Domains of Macrophage NO Synthase Have Divergent Roles in Forming and Stabilizing the Active Dimeric Enzyme†

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ABSTRACT: The cytokine-inducible NO synthase (iNOS) is a flavin-containing hemeprotein that must dimerize to generate NO. Trypsin cleaves the dimeric enzyme into an oxygenase domain fragment that remains dimeric, contains heme and H4biopterin, and binds L-arginine and a reductase domain fragment that is monomeric, binds NADPH, FAD, FMN, and catalyzes the reduction of cytochrome *c* [Ghosh, D. K. & Stuehr, D. J. (1995) *Biochemistry 34*, 801−807]. The current study investigates the isolated oxygenase and reductase domains of iNOS to understand how they form and stabilize the active dimeric enzyme. The dimeric oxygenase domain dissociated into folded, heme-containing monomers when incubated with 2−5 M urea, whereas the reductase domain unfolded under these conditions and lost its ability to catalyze NADPH-dependent cytochrome *c* reduction. Spectral analysis of the dissociation reaction showed that it caused structural changes within the oxygenase domain and exposed the distal side of the heme to solvent, enabling it to bind dithiothreitol as a sixth ligand. Importantly, the oxygenase domain monomers could reassociate into a dimeric form even in the absence of the reductase domain. The reaction required L-arginine and H4biopterin and completely reversed the structural changes in heme pocket and protein structure that occurred upon dissociating the original dimer. Together, this confirms that the oxygenase domain contains all of the determinants needed for subunit dimerization and indicates that the dimeric structure greatly affects the heme and protein environment in the oxygenase domain.

Nitric oxide (NO)[1] has emerged as an important signal and effector molecule in mammalian physiology (Nathan, 1992; Schmidt & Walter, 1994). Biosynthesis of NO is carried out by three NO synthase isoforms (NOSs) which catalyze the formation of NO and citrulline from L-arginine, O_2, and NADPH [for reviews, see Griffith and Stuehr (1995) and Marletta (1993)]. An inducible NOS isoform (iNOS) is expressed in a variety of human and animal cells upon exposure to immunostimulating cytokines, bacterial products, or infection (Nathan, 1992; Kroncke et al., 1995). The iNOS appears to be the product of a single gene whose coding sequence is highly conserved between species (Nathan & Xie, 1994; Geller et al., 1993). Expression of iNOS enables cells to control the growth of microbial pathogens and is also linked to tissue destruction that occurs in a number of diseases including arthritis, diabetes, septic shock, transplant rejection, and multiple sclerosis (Kroncke et al., 1995; Stefanovic-Racic et al., 1993; Hoffman et al., 1993; Southern et al., 1990; Bo et al., 1994).

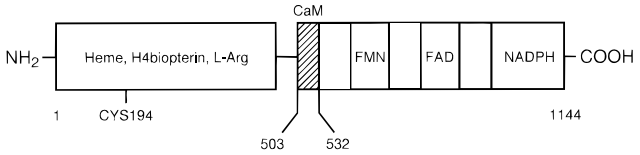

FIGURE 1: Distribution of prosthetic group and substrate binding sites in a single subunit of an iNOS dimer. The N-terminal portion of the subunit is an oxygenase domain that binds L-arginine, H4biopterin, and heme, while the C-terminal portion is a reductase domain that binds calmodulin (CaM), FMN, FAD, and NADPH. Modeling considerations predict that Cys194 may be involved in heme iron binding (Renaud et al., 1993).

