Distinct Dimer Interaction and Regulation in Nitric-oxide synthase Types I, II, and III

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ABSTRACT: Homodimer formation activates all nitric-oxide synthases (NOSs). It involves interaction between two oxygenase domains (NOSoxy) that each bind heme and 6R-tetrahydrobiopterin (H4B) and catalyze NO synthesis from L-Arg. Here we compared three NOSoxy isozymes regarding dimer strength, interface composition, and the ability of L-Arg and H4B to stabilize the dimer, promote its formation and protect it from proteolysis. Urea dissociation studies indicated that the relative dimer strengths were NOSIIIoxy >> NOSIoxy > NOSIIoxy (eNOSoxy >> nNOSoxy > iNOSoxy). Dimer strengths of the full-length NOSs had the same rank order as judged by their urea-induced loss of NO synthesis activity. NOSoxy dimers containing L-Arg plus H4B exhibited the greatest resistance to urea-induced dissociation, followed by those containing either molecule, and then by those containing neither. Analysis of crystallographic structures of eNOSoxy and iNOSoxy dimers showed more intersubunit contacts and buried surface area in the dimer interface of eNOSoxy than iNOSoxy, thus revealing a potential basis for their different stabilities. L-Arg plus H4B promoted dimerization of urea-generated iNOSoxy and nNOSoxy monomers, which otherwise was minimal in their absence, and also protected both dimers against trypsin proteolysis. In these respects, L-Arg alone was more effective than H4B alone for nNOSoxy, whereas for iNOSoxy the converse was true. The eNOSoxy dimer was insensitive to proteolysis under all conditions. Our results indicate that the three NOS isozymes, in spite of their general structural similarity, differ markedly in their strengths, interfaces, and in how L-Arg and H4B influence their formation and stability. These distinguishing features may provide a basis for selective control and likely help to regulate each NOS in its particular biologic milieu.
The free radical nitric oxide (NO) drives important physiologic and pathophysiologic functions in animals and is produced by a family of enzymes termed nitric-oxide synthases (NOSs) (1-4). NOS exists as three main isozymes, inducible NOS (iNOS or Type II), neuronal NOS (nNOS or Type I), and endothelial NOS (eNOS or Type III), as well as their splice variants (5-10). All three NOSs share between 50 and 60% sequence homology (11), and catalyze an NADPH and O$_2$- dependent oxidation of L-Arg to NO and citrulline, forming N$^\omega$-hydroxy-L-Arg (NOHA) as an intermediate (12, 13). The NOSs exhibit a bidomain structure comprised of an N-terminal oxygenase domain that is linked to a C-terminal reductase domain through a calmodulin (CaM) binding motif (14-17).

Dimerization of NOS proteins is essential for their activity (13, 18-20). The interaction of two oxygenase domains (NOSoxy) creates an extensive interface between them (21-26). The 6R-tetrahydrobiopterin (H4B) cofactor interacts with residues in both subunits of the dimer and also hydrogen bonds to the active site heme. Dimerization activates NOS in at least three ways: it sequesters heme from solvent, creates high affinity binding sites for substrate L-Arg and cofactor, H4B and enables electrons to transfer from the reductase domain flavins to the oxygenase domain heme (23-31).

NOS dimer formation may be an important point of biologic and pharmacologic regulation (32-35). However, in contrast to the three NOSs being well-distinguished in regard to their expression, Ca$^{2+}$ response, and catalytic profiles (36-43), the differences in their dimerization properties are still unclear. Several studies showed that NOSs must first incorporate heme to dimerize (18-20, 44). A rank order for dimer strength of eNOS
nNOS > iNOS was suggested by low temperature SDS-PAGE experiments (45). But to what degree H4B and L-Arg stabilize NOS dimers or promote their formation is controversial. For example, H4B and L-Arg differed in their capacity to stabilize each NOS dimer against dissociation on SDS-PAGE gels (19, 20, 45, 46). Although H4B is not required for dimerization when NOSs are overexpressed in bacteria (20, 23, 47), H4B and L-Arg promoted dimerization of a purified heme-containing iNOS monomer (23, 48), promoted dimerization of iNOS during its expression in NIH3T3 cells (49), and prevented dissociation of nNOS dimer during catalysis (50). In contrast, dimerization of heme-containing nNOS or eNOS monomer either did not require H4B or L-Arg or was promoted to lesser degrees by these molecules as compared to iNOS (19, 27, 51, 52). The discrepancies were further reflected in crystal structure analysis of iNOSoxy and eNOSoxy. Analysis of iNOSoxy monomer and dimer crystal structures led Crane et al (24, 53) to propose a role for H4B and L-Arg in favoring protein conformational changes that help form and stabilize the dimer. In contrast, crystal structure analysis of H4B-replete and H4B-free eNOSoxy as well as iNOSoxy dimers led others to propose that H4B has no role in forming a stable dimer (25, 54).

To address these issues, we compared iNOS, eNOS, and nNOS proteins expressed and purified from bacteria regarding their dimer strengths, dimer interfaces, and responses toward L-Arg and H4B in stabilizing the dimer, promoting dimer formation, or in protecting dimer against proteolysis. Our report helps clarify how each NOS differs in these respects, and discusses potential structural and biologic origins for the differences.
MATERIALS AND METHODS

Reagents: Ultrapure urea was obtained from Aldrich Chemicals. All other supplies were of highest purity grade available and from sources previously reported (23, 55).

