Subunit Dissociation and Unfolding of Macrophage NO Synthase: Relationship between Enzyme Structure, Prosthetic Group Binding, and Catalytic Function†

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ABSTRACT: Macrophage NO synthase is a homodimer of 130 kDa subunits. Each subunit contains an oxygenase domain that binds iron protoporphyrin IX (heme) and tetrahydrobiopterin (H_4biopterin) and a reductase domain that binds FAD, FMN, and calmodulin (CaM) [Ghosh & Stuehr (1995) *Biochemistry 34,* 801−807]. We have studied the dissociation and unfolding reactions of dimeric iNOS in urea to learn how enzyme structure relates to catalysis and prosthetic group binding. The iNOS dimer dissociated between 0 and 2.5 M urea, and the subunits partially unfolded at 2.5 M urea and above. Dimer dissociation was accompanied by loss of NO synthesis activity and release of bound H_4biopterin from the protein. However, the dissociated subunits maintained their cytochrome *c* and ferricyanide reductase activities and retained near stoichiometric quantities of bound heme. The subunit unfolding transition was accompanied by loss of reductase activities and partial loss of bound heme but retention of bound flavins and CaM. The heme iron in the dissociated subunits remained coordinated through axial cysteine thiolate ligation. Kinetic analysis of dimer dissociation showed that loss of NO synthesis correlated with a loss of heme Soret absorbance at 398 nm and an appearance of absorbance bands at 377 and 460 nm, which were attributed to DTT coordination to the sixth position of the heme iron to form a mixed bisthiolate complex. Subunits could reassociate into a dimer when incubated with L-arginine and H_4biopterin. Dimer formation correlated with proportional recoveries of NO synthesis and heme Soret absorbance at 398 nm. Thus, dimeric iNOS undergoes separate dissociation and unfolding transitions in urea, and each transition is accompanied by a loss of a specific catalytic function. The iNOS heme binding module appears to remain partially intact following dimer dissociation in urea, and its original structural and catalytic properties can be restored upon subunit reassociation.

Nitric oxide (NO)[1] is synthesized during immunostimulation and may play an important role in the pathogenesis of several diseases including diabetes, multiple sclerosis, septic shock, and adjuvant arthritis (Schmidt & Walter 1994; Griffith & Stuehr, 1995). Several immunoactive cytokines are released in tissues during inflammation or disease that can cause cells to express an "inducible" NO synthase isoform (iNOS) (Nathan, 1992; Nathan & Xie, 1994). The iNOS isolated from a cytokine-stimulated mouse macrophage cell line is homodimeric in its active form and catalyzes a stepwise NADPH-dependent oxidation of L-arginine to NO and L-citrulline (Stuehr et al., 1991a,b; Pufahl & Marletta, 1992). Dimeric iNOS contains 1 mol each of FAD, FMN, and iron proptoporphorin IX (heme) per subunit, variable amounts (0.1−1 mol/subunit) of tetrahydrobiopterin (H_4-biopterin), and an unspecified amount of tightly bound calmodulin (CaM) (Stuehr et al., 1991a; White & Marletta, 1992; Stuehr & Ikeda-Saito, 1992; Hevel & Marletta, 1992; Baek et al., 1993; Cho et al., 1992). Limited proteolysis studies (Ghosh & Stuehr, 1995) have located the heme, L-arginine, and H_4biopterin binding sites within an "oxygenase" domain comprised of the N-terminal portion of the iNOS polypeptide (residues 1−502). Similarly, the binding sites for CaM, flavins, and NADPH are located within a "reductase" domain that is the C-terminal portion of iNOS (residues 503−1144). These assignments are consistent with the predicted binding sites for CaM, flavin, NADPH, and heme as predicted by primary sequence analysis (Nathan & Xie, 1994; McMillan et al., 1992; Renaud et al., 1993). During catalysis, the iNOS flavins accept electrons from NADPH and transfer them across domains to the heme iron. Reduction of heme iron is associated with oxygen activation and catalysis of NO synthesis (Abu-Soud & Stuehr, 1993; Abu-Soud et al., 1994). In dimeric iNOS, the subunits appear to align in a head to head manner, with only the oxygenase domains of two subunits interacting in the dimeric structure (Ghosh & Stuehr, 1995).

Our previous studies revealed that iNOS is expressed in the macrophage-like cell line RAW 264.7 as a mixture of dimer and unassociated subunits following induction with interferon-γ and bacterial lipopolysaccharide (Baek et al.,

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[1] Abbreviations: Bis-Tris-propane, 1,3-bis[[tris(hydroxymethyl)-methyl]amino]propane; BSA, bovine serum albumin; CaM, calmodulin; NO, nitric oxide; NOS, nitric oxide synthase; H_4biopterin, (6*R*,6*S*)-2-amino-4-hydroxy-6-(L-*erythro*-1,2-dihydroxypropyl)-5,6,7,8-tetrahydropteridine; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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1993). The "native" iNOS subunits that are purified from these cells contain FAD, FMN, and CaM in amounts equivalent to those present in dimeric iNOS but do not contain bound heme or H_4biopterin. As such, native subunits do not generate NO from L-arginine but function equivalent to dimeric iNOS in catalyzing the NADPH-dependent reduction of electron acceptors such as cytochrome *c*, ferricyanide, or dichlorophenolindophenol (Baek et al., 1993). Incubation of native iNOS subunits with H_4biopterin, L-arginine, and a stoichiometric amount of heme at 37 °C enables a variable portion of subunits (10−90%, depending on the preparation) to associate into a dimer. The dimeric iNOS so generated is equivalent to native dimeric iNOS regarding its heme and H_4biopterin content, light absorbance spectrum, and NO synthesis activity (Baek et al., 1993). Given that the heme, H_4biopterin, and L-arginine binding sites are all located within the oxygenase domain, which is involved in subunit dimeric interaction (Ghosh & Stuehr, 1995), the results imply that an intact oxygenase domain forms during subunit interactions that generate an active iNOS dimer. However, the exact relationships between prosthetic group binding, oxygenase domain structure, subunit interaction, and catalytic function remain unclear.

To further explore these relationships, we carried out subunit dissociation and unfolding reactions with dimeric iNOS and native iNOS subunits in urea. Similar methods have helped to characterize a number of oligomeric multifunctional enzymes (Jaenicke & Rudolph, 1986; Pace, 1986; Bowie & Sauer, 1993; Blackburn & Noltmann, 1981). In this report, we document the separate dissociation and unfolding transitions of dimeric iNOS, characterize the iNOS species that form from each transition, and show that the transitions are linked to changes in prosthetic group binding and domain-specific catalytic functions.