The iNOS purified from mouse macrophages is a homodimeric enzyme (subunit molecular mass 130 kDa) that contains iron protoporphyrin IX (heme), FAD, FMN, H4biopterin, and calmodulin as bound prosthetic groups (Stuehr et al., 1991; White & Marletta, 1992; Stuehr & Ikeda-Saito, 1992; Cho et al., 1992). A variety of work suggests that iNOS is the product of a gene fusion between an "oxygenase" domain (residues 1−503) that contains heme, H4biopterin, and the L-arginine binding site and a "reductase" domain (residues 504−1172) that contains calmodulin, the flavins, and the binding site for NADPH (Figure 1) (Xie et al., 1992; Ghosh & Stuehr, 1995). During NO synthesis, an interdomain electron transfer occurs between the reductase and oxygenase domains that leads to reduction of the heme iron, oxygen activation, and conversion of L-arginine to citrulline plus NO (Abu-Soud & Stuehr, 1993). The domains thus have distinct but complimentary roles in electron import/transfer and in catalyzing the chemistry of NO synthesis.

Although control of iNOS protein expression occurs at the transcriptional level (Nathan & Xie, 1994; Geller et al., 1993), work by Baek et al. (1993) suggests that the protein

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[1] Abbreviations: CaM, calmodulin; DTT, dithiothreitol; H4biopterin, (6*R*,6*S*)-2-amino-4-hydroxy-6-(L-*erythro*-1, 2-dihydroxypropyl)-5,6,7,8-tetrahydropteridine; iNOS, cytokine-inducible nitric oxide synthase; NO, nitric oxide; SOD, ferric−manganese superoxide dismutase.

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is synthesized as an inactive monomer that must dimerize to generate the active enzyme. Purified iNOS monomers contain an intact reductase domain that binds calmodulin, FMN, FAD, and NADPH and reduces electron acceptors such as cytochrome *c* but do not contain an intact oxygenase domain, because they are devoid of H4biopterin and heme and cannot synthesize NO. These monomers can assemble into dimers in the coincident presence of heme, H4biopterin, and L-arginine. Dimerization is accompanied by insertion of heme and H4biopterin into the oxygenase domain and expression of an NO synthesis activity (Baek et al., 1993). Human iNOS appears to be controlled by a similar mechanism, in that H4biopterin is required to assemble the active dimer in intact cells (Tzeng et al., 1995). Together, this suggests that subunit dimerization is a critical posttranslational modification that assembles the active iNOS by generating a functional oxygenase domain.

The oxygenase and reductase domains of iNOS or neuronal NOS can be separated from one another by treating the dimeric enzyme with a limited amount of trypsin, which cuts each subunit between the domains (Ghosh & Stuehr, 1995; Sheta et al., 1994). The isolated domains of iNOS maintain their individual properties and when mixed together catalyze NO synthesis from the reaction intermediate *N*-hydroxy-L-arginine (Ghosh et al., 1995), suggesting that they can exist and function independently of one another. Importantly, the iNOS oxygenase domain fragment (amino acids 1−503) is isolated as a dimer, while the reductase domain fragment is monomeric. While this suggests that the oxygenase domains of two subunits may interact exclusively in the iNOS dimer (Ghosh et al., 1995), it does not reveal what role the reductase domain has in forming and stabilizing the dimer or how dimerization impacts on the structure of the oxygenase domain. Such information would help us understand how assembly of active iNOS is controlled at the posttranslational level and may provide an additional target for therapeutic intervention.

To investigate these questions, we examined the individual oxygenase and reductase domains of iNOS regarding their dissociation and unfolding in urea and their ability to reassociate into a dimeric form. Our results show that each domain has a distinct role in dimer assembly and stabilization and indicate that reversible structural changes occur in the oxygenase domain upon dimer dissociation and reassembly.

MATERIALS AND METHODS

Reagents. Ultra-pure urea was obtained from Aldrich Chemicals. All other supplies were of the highest purity grade available and from either Sigma or from sources previously reported (Ghosh & Stuehr, 1995; Abu-Soud & Stuehr, 1993; Baek et al., 1993).

Preparation of Oxygenase and Reductase Domains. Dimeric macrophage iNOS underwent limited trypsin digestion at 4 °C to split the enzyme into a dimeric oxygenase domain and a monomeric reductase domain (Ghosh & Stuehr, 1995). The domains were separated from one another using 2′,5′-ADP Sepharose chromatography as previously described, concentrated, and stored at −70 °C.

