination of the two compounds. Preincubated proteins were then suspended in SDS
monomer. Failure to detect dimeric iNOS on gels was probably due to both subunit dissociation during purification and to inability of the purified dimer to remain intact in denaturing sample buffer containing 3% SDS. That the enzyme was purified in at least partially dimeric form is indicated by our finding that purified enzyme had significant catalytic activity (~300 nmol of citrulline produced/mg/min) prior to suspension in denaturing buffer. Monomeric iNOS has been shown previously to be completely inactive (8, 9, 12–15). Dissociation of the iNOS dimer in SDS sample buffer is suggested further by our failure to detect dimeric iNOS on LT-PAGE even after preincubation of the purified enzyme with the combination of L-arginine (1 mM), BH4 (0.1 mM), and hemin chloride (4 μM), conditions that have been shown previously to induce iNOS dimer formation in non-denaturing buffers (9).

Expression of iNOS in the same eukaryotic expression system as eNOS and nNOS demonstrates that the iNOS dimer is the least stable of the NOS dimers. This is consistent with previous reports that iNOS loses its heme prosthetic group and dissociates into inactive monomers in the absence of BH4 and L-arginine (8, 9). Under the same conditions, nNOS and eNOS retain their dimeric structure (10, 19, 20). However, because a significant portion of the nNOS oligomer (but not the eNOS oligomer) dissociates either during purification or upon suspension in SDS sample buffer and because the eNOS dimer has a higher critical temperature for dissociation than the nNOS dimer has, we conclude that the order of stability of NOS oligomers is eNOS > nNOS > iNOS.

Quaternary Structure of Mutant Forms of eNOS Expressed and Purified from a Baculovirus System—Only BH4 (of all eNOS cofactors, prosthetic groups, and substrates) stabilizes the eNOS dimer after it has been formed. Certain ones of these factors, however, may be necessary for assembly of the dimer from its monomeric subunits in vivo. To gain further insight into which factors are involved in dimer assembly, we have expressed several mutant forms of eNOS in the baculovirus system. Expressed proteins were purified by 2',5'-ADP-Sepharose and analyzed by LT-PAGE. In a recent report, Hellerman and Solomonson (27) have concluded that binding of Ca2+/CaM by eNOS is a requirement for enzyme dimerization. In the present study, however, we have found that eNOS purified from the baculovirus system is 98% dimeric. Because the protein was purified under conditions (2 mM EGTA) that yield an enzyme that is 100% dependent on exogenous Ca2+/CaM for activity (21, 22), it is unlikely that the purified dimer contains any bound CaM. CaM binding, therefore, appears not to be a prerequisite for dimerization. In order to confirm this conclusion, we have expressed a ΔCaM mutant of bovine eNOS in the baculovirus system. This protein, which has been characterized previously (21), lacks a CaM-binding domain because residues 493–512 have been deleted from the cDNA coding sequence. ΔCaM eNOS was purified and analyzed by LT-PAGE. Like

FIG. 1. Thermal stability of the eNOS dimer expressed in SF9 insect cells and in bovine aortic endothelial cells. A, wild-type bovine eNOS was expressed and purified from a baculovirus system and incubated in SDS sample buffer for 30 min at the indicated temperatures. Samples were subjected to LT-PAGE, and monomeric (Mono-eNOS) and dimeric (Di-eNOS) forms of eNOS were visualized by Coomassie staining. B, bovine aortic endothelial cell lysates were incubated in SDS sample buffer for 30 min at the indicated temperatures. Samples were subjected to LT-PAGE, and monomeric and dimeric forms of eNOS were visualized by immunoblotting with anti-eNOS antibody. Equivalent results were obtained in four different experiments.

FIG. 2. Effect of BH4 on thermal stability of purified eNOS. Wild-type bovine eNOS was expressed and purified from a baculovirus system and incubated for 10 min with BH4 (0.1 mM) at 37°C. Chilled SDS sample buffer was added, and samples were incubated for a further 30 min at the temperatures indicated. Proteins were separated on LT-PAGE, and monomeric (Mono-eNOS) and dimeric (Di-eNOS) forms of eNOS were visualized by Coomassie staining. Similar results were obtained in three experiments.
wild-type eNOS, ΔCaM eNOS migrated on LT-PAGE in 98% dimeric form (Table I). The critical temperature for dissociation of the mutant dimer was 30–40 °C.

