Macrophage NO Synthase: Characterization of Isolated Oxygenase and Reductase Domains Reveals a Head-to-Head Subunit Interaction†

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ABSTRACT: Macrophage NO synthase (NOS) is a dimeric enzyme comprising two identical 130 kDa subunits and contains iron protoporphyrin IX (heme), tetrahydrobiopterin, FAD, FMN, and calmodulin. We have carried out limited proteolysis to locate the domains involved in prosthetic group binding and subunit interaction. Trypsin cleaved the subunits of dimeric macrophage NOS at a single locus, splitting the enzyme into two fragments whose denatured molecular masses were 56 and 74 kDa. The smaller fragments remained dimeric in their native form (112 kDa), contained heme and tetrahydrobiopterin, and could bind L-arginine, CO, or imidazole. In contrast, the larger fragments were monomeric in their native form, contained FAD, FMN, and CAM, and bound NADPH. Although neither purified fragment alone or in combination catalyzed NO synthesis from L-arginine, the flavin-containing fragment did catalyze cytochrome *c* reduction at a rate that was equivalent to that of native dimeric NOS. These results indicate that trypsin cuts macrophage NOS into two domains that can exist and function independently of one another. The domain that binds heme, H_4biopterin, and substrate is also responsible for maintaining the NOS dimeric structure, while the domain containing FAD, FMN, and CAM is not required for subunit interaction. This suggests a structural model for macrophage NOS in which the subunits align in a head-to-head manner, with the oxygenase domains interacting to form a dimer and the reductase domains existing as independent extensions.

Nitric oxide (NO)[1] has emerged as an important signal and effector molecule in mammalian physiology [for reviews, see Nathan (1992), Stuehr and Griffith (1992), and Bredt and Snyder (1994)]. NO is generated by a family of enzymes called NO synthases (NOSs; EC 1.14.13.39), which differ from each other with respect to their mode of expression, primary sequence, and dependence on exogenous Ca^{2+} and calmodulin (CAM) (Bredt et al., 1991; Lamas et al., 1992; Xie et al., 1992). The NOS isolated from mouse macrophages is expressed in cells only after their exposure to bacterial lipopolysaccharide or activating cytokines (Xie et al., 1992; Deng et al., 1993). Macrophage NOS is homodimeric in its active form (subunit molecular mass, 130 kDa) and contains 1 mol each of iron protoporphyrin IX (heme), FAD, and FMN per subunit, variable amounts (0.1−1 mol/subunit) of tetrahydrobiopterin (H_4biopterin), and an unspecified amount of tightly bound CAM (Stuehr et al., 1991a; Baek et al., 1993; Hevel & Marletta, 1992; Cho et al., 1992; White & Marletta, 1992; Stuehr & Ikeda-Saito, 1992). Cloning and modeling studies have identified both NADPH and flavin binding sites within the C-terminal half of NOS (Xie et al., 1992), a possible heme binding domain within the N-terminal half at Cys_{194} (Renaud et al., 1993; McMillan et al., 1992), and a CAM binding site near the center of the polypeptide (Xie et al., 1992) (Figure 1). The NOS flavins store NADPH-derived electrons and can transfer them to the heme iron (Abu-Soud & Stuehr, 1993), which is thought to activate oxygen and catalyze the two-step oxidation of L-arginine to NO and L-citrulline (White & Marletta, 1992; Stuehr & Ikeda-Saito, 1992; Stuehr et al., 1991b). Heme iron reduction is controlled by the tightly bound CAM, which enables a flavin-to-heme electron transfer to occur (Abu-Soud & Stuehr, 1993). Although bound H_4biopterin is required for macrophage NOS to oxidize L-arginine (Hevel & Marletta, 1992; Baek et al., 1993, Kwon et al., 1989), its precise role in catalysis remains unclear.

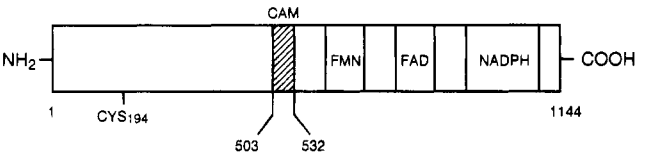

FIGURE 1: Model for prosthetic group binding domains in the macrophage NOS polypeptide. Within the C-terminal half, consensus binding sequences are present for NADPH, FAD, and FMN binding (Xie et al., 1992). This region is highly homologous with the enzyme NADPH−cytochrome P-450 reductase. In the N-terminal region, sequence and modeling considerations predict that Cys_{194} may be involved in heme iron binding (Renaud et al., 1993). A CAM consensus binding site is present near the center of the polypeptide. Its sequence (residues 503−532) with N-terminal flanking residues is R_{501}PRRREIRFRVLVKVVFFASMLMRK-VMASRVR_{532}.

