Reconstitution of the Second Step in NO Synthesis Using the Isolated Oxygenase and Reductase Domains of Macrophage NO Synthase†

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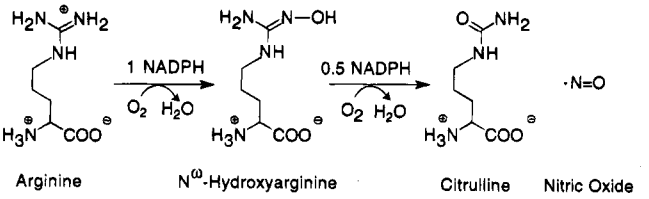
ABSTRACT: Inducible macrophage NO synthase (iNOS) is a homodimer of 130 kDa subunits. Trypsinolysis of iNOS inactivates its NO synthesis activity and cleaves the enzyme into a dimeric oxygenase fragment that contains heme, tetrahydrobiopterin, and the substrate binding site and a monomeric reductase fragment that contains FAD, FMN, calmodulin, and the binding site for NADPH [Ghosh, D. I., & Stuehr, D. H. (1995) *Biochemistry 34*, 801−807]. In this paper, we describe the reconstitution of NO synthesis activity utilizing the isolated oxygenase and reductase domains of iNOS. Mixing the domains at various ratios showed that NO was not produced from L-arginine but could be formed from the reaction intermediate N^{ω}-hydroxy-L-arginine (L-NOHA). The apparent K_m with L-NOHA in the resonstituted system was 100 μM versus 19 μM for native iNOS. D-NOHA was not a substrate. Maximum specific activity (per heme) occurred at an oxygenase to reductase molar ratio of 4:1, with higher ratios causing some inhibition. Reconstitution of activity was associated with electron transfer between the domain fragments and led to an incomplete reduction of the oxygenase domain heme iron. L-NOHA, but not L-arginine, increased NADPH consumption in the reconstituted system. Between 2.5 and 3.0 NADPH were consumed per NO formed from L-NOHA, considerably higher than the stoichiometry obtained with native iNOS (0.5 NADPH oxidized per NO formed), indicating an uncoupled electron transfer between the domain fragments. Thus, the isolated iNOS reductase and oxygenase domains each retain their separate catalytic functions but interact to catalyze only the second step of NO synthesis. In this way, they form a monooxygenase system similar to those of eukaryotic cytochromes P-450.

Nitric oxide synthases (NOSs)[1] catalyze the stepwise formation of NO and citrulline from L-arginine, O_2, and NADPH (Scheme 1) [for review, see Griffith and Stuehr (1995) and Marletta (1993)]. The cytokine-inducible NOS of mouse macrophages (iNOS) is a soluble, catalytically self-sufficient homodimeric protein (subunit molecular mass of 130 kDa) that contains one iron protoporphyrin IX (heme), FAD, and FMN prosthetic group per subunit, variable amounts of tetrahydrobiopterin (H_4biopterin), and an unspecified amount of tightly bound calmodulin (CaM) (Stuehr, 1991; White & Marletta, 1992; Stuehr & Ikeda-Saito, 1992; Cho et a., 1992).

Cloning and modeling studies have identified a flavoprotein reductase domain in the C-terminal half of iNOS and a potential heme-binding oxygenase domain in its N-terminal region, suggesting that iNOS is a bifunctional polypeptide (Xie et al., 1992; Renaud et al., 1993). Recent studies from our laboratory have generated these two functional domains by limited trypsinolysis of dimeric iNOS and have characterized them with respect to prosthetic group content, substrate binding, and their role in dimer formation (Ghosh & Stuehr, 1995). An analogous bidomain structure has been shown to exist for fatty acid hydroxylase cytochrome P-450BM3 of *Bacillus megaterium* (Nahri & Fulco, 1987) and neuronal NOS (Sheta et al., 1994) by limited proteolysis experiments. Although the isolated oxygenase domains retain their substrate binding properties in all cases, reconstitution of monooxygenase (or NO synthesis) activity by mixing of the two isolated domains was unsuccessful.