Preparation of NOSoxy proteins: Murine iNOSoxy (residues 1-503) and rat nNOSoxy (residues 1-720) with a fused C-terminal His6 tag were over-expressed in *Escherichia coli* and purified in the absence of H4B or L-Arg using Ni-chelate chromatography as described previously (23, 56). Bovine eNOSoxy was obtained by limited trypsin hydrolysis of pure bovine eNOS protein as detailed before (57, 58). This method cleaves eNOS at a point between its oxygenase domain and CaM binding sequence. In all cases, enzyme concentration was determined from the 444nm absorbance of the ferrous-CO complex using an extinction coefficient of 76 mM⁻¹cm⁻¹. All purified proteins were checked for their purity by gel electrophoresis.

Incubation of NOS Proteins with Urea: NOSoxy or full-length NOS were incubated at 15 °C for 2.5 h in 40 mM EPPS buffer, pH 7.6, containing 3mM DTT and the indicated concentrations of urea (0-7 M) in a final volume of 100 µl. Heme concentrations of the treated holo-NOS proteins in the incubation system ranged from 6 to 10 µM. In studies monitoring spectral change of urea-treated proteins over time, the whole incubation was performed in a cuvette at a final volume of 300 µl.

Activity Assays: To measure activity of NOSoxy proteins, their nitrite production from Nω-hydroxy-L-Arg (NOHA) in a H2O2-supported reaction was assayed in 96 micro
well plates at 37°C as has been previously described (29). Assay mixtures (100 µl final volume) contained 60 mM EPPS, pH 7.6, 150-300 nM NOSoxy heme, 1 mM NOHA, 1 mM DTT, 30 mM H₂O₂, 25 units/ml SOD, 50 mg/ml BSA, 0.5mM EDTA and 4 µM H₄B. The reactions were initiated by the addition of NOHA and H₂O₂ and stopped after 10 min of incubation at 37 °C by addition of 1300 units of catalase. Finally, 100 ml Greiss reagent was added into each well and the absorbance recorded at 550 nm using a Thermomax microplate reader. The amount of nitrite formed was determined based on NaNO₂ standards. Activities of full-length NOS proteins were determined in NADPH driven NO synthesis assays using the oxyhemoglobin detection method as detailed before (29, 55). NOS reductase domain activity was determined by measuring NADPH-dependent cytochrome c reduction by the CaM-bound NOS enzymes as described before (55).

**UV-Visible Spectroscopy:** Spectral data were recorded on a Hitachi U3110 Spectrophotometer.

**Gel Filtration Chromatography:** Samples were subjected to gel filtration chromatography on a 30 x 10 cm Pharmacia Superdex 200 HR column. The column was equilibrated with 40 mM EPPS buffer, pH 7.6, containing 10% glycerol, 3 mM DTT and 150-250 mM NaCl. Protein in the column effluent was detected at 280 nm using a flow-through UV-detector. Molecular mass was estimated based on elution profiles of protein standards. Typically, two copies of the gel filtration profiles were made and the overlapping dimer and monomer peaks were cut out separately from each copy and
weighed on a microbalance. The weights of the peaks were used as direct measures of their areas and used to determine the dimer : monomer ratios.

**Dimerization of Urea-Generated NOSoxy Monomers:** NOSoxy monomers were prepared by incubating the as-isolated proteins (120-150 µM) with urea at concentrations ranging from 3M to 5M for 2.5 h at 15 °C as described before (29). The samples were then diluted 10 fold with 40 mM EPPS, pH 7.6, containing 10% glycerol, and 2 mM DTT. Diluted protein samples were then incubated for 2.5 h at room temperature in the absence or presence of L-Arg (10 mM) and/or H4B (100 µM).

**NOSoxy Sensitivity to Trypsin Proteolysis:** NOSoxy protein samples were incubated overnight on ice in the absence or presence of L-Arg (10 mM) and/or H4B (100 µM). They then were treated with 10 µg of trypsin for 5 min and then immediately boiled in 20 µl gel sample buffer containing 0.125 M Tris-HCl, pH 6.8, 10% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 20% (w/v) glycerol and 0.02% (w/v) bromophenol blue and then subjected to SDS-PAGE at a constant current of 20 mA on 1mm slab gels using Protean II apparatus from Bio-Rad. Following electrophoresis, the gels were stained for protein with Coomassie Blue R-250. The apparent Mr of the proteins and their trypsin-generated fragments were calculated on the basis of Bio-Rad pre-stained SDS-PAGE standards. N-terminal sequencing of proteins excised from the gel was done at the Cleveland Clinic core facility.
Structural Analysis of eNOSoxy and iNOSoxy Dimer Interface: The numbers of pairwise contacts across the dimer interface and buried surface areas in the structures of dimeric murine iNOSoxy (24) and bovine eNOSoxy (25) were computed and analyzed (PDB codes 1DF1 and 1D1V). Pairwise contacts between atoms in the two halves of each dimer were identified using CNS (59) with a contact cutoff distance of 4 Å. We calculated solvent-accessible surface areas buried in the dimer interfaces using CCP4 (60) and calculated the molecular surface areas buried in the dimer interfaces using MS (61, 62). A 1.4 Å probe radius was used in the surface area calculations. By using two independent methods we verified that calculated differences exceeded uncertainties inherent to either surface area calculation method. Multiple structures were analyzed for each isozyme and these calculations verified that differences in the surface areas of the two isozymes could not be attributed refinement and/or co-crystallization with inhibitors.

