Domain Swapping in Inducible Nitric-oxide Synthase

ELECTRON TRANSFER OCCURS BETWEEN FLAVIN ANDHEME GROUPS LOCATED ON ADJACENT SUBUNITS IN THE DIMER

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Cytokine-inducible nitric-oxide (NO) synthase (iNOS) contains an oxygenase domain that binds heme, tetrahydrobiopterin, and L-arginine, and a reductase domain that binds FAD, FMN, calmodulin, and NADPH. Dimerization of two oxygenase domains allows electrons to transfer from the flavins to the heme irons, which enables O2 binding and NO synthesis from L-arginine. In an iNOS heterodimer comprised of one full-length subunit and an oxygenase domain partner, the single reductase domain transfers electrons to only one of two hemes (Siddhanta, U., Wu, C., Abu-Soud, H. M., Zhang, J., Ghosh, D. K., and Stuehr, D. J. (1996) J. Biol. Chem. 271, 7309–7312). Here, we characterize a pair of heterodimers that contain an L-Arg binding mutation (E371A) in either the full-length or oxygenase domain subunit to identify which heme iron becomes reduced. The E371A mutation prevented L-Arg binding to one oxygenase domain in each heterodimer but did not affect the L-Arg affinity of its oxygenase domain partner and did not prevent heme iron reduction in any case. The mutation prevented NO synthesis when it was located in the oxygenase domain of the adjacent subunit but had no effect when in the oxygenase domain in the same subunit as the reductase domain. Resonance Raman characterization of the heme-L-Arg interaction confirmed that E371A only prevents L-Arg binding in the mutated oxygenase domain. Thus, flavin-to-heme electron transfer proceeds exclusively between adjacent subunits in the heterodimer. This implies that domain swapping occurs in an iNOS dimer to properly align reductase and oxygenase domains for NO synthesis.

Nitric oxide (NO) acts as a signal and cytotoxic molecule in biology (1–3) and is synthesized from L-arginine (L-Arg) by enzymes termed NO synthases (NOS). The NOS exhibit a bi-domain structure in which a N-terminal oxygenase domain that contains binding sites for iron protoporphyrin IX (heme), tetrahydrobiopterin (H4B), and L-Arg is fused to a C-terminal reductase domain that contains binding sites for calmodulin (CaM), FMN, FAD, and NADPH (4, 5). To synthesize NO, NADPH-derived electrons must transfer from the reductase domain flavins to the oxygenase domain heme irons, which are bound to the protein via cysteine thiolate axial ligation as in the cytochromes P450 (6–10). The flavin-to-heme electron transfer is thought to be critical for catalysis because it enables each heme iron to bind and activate oxygen at two steps in the reaction sequence, resulting in oxygen insertion into L-Arg to form N^•-hydroxy-L-Arg, and subsequent oxygenation to generate NO and citrulline as products (11–13).

The NOS are only active as homodimers (4, 14), and understanding how dimerization relates to NOS catalysis is a topic of current interest. Studies with the cytokine-inducible NOS (iNOS) indicate its dimer assembly occurs with stable incorporation of one heme and one H4B into each subunit (15, 16). The dimeric interaction only requires the oxygenase domains of each subunit with the reductase domains apparently not interacting with one another (17). The NOS oxygenase and reductase domains can be expressed separately and fold and function independently of one another (17–21). This has facilitated spectroscopic (17, 20), mutagenic (22–24), and crystallographic (25, 26) characterization of the iNOS oxygenase domain (iNOSox).

Both full-length iNOS and iNOSox dimers can reversibly dissociate into folded monomers in the presence of urea (27, 28). Although the full-length iNOS monomer still binds its flavin and heme groups and can transfer electrons from NADPH into its flavins, it can no longer catalyze reduction of its heme iron (29), indicating that dimeric structure in some way is critical for the flavin-to-heme electron transfer step. To investigate how iNOS dimerization, electron transfer, and catalytic function are related, we characterized an iNOS “heterodimer” comprised of one full-length and one iNOSox subunit. The single reductase domain of this “wild-type” heterodimer transferred NADPH-derived electrons to only one of the two heme irons located in the dimeric oxygenase core, but...
this was sufficient to support a normal rate of NO synthesis by that heme (29). Although these results showed that dimerization enables the flavin-to-heme electron transfer, they did not identify which of the two hemes accepts electrons from the single reductase domain and thus did not distinguish whether electron transfer occurs between flavin and heme groups located in the same or adjacent subunits of a dimer.