EXPERIMENTAL PROCEDURES

Materials. Ultrapure urea was obtained from Bio-Rad. Interferon-γ was a gift from Genentech (South San Francisco, CA). Iron protoporphyrin IX was a gift from Dr. Masao Ikeda-Saito, Case Western Reserve University. All other supplies were of the highest purity grade available and were obtained from either Sigma or sources that have been previously reported (Baek et al., 1993).

Purification of Dimeric iNOS and Subunits. Dimeric iNOS and native iNOS subunits were purified from supernatants of interferon-γ-activated and *Escherichia coli* lipopolysaccharide-activated RAW 264.7 cells by a two-column procedure using a Pharmacia fast protein liquid chromatograph as previously described (Baek et al., 1993).

Gel Filtration Chromatography. To determine the relative amounts of dimeric iNOS and dissociated iNOS subunits present in a sample, size exclusion chromatography was carried out at 4 °C using either Pharmacia TSK-G4000 SW or Superdex 200 columns. The columns were equilibrated (at 0.2 or 0.5 mL/min, respectively), with 40 mM Bis-Tris-propane buffer, pH 7.7, containing 2 μM H_4biopterin, 2 mM DTT, and 5% glycerol. For samples obtained from urea equilibration studies, the column buffer also contained urea at concentrations that matched those used in each equilibration reaction. Injection volumes ranged from 50 to 100 μL. Protein in the column effluent was detected at 280 nm using a flow-through detector. The molecular weights of the protein peaks were estimated relative to gel filtration molecular weight standards, as previously described (Baek et al., 1993).

Measurement of NO Synthesis Activity. The initial rate of NO synthesis was determined at 37 °C using a spectrophotometric oxyhemoglobin assay for NO. Briefly, 10−35 μL aliquots were removed from enzyme incubations and transferred to a prewarmed cuvette that contained 40 mM Tris-HCl buffer, pH 7.8, supplemented with 5−10 μM oxyhemoglobin, 0.3 mM DTT, 1 mM arginine, 0.1 mM NADPH, 4 μM each of FAD, FMN, and H_4biopterin, 100 units/mL catalase, 10 units/mL superoxide dismutase, and 0.1 mg/mL bovine serum albumin, to give a final volume of 0.7 mL. The NO-mediated conversion of oxyhemoglobin to methemoglobin was monitored over time as an increase in absorbance at 401 nm and quantitated using an extinction coefficient of 38 mM^{-1} cm^{-1} at 401 nm (Kelm et al., 1988). The NO synthesis activity of column fractions was measured using a 96-well plate format and a colorimetric assay for nitrite as previously described (Baek et al., 1993).

Reductase Activities. Reactions were carried out in triplicate and analyzed using a Molecular Dynamics Thermomax 96-well microplate reader. Each microwell contained either no iNOS or 10−20 μL of diluted iNOS, 40 mM Tris-HCl, pH 7.8, 4 μM FAD, 4 μM FMN, 0.9 mM EDTA, and 100 μM cytochrome *c* or 1 mM ferricyanide in a total volume of 90 μL. The plate was preincubated for 10 min at 25 °C to oxidize residual DTT and H_4biopterin carried over in the enzyme aliquot (these compounds can nonenzymatically reduce cytochrome *c* and ferricyanide), and then the reactions were initiated by addition of 10 μL of NADPH solution to give a final concentration of 0.5 mM NADPH in each well. After NADPH addition, the plate was returned to the microplate reader, and the reaction was monitored at either 420 or 550 nm for ferricyanide or cytochrome *c* reduction, respectively (one measurement recorded per well every 18 s, with stirring). The kinetic traces were automatically collected, and the rates of absorbance change were calculated using software provided by the manufacturer. In some cases, iNOS reductase activities were quantitated in cuvettes using a conventional spectrophotometer. Reactions were run under the conditions described above, except that the final volume was 0.7 mL. Wavelengths and extinction coefficients used to quantitate cytochrome *c* and ferricyanide reductase activities were 550 nm (21 mM^{-1} cm^{-1}) and 420 nm (1.2 mM^{-1} cm^{-1}), respectively (Kurzban et al., 1990).

Equilibration of iNOS with Urea. For a typical equilibration, the purified iNOS samples (5−10 μg) were incubated at 4 °C for either 2 h or for times specified in the text in 40 mM Bis-Tris-propane buffer, pH 7.4, 2 μM FMN, 2 μM FAD, 1 mM DTT, and the indicated urea concentrations, for a final volume of 100 μL. At designated times, aliquots from the iNOS−urea incubates were removed and immediately diluted in four to nine volumes of cold 40 mM Tris-HCl buffer, pH 7.6, containing 2 μM each of FMN and FAD, and stored on ice. Aliquots of the diluted samples were then used in the NO synthesis, ferricyanide, or cytochrome *c* reductase assays within 2 h.

Spectrophotometric recording of dimeric iNOS dissociation in 2 M urea was done in cuvettes at 10 °C. Dimeric iNOS was diluted to approximately 1 μM in Bis-Tris propane

buffer, pH 7.4, containing 1 mM DTT. Concentrated urea solution was added to give a final concentration of 2 M.

Determination of Bound Prosthetic Groups. In most cases, bound heme was quantitated spectrally through formation of a pyridine hemochromogen (Stuehr & Ikeda-Saito, 1992). To quantitate heme in gel filtration fractions that contained low amounts of iNOS, a more sensitive fluorometric method was used (Morrison, 1965; Sassa, 1976). Briefly, samples were boiled for 30 min in 2 M oxalic acid and then cooled to room temperature. Porphyrin was quantitated by its fluorescence emission at 662 nm (excitation 400 nm) relative to standard curves generated with freshly prepared iron(III) protoporphyrin IX solutions (dissolved in 0.1 M NaOH) that had been subjected to the oxalic acid treatment. Enzyme-bound FAD, FMN, and H_4 biopterin were quantitated by previously published fluorometric procedures (Baek et al., 1993). Bound CaM was qualitatively determined using an anti-CaM antibody provided by Upstate Biotechnology (Lake Placid, NY) as previously described (Baek et al., 1993; Cho et al., 1992; Ghosh & Stuehr, 1995).