Incubation of Oxygenase and Reductase Domains with Urea. Each domain was incubated at 4 °C in 40 mM Tris-HCl buffer, pH 7.5, containing 2 mM DTT and the indicated urea concentrations (0−5 M) in a final volume of 125 μl. Domain concentrations ranged from 3 to 6 μM, and the incubations were run for up to 2 h. In some cases, incubations containing the oxygenase domain were carried out in quartz cuvettes at 10 °C in final volumes of 0.3 or 1.0 mL in order to monitor light absorbance or fluorescence emission over time during subunit dissociation with 5 M urea.

Cytochrome c Reductase Activity. The iNOS reductase domain was incubated with various concentrations of urea for 2 h, and the samples were diluted with 9 volumes of cold Tris buffer, pH 7.6, containing 2 μM each of FMN and FAD and were assayed in triplicate for NADPH-dependent cytochrome *c* reductase activity as described previously (Ghosh & Stuehr, 1995; Baek et al., 1993).

Spectroscopy. Light absorbance spectra were recorded at 120 nm/min with a Hitachi 3010 UV−visible spectrophotometer during iNOS oxygenase domain dissociation or dimerization. In some cases, a Thermomax kinetic plate reader was used to follow the change in absorbance at 405 nm during dimerization of oxygenase domain monomers. The change in tryptophan fluorescence during iNOS oxygenase domain dissociation or dimerization were recorded at 10 or 27 °C using a Hitachi F-2000 Spectrofluorometer set at an excitation wavelength range of 290−300 nm and an emission wavelength range of 325−335 nm. A neutral density filter that blocked 92% of the incident light was used in the fluorescence studies to reduce the intensity of light irradiating the sample during the experiment. Readings were corrected by subtracting data obtained in the absence of protein.

Gel Filtration Chromatography. Samples containing either iNOS domain were analyzed by gel filtration chromatography on a 30 × 10 cm Pharmacia Superdex 200HR column. The column was equilibrated with 40 mM Tris-HCl buffer, pH 7.7, containing 2 μM H4biopterin, 2 mM DTT, and 10% glycerol. For analyzing samples incubated with urea, the column buffer contained urea at a concentration identical to that used in each incubation. Protein in the column effluent was detected at 280 nm using a flow-through detector. The apparent molecular weights of the protein peaks were estimated relative to gel filtration molecular weight standards as previously described (Ghosh & Stuehr, 1995).

Dimerization of Urea-Generated Oxygenase Domain Monomers. Monomers were prepared by dissociating the dimeric oxygenase domain in 5 M urea for 2 h as described above. To lower the urea concentration of the monomer preparation, it was dialyzed at 4 °C against 500 volumes of 40 mM Tris-HCl buffer, pH 7.7, containing 2 M urea, 10% glycerol (v/v), and 3 mM DTT for 4 h and then dialyzed overnight against 500 volumes of 40 mM Tris-HCl buffer, pH 7.7, containing 0.1 M urea, 3 mM DTT, and 10% glycerol.

Dimerization reactions were carried out for 30−90 min at 27 °C and contained the oxygenase monomers at 0.75−1.5 μM in Tris-HCl buffer, pH 7.7, containing 3 mM DTT, 10 μM H4biopterin, 3 mM L-arginine, and 0.1 mg/mL BSA. Reactions were run in cuvettes or in a 96-well microplate in order to monitor changes in visible absorbance or tryptophan fluorescence emission that occur during the dimerization reactions. Additives were omitted from the incubation as described in the text. Samples were analyzed by gel filtration chromatography to determine the relative amounts of monomer and dimer that were present at the end of each reaction.