In the current report, we address this question by constructing a complementary pair of INOS heterodimers, each comprised of one wild-type subunit and one subunit containing a point mutation (E371A) that causes an exclusive and absolute defect in t-Arg binding (22). The catalytic, spectroscopic, and electron transfer properties of these complementary heterodimers and of a heterodimer selectively enriched in 54Fe establishes the electron transfer pathway in iNOS, and helps explain why dimer formation is essential to complete electron transfer to the heme.

**EXPERIMENTAL PROCEDURES**

**Materials**—Chemicals, culture media, and chromatography resins were obtained from sources previously reported (22, 23, 29, 30). 54Fe metal was from Cambridge Isotope Laboratories, Cambridge, MA.

**Generation of Full-length E371A iNOS**—A cDNA fragment containing the mutation E371A was removed from a pCWoRi plasmid containing E371A iNOSox (22) by restricting with BnaI (AvrII) and Eco47III (Boehringer Mannheim). This fragment was ligated into similarly restricted pCWori containing full-length iNOS (31). The ligation product was transformed into competent Escherichia coli JM109, and the mini-prep DNA prepared from a single colony was sequenced in the core facility of the Cleveland Clinic to confirm the mutation was transferred. The pCWori plasmid containing E371A full-length iNOS and a plasmid containing human CaM (31) were sequentially transformed into competent E. coli BL21 (DE3) for protein expression.

**Purification of Wild-type iNOSox and E371A iNOSox**—E. coli BL21 expressing C-terminal His$_{6}$-tagged iNOSox (amino acids 1–498) (29) and the E371A iNOSox (22) were grown in Terrific broth containing 100 µg/ml ampicillin at 37 °C with shaking at 250 rpm. The cultures were induced at $A_{600}$ = 0.8–1.2 with 1 mM isopropyl-$eta$-D-thiogalactopyranoside (IPTG) and continued to grow for an additional 14–18 h at 30 °C. The cells were lysed by freeze-thawing, followed by sonication on ice. Cell debris was removed by centrifugation at 5000 rpm at 4 °C, and resuspended in one-fifth of the culture volume in lysis buffer (40 mM EPPS, pH 7.6, containing 1 mM phenylmethylsulfonyl fluoride, 100 µM aprotinin, 1 µg/ml leupeptin, 4 µM H$_{4}$B, and 1 mM t-Arg). The cells were lysed by freeze-thawing, followed by sonication on ice. Cell debris was removed by centrifugation at 10,000 rpm for 30 min at 4 °C. Proteins were purified as described previously (22, 23) using Ni$_{2}$-chelate affinity resin equilibrated with 40 mM EPPS, pH 7.6, containing 0.25 M NaCl, 4 µM H$_{4}$B, and 1 mM t-Arg. After initial adsorption, the column was washed with the same buffer containing 150 mM imidazole. The eluted protein was concentrated using a 2′,5′-ADP-Sepharose column. Contaminating iNOSox dimer was removed by washing with ADP column buffer, and the heterodimer was eluted with 10 mM NADPH.

**Gel Filtration Chromatography**—A Superdex 200 HR column (Amersham Pharmacia Biotech) of ~25 ml bed volume was used to size fractionate heterodimer preparations as noted in the text. The column was equilibrated with 40 mM EPPS, pH 7.6, containing 10% glycerol, 0.2 M NaCl, and 4 µM H$_{4}$B. The flow rate was maintained at 0.5 ml/min using a Pharmacia FPLC. Protein in the eluate was detected using a flow-through detector set at 280 nm.

**NO Synthesis Activity**—The initial rate of NO synthesis by full-length INOSox was quantitated as described previously (34). The iNOS (10–50 ng with respect to heme iron concentration) was added to a cuvette containing 40 mM EPPS, pH 7.6, supplemented with 10% glycerol, 0.3 mM DTT, 5 mM t-Arg, 4 µM each of FAD, FMN, and H$_{4}$B, 100 units/ml catalase, 10 units/ml superoxide dismutase, 0.1 mg/ml bovine serum albumin, and 10 µM oxyhemoglobin to give a final volume of 0.3 ml. The reaction was started by adding NADPH to give 0.1 mM. The NO-mediated conversion of oxyhemoglobin to methemoglobin was monitored over time as an absorbance increase at 401 nm and quantitated using the extinction coefficient of 38 mM$^{-1}$ cm$^{-1}$.