Dimerization of Urea-Generated iNOS Subunits. Dimeric iNOS that had been dissociated by equilibration in 2 M urea was dialyzed at 4 °C overnight in 500 volumes of 40 mM Bis-Tris-propane buffer, pH 7.4, containing 0.1 M urea, 3 mM DTT, and 10% glycerol. The dialyzed subunit preparation was incubated at concentrations between 0.1 and 3 μM at 37 °C for 60−90 min in 0.1−0.3 mL of Tris-HCl buffer, pH 7.8, containing 3 mM DTT, 10 μM H_4biopterin, 40 μM NADPH, 3 mM L-arginine, 0.1 mg/mL bovine serum albumin (BSA), and heme in amounts equivalent to the subunit concentration. In some cases, certain additives were omitted as noted in the text.

Renaturation of iNOS Equilibrated in 5 M urea. iNOS preparations that expressed no reductase activity following equilibration in 5 M urea were diluted to 125 nM in 40 mM Bis-Tris-propane buffer, pH 7.4, containing 1 M urea, or diluted in buffer plus urea plus one or more of the following: 5.1 μM BSA, 1 μM FAD, 1 μM FMN, 200 nM CaM, and 100 μM Ca^{2+}. After 20 h incubation at 4 °C, aliquots were removed and assayed for cytochrome *c* reduction in the microplate assay described above.

RESULTS

Generation of New Equilibrum States by Incubation with Urea. Our initial experiments monitored the loss of both NO synthesis and cytochrome *c* reductase activities over time during incubation of dimeric iNOS with various concentrations of urea at 4 °C. Aliquots were removed at discrete time points and diluted immediately in buffer to reduce the urea concentrations to ≤0.4 M, after which assays for both NO synthesis and cytochrome *c* reduction were carried out.[2] As shown in Figure 1, we observed time-dependent decreases in NO synthesis activity (panel A) and cytochrome *c* reductase activity (panel B) during incubation of dimeric iNOS with urea. In all cases, there was an initial rapid loss of activity followed by a more gradual reduction, such that the enzyme activities either reached new equilibrium values

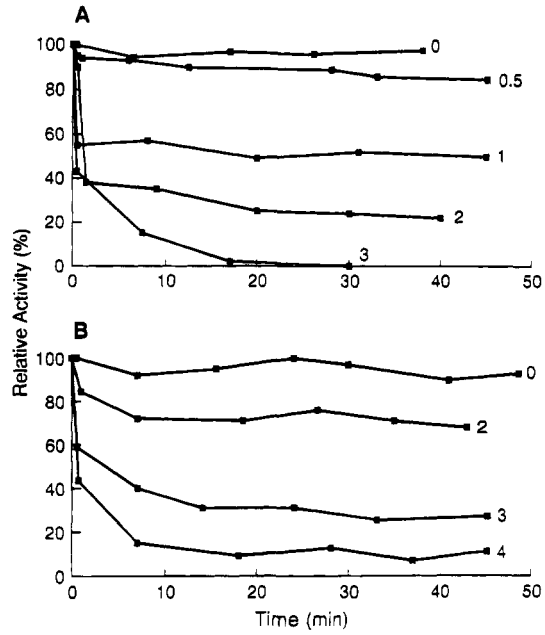

FIGURE 1: Loss of NO synthesis activity (panel A) or cytochrome *c* reductase activity (panel B) over time during incubation of dimeric iNOS with various concentrations of urea. Points represent assays done on aliquots that were removed at the indicated times and diluted immediately in 4−9 volumes of buffer that did not contain urea. The number to the right of each data line is the molar concentration of urea present in the enzyme incubations prior to dilution. The data are representative of two similar experiments.

or disappeared completely after 30−50 min of incubation with urea. The amounts of NO synthesis or cytochrome *c* reductase activities that remained after equilibrium was established were inversely proportional to the concentration of urea that was used. Also, at any given urea concentration, there was a greater loss of iNOS NO synthesis activity than cytochrome *c* reduction. Similar kinetics were observed for the urea-promoted loss of ferricyanide reductase activity of dimeric iNOS (data not shown). Control incubations that contained dimeric iNOS without urea did not lose their NO synthesis or cytochrome *c* reductase activities over the course of the experiment (Figure 1). These results indicate that incubation of iNOS with various concentrations of urea for approximately 1 h is sufficient to establish a range of new equilibrium states regarding expression of iNOS catalytic functions.

Differential Loss of Catalytic Functions Due to Equilibrium of dimeric iNOS or iNOS Subunits with Urea. We next compared NO synthesis, cytochrome *c* reductase, and ferricyanide reductase activities for dimeric iNOS and native iNOS subunits that had been equilibrated in a wide range of urea concentrations (0−5 M) and then diluted in 4−9 volumes of buffer. As shown in the upper panel of Figure 2, dimeric iNOS NO synthesis activity decreased in a linear manner between 0 and 2 M urea, and complete loss of NO synthesis activity was observed at 3 M urea. From these data, a 50% decrease in NO synthesis activity was estimated to result from equilibration with 1.75 M urea. In contrast, the cytochrome *c* and ferricyanide reductase activities of dimeric iNOS remained constant following equilibration between 0 and 2 M urea and then decreased as urea concentration increased such that a loss of 50% of either reductase activity was estimated to occur at 2.75 M urea. Full loss of reductase activities was observed following

[2] Samples were diluted in L-arginine- and H_4biopterin-free buffer to prevent possible subunit reassociation from occurring at the diluted urea concentrations. The concentrations of urea that were present in the subsequent activity assays (0−50 mM) did not inhibit NO synthesis or cytochrome *c* reduction by an authentic dimeric iNOS.

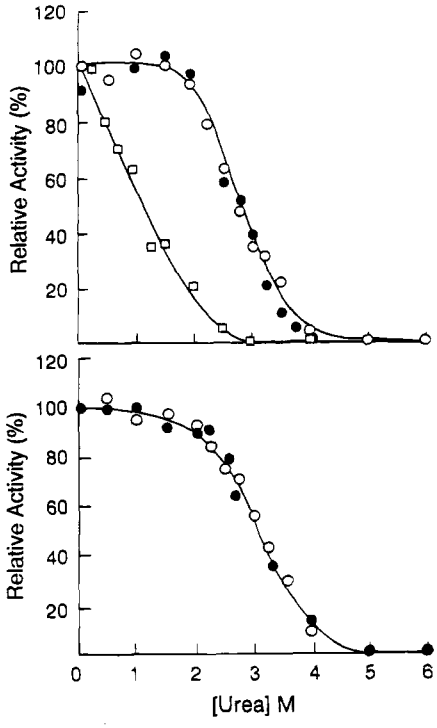

FIGURE 2: Catalytic activities of dimeric iNOS and native iNOS subunits following equilibration in various concentrations of urea. Dimeric iNOS (upper panel) or iNOS subunits (lower panel) were equilibrated at 4 °C in the indicated urea concentrations for 90 min, after which time aliquots were diluted and assayed for NO synthesis (□), cytochrome *c* reduction (●), or ferricyanide reduction (○). The data shown are representative of three similar experiments.