Scheme 1: Stepwise Catalysis of NO Synthesis by iNOS



Recently, Boddupalli et al. (1992) reported the successful reconstitution of the fatty acid hydroxylase activity of cytochrome P-450BM3 by mixing its recombinant heme and flavoprotein domains. In our earlier paper, mixing of the two iNOS domains under conditions normally used for assaying NO synthesis by full-length iNOS failed to show any activity. The present paper describes conditions that

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[1] Abbreviations: CaM, calmodulin; DTT, dithiothreitol; H_4biopterin, (6*R*,6*S*)-2-amino-4-hydroxy-6-(L-*erythro*-1,2-dihydroxypropyl)-5,6,7,8-tetrahydropteridine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; iNOS, cytokine-inducible nitric oxide synthase; NO, nitric oxide; NOHA, N^{ω}-hydroxy-L-arginine; SOD, ferric−manganese superoxide dismutase.

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reconstitute NO synthesis activity by the tryptic oxygenase and reductase domains of dimeric iNOS. We document interdomain electron transfer, compare NO and citrulline formation with NADPH consumption, and examine the relative abilities of L-arginine and the reaction intermediate N^{ω}-hydroxy-L-arginine (L-NOHA) to act as substrates in the reconstituted system.

MATERIALS AND METHODS

Materials. N^{ω}-Hydroxy-L-arginine (L-NOHA) and N^{ω}-hydroxy-D-arginine (D-NOHA) were synthesized by Dr. Paul Feldman at Glaxo Research Institute. All other reagents were obtained from Sigma or from sources that have been previously reported (Ghosh & Stuehr, 1995).

Preparation of Oxygenase and Reductase Domains. Purified dimeric iNOS was subjected to limited trypsin digestion at 4 °C as described previously (Ghosh & Stuehr, 1995). The proteolytically generated oxygenase and reductase domains were separated using 2′,5′-ADP Sepharose chromatography and characterized by catalytic activity and spectral measurements. After purification, the proteins were concentrated and kept at −70 °C until used.

Spectroscopy and Assay of Enzyme Activity. UV−visible spectra were recorded at 10 °C on a Hitachi U3110 spectrometer equipped with computer-controlled data collection software. Reduction of iNOS flavin and heme groups either in the reconstituted system containing oxygenase and reductase fragments in a 4:1 molar ratio or with the domains alone was recorded under an atmosphere of nitrogen as described previously (Abu-Soud et al., 1994). Cytochrome *c* reduction by the isolated domains was measured by following the NADPH-dependent increase in absorbance at 550 nm according to a published procedure (Ghosh & Stuehr, 1995).

The initial rate of NO synthesis was quantitated spectroscopically at 37 °C using 5−15 μM oxyhemoglobin as a NO scavenger (Abu-Soud et al., 1994; Abu-Soud & Stuehr, 1993). The reactions contained 40 mM HEPES or Tris-HCl buffer, pH 7.7, 4 μM each of H_4biopterin, FAD, and FMN, 0.2 mM DTT, and either L-arginine or L-NOHA at a final concentration of 500 μM, unless otherwise specified. The substrate was added first followed by oxygenase and reductase fragments at varying molar ratios. After preincubation for 2−3 min, 200 μM NADPH was added to initiate the reaction. The NO-mediated conversion of oxyhemoglobin to methemoglobin was monitored at 37 °C as an increase in absorbance at 401 nm and quantitated using an extinction coefficient of 38 mM^{-1} cm^{-1}. K_m and V_{max} values were derived from Lineweaver−Burk plots of $1/v$ versus $1/s$.