**NADPH Oxidation**—The initial rate of NADPH oxidation at 25 °C was quantitated spectrophotometrically at 340 nm using an extinction coefficient of 6.2 mM$^{-1}$ cm$^{-1}$ for reduced NADP$^{+}$ (34).

**Calculation of Specific Activity**—The nanomoles of NO produced (or NADPH oxidized) per min per nmol of heme iron was calculated based on the concentration of heme iron, determined from the absorbance difference between 444 and 500 nm for the ferrous-CO species and using an extinction coefficient of 74 mM$^{-1}$ cm$^{-1}$ for ferrous CO-bound iNOS (34).

**Resonance Raman Experiments**—The instrumentation for resonance Raman experiments has been described in detail previously (35). Excitation was provided by a He-Cd laser at 441.6 nm with a power at the sample of 1 milliwatt. For all experiments, 40 mM BisTris buffer, pH 7.6, containing 1 mM DTT was used. Heme concentration in the sample was between 20 and 50 µM. Ferrous-CO iNOS samples were prepared by adding a small amount of dithionite solution to the anaerobic sample cell under a CO atmosphere, and conversion to the CO-bound form was confirmed by UV-visible spectroscopy. In some cases, an anaerobic solution of t-Arg was added to give a final concentration of 3 mM.

**RESULTS**

**Characterization of Full-length E371A iNOS**—UV-visible spectra of purified E371A iNOS are shown in Fig. 1. The broad Soret absorbance centered at 405 nm in the absence of H$_{4}$B and t-Arg indicates that the ferric heme iron is a mixture of low and high spin states, as observed for wild-type full-length iNOS purified under identical conditions (36). The absorbance shoulders at 450–485 nm indicate the presence of bound flavins and are unusually prominent with respect to the Soret absorbance. Addition of t-Arg alone failed to bring about any change in the spectrum, whereas addition of H$_{4}$B alone to the ferric heme then produced a mostly high spin state. Addition of t-Arg to the pterin-bound protein did not change the spectrum (not shown).

Chemical reduction by dithionite in the presence of CO enabled formation of the thiol-ligated ferrous-CO complex absorbing at 444 nm. Thus, the E371A mutation rendered full-length iNOS incapable of binding t-Arg but did not appear to affect its Soret absorbance.

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ligation or interaction with H4B, consistent with previous mutagenic and crystallographic results (22, 25).

The gel filtration profile of E371A iNOS isolated in the absence of H4B and L-Arg (Fig. 2, panel A) indicates that the protein is a mixture of approximately 75% dimer and 25% monomer that elute at 9.5 and 10.9 ml, respectively. The protein that elutes at 13 ml was identified as the iNOS reductase domain derived from nonspecific proteolysis of full-length E371A iNOS (data not shown). The presence of the reductase domain fragment likely explains why the flavin absorbance bands are so prominent in the visible spectrum of E371A iNOS (Fig. 1). Like wild-type iNOS, the E371A dimer can be dissociated into folded monomers in 2M urea (Fig. 2, panel B), and the monomers can reassociate into dimer after being diluted 10-fold and incubated with H4B (Fig. 2, panel C).

Unlike native iNOS, the E371A iNOS dimer was unable to synthesize NO from L-Arg and did not increase its rate of NADPH oxidation in response to L-Arg (data not shown). Adding NADPH to H4B-saturated E371 iNOS led to reduction of ~90% of its heme iron as determined by formation of the ferrous-CO species absorbing maximally at 444 nm (Fig. 3), similar to wild-type iNOS (6). L-Thiocitrulline, a substrate analog that acts as a competitive inhibitor versus L-Arg and blocks NADPH-dependent heme iron reduction (37), did not do so for E371A iNOS (Fig. 3), consistent with a defect in substrate binding. In sum, the E371A mutation prevented iNOS from binding or responding to L-Arg, but did not affect iNOS dimeric interaction, H4B binding, heme environment, or electron transfer from the reductase domain to the heme. This confirms and extends results obtained with E371A iNOSox (22) and with an analogous mutant of endothelial NOS (38).