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[1] Abbreviations: BTP, Bis-Tris/propane; 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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In addition to their participating in catalysis, L-arginine, H_4biopterin, and heme also help form and maintain the dimeric structure of macrophage NOS. Specifically, purified NOS subunits must be coincubated with heme, H_4biopterin, and L-arginine to associate into a dimeric form, with heme and H_4biopterin becoming bound to the dimer during its assembly (Baek et al., 1993). This has led us to speculate that the heme, L-arginine, and H_4biopterin binding sites may participate in the subunit interaction. However, it is still unclear where these factors bind within the macrophage NOS subunit or how the subunits align to form the active dimeric enzyme.

To address these questions, we utilized limited trypsin proteolysis, which has aided in characterizing multidomain enzymes such as the bacterial flavoprotein cytochrome P-450$_{BM3}$ (Narhi & Fulco, 1987) and neuronal NOS (Sheta et al., 1994). Studies reported here identify two functional domains within the macrophage NOS polypeptide and characterize each domain regarding its prosthetic group binding, catalytic function, and role in forming the dimeric structure.

MATERIALS AND METHODS

Materials. Polyclonal antibody raised against macrophage NOS was a gift from Carl Nathan (Cornell University Medical College, New York). All other reagents were obtained from Sigma (St. Louis, MO), Amersham (Arlington Hts., IL), or Bio-Rad (Melville, NY).

Analytical Procedures. FMN and FAD bound noncovalently to the purified tryptic fragments were quantitated using C_{18} reverse phase HPLC chromatography and fluorimetric detection, as described previously (Baek et al., 1993). H_4biopterin bound within tryptic fragments was measured using a published fluorimetric method (Takeshi & Nixon, 1980) and quantitated using a standard curve generated with authentic H_4biopterin. The amount of bound heme contained in the tryptic fragments was estimated using an extinction coefficient of 71 mM^{-1} cm^{-1} at 397 nm, determined for the macrophage NOS dimer (Stuehr & Ikeda-Saito, 1992).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out using a published procedure (Laemmli, 1970). Samples were electrophoresed on 8.5% gels using a Bio-Rad minigel apparatus. The protein bands were visualized by staining with Coomassie Blue. Western blot analysis was performed as described by Towbin et al. (1979). The trans-blotted proteins were located using a rabbit polyclonal antibody developed against the macrophage NOS dimer, followed by detection using ECL reagents. Bound calmodulin in the purified macrophage NOS fragments was determined by SDS–PAGE on a 15% gel. Trans-blotted proteins were located with a monoclonal anti-calmodulin antibody obtained from Upstate Biotechnology (Lake Placid, NY), using authentic calmodulin as a control. Protein concentration was measured colorimetrically using the Bio-Rad assay kit and bovine serum albumin as a standard.

Proteolysis of Dimeric Macrophage NOS. Macrophage NOS was purified from cultures of the RAW 264.7 macrophage cell line, as previously described (Baek et al., 1993; Stuehr & Ikeda-Saito, 1992). Limited trypsin digestion of pure dimeric macrophage NOS was performed on ice in 40 mM Tris-HCl buffer (pH 7.9) containing 1 mM DTT, 1 mM L-arginine, and 1 μM BH4. To determine the kinetics of trypsin digestion, the protein was incubated with trypsin at a 40:1 (w/w) ratio. For purification of the proteolytic fragments, 200–300 μg of macrophage NOS dimer was incubated with trypsin as above, and the kinetics of the digestion was followed by assaying NO synthesis activity. The reaction was stopped by adding a 10-fold excess of soybean trypsin inhibitor as soon as the activity became close to zero. This typically took about 2.5 h. The fragments were purified by chromatography on a 2′,5′-ADP Sepharose column (10 × 100 mm) using a Pharmacia-LKB FPLC chromatography system. The column was equilibrated with 40 mM Bis-Tris/propane (BTP), 1 mM DTT, 4 μM H_4-biopterin, 1 mM L-arginine, and 10% glycerol (pH 7.5). After injection of the digest, the flow-through fraction was collected, and the bound protein was eluted with 1 mL of column buffer containing 10 mM NADPH. The flow-through and NADPH-eluted fractions were concentrated separately using Centricon-30 microconcentrators (Amicon, Danvers, MA). Gel filtration chromatography of the purified N-terminal and C-terminal trypsin fragments was carried out using a Superdex-200 HR 10/30 column (Pharmacia) equilibrated with 40 mM BTP (pH 7.5) containing 1 mM DTT, 1 mM L-arginine, 4 μM H_4biopterin, 10% glycerol, and 200 mM NaCl.