NADPH oxidation either by full-length dimeric iNOS, by its isolated domains, or in the reconstituted system was determined spectrophotometrically at 340 nm, using an extinction coefficient of 6220 M^{-1} cm^{-1}. The reaction was started by addition of 100 μM NADPH followed by addition of the desired amounts of oxygenase and reductase domains. To determine substrate-dependent NADPH oxidation, either L-arginine or L-NOHA was added last at 500 μM final concentration. Substrate-specific NADPH oxidation was determined by subtracting the rate of NADPH oxidation in the reconstituted system under substrate-free conditions from that obtained in the presence of substrate.

Kinetics of Citrulline Production in the Reconstituted System. Enzymatic conversion of L-NOHA or L-arginine was followed in the reconstituted system containing 0.2 μM oxygenase and 0.05 μM reductase domains by measuring citrulline production. In a typical experiment, the reactions (50−125 μL) were carried out in HEPES buffer under the conditions used for the oxyhemoglobin assay (omitting oxyhemoglobin) and contained 5−40 nmol of L-NOHA or L-arginine and 200 μM NADPH at 37 °C. At specified times, 10 μL aliquots were transferred to microfuge tubes kept on dry ice. Citrulline and unreacted L-NOHA or L-arginine were derivatized with *o*-phthaldialdehyde reagent (OPA) and analyzed by reverse-phase HPLC with fluorescent detection using a slight modification of the methods of Joseph and Marsden (1986). Amounts of citrulline and unreacted substrates were calculated from standard curves generated with authentic compounds.

Generation of an Intermediate L-Arginine Metabolite. Reactions were run in duplicate (50 μL), containing 0.42 μM oxygenase domain, 0.084 μM reductase domain, 1 mM NADH, [^{14}C]-L-arginine (200 nM, 1.2×10^6 cpm), and 200 μM cold L-arginine, in Tris-HCl buffer, pH 7.7, containing all the cofactors for NO synthesis as described earlier. After incubation at 37 °C for 90 min, the reaction was stopped by cooling on ice, and L-citrulline and L-NOHA were added at 1 mM to the reaction mixture. A portion of the reaction mixture (20 μL) was analyzed by thin-layer chromatography using an ethanol/acetic acid/water (4/1/1) solvent system, which separates L-arginine, L-NOHA, and citrulline (Stuehr et al., 1991b). Samples were visualized on the plate with ninhydrin spray. The areas on the plate comigrating with standard L-citrulline and L-NOHA were analyzed for ^{14}C by liquid scintillation counting.

RESULTS

The oxygenase and reductase fragments used for this study were similar to those previously characterized (Ghosh & Stuehr, 1995), in that the oxygenase fragment was dimeric, contained heme and H_4biopterin, and could bind L-arginine, while the reductase domain was monomeric, contained FAD, FMN, and CaM, and catalyzed electron transfer from NADPH to cytochrome *c* at a rate of 2.8−3.0 mol of cytochrome *c* reduced per minute per millimole of bound FAD. Our initial work revealed that mixtures of the two domains did not convert L-arginine to NO but could catalyze some NO synthesis from L-NOHA. The ability of the mixed domains to catalyze NO synthesis and NADPH oxidation in the presence of L-NOHA or L-arginine at various oxygenase:reductase ratios is summarized in Figure 1.

As shown in panel A, the rate of NO synthesis from L-NOHA increased as the oxygenase:reductase ratio was increased, peaking at a 6:1 ratio and then falling off at a higher ratio. In contrast, there was no measurable rate of NO synthesis from L-arginine at either of two ratios. The increase in NOHA-dependent NO synthesis that was observed at the higher oxygenase:reductase ratios correlated with an increase in NADPH oxidation by the mixed system (panel B). However, an examination of the rates of NADPH oxidation observed in the absence of substrate shows that approximately half of the increase in NADPH oxidation was independent of substrate at all ratios tested. In fact, simple addition of the oxygenase domain markedly stimulated NADPH oxidase activity in the system over that observed for the reductase domain alone, which is also shown in panel