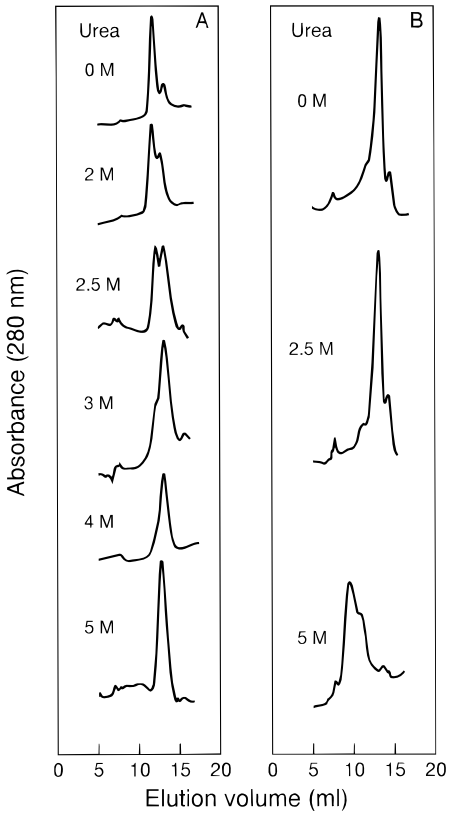

FIGURE 2: Structural changes in the iNOS oxygenase and reductase domains upon their incubation with urea. The dimeric oxygenase domain (A) or monomeric reductase domain (B) were incubated with the indicated concentrations of urea at 4 °C for 2 h and then subjected to analysis by gel filtration chromatography. The protein peaks represent the dimeric, monomeric, or unfolded forms of the domains. The data are representative of two separate experiments.

RESULTS

We utilized urea to dissociate the dimeric oxygenase domain fragment into monomers, given that this method has been successful for dissociating full-length dimeric iNOS (Abu-Soud et al., 1995). Figure 2, panel A, depicts gel filtration profiles of oxygenase domain dimer preparations that were incubated for 2 h in buffer containing different concentrations of urea. At 0 M urea, a very small percentage of the oxygenase domain was present in monomeric form, as indicated by the peak eluting at 14 mL (estimated molecular mass, 55 kDa). At 2 M urea, the monomer peak appears as a shoulder on the right side of the dimer peak. Samples incubated with 2, 2.5, 3, 4, and 5 M urea show a concentration-dependent shift toward monomer, such that the dimeric species is estimated to represent about 80, 50, 30, 20, and 10% of the total oxygenase protein at the indicated urea concentrations, respectively. In no case did we observe formation of a species with a shorter retention time than the dimer, indicating that unfolding of the oxygenase domain does not occur even at 5 M urea. This contrasts with full-length dimeric iNOS, which partially unfolds at this urea concentration (Abu-Soud et al., 1995). Thus, equilibration in 5 M urea was required to dissociate the oxygenase domain dimer into folded monomers.

Given the above, we investigated the effect of urea on the physical and catalytic properties of the iNOS reductase domain. The reductase domain is generated by trypsin cleavage of dimeric iNOS and is isolated as a monomer that can catalyze electron transfer from NADPH to cytochrome *c* (Ghosh & Stuehr, 1995). As depicted in panel B of Figure 2, incubation with urea at concentrations up to 2.5 M did not increase the apparent hydrodynamic volume of the reductase domain, indicating denaturation had not occurred. However, incubation with 5 M urea did cause unfolding of the reductase domain. Loss of native protein structure was associated with loss of catalytic function, as measured by cytochrome *c* reductase activity (Figure 3). Thus, the iNOS oxygenase and reductase domains differ regarding the stability of their tertiary structures in the presence of chaotropic agents like urea.