**Generation and Purification of Complimentary E371/Wild-type Heterodimers**—We next utilized the E371A full-length and oxygenase domain mutants to prepare complimentary heterodimers with wild-type partners. As shown in Fig. 4, these were a full-length wild-type iNOS subunit paired with an E371A oxygenase domain subunit (FLWT/OXYE371A); and a full-length E371A subunit paired with a wild-type oxygenase domain subunit (FLWT/OXYWT). A completely wild-type heterodimer was also prepared as a control (FLWT/OXYWT). To generate heterodimers, the original full-length and oxygenase domain homodimers were first dissociated into monomers using urea, mixed, and then induced to dimerize with H4B and

**FIG. 1. Light absorbance spectra of E371A full-length iNOS.** The cuvette contained 2.7 μM of E371A full-length iNOS in 40 mM EPPS, pH 7.7, containing 10% glycerol at 25 °C. Spectra were recorded prior to (—) and following (---) a 30 min incubation with 5 μM H4B. A replica sample was incubated for 15 min with 5 mM L-Arg in place of H4B (-----). Subsequently, the sample was given CO and reduced with dithionite to form the ferrous-CO complex (-----).

**FIG. 2. Gel filtration profiles of E371A full-length iNOS.** Panel A, 40 μg of purified protein was loaded on a Superdex 200 HR column equilibrated with 40 mM EPPS, pH 7.6, containing 10% glycerol and 0.2 M NaCl. From left to right the four peaks represent protein aggregate, homodimer, monomer, and a proteolytically derived reductase domain fragment. Panel B, the profile after equilibrating the purified protein in 2 M urea to dissociate the homodimer into folded monomers. Panel C, the profile after incubating the urea-generated monomer with 10 μM H4B for 1 h at 25 °C to reform the homodimer. Results shown are representative of four similar experiments.
Each heterodimer was purified by sequential chromatography on Ni$_2$-chelate, 2',5'-ADP-Sepharose, and Superdex gel filtration columns. A typical gel filtration profile for a heterodimer is shown in Fig. 5, along with gel filtration profiles for the two principle contaminants (full-length and oxygenase domain homodimers). After gel filtration chromatography the heterodimer fractions were greater than 80% pure and were biochemically characterized at this level of purity.

**L-Arg Binding**—We first determined how the E371A mutation affected L-Arg binding by each heterodimer. We utilized a spectroscopic perturbation method that is based on the ability of L-Arg to displace heme-bound imidazole and convert the iNOS heme iron from a low spin to high spin state (28, 39). As shown in Fig. 6, panels A–C, addition of 0.4 mM imidazole to each of the three H$_4$B-saturated heterodimers converted their predominantly high spin heme (Soret absorbance at 400 nm) to a fully low spin, imidazole-bound form with Soret absorbance at 426 nm. L-Arg was then added in graded amounts, and a spectrum was recorded after each addition until no further spectral change was observed. For the FLwt/OXYwt heterodimer control (panel A), adding a saturating concentration of L-Arg (5 mM) caused an almost complete displacement of bound imidazole and generated high spin heme, which absorbs maximally at 390 nm. In contrast, a saturating concentration of L-Arg only displaced imidazole from about half of the heme iron in either mutant heterodimer (panels B and C). The difference spectra for the three heterodimers are shown in Fig. 6, panel D, and confirm that L-Arg saturation displaced approximately half of the imidazole in each E371A mutant heterodimer as compared with the wild-type control. Thus, the E371A mutation only prevented L-Arg binding to one of two oxygenase subunits present in the mixed heterodimers.

To determine if the E371A mutation affected the affinity of the oxygenase partner that still could bind L-Arg, we examined plots of absorbance change (390–430 nm) versus L-Arg concentration (Fig. 7). A similar concentration range of L-Arg ap-
peared to saturate all three heterodimers. Calculated $K_s$ values for L-Arg binding were 32, 40, and 41 m$M$ for FL WT/OXYWT, FLWT/OXYE371A, and LE371A/OXYWT, respectively. This indicates the E371A mutation did not affect L-Arg affinity in one of the two subunits of each mixed heterodimer. Thus, our spectral analysis shows that the mutation preserves its phenotype in one subunit of a mixed heterodimer without affecting the other subunit’s affinity toward L-Arg.