RESULTS

We studied and compared subunit dissociation and reassociation among three NOSoxy and utilized full-length NOS dimers in a subset of experiments. We selected urea as the chaotrope for dimer dissociation because it has been used extensively to reversibly dissociate iNOSoxy and full-length iNOS into heme-containing monomer (28, 30, 48, 55, 63), and nNOS into heme-containing monomer (29, 31, 64). We also examined roles of L-Arg and H4B in stabilizing the three NOS dimers and in promoting reassociation of their urea-generated monomers.
Stability of NOSoxy dimers and effect of L-Arg and H4B- The three NOSoxy proteins prepared in the absence of H4B and L-Arg were incubated overnight at 4 °C with or without L-Arg and /or H4B prior to treatment with urea. Fig. 1 shows their gel filtration profiles after being preincubated under the various conditions and then treated with 0 M, 3 M and 5 M urea. It is clear that in the absence of both L-Arg and H4B the iNOSoxy and nNOSoxy dimer were highly vulnerable to subunit dissociation compared to the eNOSoxy dimer. The iNOSoxy dimer exerted least resistance to urea-induced dissociation as it formed over 94% monomer with 3 M urea and completely dissociated with 5 M urea. This was followed by nNOSoxy, which despite undergoing some aggregation after dilution from high concentrations of urea was nearly 80% monomeric at 3 M urea and almost totally monomeric at 5 M urea. However, gel filtration analysis of fractions of such proteins show that a substantial part of such aggregated proteins are active with P450 spectral characteristics and not necessarily denatured. In comparison, eNOSoxy exhibited highest resistance in that we observed 18% monomer at 3M urea, 25% monomer at 5 M urea, and failed to observe a predominant monomer population even at 7 M urea (data not shown). Fig.1 also demonstrates that coincident preincubation with L-Arg and H4B imparted the strongest resistance to urea dissociation in all three NOSoxy dimers. Thus, conditions supporting the most stable dimer interaction match those that support full enzymatic activity in the NOS isozymes. Experiments that examined the individual contribution of L-Arg or H4B suggested that H4B was a bit more effective than L-Arg in stabilizing dimer against urea dissociation (Fig.1). Together, the results suggest: (i) dimer stability in L-Arg and H4B-free NOSoxy dimers follows the rank order eNOS >> nNOS > iNOS, (ii) L-Arg and H4B together or separately increase
resistance towards urea-induced subunit dissociation in all three NOSoxy, and (iii) the same rank order of dimer stabilities is maintained in the presence of L-Arg and H4B.

Fig.2, Left Panels, show activity curves for each NOSoxy protein after being treated with various concentrations of urea (0-5 M) in the absence or presence of L-Arg (10 mM) and/or H4B (100 µM). Treatment with urea caused a concentration dependent loss of activity in almost all cases. The activity loss was most prominent when L-Arg and H4B were absent. The activity loss, in each case, can be explained by the singular or combined effect of (i) increased dimer dissociation into monomer, as seen in Fig.1, and/or (ii) deactivation of the protein from urea-induced denaturation. Protein deactivation is manifested by conversion of the NOSoxy heme from a UV visible spectrum resembling cytochrome P450 to one resembling P420 chromophore as shown in the right panels of Fig. 2. This change was measured by recording the dithionite-reduced CO-bound spectrum of each NOSoxy sample under the specific conditions of treatment.

The activity response of the three NOSoxy that were treated with urea in the absence of L-Arg and H4B is qualitatively similar. Typically, a sharp drop is observed at 2 or 3 M urea treatment with a subsequent gradual decline (Fig.2, left panels). For iNOSoxy and nNOSoxy, this drop is primarily associated with loss of dimer content (see Fig. 1), whereas for eNOSoxy it is instead associated with protein denaturation as detected by the P450 measurement.

Interestingly, behavior of the three NOSoxy was different toward urea-induced deactivation or denaturation in the presence of L-Arg and/or H4B. For example, in the
presence of L-Arg plus H4B, nNOSoxy showed total resistance in both respects, eNOSoxy showed near total resistance, while iNOSoxy activity remained susceptible. This correlated with iNOSoxy losing the most dimer content under this condition (Fig. 1). Although eNOSoxy underwent virtually no dissociation at 5 M urea in the presence of L-Arg and H4B, it did show some drop in activity over the treatment range, and this correlated with a similar degree of spectroscopic transition to cytochrome P420 (Fig. 2). H4B was better than L-Arg at stabilizing activities and P450 contents of the eNOSoxy and nNOSoxy dimer, whereas for the iNOSoxy dimer H4B and L-Arg provided similar protections (Fig. 2).

**Stability of full-length NOS** - We examined whether the full-length NOS isozymes would behave similarly to their NOSoxy counterparts in response to chaotropic stress from urea. We measured one dimer-dependent and one dimer-independent catalytic activity (NO synthesis and cytochrome c reductase activity, respectively) after incubating NOS proteins with different concentrations of urea. The full-length NOS proteins used here were bacterially-expressed and purified in the absence of L-Arg and H4B. All three were predominantly dimeric as purified (data not shown), consistent with previous reports (23, 47, 65). Fig. 3, upper panel shows that urea induced differential loss of NO synthesis activity among the three NOS isozymes. Their resistance toward urea displayed the same rank order as seen for NOSoxy proteins (eNOS >> nNOS > iNOS) but there was quantitatively decreased dimer dissociation-denaturation at lower urea concentration compared to the NOSoxy proteins (see Fig. 2). This is consistent with our previous studies showing that full-length iNOS dimer isolated with H4B dissociates at a lower
urea concentration than does H4B-bound iNOSoxy dimer (28, 55). Our data suggest that attached reductase domains may also destabilize nNOS and eNOS dimers compared to their oxygenase domain counterparts. We also examined whether bound CaM had any effect in stabilizing the dimers in the three NOS isoforms and found that the presence of CaM does not resist or assist urea-induced monomerization of the NOS proteins (data not shown).