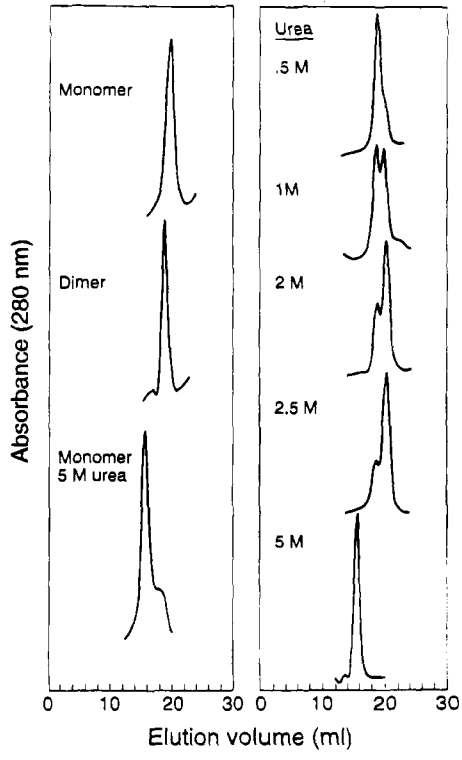

FIGURE 3: Gel filtration profiles for dimeric iNOS equilibrated with various concentrations of urea. The left panel shows the elution profiles of native iNOS subunits (monomer), dimeric iNOS (dimer), and native iNOS subunits that had been equilibrated with 5 M urea. The right panel shows the elution profiles of dimeric iNOS preparations that had been equilibrated with the indicated concentrations of urea. Chromatography was carried out on a TSK G-4000 SW gel filtration column at 4 °C. The results are representative of two similar experiments.

equilibration of dimeric iNOS with 5 M urea. Identical experiments carried out with isolated iNOS subunits (Figure 2, lower panel) showed that subunit cytochrome *c* and ferricyanide reductase activities remained unchanged following equilibration with up to 2 M urea and then decreased concomitantly as urea concentrations increased in a manner identical to that observed for dimeric iNOS, giving an estimated 50% inactivation at 2.8 M urea and full inactivation at 5 M urea. Together, the data indicate that the transitions responsible for loss of NO synthesis and reductase activities occur in different urea concentration ranges.

Relation between iNOS Structure and Expression of Catalytic Functions. To determine whether the urea-promoted loss of catalytic functions described above was associated with iNOS subunit dissociation and/or unfolding transitions, we utilized gel filtration chromatography to quantify the relative amounts of dimeric iNOS, dissociated subunits, and unfolded subunits present at equilibrium at the various urea concentrations. To maintain sample equilibrium during gel filtration, the column equilibration buffer also contained urea at a concentration that matched the injected samples, as well as cofactors previously identified (Baek et al., 1993) to maintain iNOS dimeric structure in the absence of urea. As shown in Figure 3, left panel, authentic samples of native iNOS subunits (monomer) and dimeric iNOS that were run on the gel filtration column in the absence of urea eluted as single, symmetrical peaks with elution volumes of 20 and 18 mL, respectively. Native iNOS subunits that had been equilibrated with 5 M urea prior to chromatography exhibited a reduced elution volume of 15 mL. This apparent increase in iNOS hydrodynamic volume at 5 M urea indicates that iNOS, like several other enzymes (Jaenicke & Rudolph, 1986; Pace, 1986; Bowie & Sauer, 1993; Blackburn & Noltmann, 1981), partially unfolds under this condition.

The elution profiles for dimeric iNOS samples that had been equilibrated with 0.5, 1, 2, 2.5, or 5 M urea prior to chromatography are illustrated in the right panel of Figure 3. At 0.5 M urea, a new peak with a retention time identical to authentic iNOS subunits appeared as a shoulder on the right side of the dimeric iNOS peak. In samples equilibrated at higher urea concentrations, the size of the iNOS subunit peak increased coincident with a corresponding decrease in the dimeric iNOS peak, such that dimeric iNOS was estimated to represent ~90%, 50%, 20%, and 12% of the total iNOS protein present at 0.5, 1.0, 2.0, and 2.5 M urea, respectively. Dimeric iNOS that had been equilibrated with 5 M urea exhibited a decreased elution volume that was identical to the elution volume of native iNOS subunits that had been equilibrated with 5 M urea.

These equilibrium studies show that, in the range of 0.5–2.5 M urea, the iNOS dimer dissociates to generate an increasing population of folded subunits. At 5 M urea, both dimeric iNOS and iNOS subunits appear to partially unfold and form a common species whose hydrodynamic volume is increased relative to either native dimeric iNOS or the folded subunits.

Relationship between iNOS Structure and Prosthetic Group Binding. Because native iNOS subunits isolated from cells do not contain bound heme or H_4biopterin (Baek et al., 1993), we investigated whether H_4biopterin and heme remained bound following dissociation of dimeric iNOS in

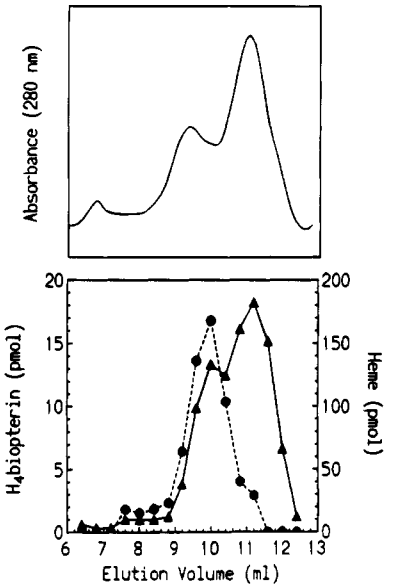

FIGURE 4: Heme and H_4biopterin content of iNOS following urea-promoted subunit dissociation. Dimeric iNOS (78 μg, 600 pmol of subunits) was dissociated at 4 °C by incubation in 2 M urea for 90 min. The preparation was then dialyzed in buffer containing 0.1 M urea and subjected to gel filtration chromatography on a Superdex 200 column at 4 °C. The upper panel shows the protein elution profile, which contains three peaks representing (left to right) an iNOS aggregate, dimeric iNOS, and iNOS subunits. The lower panel shows the heme (▲) and H_4biopterin (●) contents of the corresponding 0.4 mL column fractions. The data are representative of two similar experiments.