Two additional eNOS mutants were expressed in the baculovirus system and analyzed by LT-PAGE. A myristoylation-deficient mutant of bovine eNOS, designated (–myr) eNOS, has also been characterized previously (21). This mutant, in which glycine 2 has been mutated to an alanine, differs from wild-type eNOS in that it is neither myristoylated nor palmitoylated and, consequently, is not membrane-associated (21, 28, 29). Like wild-type eNOS, purified (–myr) eNOS migrated on LT-PAGE in 98% dimeric form with a critical temperature for subunit dissociation of 30–40 °C (Table I). Neither fatty acylation nor membrane association of eNOS, therefore, appears to be required for dimer formation. A final mutant form of bovine eNOS to be analyzed was designated (–heme) eNOS. In this expressed protein, we have changed the cysteine 186 codon (TGC) to an alanine (GCC). Cysteine 186 of bovine eNOS is equivalent to the cysteine 184 heme ligand of human eNOS. Mutation of this residue has been shown to result in loss of incorporation into eNOS of the heme prosthetic group (30). Klatt et al. (19) have recently shown that heme-deficient wild-type nNOS is unable to form dimers. These authors concluded that incorporation of the heme prosthetic group into nNOS is a requisite for dimerization. In the present study, (–heme) eNOS expressed and purified from the baculovirus system was also found to exist exclusively in monomeric form when analyzed by LT-PAGE (Table I). Incorporation of the heme prosthetic group, therefore, appears to be a requirement for dimerization not only of nNOS but also of eNOS.

Role of BH₄ in eNOS Dimerization—Bovine eNOS expressed in E. coli lacks the posttranslational modifications of the native enzyme and is BH₄-free. This purified enzyme, however, migrates on LT-PAGE in 50% dimeric form (20). It has been concluded that BH₄ binding is not required for eNOS dimer formation and that eNOS is thus fundamentally different from both iNOS and nNOS (20). Whether BH₄ independent eNOS dimer formation occurs with native eNOS or whether this previous observation is an artifact of prokaryotic expression is not clear. We have, therefore, examined the BH₄ dependence of eNOS dimerization in the baculovirus expression system, where eukaryotic posttranslational modifications such as fatty acylation and phosphorylation are known to occur. Because the BH₄ binding site of eNOS has not yet been identified, expression of a BH₄-free mutant of eNOS in the baculovirus system was not feasible. In order to manipulate the BH₄ content of eNOS, we expressed the wild-type protein in the absence or presence of 10 mM 2,4-diamino-6-hydroxyprymidin (DAHP), an inhibitor of GTP cyclohydrolase I, the rate-limiting enzyme in BH₄ biosynthesis (31). It has been shown previously that DAHP treatment of SF9 cells expressing rat nNOS results in a reduction of the BH₄ content of the purified enzyme from 0.47 to 0.07 mol of BH₄/mol of subunit. Reduction of BH₄ content was correlated with a proportional decrease in the specific activity of the enzyme (32). Other investigators have also reported that activities of purified NOS enzymes are directly proportional to the amount of bound BH₄ with full activity requiring 1 mol of BH₄/mol of subunit (6, 33). In the present and previous (21) studies, purified eNOS was found to be 45–50% independent of exogenous BH₄ for activity. The purified enzyme expressed in the absence of DAHP, therefore, contains 0.45–0.50 mol of bound BH₄/mol of subunit. However, when the enzyme was expressed in the presence of DAHP, the purified protein was found to have only 3% of full activity in the absence of exogenously added BH₄, indicating that this enzyme preparation was virtually BH₄-free. Analysis of the BH₄-free enzyme on LT-PAGE showed that it was 98% dimeric (Table I). Thus, BH₄ binding is not required for eNOS dimer formation in the baculovirus system. Similar conclusions have been reported by Rodriguez-Crespo et al. (20) following expression of the enzyme in an E. coli expression system. These authors concluded further that eNOS is unique in this respect as compared with both iNOS and nNOS. In order to determine whether the BH₄ re-
Interactions between NOS oxygenase and reductase domains in a yeast two-hybrid system

Pairwise combinations of hybrid plasmids were used to cotransform SFY526 yeast cells. Cotransformants were assayed for β-galactosidase activity by the colony lift filter method using X-Gal as substrate and by liquid culture assay with chlorophenol red-β-D-galactopyranoside as substrate. β-Galactosidase activity is expressed in Miller units (25). Results shown are mean ± S.D. from three separate transformations.