The cytochrome c reductase activities of the three NOS also show differential loss in response to urea, with rank order of resistance being eNOS >> nNOS > iNOS (Fig. 3, lower panel). The eNOS reductase activity was remarkably more stable than the other NOS, requiring 5 M urea and above to lower its activity. In all cases, higher urea concentrations were needed to cause loss of cytochrome c reductase activity compared to the NO synthesis activity. This insures that loss of NO synthesis activities were not due to unfolding of the reductase domains, but instead were likely due to dimer dissociation, as has been directly shown for iNOS (55).

Recovery of dimeric structure, cytochrome P450-like spectra, and catalytic activity—We examined the ability of urea-treated NOSoxy proteins to reassociate into dimers and recover their catalytic activity and cytochrome P450–like spectrum. Urea-treated protein samples were diluted so that the urea concentration fell below 0.1M, and then they were incubated at 30 °C for 2.5 h in buffer plus DTT alone or buffer containing L-Arg and/or H4B along with DTT. As shown in Fig. 4, greatest recovery of activity and cytochrome P450-like spectrum was obtained in all three NOSoxy when urea-treated
proteins were incubated with L-Arg plus H4B. The magnitude of the recoveries correlated with the degree of induced dimerization in the iNOSoxy and nNOSoxy samples (Table I). iNOSoxy recovery of activity and cytochrome P450-like spectrum was nearly complete, while recovery was submaximal in nNOSoxy and eNOSoxy samples. In nNOSoxy and eNOSoxy, L-Arg alone was clearly more effective at inducing recoveries than H4B alone, while for iNOSoxy the opposite was true (Fig. 4 and Table I). In fact, in recovery of urea-treated nNOSoxy, the effect of L-Arg alone almost mimicked that of L-Arg plus H4B. For eNOSoxy samples, differences among responses to the various treatment conditions were not as prominent as for nNOSoxy (Fig. 4) and were not associated with changes in eNOSoxy dimer-monomer content (Table I). Together, we conclude (i) recovery of dimer (including the little observed in eNOSoxy), catalytic activity, and cytochrome P450 spectral-character was possible in the urea-treated proteins and such cumulative recovery followed a rank order of iNOSoxy > nNOSoxy > eNOSoxy, (ii) the greatest recoveries were obtained by incubation with L-Arg plus H4B in all three proteins, and (iii) L-Arg itself best promoted recoveries for nNOSoxy and eNOSoxy, while H4B itself best promoted recoveries for iNOSoxy.

Kinetics of recovery—We compared the three NOSoxy regarding kinetics of activity recovery and how this correlated with spectral absorbance changes during a 2 h incubation period. Fig. 5 shows the time-dependent changes in activity (left panels), as well as the spectral transitions that occurred (center and right panels) during incubation of urea-treated, diluted proteins under various conditions. Activities increased and then approached a plateau after 15 to 30 min for iNOSoxy and after 30 min for nNOSoxy,
while for eNOSoxy the activities either increased slowly or remained unchanged over the 2 h period. As seen before, L-Arg was more effective at promoting recovery in nNOSoxy and eNOSoxy than was H4B. The kinetics and degree of reactivation correlated with an absorbance increase at 393 nm versus time in all cases (center panels). Absorbance change at this wavelength represented the transition of NOSoxy species from a 6-coordinate, primarily DTT-bound ferric form to a 5-coordinate high spin ferric form during the incubation (right panels). H4B was more effective than L-Arg at promoting the spectral transition in iNOSoxy, whereas the opposite was true for nNOSoxy and eNOSoxy.

**Comparative susceptibility to proteolysis** - We also compared the sensitivity of the three NOSoxy to trypsin proteolysis in the absence or presence of L-Arg and/or H4B. For nNOSoxy (Fig. 6, left panel), trypsin treatment in the absence of L-Arg and H4B removed about 40 kDa from the protein to generate one major 40 kDa fragment. L-Arg alone or combined with H4B protected a cleavage site in the protein and led to generation of a larger 49 kDa fragment (lanes 3 and 5), whereas H4B alone had no protective effect (lane 4). N-terminal sequence analysis showed that the cleavage sites were at R296 and R349, with the latter site being shielded in the L-Arg-bound dimer (Fig.7). Fig. 6, middle panel shows that in the absence of L-Arg or H4B, trypsin removed about 11 kDa from iNOSoxy to generate one major fragment of around 42 kDa (lanes 1 and 2). The presence of L-Arg did not provide protection from this proteolysis (lane 3), whereas H4B alone (lane 4) or H4B combined with L-Arg (lane 5) did provide protection. N-terminal sequence analysis showed that the cleavage occurred at Lys 117 (Fig.7). This confirmed
our previous results with iNOSoxy (23). In marked contrast, eNOSoxy was insensitive to trypsin cleavage irrespective of L-Arg and H4B (Fig. 6, right panel), as previously reported (57).