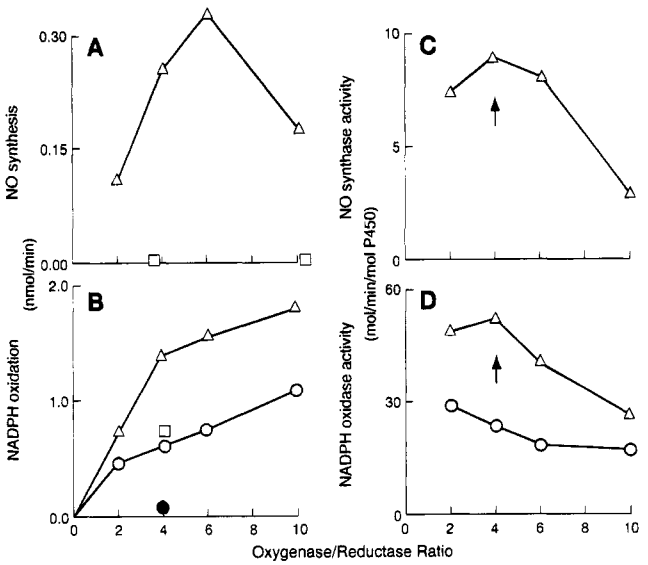

FIGURE 1: NO synthesis and NADPH oxidase activity in a reconstituted system at different molar ratios of iNOS oxygenase and reductase domains. NO synthesis and NADPH oxidation were measured in 400 μL assays containing either no substrate (○), 500 μM L-arginine (□), or 500 μM L-NOHA (△), 200 μM NADPH, and various concentrations of iNOS oxygenase domain (0−250 nM) with a fixed concentration of reductase domain (25 nM). Panel A shows the rates of NO synthesis with L-NOHA or L-arginine acting as substrate. Panel B depicts rates of NADPH oxidation in the absence of substrate or in the presence of L-NOHA or L-arginine or by the reductase fragment alone (●). Panels C and D show the specific activities of NO synthesis and NADPH oxidation in the presence of L-NOHA (△) or in the absence of substrate (○). The arrow indicates data obtained at the ratio used in all subsequent studies. The data shown are representative of three similar experiments.

B. The oxygenase domain alone exhibited no NADPH oxidase activity under these conditions (data not shown). The substrate-independent increase in NADPH consumption due to oxygenase domain addition was not prevented by agents known to block heme iron reduction in NOS, such as imidazole and thiocitrulline (Abu-Soud et al., 1994) (data not shown). In contrast to L-NOHA, neither L-arginine (panel B) nor D-NOHA (not shown) stimulated NADPH oxidation at a 4:1 oxygenase to reductase ratio, consistent with their not functioning as substrates for NO synthesis in the reconstituted system. By subtracting the substrate-independent rates of NADPH consumption from the rates observed in the presence of L-NOHA, we calculate an average stoichiometry of 3 ± 0.6 mol of NADPH oxidized per mole of NO formed from L-NOHA at oxygenase to reductase ratios of 2, 4, and 6. This value is considerably higher than the stoichiometry obtained for full-length dimeric iNOS (0.5 mol of NADPH per NO) (Stuehr et al., 1991b), indicating L-NOHA-dependent NADPH oxidation by the mixed domains is relatively uncoupled with regard to NO synthesis. To check if the superoxide generated due to uncoupled NADPH oxidation led to scavenging of NO (Beckman et al., 1990), we examined whether an increased SOD concentration would boost the detection of NO synthesis from L-NOHA. However, addition of SOD at levels up to 100 units/mL (10 units/mL is normally used in our experiments) did not increase NO detection by oxyhemoglobin, indicating that the values reported in Figure 1 are accurate with regard to NO synthesis and NADPH oxidation stoichiometry. Conversion of the L-NOHA data in panels A and B to