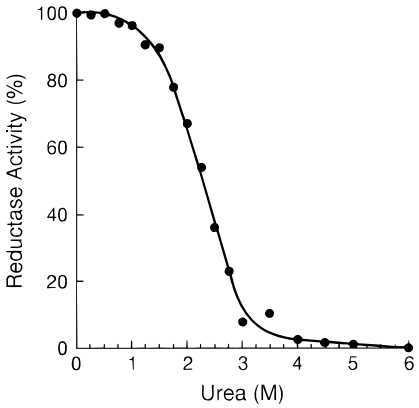

FIGURE 3: Loss of reductase domain catalytic function following incubation in various concentrations of urea. The reductase domain was incubated for 2 h at 4 °C with the indicated concentrations of urea and then diluted and assayed in triplicate for NADPH-dependent cytochrome *c* reductase activity. The points shown are the average of two separate experiments.

We next characterized the changes that occur in the dimeric oxygenase domain during its dissociation in 5 M urea. Figure 4 panels A and B show that dissociation in the presence of DTT is accompanied by time-dependent change in the oxygenase domain's visible spectrum, resulting in a loss of absorbance near 400 nm and a gain in absorbance at 460 nm. This spectral change indicates that DTT is binding as a sixth ligand to the oxygenase domain heme iron, as occurs during dissociation of the full-length iNOS dimer (Abu-Soud et al., 1995). The inset of panel A shows a spectrum of the dithionite-reduced, CO-bound oxygenase domain monomer. The absorbance peak at 444 nm indicates that its heme iron remains bound in the protein through ligation to a cysteine thiolate, as is the case for the dimeric oxygenase domain and native iNOS (Ghosh & Stuehr, 1995; Stuehr & Ikeda-Saito, 1992). Panel C shows that a time-dependent increase in tryptophan fluorescence emission also occurs during dissociation of the oxygenase domain dimer in 5 M urea. Control reactions that contained the dimer without urea maintained the same fluorescence intensity over the experimental period (not shown).

Previous studies from our laboratory indicate that dimerization of native iNOS subunits requires the presence of added heme, H4-biopterin, and L-arginine (Baek et al., 1993). In contrast, dimerization of urea-generated iNOS subunits requires only H4biopterin and L-arginine, due to their containing endogenous heme (Abu-Soud et al., 1995). The availability of oxygenase domain monomers now enabled us to test whether the oxygenase domain itself would dimerize in the absence of the iNOS reductase domain, to determine what cofactors were required for this transformation and to observe which spectral changes that occur upon