2 M urea. As noted above, this procedure typically generated a preparation that is predominantly (~80%) dissociated subunits. The H_4biopterin content of three preparations ranged from nonmeasurable (<0.01 mol/mol of subunit, n = 2) to 0.04 mol/mol of subunit (n = 1), suggesting that bound H_4biopterin is released from dimeric iNOS during subunit dissociation.[3] In contrast, the urea-generated subunit preparations all contained near normal quantities of bound heme (an average of 0.83 mol/mol of subunit, n = 3). These results were confirmed by performing gel filtration chromatography on a 2 M urea-treated dimeric iNOS preparation and analyzing the eluted proteins for bound H_4biopterin or heme. As shown in the upper panel of Figure 4, the urea treatment caused approximately 75% of dimeric iNOS to dissociate into its subunits. Bound H_4biopterin was present only in the column fractions that contained dimeric iNOS. In contrast, bound heme was present in both the iNOS dimer and iNOS subunit peak fractions. Thus, unlike native iNOS subunits isolated from cells (Baek et al., 1993), the urea-generated subunits maintain most of their bound heme following dissociation.

To investigate which prosthetic groups remain bound to iNOS after equilibration with urea at concentrations greater than 2 M, we dialyzed a sample of dimeric iNOS in buffer containing 5 M urea for 6.5 h and analyzed the resultant protein for bound CaM, flavins, and heme. The 5 M urea-treated iNOS still contained bound CaM, FAD, and FMN but contained reduced quantities of bound heme (0.3 mol/subunit). Maintenance of bound flavins and CaM following equilibration in 5 M urea is consistent with urea not causing

[3] The dimeric iNOS preparations used in these experiments contained between 0.12 and 0.4 mol of H_4 biopterin bound per mole of subunit.

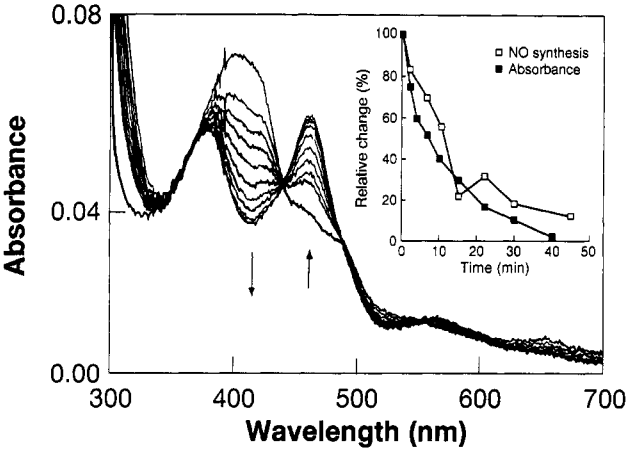

FIGURE 5: Spectral change during dimeric iNOS subunit dissociation in 2 M urea. The spectrum of a dimeric iNOS solution (1 μM) was recorded both prior to and 1, 3, 6, 10, 15, 22, 30, 40, and 45 min following addition of 2 M urea. The arrows show where the absorbance decreased or increased over time during the experiment. The inset compares the time-dependent loss of absorbance at 400 nm with the loss in NO synthesis activity measured on aliquots that were removed from the cuvette at the indicated times and immediately diluted to stop the dissociation reaction. The results shown are representative of two identical experiments.

a complete loss of protein structure (Jaenicke & Rudoph, 1986; Pace, 1986).

Spectral Changes Associated with iNOS Subunit Dissociation. Because the urea-generated iNOS subunits maintained most of their bound heme, we examined if changes in heme Soret absorbance would occur during dissociation of dimeric iNOS in 2 M urea. As shown in Figure 5, there was a time-dependent loss of heme Soret absorbance near 400 nm along with a coincident increase in longer wavelength absorbance at 460 nm, such that at equilibrium two new absorbance bands of approximately equal intensity were present at 385 and 460 nm. The dithionite-reduced, CO-bound complex exhibited a Soret maximum at 444 nm (data not shown).[4] This indicated that the heme iron in the dissociated subunits was ligated to a cysteine thiolate, as is the case for native dimeric iNOS (Stuehr & Ikeda-Saito, 1992; Wang et al., 1993). The inset of Figure 5 shows that the progressive loss of Soret absorbance at 400 nm that occured during subunit dissociation correlated with a loss of dimer-specific catalytic function (NO synthesis).

Reassociation of urea-Generated iNOS Subunits into a Dimeric Enzyme. The association of native iNOS subunits into an active dimer is promoted by added H_4biopterin, L-arginine, and a stoichiometric amount of heme and is inhibited by millimolar concentrations of NADPH (Baek et al., 1993). To determine if urea-dissociated subunits could reassociate into active dimers, we incubated the subunits under conditions known to induce dimerization of native iNOS subunits and monitored dimer formation by gel filtration chromatography and recovery of NO synthesis. Results from a typical experiment are illustrated in Figure 6. Trace A shows the gel filtration profile of a urea-dissociated, dialyzed iNOS preparation prior to its incubation

[4] The estimated extinction coefficient for the ferrous−CO complex of dimeric iNOS is 74 mM^{-1} cm^{-1} at 444 nm (Stuehr & Ikeda-Saito, 1992). Using this value, we estimate that the iNOS subunit preparation used in Figure 5 contained 0.9 mol of heme/mol of subunits.