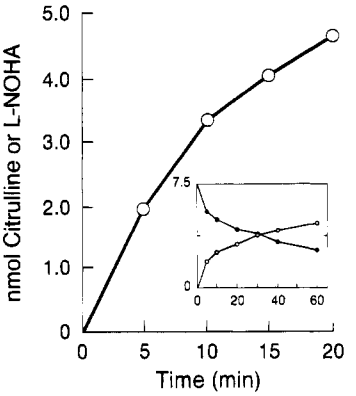

FIGURE 2: Citrulline production (○) from L-NOHA over time in the reconstituted system. The 125 μL reaction was run at 37 °C and contained 37.5 nmol of L-NOHA and 200 μM NADPH. Samples were withdrawn at the indicated times and reacted with OPA for fluorometric detection using HPLC as described in Materials and Methods. The inset compares citrulline production (○) and L-NOHA disappearance (●) over time in a similar reaction that initially contained 7.5 nmol (50 μM) of L-NOHA. Results shown are representative of two or three similar experiments.

specific activities (panels C and D) shows that maximum NO synthesis and corresponding NADPH oxidation occurs at an oxygenase to reductase ratio of 4:1. Therefore, this ratio was used for the remaining experiments in the study.

As shown in Figure 2, conversion of L-NOHA to citrulline in the reconstituted system under V_{max} conditions was nearly linear for 10 min. The inset shows that citrulline formation could completely account for the amount of L-NOHA consumed, indicating that there were no other significant routes of metabolism for L-NOHA. Also, the initial rate of citrulline production (0.4 nmol/min) approximated the initial rate of NO synthesis (0.3 nmol/min) as determined under identical conditions with the oxyhemoglobin assay. The apparent K_m for L-NOHA in the reconstituted system (80−100 μM) was higher than that obtained with native enzyme (15 μM) under identical conditions. The corresponding V_{max} values were 8 and 100 mol of NO per minute per mole of P450 in the reconstituted and native systems, respectively.

To check whether there was trace metabolism of L-arginine to L-NOHA or citrulline, we monitored the oxidation of [^{14}C]-L-arginine in the reconstituted system using thin-layer chromatography. Analysis of an 90 min reaction revealed that only 0.04% of the initial L-arginine (10 nmol) was converted to L-NOHA or citrulline. These results are consistent with our NO synthesis measurements and confirm the relative inability of the reconstituted system to metabolize L-arginine.

To further understand catalysis in the reconstituted system, we investigated whether the reductase domain could transfer electrons from NADPH to the oxygenase domain heme iron, as occurs in native full-length iNOS (Baek et al., 1993; Abu-Soud & Stuehr, 1993). Reduction of the heme iron was monitored by CO binding under an anaerobic atmosphere. As shown in panel A of Figure 3, about 22% of the heme iron was reducible by NADPH in the reconstituted system relative to that obtained using dithionite, which completely reduces the iNOS heme iron independent of the reductase domain (Ghosh & Stuehr, 1995). The inset shows the difference spectrum for the NADPH-dependent reduction data in panel A. Adding L-NOHA did not increase NADPH-dependent heme iron reduction over the substrate free value