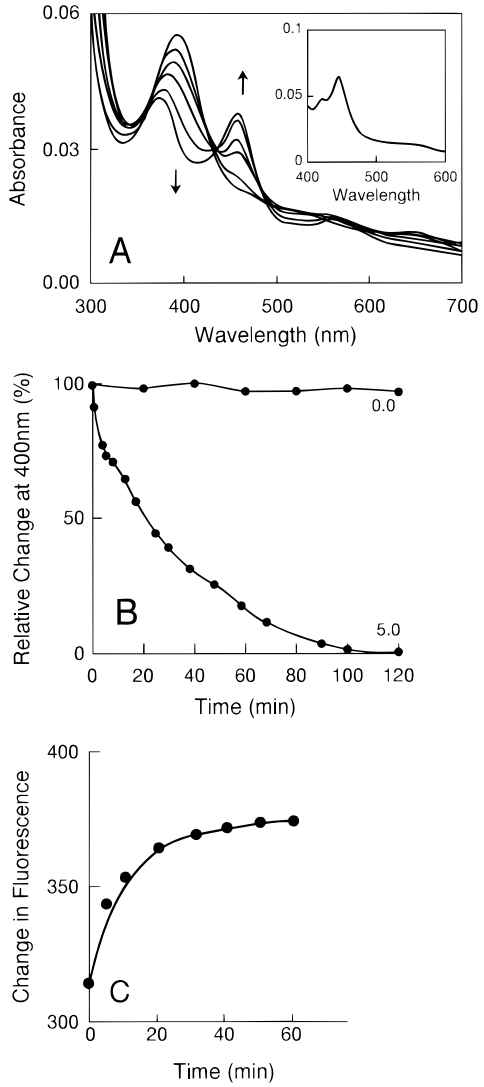

FIGURE 4: Changes in dimeric oxygenase domain light absorbance and tryptophan fluorescence during its dissociation in 5 M urea at 10 °C. (A) UV−visible spectra of dimeric iNOS oxygenase domain (1 μM) in the presence of 1.5 mM DTT recorded both prior to and 1, 10, 20, 30, 60, or 120 min after adding urea. The arrows indicate the direction of absorbance change over time. The inset of panel A contains the spectrum of the dithionite-reduced, CO-bound oxygenase domain formed after its incubation for 120 min with 5 M urea. The data are representative of three experiments. (B) The kinetics of absorbance loss at 400 nm during incubation of the oxygenase domain with 0 or 5 M urea. (C) The change in protein tryptophan fluorescence over time under the same conditions.

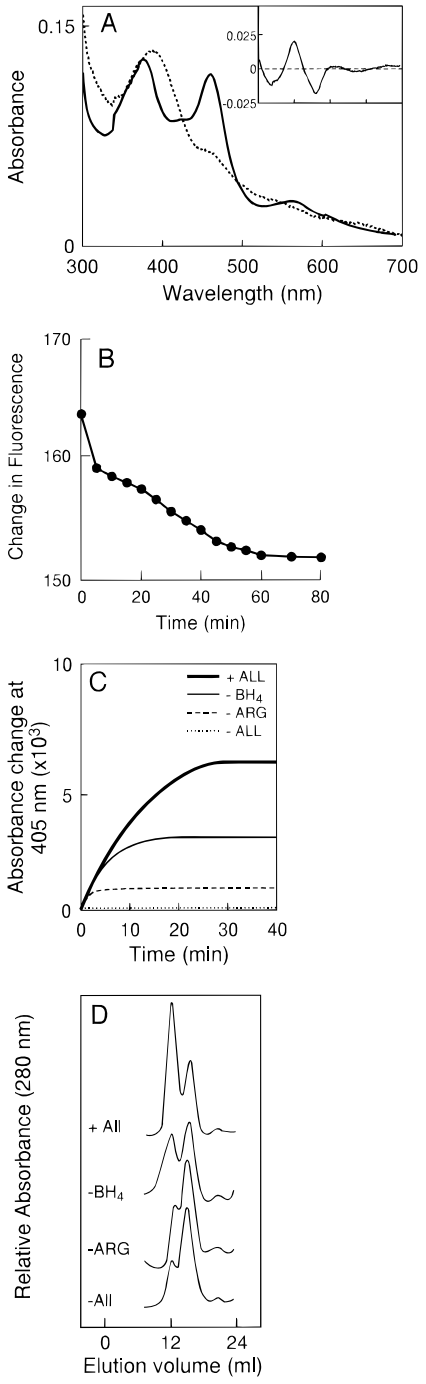

FIGURE 5: Light absorbance and tryptophan fluorescence changes associated with dimerization of the oxygenase domain monomer. (A) Light absorbance spectra of the oxygenase domain monomer (1.5 μM) before (—) and after (---) a 60 min incubation with 5 mM L-arginine and 10 μM H4biopterin at 27 °C. The inset depicts the difference spectrum obtained by subtracting the initial spectrum from the 60 min spectrum. The data are representative of two similar experiments. (B) Decrease in oxygenase domain tryptophan fluorescence during incubation of the oxygenase domain monomer under the same conditions as panel A. Results shown are one of two experiments. (C) Time course of absorbance change at 405 nm during incubation of the oxygenase monomer under various dimerization conditions. The absorbance change in incubations containing both H4biopterin and L-arginine (+all), or in incubations that omitted either H4biopterin (−BH$_4$), L-arginine (−ARG), or both (−all) are also shown. The data are representative of three experiments. (D) Gel filtration profiles of the incubates from panel C.

dissociation would be reversed upon recovery of dimeric structure.