**NADPH-dependent Heme Iron Reduction**—We next compared the heterodimers regarding NADPH-dependent heme iron reduction under anaerobic conditions and in the presence of CO (Fig. 8). Under these conditions the wild-type heterodimer had previously been shown to reduce only one of its two heme irons (29). This was confirmed in Fig. 8, panel A, where addition of excess NADPH caused reduction of approximately 50% of the heme iron in the FLWT/OXYWT heterodimer as judged by the NADPH-dependent buildup of a ferrous-CO peak 444 nm relative to the peak intensity obtained upon reducing all of the heme iron with dithionite. Similar results were obtained with the FLWT/OXYE371A and FL E371A/OXYWT mutant heterodimers, indicating that only half of their heme iron was reduced by NADPH (Fig. 8, panels B and C, respectively). Thus, electron transfer between the single reductase domain and one oxygenase domain heme iron was not disabled when the E371A mutation was present in the same or adjacent subunit relative to the reductase domain.

**Catalytic Properties**—Table I compares the NO synthesis and NADPH oxidation activities of the three heterodimers. The FLWT/OXYWT control heterodimer synthesized NO at a rate that was approximately half that of a full-length iNOS homodimer on a per heme basis, consistent with our previous studies.
FL E371A/OXYwt heterodimer preparations, containing 10% glycerol, 0.05 mM DTT, 4 μM FLE371A/OXYWT heterodimers, respectively. An initial spectrum of each mol of heme. The values represent the mean and standard error for experimental Procedures." Under these conditions the NO synthesis activity for the parent full-length iNOS homodimer was 181 mol/min per experimental medium. The NO synthesis rate of the E371A mutation was lower than that of the wild-type homodimer, and its rate of NADPH oxidation was also increased by L-Arg and decreased by L-thiocitrulline in a manner similar to the control. In contrast, the FL WT/OXYWT heterodimer only exhibited residual NO synthesis compared with the control (9%), and its rate of NADPH oxidation was affected only slightly by L-Arg and not at all by L-thiocitrulline. Thus, the E371A mutation only affected heterodimer NO synthesis and responses to L-Arg or L-thiocitrulline when it was present in the oxygenase domain located opposite to the reductase domain.

**Heme-L-Arg Interaction**—At this point, our heterodimer data is consistent with two possibilities. (1) Electron transfer occurs between reductase and oxygenase domains located on adjacent subunits if the E371A mutation affects L-Arg binding in the subunit in which the mutation resides. (2) Electron transfer occurs between reductase and oxygenase domains in the same subunit if the E371A mutation affects L-Arg binding by the adjacent wild-type subunit. To differentiate between possibilities 1 and 2, we examined the nature of the heme-L-Arg interaction using resonance Raman spectroscopy and a FL E371A/OXYWT heterodimer whose OXYWT heme was selectively enriched in 54Fe (Fig. 9). This mixed heterodimer contains only one functional L-Arg binding site and two nonequivalent heme irons, and thus enabled us to view the interaction that occurs between a single L-Arg and a single reduced, CO-bound heme iron. The method relies on the fact that the lighter 54Fe isotope causes the νFe-CO stretching vibration, which is sensitive to L-Arg (Fig. 10, compare traces a-d) to shift 3 cm⁻¹ toward higher frequency compared with natural abundance Fe (designated 56Fe in Fig. 10, compare traces d and c).

The resonant Raman spectrum of the reduced, CO-bound, 54Fe-enriched FL E371A/OXYWT heterodimer (Fig. 10, trace f) contains both a broad νFe-CO band centered at 486 cm⁻¹ and a sharp νFe-CO band centered at 514 cm⁻¹. The former is attributable to a 56Fe-CO species that is not interacting with bound L-Arg, whereas the latter is attributable to a 54Fe-CO species that is interacting with L-Arg. Because the E371A mutation and the 54Fe heme are located in opposite subunits of the FL E371A/OXYWT heterodimer, we conclude that the E371A mutation must block L-Arg binding in the same subunit in which the mutation is present. Thus, interactions between L-Arg and the 54Fe heme-CO complex must occur within a single subunit, eliminating “possibility 2” above.