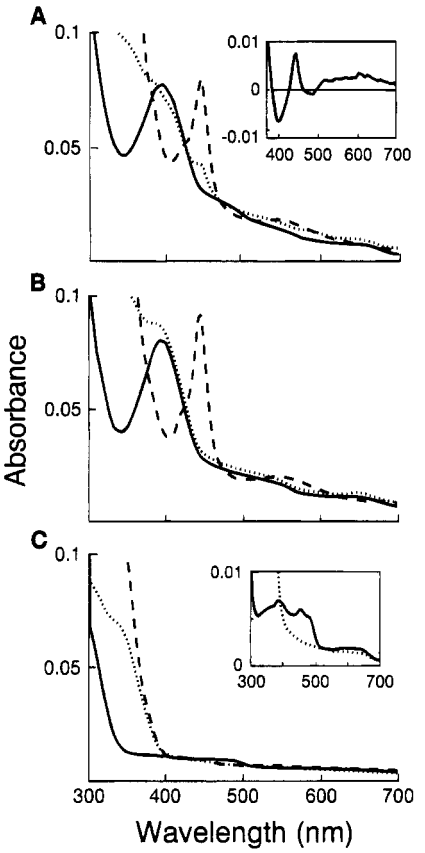

FIGURE 3: Reduction of the oxygenase domain heme iron in the reconstituted system as determined by CO binding. All samples were equilibrated at 10 °C under anaerobic conditions in buffer saturated with CO. Panel A: The cuvette contained 1 μM oxygenase domain and 0.25 μM reductase domain in 2.5 mL of 40 mM Bis-Tris-propane, pH 7.4. Spectra were collected prior to (—) and following (•••) addition of 20 μM NADPH and a 60 min equilibration period or following addition of 60 μM dithionite (- - -). The inset shows the difference spectrum generated by subtracting the spectrum obtained following NADPH addition from the initial spectrum prior to NADPH addition. Panel B: An experiment run under the same conditions as in panel A except it contained the oxygenase domain alone. Panel C: The same experiment as above except it contained the reductase domain alone. The inset shows the details of spectra recorded prior to and following addition of NADPH to the reductase domain alone. Results shown are representative of two or three similar experiments.

(data not shown). Panel B shows that NADPH was completely unable to reduce the oxygenase domain heme iron in the absence of added reductase domain, as judged by a lack of CO complex absorbance at 444 nm, while dithionite addition resulted in complete reduction. This indicated that there was no contaminating full-length iNOS in the oxygenase preparation. Panel C shows that a CO complex also did not form upon reduction of the reductase domain with either NADPH or dithionite, indicating the absence of contaminating full-length iNOS. However, the inset shows that NADPH was able to completely reduce the reductase domain flavins, as judged by the loss of their visible absorbance between 400 and 500 nm.

DISCUSSION

Previous work with native full-length iNOS has shown that NO synthesis requires an intact dimeric oxygenase domain (Baek et al., 1993; Ghosh & Stuehr, 1995) and involves electron transfer between the reductase and the oxygenase domains (Abu-Soud & Stuehr, 1993). Our current results establish that the iNOS tryptic domains can carry out partial catalysis of the NO synthesis reaction, in that they convert L-NOHA, but not L-arginine, to NO. This conversion represents the second step in the enzymatic oxidation of L-arginine to NO (Stuehr et al., 1991, Pufahl & Marletta, 1992). Nonenzymatic processing of L-NOHA in our system was ruled out by the finding that D-NOHA was not metabolized and conversion of L-NOHA to citrulline and NO was independent of superoxide formation.

Maximum activity in the reconstituted system required that the oxygenase domain be present in excess over the reductase domain. However, even under this condition the specific rate of NO synthesis was approximately one-tenth that of native full-length iNOS, when compared on a per heme basis. The reduced rate was not due to an inability of the oxygenase domain to bind L-arginine (Ghosh & Stuehr, 1995) or L-NOHA nor due to its conversion to an inactive P420-like species (see Figure 3, panel A). Likewise, the tryptic reductase domain was able to bind NADPH, reduce its flavins, and catalyze reduction of cytochrome *c* at a rate identical to native full-length iNOS (Baek et al., 1993). Instead, there appeared to be an inefficiency regarding electron transfer to the oxygenase domain heme iron in the reconstituted system. Only 20–25% of the oxygenase domain heme iron was susceptible to NADPH-dependent reduction as mediated by the iNOS reductase domain, as opposed to the 90–100% reduction that is typically observed for full-length iNOS (Abu-Soud & Stuehr, 1993). If one assumes that only the fraction of heme iron that can be reduced participates in NO synthesis in the reconstituted system, then the specific rate of NO synthesis approaches 50% of that observed with full-length native iNOS. Thus, it is likely that incomplete reduction of the oxygenase domain heme iron limits catalysis in our reconstituted system.