Spectroscopy and Assay of Enzyme Activity. UV–visible spectra were obtained at 10 °C on a Hitachi U3110 spectrometer equipped with computer-assisted data collection software. In some cases, spectra were recorded under anaerobic conditions, as described previously (Abu-Soud & Stuehr, 1993). Cytochrome *c* reductase activity was measured at 37 °C by following the NADPH-dependent increase in absorbance at 550 nm and quantitated using an extinction coefficient of 21 mM^{-1} cm^{-1}, according to a published procedure (Baek et al., 1993). NO synthesis activity was measured at 37 °C using the spectrophotometric oxyhemoglobin assay for NO, as reported (Abu-Soud & Stuehr, 1993).

Liquid Chromatography/Mass Spectrometry (LC/MS) Sequence Analysis. Sequence determination of the purified tryptic fragments was carried out using liquid chromatography/electrospray ionization mass spectrometry (LC/ESIMS). The N-terminal and C-terminal macrophage NOS fragments were subjected to additional trypsin digestion, and the oligomers generated from either fragment were separated on a C_{18} reverse phase Applied Biosystems capillary column (1/100 mm) at a flow rate of 40 μL/min, with 0.05% TFA and a gradient of 5–95% acetonitrile over 60 min. The column effluent was fed directly to the electrospray interface of a VG Quattro mass spectrometer, which was set to scan from *m/z* 300 to 2000 over 5 s.

RESULTS

Trypsin Cleavage of Macrophage NOS. Limited digestion of dimeric macrophage NOS was carried out at a trypsin to NOS ratio of 1:40, and the reaction was followed by performing SDS gel analysis and catalytic activity measurements on aliquots removed over the course of the reaction. Gel analysis (Figure 2B) showed that, after 30 min of digestion, two cleavage peptides of ~55 and ~70 kDa were present along with the remaining intact NOS (130 kDa). After 150–180 min, all of the native NOS dimer had apparently been cleaved into the ~55 and ~70 kDa peptide fragments. Thus, under these conditions, trypsin predominantly cut the macrophage NOS polypeptide once into two fragments of unequal mass.