### Table I

**Catalytic activities of the iNOS heterodimers**

| Heterodimer     | Conditions | NO synthesis | NADPH oxidation (mol product/min per mol heme) |
|-----------------|------------|--------------|---------------------------------------------|
| FL wt/OXYwt     | -L-Arg     | 13 ± 0.3     |                                             |
|                 | +L-Arg     | 92 ± 10      | 58 ± 4                                      |
|                 | +L-thiocitrulline | 3 ± 0.1        |                                             |
| FL E371A/OXYwt  | -L-Arg     | 11 ± 0.1     |                                             |
|                 | +L-Arg     | 69 ± 1       | 59 ± 4                                      |
|                 | +L-thiocitrulline | 4 ± 0.1        |                                             |
| FL wt/OXY E371A | -L-Arg     | 8 ± 5        | 24 ± 0.1                                    |
|                 | +L-Arg     | 15 ± 0.4     |                                             |
|                 | +L-thiocitrulline | 15 ± 1.1        |                                             |

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4 The small rate of NO synthesis and increase in NADPH oxidation was likely attributed to residual contamination by full-length wild-type iNOS homodimer.

5 The full-length E371A subunit partner contained natural abundance iron, which averages to 55Fe.

6 In an iNOS homodimer, L-Arg sharpens the Fe-CO bending vibration and increases its frequency by a value that is specific for that isofrom and substrate (54).
DISCUSSION

Because NOS heme iron reduction is critical for NO synthesis, it is important to understand the path of electron transfer and how it is controlled. During NO synthesis the reductase domain flavins accept electrons from NADPH and transfer them to the heme iron located in the oxygenase domain. However, the homodimeric structure of iNOS introduces complexity to this system and creates three possibilities regarding electron transfer from a single reductase domain. Electrons could flow to both hemes, only to the heme located on the same subunit, and how it is controlled. During NO synthesis the reductase domain flavins accept electrons from NADPH and transfer them to the heme iron located in the oxygenase domain. However, the homodimeric structure of iNOS introduces complexity to this system and creates three possibilities regarding electron transfer from a single reductase domain. Electrons could flow to both hemes, only to the heme located on the same subunit, or only to the heme located on the opposite subunit. Our previous work with an iNOS heterodimer revealed that its arrangement possibly circumvents a physical barrier for electron transfer between reductase and oxygenase domains to generate NO by oxidizing the \( \text{L-Arg} \) molecule bound within it.

Our conclusion derives from experiments that detailed the basis of each mutant heterodimer’s catalytic profile. Such analysis is essential when using mutagenesis to investigate structure-function aspects of any NOS dimer, because in principle a single oxygenase point mutation could affect the functioning of the domain in which the mutation is present, the adjacent wild-type domain, or both domains. The E371A mutation completely prevents \( \text{L-Arg} \) binding when present in both oxygenase domains of an iNOSox (22) or full-length iNOS homodimer. When the E371A mutation was present in only one of two oxygenase domains of a heterodimer, it completely prevented \( \text{L-Arg} \) binding to one oxygenase domain in each heterodimer, but did not affect the \( \text{L-Arg} \) affinity of the oxygenase domain partner and did not prevent heme iron reduction in any case. The E371A mutation prevented NO synthesis when it was located in the oxygenase domain opposite to the reductase domain but had no effect on NO synthesis when it was located in the same subunit as the reductase domain. Our \( ^{54}\text{Fe} \) resonance Raman study showed that the E371A mutation only affected \( \text{L-Arg} \) binding within the same oxygenase domain in which the mutation was present, and consequently revealed that a bound \( \text{L-Arg} \) molecule interacts only with the heme that is located in the same oxygenase component to which \( \text{L-Arg} \) is bound. Together, this led us to conclude that heme iron reduction in the heterodimer must occur entirely in the oxygenase domain adjacent to the full-length subunit, and this enables the oxygenase domain to generate NO by oxidizing the \( \text{L-Arg} \) molecule bound within it.