NADPH consumption measurements showed that the presence of the oxygenase domain greatly stimulated NADPH oxidation by the reductase domain relative to its basal value. However, this increase in electron flux did not appear to involve electron transfer to the oxygenase domain heme iron, because it was not prevented by agents that block heme iron reduction in NOS (imidazole and thiocitrulline; Abu-Soud et al., 1994). That the oxygenase domain promotes heme-independent NADPH oxidation in the mixed domain system is intriguing and deserves additional study.

Adding L-NOHA further boosted NADPH consumption in the reconstituted system in conjunction with NO synthesis. It is impossible to tell whether the increased electron flux in this case occurred through the heme iron. However, only a fraction (one-sixth) of the substrate-dependent electron flux was coupled to NO synthesis, as judged by the stoichiometry observed in the reconstituted system (3 NADPH per NO) relative to native full-length iNOS (0.5 NADPH per NO; Stuehr et al., 1991b). Thus, the tryptic domains of iNOS can electronically interact but catalyze an inefficient interdomain electron transfer relative to full-length native enzyme. Similar uncoupled electron transfer has been observed upon mixing the recombinant flavoprotein and heme domains of cytochrome P-450BM3 (Boddupalli et al., 1992).

In studies with recombinant heme and flavin domains of cytochrome P-450BM3, Boddupalli et al. (1992) point out

that the rate-limiting step in the reconstituted system may be the productive collision of the individual domains. To maximize productive collisions, they increased the heme domain concentration over that of flavoprotein domain, as we did in our current system. In multicomponent P-450 monooxygenase systems, individual reductase and oxygenase proteins must interact to form a catalytically active complex. However, in proteins that contain oxygenase and reductase domains on the same polypeptide (P-450BM3 and iNOS), selective pressure to maintain a strong interdomain binding affinity may have been lost over time. Thus, once separated from each other, it may be difficult for the iNOS domains to associate in a correct orientation for productive electron transfer.

On the basis of the above discussion and our current understanding of the enzyme reaction mechanism, we can begin to discern why catalysis of the second step is favored in the reconstituted system. A variety of evidence indicates that conversion of L-arginine to L-NOHA occurs via mixed function hydroxylation, which requires that two electrons derived from NADPH be transferred from the reductase domain to the oxygenase domain for oxygen activation by the heme iron (Stuehr et al., 1991b; Griffith & Stuehr, 1995; Marletta, 1993; Feldman et al., 1993). In contrast, our stoichiometry studies of the second step in NO synthesis (Stuehr et al., 1991b) indicate that only one electron need be transferred from the reductase domain for conversion of L-NOHA to NO and citrulline, leading to the proposal that L-NOHA donates one electron to NOS during its own oxidation (Stuehr & Griffith, 1992; Marletta, 1993; Feldman et al., 1993). In our reconstituted system, we speculate that the two- or three-electron transfers required for conversion of L-arginine to L-NOHA or to citrulline plus NO are less likely to occur than single electron transfer between temporarily interacting tryptic domains. Thus, catalysis in the reconstituted system is limited to the second step in NO synthesis.

A related point is that the separated iNOS domains now behave more like typical cytochrome P-450's in their catalyzing only a single monooxygenation. This provides experimental support for sequence and modeling studies that suggest NOS arose through a gene fusion between an NADPH-dependent flavoprotein reductase and a cytochrome P-450-like heme protein. Indeed, the specific activity of our reconstituted enzyme system [8−10 mol min^{-1} (mol of P-450)$^{-1}$] is significantly greater than that observed for many reconstituted mammalian cytochrome P-450 systems (Guengerich, 1991; Wolf et al., 1988), which are typically about 1 mol min^{-1} (mol of P-450)$^{-1}$] for many substrates.

In summary, the isolated domains of iNOS were found to interact electronically but could only catalyze the second step in NO synthesis, using the reaction intermediate L-NOHA as substrate. Their inability to catalyze the two-step oxidation of the physiologic substrate L-arginine appeared to be related to an inefficient electron transfer between the two separate domains. This suggests that structural integrity is important for the interdomain electron transfers leading to NO synthesis and may help explain why iNOS developed and maintains its bidomain structure.

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