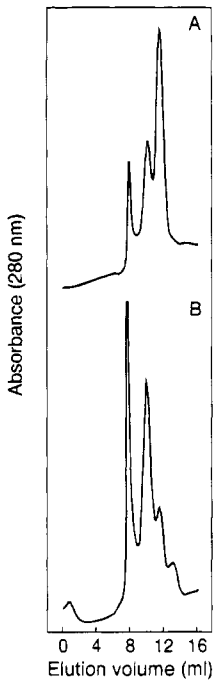

FIGURE 6: Reassembly of urea-generated iNOS subunits into a dimeric enzyme. A dimeric iNOS preparation was dissociated by equilibration in 2 M urea and then dialyzed in buffer containing 0.1 M urea. Shown are the gel filtration profiles of the dialyzed iNOS subunit preparation before (trace A) or after (trace B) its incubation under dimerization conditions. The protein peaks and their estimated molecular weights are (from left to right) iNOS aggregates plus injected air (>670 000), dimeric iNOS (~260 000), and iNOS subunits (~130 000). The experiment shown is representative of five similar experiments.

under dimerization conditions. Each of the three resolved peaks contained iNOS protein as determined by SDS–PAGE analysis (not shown), and they were identified on the basis of their elution volumes to be (from left to right) iNOS aggregates plus injected air, dimeric iNOS, and iNOS subunits. A portion of the urea-dissociated, dialyzed iNOS preparation was then incubated for 90 min under dimerization conditions, followed by gel filtration analysis. As shown in trace B, this resulted in an apparent increase in the amount of iNOS aggregates, an increase in dimeric iNOS, and a decrease in the amount of iNOS subunits. SDS–PAGE analysis revealed that the amounts of iNOS protein in the aggregate peaks of trace A and B were approximately similar, suggesting that most of the apparent increase in aggregation was due to injected air (data not shown). On the basis of SDS–PAGE analysis and the monomer and dimer peak areas in the two gel filtration profiles, the amount of dimer was estimated to increase from ~15% to 60% of the total iNOS protein during incubation under the dimerization conditions. Dimer formation in this experiment was accompanied by a recovery of NO synthesis activity from 2 to 323 nmol of NO min^{-1} mg^{-1}. This represents an ~30% recovery in total NO synthesis activity, as estimated using a specific activity of 1.2 μmol min^{-1} mg^{-1} for pure dimeric iNOS (Stuehr et al., 1991a). Assays done on the gel filtration column fractions showed that only those fractions containing dimeric iNOS were able to synthesize NO (data not shown). Thus, like native iNOS subunits isolated from cells (Baek et al., 1993), the urea-generated subunits can reassemble and recover their NO synthesis in the presence of added L-arginine, H_4biopterin, and heme.

Table 1: Effect of Incubation Conditions on Reconstitution of Subunit NO Synthesis Activity[a]

condition	NO synthesis activity (nmol of NO min^{-1} mg^{-1})
omitted	
all three	<10
none	400
H_4biopterin	140
L-arginine	100
heme	680

[a] iNOS subunits (1 μM) were incubated at 37 °C under dimerization conditions described in Experimental Procedures or were instead incubated under conditions where added H_4biopterin, L-arginine, and heme were omitted as noted. After 90 min, 35 μL aliquots were removed and assayed for NO synthesis activity in duplicate. Data shown are the average values and are representative of three similar experiments. The urea-treated iNOS preparation used in the above experiment contained a mixture of subunits and dimeric enzyme at a ratio of 4 to 1 and displayed a specific activity of 48 nmol of NO min^{-1} mg^{-1} prior to use in the dimerization incubations.

We next performed experiments in which L-arginine, H_4-biopterin, or heme was omitted from the dimerization reactions. As shown in Table 1, recovery of NO synthesis did not occur if subunit dimerization was attempted in the absence of L-arginine, heme and H_4biopterin. In the presence of these three additives plus NADPH,[5] the subunits recovered approximately 30% of their maximum possible NO synthesis. In the absence of H_4biopterin or L-arginine, recovery was decreased to 35% and 25% the value of the fully supplemented reaction, respectively. In contrast, when added heme was omitted from the incubation, recovery was actually increased to 170% the value of the fully supplemented system. The inhibition by added heme is consistent with previous results that showed excess heme inhibits the dimerization of native iNOS subunits (Baek et al., 1993).[6] Gel filtration chromatography performed on aliquots taken from the completed dimerization incubations showed that the degree of subunit dimerization correlated with the percent recovery of NO synthesis in each case (data not shown).

The fact that urea-generated iNOS subunits could reassociate into an active dimer in the absence of added heme suggests that the heme which remained bound to the subunit after dissociation could recover its original structural and catalytic properties as a consequence of dimerization. To examine this possibility, we recorded light absorbance spectra of a urea-generated iNOS subunit preparation as it underwent dimerization in the absence of added heme. The lower panel of Figure 7 depicts spectra that were recorded immediately after addition of L-arginine and H_4biopterin to initiate dimerization of urea-generated iNOS subunits and after an additional 20 min had elapsed. An absorbance increase between 350 and 420 nm and an absorbance decrease between 420 and 480 nm occurred over this 20 min time period. A difference spectrum generated by subtracting the initial spectrum from the spectrum obtained at 20 min is depicted in the upper panel of Figure 7. It shows the buildup

[5] Related experiments showed that dimerization of both natural and urea-generated iNOS subunits was sometimes increased in the presence of micromolar concentrations of NADPH, with a maximal effect obtained at ~40 μM (M. Loftus and D. J. Stuehr, unpublished results).

[6] For the dimerization experiment in Table 1, the total heme present (bound plus added) would be approximately twice the concentration of the urea-generated subunits.

of a typical dimeric iNOS heme Soret absorbance at 398 nm (Stuehr & Ikeda Saito, 1992) and a decrease in absorbance at 460 nm, thus partially reversing the spectral changes that occurred upon urea-promoted iNOS subunit dissociation (see Figure 5). Using the absorbance ratio between urea-dissociated iNOS subunits and purified dimeric iNOS at 398 nm (0.45; see Figure 5) and the dimeric iNOS extinction coefficient at 398 nm (71 mM^{-1} cm^{-1}; Stuehr & Ikeda-Saito, 1992), we estimate that the change in 398 nm absorbance obtained in the 20 min dimerization period (0.0035 absorbance unit) corresponds to formation of ~110 nM dimeric iNOS, which is approximately 27% of the maximum possible dimerization. This value is consistent with the ~30% recover in NO synthesis specific activity (17−323 nmol of NO min^{-1} mg^{-1}) that was obtained over the entire 60 min incubation under dimerization conditions. Thus, a portion of the heme that remained bound to the dissociated subunits was able to reestablish its normal spectral and catalytic properties during the subunit dimerization reaction.