**Structural analysis of NOSoxy dimer interfaces** - We analyzed the dimer interfaces by comparing sequence conservation of residues in the dimer interfaces and calculating the buried surface areas. The NOSoxy dimer interface is comprised primarily of residues in the N-terminal*-hooks, Zn-binding loop, Helical Lariat, and Helical-T, as well as the H4B cofactor from each subunit (Fig. 7 & 8). The N-terminal*-hooks and Zn-binding loop contain dimer interface residues that are primarily charged or polar and are least conserved of the dimer interface residues among the three isozymes (rat nNOS, bovine eNOS, and murine iNOSoxy) (Fig. 7, top). In contrast, the Helical Lariat and Helical-T contain dimer interface residues that are primarily hydrophobic and conserved among the three isozymes (Fig. 7, bottom).

We calculated the surface areas buried in eNOSoxy and iNOSoxy dimer interfaces using MS (61, 62). The calculated molecular surface area buried in the eNOSoxy dimer interface is 320 Å² larger than the corresponding buried surface area of the murine iNOSoxy dimer (eNOSoxy 4980 Å², iNOSoxy 4650 Å²). By calculating surface areas for multiple independently refined structures of both isozymes (unpublished results), we were able to verify that a difference of 320 Å² was significant. We used only structures that had H4B-bound and Zn bound with the N-terminal hooks unswapped, in order to use structures that were as similar as possible. The surface area buried by the
H4B cofactor (range: 290 – 340 Å²; average 320 Å²) was strikingly similar to the magnitude of the differences observed between eNOSoxy and iNOSoxy (330 Å²). Of the three isozymes, iNOSoxy also has the smallest number of pairwise contacts across the dimer interface (distance cutoff 4 Å). iNOSoxy has approximately 20% fewer pairwise contacts (302 contacts) than eNOSoxy (364 contacts). Analysis of the dimer surface area and contacts across the dimer interface lend structural support to our biochemical findings that: (i) the eNOSoxy dimer is stronger than the iNOSoxy dimer (ii) the H4B cofactor can play a significant role in stabilizing the iNOSoxy dimer, and that (iii) eNOSoxy dimer stability may not depend upon H4B binding as critically as iNOSoxy.

**DISCUSSION**

Subunit dimerization is essential for activating NOS as well as serves as both a point of biologic regulation (32) and pharmacologic intervention (33-35). Our results reveal some important similarities and differences among the three NOS regarding strength and control of their dimeric interaction. These facets and their potential structural and biologic foundations are discussed below.

**Dimer stabilities** - The urea dissociation study suggested that dimeric interaction is strongest in eNOSoxy followed by nNOSoxy and then iNOSoxy. The dimer interaction of eNOSoxy was so strong that we observed only minor dissociation at urea concentrations up to 7 M. The full-length NOSs appeared to follow the same rank order of dimer strength, based on the urea concentrations that were required for loss of their NO synthesis activities (see Fig. 3). Our results suggest that attached CaM binding sites
and reductase domains have little influence on the dimeric interactions of all three NOS. Instead, the attached reductase domains may destabilize the dimeric interactions between oxygenase domains. In fact, we found that CaM binding does not help to strengthen the existing dimer interaction in nNOS and eNOS.

Venema and colleagues observed the same rank order of dimer strengths when subjecting eukaryotic-expressed NOS dimers to low-temperature SDS-PAGE (45). In their study, eNOS dimer was 100% resistant to dissociation induced by the 3% SDS, while nNOS dimer partitioned into a 60:40 dimer-monomer ratio, and iNOS dimer converted completely to monomer. The eNOS dimer exhibited the highest critical temperature for dissociation (30-40 °C) in this system, followed by nNOS (0-20 °C), and then iNOS (< 4 °C). The fact that our laboratories observed identical results in such dissimilar systems boosts confidence in a rank order of dimer strength of eNOS > nNOS > iNOS.

Bound H4B plus Arg increased the stability of all three NOSoxy dimers as evidenced by their increased resistance to urea dissociation. The effect was particularly evident in nNOSoxy and iNOSoxy, and was manifested by their maintaining greater NOHA oxidation activity at the higher urea concentrations (see Figs. 1 and 2). H4B was more effective than L-Arg at maintaining dimeric structure of nNOSoxy, and in maintaining the activity of both nNOSoxy and eNOSoxy. These results are consistent with low-temperature SDS-PAGE studies that showed inclusion of H4B alone or with L-
Arg stabilized nNOS dimer against dissociation, whereas L-Arg alone, or H4B alone in the case of eNOS, had less effect (20, 45, 46).

Our work suggests there are important similarities and differences among the NOS isozymes regarding the structural states of their dimers in solution. When L-Arg and H4B are bound, all three NOSoxy dimers are similar in that they are resistant to urea dissociation and to trypsin proteolysis at a basic site near a flexible region located just beyond the Zn-binding loop (Fig. 7). The protection brought on by L-Arg and H4B binding is analogous to that observed in cytochrome P4502B1, where substrate binding prevents proteolysis within a flexible region of that enzyme (66). The crystallographic structures of NOSoxy dimers with L-Arg and H4B bound are likewise very similar (24, 25). The dimer interfaces bury approximately 15% of the solvent accessible surface area of each monomer. Both L-Arg and H4B form extensive hydrogen-bonding networks that stabilize the surrounding protein, explaining why removal of the substrate and cofactor is destabilizing.

