Heme Iron Reduction and Catalysis by a Nitric Oxide Synthase Heterodimer Containing One Reductase and Two Oxygenase Domains*

(Received for publication, December 26, 1995, and in revised form, January 24, 1996)

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Inducible nitric oxide (NO) synthase (iNOS) is comprised of an oxygenase domain containing heme, tetrahydrobiopterin, the substrate binding site, and a reductase domain containing FAD, FMN, calmodulin, and the NADPH binding site. Enzyme activity requires a dimeric interaction between two oxygenase domains with the reductase domains attached as monomeric extensions.

To understand how dimerization activates iNOS, we synthesized an iNOS heterodimer comprised of one full-length subunit and one histidine-tagged subunit that was missing its reductase domain. The heterodimer was purified using nickel-Sepharose and 2',5'-ADP affinity chromatography. The heterodimer catalyzed NADPH-dependent NO synthesis from L-arginine at a rate of 52 ± 6 nmol of NO/min/mmol of heme, which is half the rate of purified iNOS homodimer. Heterodimer NO synthesis was associated with reduction of only half of its heme iron by NADPH, in contrast with near complete heme iron reduction in an iNOS homodimer. Full-length iNOS monomer preparations could not synthesize NO nor catalyze NADPH-dependent heme iron reduction. Thus, dimerization activates NO synthesis by enabling electrons to transfer between the reductase and oxygenase domains. Although a single reductase domain can reduce only one of two hemes in a dimer, this supports NO synthesis from L-arginine.

Nitric oxide (NO) has important roles as a signal and cytotoxic molecule in biology (1–3) and is synthesized from L-arginine (4–7). NOS binds near enough to the heme to influence its reduction and its interactions with small ligands such as CO or NO (8–10). L-Arginine or substrate analog binding to the oxygenase domain is also influenced by H₂bipterin, which may bind near the heme (11–13).

Conversion of L-arginine to NO occurs in two steps with N^⁶-hydroxy-L-arginine forming as an enzyme-bound intermediate (for review see Refs. 1, 14, and 15). Both steps require that NADPH-derived electrons be transferred from the reductase domain flavins to the heme group in the oxygenase domain, which is bound to the protein via a cysteine thiolate as in the cytochromes P-450 (16–19). The flavin to heme electron transfer is critical for catalysis because it is thought to enable the heme iron to bind and activate oxygen at both steps in the mechanism, resulting in oxygen insertion into L-arginine and into N^⁶-hydroxy-L-arginine.

In spite of each subunit containing the required flavin and heme groups, the NOSs appear to act only in dimeric form (20, 21). Dimeric structure does not seem to influence the electron transfer properties of the NOS reductase domain, because it reduces electron acceptors like cytochrome c with equal activity when present in a NOS dimer or a monomer (20, 22).

Indeed, recent studies with the cytokine-inducible NOS (iNOS) suggest that the subunit dimeric interaction only involves the oxygenase domains of each subunit, with the reductase domains existing as independent monomeric extensions (7, 24) (Fig. 1). The reductase domains can be removed with trypsin to generate a dimer comprised exclusively of two oxygenase domains. This oxygenase domain dimer maintains all of its native properties, including the ability to bind substrate and catalyze NO synthesis from N^⁶-hydroxy-L-arginine when supplied with electrons from an external reductase protein (7, 23, 24).

Although the proposed subunit alignment for iNOS raises intriguing possibilities regarding electron transfer and cooperative catalysis between the oxygenase domains during NO synthesis (7), exactly how dimerization activates iNOS catalysis remains unclear.

We recently showed that full-length and oxygenase domain dimers can both be dissociated into heme-containing monomers when treated with urea (22, 24). Each monomer can reassemble into its respective homodimer in the presence of L-arginine and H₂bipterin. Besides confirming that all of the determinants for dimerization reside within the oxygenase domain, these results suggest that it may be possible to construct an iNOS heterodimer that contains a single reductase domain linked to a dimeric oxygenase core (Fig. 1). This species, in conjunction with a full-length iNOS monomer, could be used to investigate how dimerization influences electron transfer and catalytic activity in the oxygenase domain. In this report, we describe a method to synthesize and purify such an iNOS heterodimer and report on its catalytic and electron transfer properties.

EXPERIMENTAL PROCEDURES

Materials—All materials were obtained from Sigma or from sources previously reported (7, 20, 22).