| Binding domain hybrid | Activation domain hybrid | Colony color | β-Galactosidase activity |
|----------------------|--------------------------|--------------|-------------------------|
| nNOS (1–744)         | nNOS (1–744)             | Blue         | 149 ± 30                |
| nNOS (745–1433)      | nNOS (745–1433)          | Light blue   | 149 ± 30                |
| nNOS (1–744)         | nNOS (1–744)             | Blue         | 109 ± 7                 |
| nNOS (745–1433)      | nNOS (745–1433)          | Blue         | 124 ± 10                |
| nNOS (239–744)       | nNOS (239–744)           | Blue         | 113 ± 12                |

- **Table II**

Interactions of eNOS, nNOS, and iNOS Oxygenase and Reductase Domains in a Yeast Two-hybrid System—Previous studies have concluded that all of the determinants for iNOS dimer formation are contained in the oxygenase domain of the enzyme (4, 16, 17). However, evidence has been presented for eNOS that suggests that both the N-terminal oxygenase domain and the C-terminal reductase domain are involved in dimerization (20). At present, it is not known whether these divergent results reflect intrinsic differences between iNOS and eNOS oligomerization or whether they are due to differences in experimental approach. In order to gain additional insight into which domains of the NOS isoforms contribute to subunit interactions, we have utilized the yeast two-hybrid system developed originally by Fields and Song (34). The two-hybrid system takes advantage of the modular nature of the yeast GAL4 transcriptional activator. The GALA protein consists of a site-specific DNA-binding domain that is distinct from the domain responsible for transcriptional activation. Various proteins are expressed in the two-hybrid system as fusions (or hybrids) with either the GAL4 DNA-binding domain or the GALA activation domain. The capacity of the two fusion proteins to interact is determined by cotransformation into a strain of S. cerevisiae, which contains an integrated copy of a lacZ reporter gene. If the two proteins interact, the two domains of GALA are brought into close physical proximity, resulting in reconstitution of GALA activity and β-galactosidase reporter gene transcription.

GALA DNA-binding domain and activation domain fusion plasmids were constructed of the eNOS, iNOS, and nNOS oxygenase and reductase domains using the shuttle/expression vectors, pGBT9 and pGAD424. Various pairwise combinations of plasmid constructs were then introduced into the yeast strain, SFY526. Interactions of fusion proteins in *vivo* were first determined by β-galactosidase activity assay of cotransformants by a colony lift filter assay using X-Gal as substrate. Fusion proteins that interact in this system give a positive (blue) signal on filters. Fusion proteins that do not interact give a negative (white) signal. The strength of the two-hybrid interaction was quantitated further by liquid culture assay using chlorophenol red-β-D-galactopyranoside as substrate. As shown in Table II, eNOS oxygenase domain (residues 1–505) fusion proteins (fused to either the GAL4 DNA-binding domain or the GAL4 activation domain) interacted strongly in the two-hybrid system due to hemidimer formation. Strong interactions were also detected between both of two different combinations of eNOS oxygenase domain and the eNOS reductase domain (residues 506–1205) hybrids. Weaker but significant interactions were detected between pairs of eNOS C-terminal reductase domain fusion proteins. However, in marked contrast to the results with eNOS hybrids, iNOS oxygenase domain (residues 1–498) and reductase domain (residues 499–1144) fusion proteins interacted in the two-hybrid system only through head to head interactions of N-terminal oxygenase domains (Table II). No head to tail or tail to tail interactions were detected with iNOS hybrids. Differences between eNOS and iNOS demonstrated in this system were not due to insufficient concentrations of endogenous BH4 in yeast because identical results were obtained when cells were grown on plates containing added BH4 (0.1 mM).

When nNOS oxygenase domain (residues 1–744) and reductase domain (residues 745–1433) fusions were tested in the two-hybrid system, interactions were very similar to those detected for eNOS. nNOS oxygenase and reductase domains interacted strongly in both head to head and head to tail fashion and weakly in a tail to tail manner (Table II). Interestingly, hybrids of the nNOS oxygenase domain (residues 239–744), which lacked the nNOS N-terminal extension known as the PDZ domain, did not interact in the two-hybrid system. The PDZ domain of nNOS interacts with α-1-syntrophin in the dystrophin complex in skeletal muscle (35) and with the postsynaptic density protein, PSD-95, in brain (36). The results of the present study suggest that the PDZ domain may also be involved in nNOS dimer formation. Alternatively, attachment of the PDZ domain to the nNOS oxygenase domain may alter the folding or conformation of the oxygenase domain and thereby affect dimer formation indirectly. Furthermore, our comparative analysis of NOS oxygenase domain and reductase domain interactions in the two-hybrid system provides an explanation for differences in dimer stability of the NOS isoforms. eNOS and nNOS dimers are very likely more stable than iNOS dimers due to more extensive subunit interactions of the en-
enzymes involving, not only head to head interactions of oxygenase domains but also tail to tail interactions of reductase domains and head to tail interactions between oxygenase and reductase domains.