Renaturation of Partially Unfolded Inactive Subunits. We next attempted to renature the cytochrome *c* reductase activity of an iNOS subunit preparation that had been inactivated by equilibration in 5 M urea. A general method for renaturating unfolded enzymes was adapted from the literature (Jaenicke & Rudolph, 1986; Pace, 1986) in which the inactive iNOS was diluted in buffer to give 1 M urea and incubated at 4 °C overnight in the presence or absence of flavins, Ca^{2+}, CaM, or BSA. Results of a typical experiment are shown in Table 2. Denatured iNOS incubated overnight in the absence of all additives recovered only 3% of its cytochrome *c* reductase activity when compared to the non-urea-inactivated control. Approximately 21% and 24% recovery of reductase activity was observed for overnight incubation in the presence of BSA alone or BSA plus FAD and FMN, respectively. Adding Ca^{2+} and CaM did not further increase recovery. The ~20−25% recovery in iNOS reductase activity suggests that the unfolding due to equilibration in 5 M urea is at least partially reversible.

DISCUSSION

Macrophage iNOS is a homodimeric, bidomain, flavin-containing heme protein. In this report, we investigated the subunit dissociation and unfolding reactions of dimeric iNOS in urea in order to more clearly understand how enzyme structure relates to prosthetic group binding and catalytic functioning of the domains.

Our results show that a dimer to monomer transition occurs when dimeric iNOS is equilibrated at urea concentrations between 0.5 and 2.5 M. In this range, two protein species are present at equilibrium which coelute on gel filtration columns with authentic iNOS dimer and folded iNOS subunits. As urea concentrations are increasd to 2−2.5 M, dimer dissociation also increases such that folded iNOS subunits become the predominant species at equilibrium. That dimeric iNOS can dissociate into folded monomers is consistent with our previous work that showed significant amounts of folded iNOS subunits are present along with dimeric iNOS in activated macrophage cell lysates (Baek et al., 1993). Equilibration of either dimeric iNOS or iNOS subunits at 5 M urea generated a common species whose reduced elution volume on gel filtration columns identified it as a denatured form of the iNOS subunit. Together, these

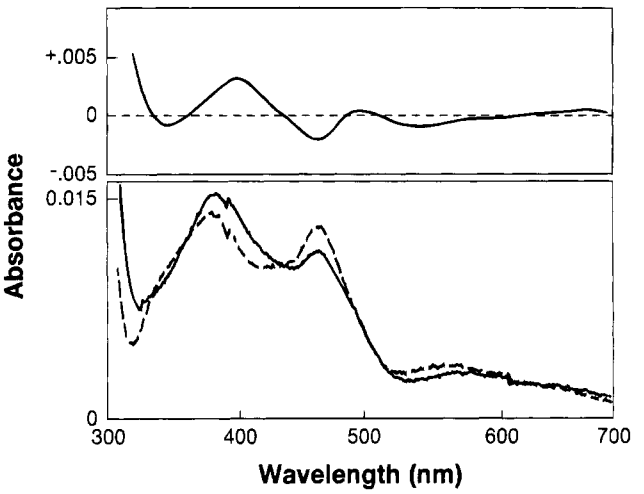

FIGURE 7: Spectral change during dimerization of iNOS subunits in the absence of added heme. Urea-generated iNOS subunits were diluted to 0.4 μM in a cuvette, and subunit dimerization was initiated by adding 3 mM L-arginine and 10 μM H_4biopterin. The lower panel shows the light absorbance spectra that were recorded just after addition of L-arginine and H_4biopterin (- - -) and recorded 20 min afterward (—). The upper panel is the difference spectrum (smoothed) generated by subtracting the initial spectrum from the 20 min spectrum. The data shown are representative of three similar experiments.

Table 2: Effect of Incubation Conditions on Recovery of Subunit Cytochrome *c* Reductase Activity[a]

condition	cytochrome *c* reduction [mol min^{-1} (mmol of subunit)$^{-1}$]
positive control	2.9 ± 0.1
urea-denatured iNOS	
+none	0.1 ± 0.02
+BSA	0.6 ± 0.1
+BSA, FAD, and FMN	0.7 ± 0.1
+BSA, FAD, FMN, Ca^{2+}, and CaM	0.7 ± 0.1

[a] Dimeric iNOS (1 μM) that had been equilibrated in 5 M urea at 4 °C was diluted to 125 nM in 40 mM Bis-Tris-propane buffer, pH 7.4, containing 1 M urea, or diluted in buffer plus urea plus one or more of the following: 5.1 μM BSA, 1 μM FAD, 1 μM FMN, 200 nM CaM, and 100 μM Ca^{2+}. After overnight incubation at 4 °C, aliquots were removed and assayed for cytochrome *c* reduction in a microplate assay described in Experimental Procedures. Recovery was assessed relative to the activities of a dimeric iNOS preparation that had not been denatured with urea but otherwise was similarly treated (positive control) and to an enzyme-free control. Values reported are the mean of three measurements and are representative of four similar experiments.

results show that dimeric iNOS behaves similarly to certain other oligomeric proteins that undergo separate subunit dissociation and unfolding transitions when exposed to denaturants (Jaenicke & Rudolph, 1986; Wente & Schachman, 1982; Jaenicke et al., 1981), as opposed to concerted subunit dissociation and unfolding transitions (Bowie & Sauer, 1993; Blackburn & Noltmann, 1981; Grant et al., 1992).

Catalytic measurements obtained after equilibration of dimeric iNOS at different urea concentrations suggest that loss of NO synthesis and reductase activities can serve to indicate the separate subunit dissociation and unfolding transitions, respectively. For example, as shown in Figure 2, dimeric iNOS equilibrated in 1 M urea maintained ~50% of its NO synthesis and 100% of its reductase activities, suggesting that 50% of dimeric iNOS had dissociated into folded iNOS subunits. This is exactly what was observed

by gel filtration analysis. Equilibration at urea concentrations above 2 M caused reductase activities to decrease such that they became completely absent at 5 M urea. Corresponding gel filtration results showed that the iNOS subunits undergo an unfolding transition under these conditions. Thus, the iNOS dimer undergoes two separate structural transitions in urea, and each is linked to the loss of a distinct catalytic function.

The insensitivity of iNOS reductase activities to the dimer dissociation transition is evidenced by the identical monophasic curves we obtained for dimeric iNOS and iNOS subunit reductase activities, as shown in Figure 2. This in turn supports our previous results which showed that the reductase activities of the purified dimer and natural subunits are equivalent (Baek et al., 1993). Thus, both previous work with natural iNOS subunits and the current dissociation studies support a model in which NO synthesis is dependent on iNOS dimeric structure, while reductase activities are not.