In the absence of L-Arg and/or H4B, the stabilities of the NOSoxy dimers differ (Fig. 6). In the absence of both L-Arg and H4B, nNOSoxy and iNOSoxy dimers are sensitive to urea dissociation and to proteolysis. In contrast, eNOSoxy dimers remain stable and resistant to proteolysis despite a trypsin-cleavable sequence motif (KFPRVK) that is similar to a proteolytic site in nNOS (RFLKVK) (Fig. 7). Although the dimers are best stabilized by the combination of L-Arg and H4B, the isozymes differ in which of these two compounds has the most significant stabilizing effects. Specifically, iNOSoxy is more stabilized by H4B, whereas nNOSoxy is more stabilized by L-Arg. Crystal
structures have shown that L-Arg can bind in the H4B-binding site of eNOSoxy (25). Whether L-Arg protects nNOSoxy dimer from proteolysis by binding to its natural site above the heme, or acts instead by binding within the H4B site as can occur in eNOS, remains to be determined.

**Analysis of the dimer interface** - In light of the isozyme differences in NOSoxy dimer stability, the crystal structures of the H4B-replete NOSoxy dimers are remarkably similar (24-26). However, close examination of dimer interfaces supports our biochemical findings that eNOSoxy forms a stronger dimer than iNOSoxy. The surface area buried in the eNOSoxy dimer interface is larger than that in the iNOSoxy dimer interface. The larger buried surface area and increased number of pairwise contacts in the eNOSoxy dimer are not localized to a single structural element, but rather arise from cumulative differences located throughout the interface. The structural elements in the dimer interface that have the greatest sequence variation among the three NOS isozymes are the N-terminal *-hooks and Zn-binding loops (Fig. 7, top). This suggests that isozyme-specific dimer stabilities could emanate primarily from differences in the N-terminal *-hooks and Zn-binding loops.

Both the N-terminal *-hooks and Zn binding can both help stabilize NOS dimers. Mutation of certain residues in the N-terminal hook region of nNOS or iNOS abrogated their ability to form catalytically active dimers (63,67). Also, biochemical and crystallographic studies of iNOSoxy suggest that its N-terminal *-hooks can swap across the dimer to interact with their partner subunits and thus stabilize the dimer (63, 68). A
role for the N-terminal hook swapping in stabilizing the iNOSoxy dimer is consistent with biochemical results showing that progressive deletion of N-terminal hook elements disrupts iNOSoxy dimers more than eNOSoxy or nNOSoxy dimers (23, 65, 69).

The Zn-binding loop in iNOSoxy generally has more conformational heterogeneity than other regions in the iNOSoxy structure or in the Zn-binding loops of eNOSoxy dimers (Fig. 7) (24-26, 54, 68). For instance, in Zn-free structure of murine iNOSoxy a disulfide bond was formed between Zn-ligand Cys109 and its symmetry mate (Cys109) in the partner subunit and the N-terminal*-hooks were swapped across iNOSoxy dimer (24). Therefore, structural heterogeneity may arise from differences in the amount of Zn bound in recombinantly expressed NOSoxy isozymes. Regarding Zn binding, mutagenesis studies have established that an intact Zn$^{2+}$(Cys)$_4$ is not required for dimerization of iNOS, nNOS, or eNOS, or for their consequent catalytic activity (63, 65, 70, 71). Further evidence that Zn is not required for dimerization is that Deinococcus radiodurans NOS does not have the N-terminal Zn-binding sequence but is still dimeric (72). However, Mayer’s group has demonstrated that a functional Zn-binding site does help to stabilize the nNOS dimer against dissociation in low-temperature SDS-PAGE experiments (73). The relative importance of Zn binding to NOS dimer assembly and stabilization for each isozyme in vivo remains to be tested.

**Dimerization of NOS monomers**- Two noteworthy characteristics of urea-generated NOS monomers are their ability to retain bound heme and to reassociate into active dimeric NOS (28, 55, 64). This allowed us to investigate the relative effects of L-
Arg and H4B in promoting dimerization of nNOSoxy versus iNOSoxy. Incubating heme-containing iNOSoxy and nNOSoxy monomers in buffer alone led only to minor dimer reassembly (10-25%). This result matched our previous work with iNOS or iNOSoxy monomers (18, 28, 48, 49, 55). However, it contrasted with work showing that heme-free nNOS monomer expressed in insect cells undergoes substantial dimerization when incubated with heme alone (19). The reason for these differences is unclear. Our urea generated nNOSoxy monomer, in spite of its good heme content, may not undergo dimerization after being diluted in buffer because of an altered heme-thiolate bond. This is suggested by the majority of monomer forming a cytochrome P420 chromophore when it is reduced in the presence of CO. Hemmens et al (74) have shown that heme-thiolate bond formation is critical for nNOS to dimerize, and the cytochrome P420 form of nNOS contains a weakened heme-thiolate bond (75). This characteristic is not reversed when heme-containing nNOSoxy monomer is incubated in buffer alone (see Figs. 2 and 4). However, normal heme-thiolate bond characteristics were recovered when we incubated urea-generated nNOSoxy monomer with L-Arg plus H4B, and recovery was coincident with dimerization. Identical behavior for urea-generated nNOS monomer was recently reported by Jiang et al (64).