**Heterooligomerization of eNOS and iNOS**—Within certain mammalian cell types, including cardiac myocytes (37) and microvascular endothelial cells (38), eNOS and iNOS are coexpressed. It has recently been speculated (18) that heterooligomerization of the two enzymes could provide a novel mechanism for regulation of NO production in vivo. Because eNOS and iNOS are 50–60% identical in primary structure (2) and because certain proteins with this degree of homology (for example, vascular endothelial and placenta growth factors (39)) are known to form functional heterodimers in situ, we have tested whether eNOS and iNOS subunits can form eNOS/iNOS heterodimers in Sf9 cells. eNOS and iNOS were coexpressed in the baculovirus system under conditions of high expression that is expected to favor heterodimer formation. After 3 days of co-infection, Sf9 cell lysates were immunoprecipitated with either anti-eNOS or anti-iNOS antibodies. Proteins precipitated by anti-eNOS were separated by gel electrophoresis, transferred to nitrocellulose, and immunoblotted with anti-iNOS. Anti-iNOS immunoprecipitates were immunoblotted with anti-eNOS. Antibody directed against eNOS did not co-precipitate iNOS in these experiments, and antibody directed against iNOS did not co-precipitate eNOS. Many physiologically important protein-protein interactions, however, are too transient or too weak to be detected in co-immunoprecipitation experiments. Interactions of the known protein partners Ras and Raf, for example, are not detectable by co-precipitation but are detectable in the yeast two-hybrid system (40). Therefore, we have also tested whether eNOS and iNOS oxygenase and reductase domains can form heterodimers in this system. eNOS/iNOS oxygenase domain hetero-hemidimer interactions produced positive (blue) signals in the two-hybrid colony lift filter assay. In liquid culture assays, the strength of the interaction was found to be approximately 50% of that of homodimer formation. Oxygenase domains of both isoforms are thus similar enough structurally to interact in vivo. However, no interactions were detected between eNOS and iNOS reductase domains and no hetero-hemidimer interactions were detected of the head to tail type that occur between eNOS oxygenase and reductase domains. Interactions of the eNOS polypeptide with itself (homo-dimer formation) are thus expected to be much stronger than those between eNOS and iNOS. Furthermore, because eNOS exists in intact cells in 100% dimeric form, it is unlikely that eNOS and iNOS form heterooligomers in vivo.

**Amino Acid Residues of eNOS and iNOS Oxygenase Domains Involved in Subunit Interactions**—Mutational analysis by Cho et al. (12) has shown that conservative amino acid substitutions at either glycine 450 or alanine 453 of murine iNOS abolishes NO production, dimer formation, and BH$_4$ binding of the enzyme. These two residues are conserved in all 10 reported NOS sequences including mammalian eNOS, nNOS, and iNOS, as well as avian and insect NOS enzymes. Significant homology of iNOS residues 448–480 to regions within three different BH$_4$-utilizing aromatic amino acid hydroxylases has led to the conclusion that residues 450 and 453 of iNOS are directly involved in binding of BH$_4$. It has been assumed that loss of dimer formation by mutation of either of these residues is a direct consequence of loss of BH$_4$ binding. However, as demonstrated in the present study, eNOS does not require BH$_4$ for dimer formation. Therefore, we hypothesized that, whereas mutation of Gly-450 or Ala-453 in iNOS oxygenase domain hybrids should reduce or abolish interactions in the two-hybrid system, mutation of equivalent residues in eNOS should be without effect. As shown in Table III, Gly-450 and Ala-453 of murine iNOS correspond to Gly-442 and Ala-445 of bovine eNOS. We therefore created equivalent Gly → Ala and Ala → Ile mutations in GAL4 activation domain/NOS oxygenase domain hybrids of eNOS and iNOS and determined whether there was an effect of mutation on interactions of the hybrid proteins with the corresponding wild-type oxygenase domains in the two-hybrid system. Each of the four mutations was found to significantly reduce the strength of the oxygenase domain interactions (Table IV). Because eNOS does not require BH$_4$ for dimer formation, these results suggest that site-specific mutation of these residues may affect dimer formation directly, rather than indirectly due to loss of BH$_4$ binding. The regions in eNOS and iNOS in which these residues are located may thus represent an important subunit interface of the enzymes that is involved in subunit-subunit interactions rather than in subunit-BH$_4$ interactions.

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