Generation and Purification of Full-length and Oxygenase Domain

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Monomers—Full-length iNOS dimer was isolated and purified from the RAW 264.7 macrophage cell line as described previously (17, 20). The iNOS oxygenase domain (amino acids 1-503) containing a six-histidine tag at its C terminus was overexpressed in Escherichia coli using a modified version of the PCWori vector, which has been used to express the oxygenase domain of neuronal NOS (12). The iNOS oxygenase domain was isolated by binding it to nickel affinity resin (Ni-Nitrilotriacetic-TEPharose CL4B resin, Pharmacia Biotech Inc.), washing the resin to remove unbound proteins, and eluting with imidazole according to the manufacturer’s directions. Characterization of the domain showed that it was dimeric and had the same spectral, binding, and catalytic properties as the native iNOS oxygenase domain that is formed by trypsinolysis of full-length dimeric iNOS (23, 24). Purified oxygenase domain dimers were dialyzed against 40 mM N-(2-hydroxyethyl)piperazine-N’-3-propane sulfonic acid (EPPS), pH 7.6, containing 10% glycerol and 0.5 mM dithiothreitol prior to use. Full-length (~15 μM) or oxygenase domain dimers (~30 μM) were dissociated using 2 or 5 mM urea, respectively (22, 24). The dissociation reaction was carried out at 28°C for 90 min and was terminated by a 10-fold dilution with 40 mM EPPS buffer, pH 7.6, containing 10% glycerol. The monomers so prepared were checked by gel filtration chromatography on Superdex 200. In general, this method yielded 70–90% full-length monomers and 50–60% oxygenase domain monomers.

Formation and Purification of the iNOS Heterodimer—Nickel affinity resin previously charged with 50 mM NISO₄ and equilibrated with 40 mM EPPS, pH 7.6, containing 10% glycerol was saturated with the urea-dissociated oxygenase domain monomer preparation. The resin was separated from the unadsorbed protein, washed with a bed volume of equilibration buffer, and incubated with the urea-dissociated full-length monomer preparation at room temperature for 35 min. L-Arginine (5 mM) and H₄bipterin (10 μM) were then added to the mixture to induce dimerization and incubated for a further 45 min with gentle shaking. The resin was packed into a column, and the unadsorbed proteins were removed by washing thoroughly with 5 column volumes of the equilibration buffer. The resin-bound protein fraction was then eluted with equilibration buffer containing 100 mM imidazole and then dialyzed against 500 volumes of equilibration buffer that also contained 1 mM dithiothreitol, 4 μM H₄bipterin, 2 μM FAD, and 1 mM l-arginine. The protein was then incubated for 1 h at 4°C with 2,5'-ADP-Sepharose CL4B resin that had been equilibrated with the above mentioned dialysis buffer. The beads were washed thoroughly to remove unbound material, and the bound protein was eluted with 1 bed volume of 10 mM NADPH. The eluate was concentrated to 0.1–0.2 ml in a Centricron 30 microconcentrator and loaded onto a Superdex 200 gel filtration column equilibrated with the above mentioned dialysis buffer. The eluted protein was collected in 0.3-ml fractions, pooled, concentrated, and stored at ~70°C.

Measurement of NO Synthesis, NADPH Oxidation, and Heme Iron Reduction—NO synthesis and NADPH oxidation were assayed by spectrophotometric methods as previously reported (17, 20). The initial rate of NO synthesis was determined by monitoring the NO-mediated conversion of oxyhemoglobin to methemoglobin at 401 nm. The assays were carried out at 37°C in buffer containing 40 mM Tris-HCl buffer, pH 7.8, supplemented with 5–10 μM oxyhemoglobin, 0.3 mM dithiothreitol, 1 mM arginine, 0.1 mM NADPH, 4 μM each of FAD, FMN, and H₄bipterin, 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.

NADPH oxidation was monitored at 340 nm in the same system except oxyhemoglobin was omitted. Anaerobic spectroscopy was carried out in septum-sealed cuvettes as detailed in previous reports (7, 25).

Calculations—iNOS concentrations are reported on a per heme basis and were determined using an estimated extinction coefficient of 71 M⁻¹ cm⁻¹ at 398 nm for macrophage iNOS (17). The percentage of iNOS ferrous heme iron was estimated based on CO binding and was obtained from the absorbance difference between 444 and 490 nm, using an estimated extinction coefficient of 74 M⁻¹ cm⁻¹ (17).