Reassociation of NOSoxy monomers was promoted the most by adding H4B and L-Arg in combination. However, if only one of these were added, nNOS association depended more upon L-Arg, whereas iNOS association depended more upon H4B. The L-Arg substrate-binding site is above the heme and L-Arg does not directly participate in the dimer interface, whereas the H4B cofactor bridges across the dimer interface, pi-
stacking and hydrogen-bonding with residues in both subunits of the dimer (24, 76). In crystal structures of eNOSoxy, but not iNOSoxy, L-Arg has been found to bind not only in the substrate-binding site, but also in the H4B-binding site (25). Whether L-Arg can promote isozyme-specific dimer assembly of eNOSoxy and nNOSoxy by binding at both the substrate and the H4B binding site remains an intriguing possibility for further study.

The ability of H4B to promote association of NOSoxy monomers is entirely consistent with crystal structures of dimeric NOSoxy. Although H4B makes nearly identical interactions in structures of dimeric iNOSoxy and eNOSoxy (25), iNOSoxy appears to require pterin for crystallization, whereas eNOSoxy does not. In fact, all published structures of dimeric iNOSoxy have H4B or a H4B-analogue bound at this site (24, 26, 54, 68, 76, 77). In structures of eNOSoxy with and without bound H4B, no differences at the dimer interface were observed (25). This may be because crystallization selects for the most ordered conformational state of several available for NOSoxy dimers in solution. The dimeric conformation indicated by the H4B-bound crystal structure of iNOSoxy may not be the predominant conformation of dimeric H4B-free iNOSoxy in solution. Subtle variations in NOS conformation caused by cofactor and substrate binding may differentially tune the activity of NOS isozymes in vivo by regulating dimer association and stability.

The isozyme-specific response toward H4B and L-Arg regarding dimer assembly appears to match the different cellular availabilities of these molecules. For example, in cells expressing iNOS, a chronic depletion of L-Arg can occur due to coinduction of
arginase (78, 79), whereas H4B levels actually increase due to coinduction of GTP
cyclohydrolase (80, 81). In contrast, low H4B levels have been measured in cells and
tissues expressing eNOS or nNOS both in physiologic and pathologic settings (82-85).
Thus, differential control of NOS dimer assembly may have evolved in response to the
varied cellular environments in which each isozyme is expressed.

**Summary**—Although differential regulation of NOS isozymes regarding their
expression, localization, and catalytic tuning is well appreciated, very little is known with
respect to the structural regulation that might distinguish them. In this respect, our present
work confirms their different dimer interaction strengths and reveals its potential
structural basis, and brings to light hitherto poorly understood roles for substrate and
pteridine cofactor in isozyme-specific regulation of dimer assembly.

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FOOTNOTES

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1. Abbreviations: NO, nitric oxide; NOS, nitric-oxide synthase; NOSoxy, nitric-oxide synthase oxygenase domain; NOS TypeI or nNOS, neuronal nitric-oxide synthase; NOS Type II or iNOS, inducible nitric-oxide synthase; NOS Type III or eNOS, endothelial nitric-oxide synthase, L-Arg, L-arginine, H4B, (6R)-5,6,7,8—tetrahydro-L-biopterin; NADPH, β-nicotinamide adenine dinucleotide phosphate (reduced form); CaM, calmodulin; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; EPPS, 4-(2-hydroxyethyl)-1-
piperazinepropanesulfonic acid; DTT, dithiothreitol; NOHA, Nω-hydroxyarginine; H₂O₂, hydrogen peroxide.

2. That L-Arg and H4B did not prevent proteolytic cleavage of nNOSoxy at the junction of its N-terminal leader sequence and the rest of the protein is consistent with this structural element functioning as a separate subdomain that binds nNOS to specific cellular protein motifs (37).

3. We assumed un-swapped N-terminal hooks for each NOSoxy in the analysis. Also, to make direct comparisons, bound pteridine was excluded from the calculation.

4. The limited dissociation of eNOSoxy dimer even at high urea concentration (7M) precluded our investigating its reassembly.

5. Our experiments using heme-free iNOS monomer expressed in macrophages showed that little dimer assembly took place when incubated with heme alone (18).
Table I

Profile of dimer and monomer content of 3M urea-treated NOSoxy proteins, before and after incubation in the presence and absence of L-Arg and H4B. Oxygenase domains of the three NOS isoforms were treated with 3M urea at 15°C for 2.5 h following which they were diluted and incubated with or without L-Arg (10mM) and / or H4B (100µM) for 2.5 h at room temperature. 50µg of the incubated protein was then subjected to Gel Filtration as described under ‘Experimental Procedures’. Results are representative of two independent experiments.

| Additives     | nNOSoxy | iNOSoxy | eNOSoxy |
|---------------|---------|---------|---------|
|               | Dimer   | Monomer | Dimer   | Monomer | Dimer   | Monomer |
|               | %       | %       | %       | %       | %       | %       |
| None (before incubation) | 20  | 80  | 6  | 94  | 82  | 18  |
| None (Incubated)         | 10  | 90  | 27 | 73  | 82  | 18  |
| + Arg                  | 42  | 58  | 44 | 56  | 87  | 13  |
| + H4B                  | 25  | 75  | 62 | 38  | 83  | 17  |
| + Arg+H4B              | 45  | 55  | 84 | 16  | 89  | 11  |
Figures and Legends

**Fig. 1** Gel Filtration profiles demonstrating effect of H4B and L-Arg on urea-induced dissociation of already formed dimers of the three NOSoxy. Proteins were pre-incubated overnight in 40mM EPPS buffer (−Arg, −H4B), 10mM L-Arg (+Arg), 100µM H4B (+H4B) or both 10mM L-Arg and 100µM H4B (+Arg, +H4B) before being treated with given concentrations of urea for 2.5h at 15 °C. Following this urea-treatment, 50µg of the treated proteins were loaded on a Superdex 200 HR column as described under ‘Experimental Procedures’. Results shown are representative of two similar experiments.