Our findings establish a critical link between iNOS dimeric structure and heme reduction that helps explain how dimerization activates iNOS. Previous studies had shown that only the oxygenase domains of iNOS participate in the dimeric interaction (17, 28). We now propose that the oxygenase-oxygenase interaction enables the enzyme to engage in a type of “domain swapping” (40) shown in Fig. 11, which in turn allows NADPH-derived electrons to transfer between reductase and oxygenase domains that are located on adjacent subunits. This arrangement possibly circumvents a physical barrier for electron transfer between reductase and oxygenase domains located on the same subunit (29).

Dimerization also enables the oxygenase domains to stably incorporate \( \text{H}_{4}\text{B} \) and to bind \( \text{L-Arg} \) with high affinity (23, 27). This impacts on iNOS electron transfer in a different way.

\footnote{The resonance Raman data is consistent with recent crystallographic structures of the iNOSox monomer and dimer that show Glu-371 is located near the heme that is bound within the same subunit, and binds to the guanidino nitrogens of \( \text{L-Arg} \) such that they are held above that same heme iron (25, 26).}
through an effect of H\textsubscript{2}B and L-Arg on the heme. In the absence of bound L-Arg and H\textsubscript{2}B, the heme iron in an iNOS dimer are solvent-exposed (23) and are poised at such a low reduction potential (~350 mV) that they appear to be thermodynamically unable to accept electrons from the reductase domain flavins (41).\textsuperscript{6} Binding either H\textsubscript{2}B or L-Arg to the dimer alters the heme environment and increases the heme iron reduction potential by 50 or 100 mV, respectively (41). Thus, dimerization properly orients iNOS reductase and oxygenase domains for electron transfer, and creates an environment where substrate and pterin can positively modulate the thermodynamics of heme iron reduction.

Given our iNOS structure-function model in Fig. 11, it is important to consider the model’s limitations and the implications of the domain swapping interaction. Although our data support an exclusive interaction between adjacent reductase and oxygenase domain pairs in an iNOS homodimer, it is still possible that electrons could exchange between the two reductase domains in the homodimer prior to transfer to an individual oxygenase domain. Such “short circuiting” of electron transfer would result in both hemes receiving electrons that were originally provided to a single reductase domain by NADPH. Present evidence suggests that short circuiting may not occur. For example, during titrations with NADPH, electron transfer between individual reductase domains in solution occurs only slowly.\textsuperscript{9} Also, the crystal structure of the iNOSox dimer shows that the two reductase domains would be attached on opposite sides of the dimeric oxygenase core with each one positioned to interact with an exposed heme edge located on the backside of the adjacent subunit’s oxygenase domain (26).

Which protein residues facilitate electron transfer between reductase and oxygenase domains are still unknown. However, putative domain contact regions have already been suggested based on the crystal structures of iNOSox (25, 26) and of NADPH-cytochrome P-450 reductase (42), which is a protein homologous to the reductase domain of iNOS. Interestingly, the proposed contact region on the surface of iNOSox may only become exposed upon dimerization (26), implying another key role for this process. Molecules that antagonize the reductase-oxygenase interaction probably exist both in nature and in the laboratory. For example, cytochrome c (43), caveolin (44–46), and certain oxygenase domain peptides (47) may bind to the oxygenase or reductase contact sites and inhibit NO synthesis by blocking domain interactions required for electron transfer to the heme.

Electron transfer as depicted in Fig. 11 takes place between noncovalently linked reductase and oxygenase domains of the dimer. However, their productive interaction probably requires that the reductase domains still remain covalently linked to the dimeric core, because mixing detached reductase monomers with iNOSox dimers promotes only inefficient heme reduction and slow NO synthesis (48). The reductase-oxygenase interaction does not endow the full-length iNOS dimer with greater stability than an iNOSox dimer (27, 28) and, unlike the oxygenase-oxygenase domain interaction, does not remain intact after the reductase and oxygenase domains are separated by limited proteolysis (17). CaM is likely to have an important role in the reductase-oxygenase domain interaction of iNOS, because it controls electron transfer between these domains in the neuronal and endothelial NOS isoforms (6, 49). Interaction between neuronal NOS and the “latch region” of CaM appears

\textsuperscript{6} The iNOS flavin reduction potentials are unknown. However, NADPH-cytochrome P-450 reductase, which is structurally homologous to the NOS reductase domain, exhibits flavin midpoint potentials of −270 and −290 mV for its one- and two-electron-reduced forms (55).

\textsuperscript{9} L. Huang and D. J. Stuehr, unpublished results.

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