To form a heterodimer we utilized a full-length iNOS monomer preparation that was generated from dimeric iNOS and an oxygenase domain monomer preparation that was generated from an oxygenase domain dimer that was expressed in E. coli and contained a six-histidine tag at its C terminus. Preliminary experiments with the recombinant oxygenase domain dimer showed that the histidine tag did not alter the spectral or binding properties of the protein relative to native oxygenase domain dimer (7, 24). The histidine tag also did not prevent dimerization between two oxygenase domain monomers in solution or when one monomer was bound to nickel affinity resin (data not shown). As detailed under “Experimental Procedures,” the dimerization reaction that formed the heterodimer was carried out between oxygenase domain monomers bound to nickel affinity resin and full-length monomers in solution. The nickel affinity resin enabled us to separate bound heterodimers from most of the unreacted full-length monomers and full-length homodimers that had formed during the dimerization reaction. After eluting the immobilized heterodimer with imidazole, it was separated from contaminating oxygenase domain monomers and homodimers by virtue of its containing a NADPH binding site, using 2,5'-ADP affinity chromatography. The heterodimer underwent analytical gel filtration as a final step to ensure full-length and oxygenase domain homodimers (Fig. 2). The protein eluted at 11.7 ml, which was between the elution volumes of authentic full-length iNOS homodimer (10.7 ml) and the oxygenase domain homodimer (13.2 ml). Analysis of the indicated protein peak fraction by SDS-polyacrylamide gel electrophoresis (Fig. 2, inset) showed that it was comprised of both full-length and oxygenase domain subunits whose relative staining densities were 2.4 to 1. Based on this densitometric data and the relative molecular mass of each subunit (130 and 55 kDa), a 1:1 molar ratio of full-length and oxygenase domain subunits was present in the peak fraction. Thus, we conclude that the isolated protein is an iNOS heterodimer. In the example shown, 0.52 nmol of pure heterodimer was obtained from reacting 9.2 nmol of immobilized oxygenase domain with an equivalent amount of full-length monomer preparation.

Activity measurement by the oxyhemoglobin assay showed that the heterodimer could synthesize NO from L-arginine or from N⁶-hydroxy-L-arginine. For three separate heterodimer preparations, the catalytic turnover number with L-arginine as substrate was 52.0 ± 5.8 nmol of NO/min/nmol of heme. This is about half of the activity obtained with a purified full-length homodimer when assayed under identical conditions (125 nmol of NO/min/nmol of heme). In contrast, full-length monomer preparations generate either no measurable NO or some small amount of NO in direct proportion to their residual dimer content (22). The average rate of NADPH oxidation by the three heterodimer preparations during NO synthesis from L-arginine was 7310.

3 Densitometric analysis of protein bands contained in fractions that eluted before or after the central heterodimer peak revealed that these fractions also contained both full-length and oxygenase domain proteins but at ratios that indicated some full-length iNOS homodimer and full-length oxygenase dimer eluted before and after the heterodimer peak, respectively.
arginine was 77.6 ± 4.3 nmol of NADPH/min/nmol of heme. This gives a calculated stoichiometry of 1.5 NADPH oxidized per NO formed, which is equal to the minimum stoichiometry for NO synthesis from L-arginine (26, 27) and indicates that NO synthesis by the heterodimer was tightly coupled to its NADPH oxidation. We conclude that dimerization enabled the oxygenase domain subunit (~130 kDa) and oxygenase domain subunit (~55 kDa). The results shown are representative of three purifications.

To examine how dimerization activated NO synthesis in the heterodimer, we utilized CO binding as a means to quantitate NADPH-dependent heme iron reduction. In previous work with a full-length iNOS homodimer, we observed that the addition of NADPH in the presence or the absence of L-arginine results in rapid reduction of 90–95% of the heme iron, as determined by build-up of a characteristic ferrous-CO Soret peak at 444 nm (25, 28). Because NADPH-derived electrons can only reduce the heme iron by passing through the reductase domain (6, 23), we performed an analogous experiment with the heterodimer to determine what portion of its heme is accessible to electrons coming from the single reductase domain. The experiment was carried out under anaerobic conditions in buffer that contained H₄biopterin and L-arginine and was saturated with CO. As shown in Fig. 3 (solid line), the light absorbance spectrum of the heterodimer displays a broad Soret absorbance centered at 398 nm, indicative of predominantly high spin ferric iNOS, as observed for a full-length iNOS homodimer under similar conditions (17, 25, 28). There also is an absorbance shoulder between 425 and 500 nm attributable to bound flavins in the heterodimer. The spectrum obtained immediately after addition of excess NADPH (dashed line) shows that a portion of the heterodimer’s heme iron remained ferric, as indicated by the absorbance remaining at 380–420 nm, and a portion had become reduced, as indicated by the new absorbance peak at 444 nm. The relative proportion of oxidized and reduced heme iron did not change with time or upon adding more NADPH (data not shown). Dithionite was then added to completely reduce all of the heme iron present (dotted line), resulting in a further absorbance decrease in the 380–420 nm region and an increase at 444 nm. Quantitation of the amount of heme iron reduced with NADPH relative to the amount reduced with dithionite indicates that 55% of the heme iron was reduced by NADPH in the heterodimer. The percentage of heme iron reduction obtained in two replicate experiments using different heterodimers was calculated to be 56 and 50% (data not shown). Thus, these results clearly distinguish the heterodimer from a full-length iNOS homodimer, where 90–95% of the available heme iron is reduced upon NADPH addition under identical circumstances (25, 28).