**Fig.2** Relative resistance of different NOSoxy isoforms to urea-induced dissociation and denaturation in the presence and absence of L-Arg and H4B. The left panel depicts the activity profile of the different NOS oxygenase isoforms after treatment with different concentrations of urea for 2.5 h at 15 °C in the complete absence and in the individual and combined presence of L-Arg (10mM) and H4B (100µM) as described. Activity was measured by H$_2$O$_2$-dependent L-NOHA oxidation and the nitrite formation was determined after a 10 min reaction as described under ‘Experimental Procedures’. The right panel shows the corresponding change in the P450 content of the same proteins. The data are representative of three independent experiments.
**Fig. 3** Catalytic activities of the different NOS full-length proteins after treatment with increasing concentrations of urea. Full-length NOS isoforms prepared in the absence of L-Arg and H4B were equilibrated at 15°C in the indicated concentrations of urea for 2.5h after which aliquots were diluted and assayed for NO synthesis (upper panel) or cytochrome C reduction (lower panel) as described under ‘Experimental Procedures’. The data shown are representative of three similar experiments.

**Fig. 4** Effect of L-Arg and H4B on activity and P450 spectral character recovery of the three NOSoxy after treatment with different concentrations of urea. The left panels, show the activity profiles of the urea-treated (2.5h at 15 °C) NOSoxy isoforms after 2.5 h of incubation with L-Arg (10mM), H4B (100µM) or both at room temperature (25°C). The right panels show the corresponding P450 content profiles of the recovered proteins at relevant urea concentrations. Activity assay for the proteins were done as in Fig.1. The data are representative of three independent experiments.

**Fig. 5** Time-dependent recovery of 3M urea-treated NOSoxy isoforms with and without treatment with L-Arg and H4B. The left panels show the activity recovery over the initial 2h of incubation. The center panels depict the time-dependent spectral shift to high spin state of the treated proteins while the right panels show the final wavelength scans of the treated proteins after the initial 2h incubation period. Further details are given in ‘Experimental Procedures’. The data are representative of three similar experiments.
Fig. 6. Effect of L-Arg and H4B on protection of different NOSoxy dimers from limited trypsin digestion. The proteins were dialysed against 40mM EPPS containing 10% glycerol and 1mM DTT and then preincubated for 16h in ice either in this buffer alone or in buffer containing 10mM L-Arg, 100 µM H4B or 10mM L-Arg plus 100µM H4B. The light absorbance spectrum of each protein sample was recorded after preincubation to ensure that the spectral changes associated with the binding of L-Arg and/or H4B have occurred. Proteolysis was then carried out at room temperature for 10 min using 1µg trypsin. Following incubation with trypsin the samples were boiled with Laemmli buffer and about 30µg protein of the treated buffer were analysed by SDS-PAGE. Lane 1, native enzymes; lane 2, enzymes treated with trypsin in the absence of L-Arg and H4B; lane 3, enzymes treated with trypsin in the presence of L-Arg (10mM); lane 4, enzymes treated with trypsin in the presence of H4B (100 µM); lane 5, enzymes treated with trypsin in the presence of both L-Arg (10mM) and H4B (100µM). Further details are given under Experimental Procedures’. The three panels from left to right represent nNOSoxy, iNOSoxy and eNOSoxy respectively. The figure shown is representative of three similar experiments.

Fig. 7. Sequence alignment of rat nNOS, bovine eNOS, and murine iNOS highlighting dimer interface residues. Residues that are identical in all three isozymes are shown as white letters on a red background and residues that are similar are shown with a yellow background. Residues that contribute at least 5 Å² to the iNOSoxy dimer interface are boxed in cyan. The cleavage sites we determined for nNOS and iNOS are marked with pink triangles, above and below the sequences, respectively. eNOS is
resistant to proteolysis, despite containing a sequence homologous to an nNOS cleavage site (black underline). Green boxes are drawn around residues that are either disordered and/or exist in multiple conformations in crystal structures. Solid diamonds above mark every 10th position in rat nNOS from residue 300 to 350 of the N-terminus (top) and residue 600-710 of the C-terminus (bottom).

**Fig. 8.** Ribbon diagram of one monomer from the crystal structure of dimeric murine iNOSoxy, as viewed from the dimer interface. Side chains of residues that contribute at least 5 Å² of surface area to the dimer interface are shown as ball and stick. Dimer interface residues that are identical among rat nNOS, bovine eNOS, and murine iNOS are shown with red carbon atoms. Non-conserved dimer interface residues are shown with yellow carbon atoms. The most sequence variation among dimer interface residues is found in the N-terminal*-hooks and the Zn-binding loop (cyan ribbons). In contrast, dimer interface residues in the substrate-binding helix, the helical-lariat, and the helical-T are largely conserved (purple ribbons). Heme, H4B, and Zn are shown in green.
Fig. 3

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Fig. 4

Cytochrome P450 Content (µM)

Moles of nitrite / mole heme

Urea (M)
Fig. 6

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Panda et al.

Fig. 7
Heme Helical-T Helical-Lariat Zn-Binding Loop H4B Substrate-Binding Helix H4B-Binding Loop Zn-Binding Loop N-Terminal hooks N-term
Distinct dimer Interaction and regulation in nitric-oxide synthase types I, II, and III
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