Dimerization of oxygenase domain monomers was studied after lowering the urea concentration to 0.1 M in all cases. As depicted in Figure 5, panels A and B, incubating the oxygenase domain monomers with L-arginine and H4biopterin for 60 min almost completely reversed the spectral and fluorescence changes that were observed during dissociation of the oxygenase dimer in 5 M urea. The inset of panel A shows the absorbance difference between the spectra obtained prior to or after incubation of oxygenase monomers with L-arginine and H4biopterin and identifies 405 nm as a point of maximum change. Panel C shows the increase in absorbance at 405 nm over time for incubations containing oxygenase monomers plus both L-arginine and H4biopterin, either with additive alone or without either additive. In each case, approximately 30−40 min were required to reach equilibrium. The absorbance increase was reduced 60% in

the absence of H4biopterin and reduced 85% in the absence of L-arginine. The spectral recovery did not occur when both L-arginine and H4biopterin were omitted. Panel D depicts the gel filtration profiles of the oxygenase monomer incubations of panel C. They show that the increases in absorbance are associated with formation of oxygenase domain dimers (eluting at 12.8 mL) from monomers (eluting at 14 mL). A comparison of panels C and D reveals that the absorbance increases correlate well with the amounts of oxygenase dimers formed under each incubation condition.

DISCUSSION

The present study sought to clarify what role each domain plays in forming and maintaining the active quaternary structure of iNOS. We found that oxygenase domain monomers can associate into a dimer in the absence of the reductase domain. This confirms that all of the determinants that enable full-length iNOS subunits to dimerize are located within the oxygenase domain, and this domain is therefore responsible for assembling the active form of the enzyme.

Dimerization of oxygenase domain monomers required added L-arginine and H4biopterin. This is consistent with these factors promoting dimerization of full-length iNOS monomers (Baek et al., 1993; Abu-Soud et al., 1995) and with their binding sites being located within the oxygenase domain (Ghosh & Stuehr, 1995). Omission of H4biopterin from the dimerization reaction resulted in only a partial decrease (60%) in oxygenase dimer formation, whereas the reaction was almost completely prevented in the absence of L-arginine. This is consistent with some dimerization of full-length iNOS subunits occurring in the absence of H4biopterin (Baek et al., 1993).

Although the reductase domain is not required for subunit dimerization, its presence has a marked effect on the stability of the dimer once it has formed. Consider that it takes 2.5 M urea to cause 50% dissociation of the oxygenase domain dimer and 5 M urea to cause near complete dissociation into monomers. These urea requirements are much higher than those of the full-length iNOS dimer, which almost completely dissociates in 2.5 M urea (Abu-Soud et al., 1995). This suggests that attached reductase domains actually destabilize the dimeric structure that forms between two oxygenase domains. Destabilization is also consistent with the reductase domains existing as monomeric extensions in the full-length dimer (Ghosh & Stuehr, 1995).

Gel filtration analysis showed that the iNOS reductase domain unfolds at urea concentrations above 2.5 M, with an accompanying loss of its reductase activity. Remarkably, its reductase activity versus urea concentration curve (Figure 3) is identical to the curves obtained with full-length iNOS monomer or dimer, which also unfold in 5 M urea (Abu-Soud et al., 1995). This suggests that an inherent instability of the reductase domain is probably responsible for the loss of reductase activity and unfolding that occurs for full-length iNOS at high urea concentrations. Apparently, the reductase domain is not stabilized by an attached oxygenase domain.