We next investigated heme iron reduction in three iNOS monomer preparations that were generated by dissociating a full-length iNOS homodimer with urea (22). The addition of NADPH in these cases resulted in very little⁴ or no heme iron reduction, as judged by the total loss of Soret absorbance in the 400 nm region and an immediate buildup of the ferrous-CO complex at 444 nm (data not shown). These results indicate that electron transfer between the reductase domain and heme iron in a full-length subunit does not occur when the subunit is in monomeric form.

**DISCUSSION**

A major finding of our study is that dimerization may activate iNOS NO synthesis by enabling the reductase domain to transfer electrons to the heme iron. This is consistent with
heme iron reduction being an essential first step that leads to oxygen activation and insertion into the substrate (1, 14, 15). As evidenced with the iNOS heterodimer, transfer of NADPH-derived electrons to the heme can occur even when only one reductase domain is attached and is sufficient to support NO synthesis. Although the heterodimer synthesized NO at a rate that was half-maximal on a per heme basis when compared with the native iNOS homodimer, only half of the heterodimer heme iron was susceptible to reduction by NADPH. This implies that the portion of heterodimer heme iron that was reducible participates in NO synthesis at a rate that equals the heme iron in a full-length iNOS homodimer.

It is remarkable that only half of the heme iron in the heterodimer was susceptible to reduction by NADPH. The partial reduction does not appear to reflect a point in a redox equilibrium, because adding extra reductant (NADPH) did not cause an increase in the amount of heme iron reduced. Furthermore, when the same experiment is carried out using a full-length iNOS homodimer, 90–95% of heme iron is reduced (25, 28). Thus, our results suggest that a kinetic barrier exists in the heterodimer toward reduction of half of its heme iron. Based on the available evidence, there is little reason to suspect that the kinetic barrier arises from an inequality between the hemes contained in the oxygenase and full-length subunits of the heterodimer. For example, a homodimer formed from two oxygenase domains has the capacity to bind L-arginine and H$_4$biopterin, accept electrons from an added reductase domain, and synthesize NO from N$^\cdot$-hydroxy-L-arginine (7, 23, 24). In addition, the light absorbance spectrum of the heterodimer indicates that both of its heme iron are predominantly high spin in the presence of L-arginine and H$_4$biopterin, as is the case with both full-length and oxygenase domain homodimers (7, 17). Thus, the most likely explanation for why NADPH reduces only half of the heterodimer heme iron is that the single reductase domain can provide electrons to only one of the two heme irons.

If the reductase domain can only transfer electrons to one of the two hemes in the heterodimer, which heme becomes reduced? The apparent inability of a full-length iNOS monomer to transfer NADPH-derived electrons to its heme iron implies that electron transfer in the heterodimer may occur in trans, i.e. electrons transfer from the reductase domain in the full-length subunit to the adjacent oxygenase domain heme iron. However, alternative mechanisms that invoke electron transfer within the full-length subunit could also explain the results. For example, dimerization may change the reactivity of the heme iron in the full-length subunit such that it can accept electrons from its reductase domain. Indeed, our related work (22, 24) indicates that the heme iron in an iNOS monomer is exposed to solvent and as a result is predominantly six-coordinate low spin, which in cytochrome P-450 is associated with a decrease in heme iron reduction potential (29, 30). Dimerization, which shifts the iNOS heme iron spin equilibrium back toward high spin (22, 24), could thus enable electron transfer by increasing the reduction potential of the heme iron in the full-length subunit. Alternatively, dimerization may cause a protein conformational change in the full-length subunit that creates a path for electrons to pass between its reductase and oxygenase domains. In any case, our current results suggest that dimerization enables electrons to transfer to a single heme in the heterodimer, and this is sufficient for activating a normal rate of NO synthesis from L-arginine. Thus, mechanisms that invoke electron transfer between two adjacent oxygenase domains, participation of both hemes in oxidizing a single molecule of L-arginine, or concerted catalysis between the subunits can be ruled out. In addition, the general method we outline here to create and purify iNOS heterodimers should now make it possible to study how sequence and structural alterations in a single subunit will affect dimerization, electron transfer, H$_4$biopterin binding, and other aspects of the reaction mechanism.

Acknowledgments—We thank Dr. John Parkinson and colleagues at Berlex Biosciences for helping construct the oxygenase domain expression plasmid, Pam Clark for expert technical assistance, and Dr. Denis Rousseau for reviewing the manuscript.

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J. Biol. Chem. 1996, 271:7309-7312.
doi: 10.1074/jbc.271.13.7309

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