A recent investigation of dimeric iNOS domain composition and subunit interaction revealed that the subunits are aligned in a head-to-head manner, with the oxygenase domains interacting to form a dimer and the reductase domains existing as independent monomeric extensions (Ghosh & Stuehr, 1985). Urea-promoted dissociation of dimeric iNOS can therefore be viewed as an oxygenase domain-specific event. Dissociation of dimeric iNOS is accompanied by release of bound H_4biopterin, a perturbation of the heme environment, and loss of NO synthesis activity. These changes are consistent with the binding sites for heme, H_4biopterin, and L-arginine all being located within the iNOS oxygenase domain (Ghosh & Stuehr, 1995). In contrast, subunit dissociation had no effect on catalysis by the iNOS reductase domain, consistent with this domain existing and functioning independently of dimeric structure (Baek et al., 1993) or in the absence of an oxygenase domain (Ghosh & Stuehr, 1995). Although a loss of reductase activities was observed at urea concentrations that caused unfolding of the iNOS subunits, this transition was not associated with release of bound flavins or CaM. Therefore, the loss in catalytic function might instead involve a more subtle structural change that possibly disables electron transfer and/or NADPH or acceptor binding.

Urea-generated iNOS subunits share a number of characteristics with native iNOS subunits that are isolated from activated macrophages (Baek et al., 1993). For example, both contain FAD, FMN, and CaM, are devoid of H_4biopterin, bind NADPH, and catalyze electron transfer to cytochrome *c* or ferricyanide. Both require L-arginine and H_4biopterin to associate into an active dimeric iNOS. However, the urea-generated subunits differ from natural subunits in their containing near stoichiometric quantities of bound heme. The spectrum of the ferrous−CO complex indicates that the heme iron in urea-generated subunits is coordinated to the protein through a cysteine thiolate axial ligation, as is the case for native dimeric iNOS (Stuehr & Ikeda-Saito, 1992; Wang et al., 1993). This suggests that the proximal cysteine ligand is maintained during subunit dissociation. The light absorbance spectrum of the dissociated iNOS subunits is reminiscent of bisthiolate ferric heme complexes of cytochrome P-450, which also show absorbance maxima near 380 and 460 nm (Sono et al., 1982; Yu et al., 1974). Thus, we speculate that a DTT sulfhydryl coordinates as a sixth ligand to the heme iron during subunit

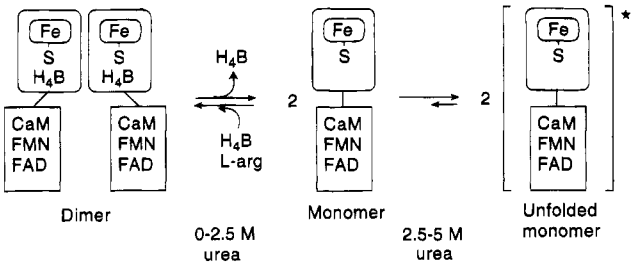

FIGURE 8: Model for urea-promoted subunit dissociation and unfolding of dimeric macrophage iNOS. Subunit dissociation and unfolding occur within two distinct urea concentration ranges. The first transition involves a dissociation between the oxygenase domains of two interacting subunits. This is accompanied by a loss of NO synthesis activity and release of bound H_4biopterin (H_4B) but retention of the cysteine thiolate-ligated heme, flavins, CaM, and full reductase activities. The sixth coordination position of the heme iron becomes exposed to solvent during subunit dissociation and can form a bisthiolate complex with DTT. The unfolding transition (iNOS*) is associated with a loss of reductase activities and partial release of bound heme but retention of bound flavins and CaM. The dissociated subunits can re-form an active dimer in the presence of added H_4biopterin and L-arginine. The loss of reductase activities associated with the unfolding transition can be partially reversed by prolonged incubation in the presence of BSA.

dissociation.[7] Adding imidazole to this complex generates a low-spin species with absorbance maxima at 417 nm, as observed for dimeric iNOS (Ghosh & Stuehr, 1995; Wolf & Gribin 1994), indicating that imidazole can displace the DTT thiolate ligand (data not shown). Together, these results suggest that the sixth coordination site of the heme iron becomes exposed to solvent during dissociation of dimeric iNOS in urea. Whether this takes place due to release of H_4biopterin and/or a change in heme pocket structure is under current investigation.

A related feature of the urea-generated subunits is their ability to reassociate into active dimeric iNOS in the absence of added heme. Dimerization of native iNOS subunits requires that exogenous heme be added in amounts that match but do not greatly exceed the subunit concentration (Baek et al., 1993). The independence toward added heme is apparently explained by our finding that near stoichiometric amounts of heme remain bound to the urea-generated subunits. The bound heme appears capable of replacing added heme in the subunit dimerization reaction and in doing so regains its original spectral characteristics and its ability to function catalytically in NO synthesis. This suggests that the changes in heme environment that occur during subunit dissociation are reversed during L-arginine- and H_4biopterin-promoted subunit reassociation.

The current study, when combined with previous work that characterized the natural iNOS monomers and the domain composition and subunit alignment in dimeric iNOS (Baek et al., 1993; Ghosh & Stuehr, 1995), helps to clarify how iNOS structure relates to prosthetic group binding and catalysis. A model that is consistent with the results to date is illustrated in Figure 8.

It is intriguing that dimeric iNOS dissociates in urea to form heme-containing subunits instead of the heme-free

[7] The heme iron in dimeric iNOS is predominantly five-coordinate high spin and does not bind DTT in the absence of 2 M urea. Also, the spectral changes reminiscent of bisthiolate complexation do not occur when dimeric iNOS is dissociated in the absence of DTT (H. M. Abu-Soud and D. J. Stuehr, unpublished results).

subunits that are found in activated macrophage lysates. This finding implies that newly synthesized iNOS subunits may be capable of binding heme independent of dimerization but for some reason do not in the activated macrophages. It will be important to determine if significant populations of iNOS monomers exist in other types of activated cells, whether the monomers are also devoid of bound heme, and what factors may limit heme insertion into iNOS. Regarding iNOS biochemistry, the availability of heme-containing subunits will now enable us to investigate how dimeric structure influences heme pocket architecture, interdomain electron transfer to the heme iron, and its catalysis of O_2 reduction and NO synthesis.

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