Our current findings, combined with previous results (Ghosh & Stuehr, 1995; Baek et al., 1993; Abu-Soud et al., 1995), clarify the process by which iNOS assembles to form an active enzyme in macrophages, as summarized in Figure 6. The iNOS protein as synthesized first binds FAD, FMN, and CaM to form a monomer that contains a functional

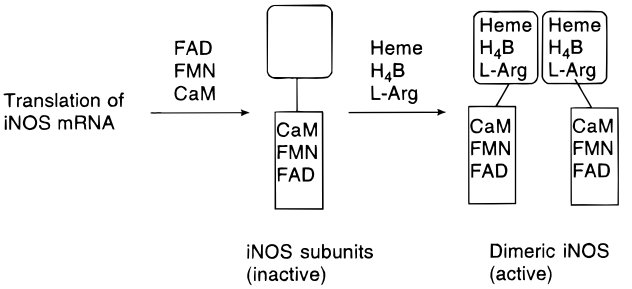

FIGURE 6: Two-step model for assembly of iNOS into an active dimeric enzyme.

reductase domain but cannot synthesize NO. Availability of H4biopterin (H4B), L-arginine, and heme[2] enables the oxygenase domains of two monomers to interact, incorporate H4biopterin and heme, and form a dimer. The dimer has its subunits aligned in a head-to-head manner with the reductase domains attached as monomeric extensions and is active regarding NO synthesis. Thus, assembly of an active iNOS requires two sequential posttranslational steps that can occur independently of one another and involve a distinct domain of the enzyme. This further supports the contention that iNOS arose from an ancestral gene fusion between a dual-flavin reductase and a cytochrome-P-450-like heme protein.

How the heme remains bound in the protein following dissociation of the oxygenase domain is an interesting question. The spectral data show that dissociation does not alter the heme's proximal ligand, which remains a cysteine thiolate as in the cytochromes P-450. Indeed, modeling studies (Renaud et al., 1993) indicate a nine amino acid sequence homology between cytochrome $P\text{-}450_{3A}$ and iNOS centering around the putative iNOS heme binding cysteine 194, suggesting that the iNOS proximal heme pocket may be similar to that in the cytochromes P-450. In cytochrome P450-type hemeproteins, the heme is buried in a hydrophobic cleft that is not accessible from the surface of the protein (Poulos et al., 1985). Interactions are also maintained between the porphyrin ring and amino acid residues present in the hydrophobic pocket after treatment with urea or guanidine-HCl (Yu et al., 1995). Thus, it seems reasonable that heme is not lost from iNOS during treatment with denaturants such as urea.

The bound heme group provides a spectral probe to monitor how the heme environment changes during dissociation of the oxygenase domain dimer. Dissociation clearly alters the heme's distal environment, which is protected from solvent in the dimer such that the heme iron is maintained in a predominantly five-coordinate high-spin state (Stuehr & Ikeda-Saito, 1992). During dissociation of the oxygenase domain dimer, the distal heme pocket becomes exposed to solvent, allowing DTT to bind as a sixth ligand to the heme iron to form a mixed bis-thiolate complex, as can be formed with cytochrome P-450 cam (Sono et al., 1982). Whether exposure of the distal pocket to solvent results from loss of bound H4biopterin remains an intriguing possibility (Wang et al., 1995). The change in heme pocket architecture may be accompanied by more global changes in oxygenase domain tertiary structure, as evidenced by an

[2] Added heme is not required if the monomers already contain bound heme.

increase in protein tryptophan fluorescence. Remarkably, the optical and fluorescence changes that occur during dissociation of the oxygenase domain dimer are reversed when the monomers reassociate in the presence of L-arginine and H4biopterin. This argues that dimerization reforms the original heme pocket and oxygenase domain structure.

Although this work confirms that the oxygenase domain is solely responsible for iNOS subunit dimerization, how this activates iNOS for NO synthesis is still an open question. It is intriguing to speculate that protein and heme pocket structural changes that occur during oxygenase domain dimerization are somehow linked to the process of enzyme activation. Structural changes could enable insertion of H4biopterin into the protein, modulate the redox chemistry of the heme iron, or create a path for interdomain electron transfer between the reductase and oxygenase domains. These and other possibilities can now be addressed.

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