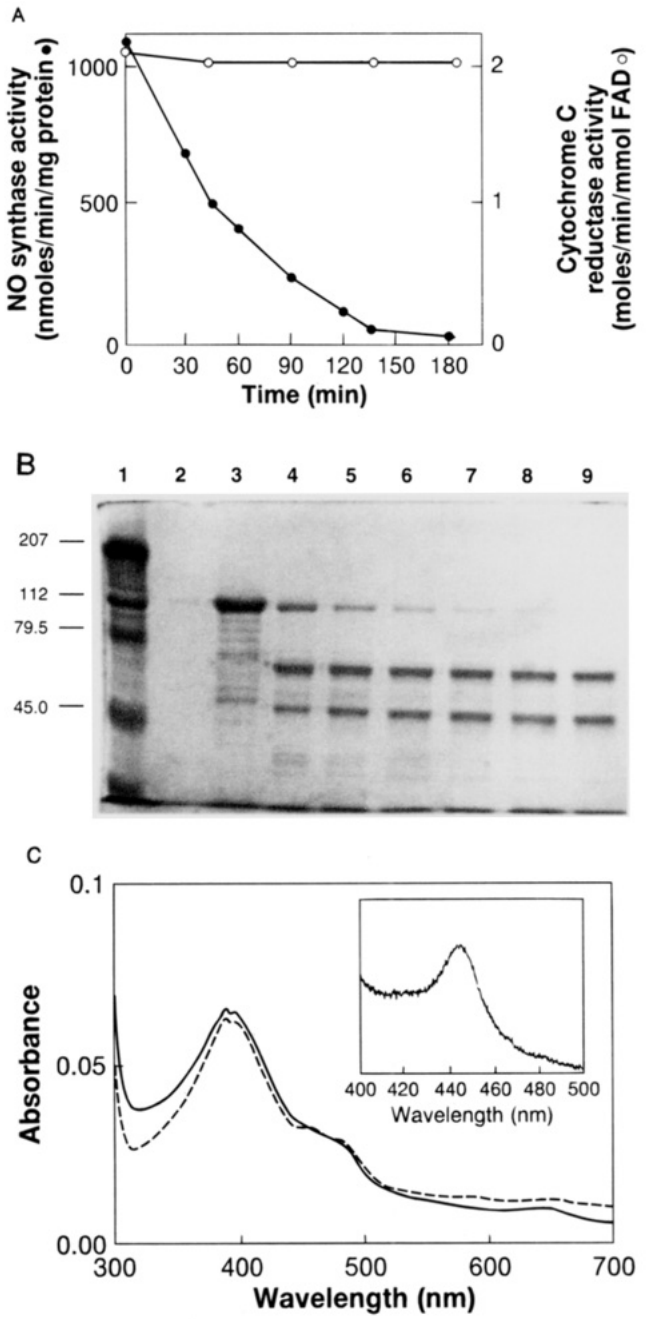

FIGURE 2: Limited trypsin digestion of dimeric macrophage NOS. The kinetics of trypsin digestion at 10 °C was followed by SDS−PAGE analysis (panel B) or by measuring NO synthesis (●) and cytochrome *c* reductase activities (○) on aliquots removed over the course of the digestion (panel A). Aliquots removed at the inidicated time intervals were assayed for enzyme activity as described in Materials and Methods. For gel analysis, each lane contained approximately 4−5 μg of protein. Lane 1 shows protein molecular weight standards, while lanes 3−9 represent samples removed at 0, 30, 60, 90, 120, 150, and 180 min, respectively. Protein was visualized with Coomassie Blue. Panel C: Light absorbance spectra of the dimeric macrophage NOS sample prior to (—) or after (− −) 150 min of trypsin digestion. Spectra were recorded at 10 °C. Inset: Spectra obtained after bubbling CO into the trypsin-digested NOS, followed by reduction with a few grains of sodium dithionite. The reference cuvette contained digest buffer only.

Catalytic measurements performed during the digestion showed that the NO synthesis activity gradually decreased to 2−5% of the original value by 2.5 h, while cytochrome *c* reductase activity remained constant throughout (Figure 2A). In comparison, control reactions in which trypsin was omitted maintained 85% of their NO synthesis activity over an identical period (not shown). Spectral analysis of the trypsin reaction at 2.5 h showed that the fragments completely maintained the spectral features of the original uncut dimeric NOS (Figure 2C), with Soret absorbance at 397 nm and flavin absorbances at 456 and 484 nm. The trypsin-cleaved NOS also bound CO upon dithionite reduction to generate a ferrous CO absorbance at 445 nm (Figure 2C, inset). Thus, although the trypsin cleavage caused macrophage NOS to lose its NO synthesis activity, the two cleavage products still maintained a cysteine-bound heme and expressed full NADPH-dependent cytochrome *c* reductase activity.

Purification and Characterization of Isolated NOS Fragments. We next attempted to separate the two trypsin-generated fragments by chromatography on 2′,5′-ADP Sepharose. Analysis of the column fractions by SDS−PAGE (Figure 3A) showed that the ~55 kDa fragment did not bind to the resin and could be recovered in the flow-through fraction, while the ~70 kDa fragment was retained by the resin and could be eluted separately with NADPH. Both fragments were recognized by a polyclonal antibody raised against authentic dimeric macrophage NOS (Figure 3B). Reaction of the purified fragments with an anti-CAM antibody revealed that only the ~70 kDa fragment contained tightly bound CAM (Figure 3C). The presence of CAM and NADPH binding sites suggested that the ~70 kDa fragment was the C-terminal domain of macrophage NOS and that the ~55 kDa fragment was the N-terminal domain. LC/MS sequence analysis of the two purified fragments confirmed this assignment, in that it identified 87% of amino acid residues 1−503 within the ~55 kDa fragment and 90% of residues 504−1144 within the ~70 kDa fragment (data not shown). N-Terminal amino acid analysis of the ~70 kDa fragment identified 17 of its first 19 amino acids (R_{504}-RE*RFRVLV*VVFFASML). Thus, trypsin cut each subunit in dimeric macrophage NOS once near the N-terminal side of the CAM binding domain at residue 503, as depicted in Figure 1. This is consistent with the presence of multiple trypsin-sensitive sites in this region of the polypeptide and is similar to the cut site observed upon trypsin cleavage of neuronal NOS (Sheta et al., 1994).

Spectral and Catalytic Properties of the Fragments. Figure 4 compares the light absorbance spectra of the purified ~55 and ~70 kDa fragments, along with the spectrum of the macrophage NOS trypsin digest prior to column chromatography. The ~55 kDa fragment exhibited a characteristic Soret band at 395−400 nm, indicating the presence of heme, but had no visible absorbance due to flavins, which were present exclusively in the spectrum of the purified ~70 kDa fragment. Together, the spectral features of each purified fragment gave the spectrum and the native dimeric NOS. Quantitation of prosthetic group binding and catalytic activities in the isolated fragments (Table 1) showed that the ~55 kDa fragment contained one heme and variable amounts of bound H_4biopterin, but no flavins, while the ~70 kDa fragment contained one FAD, one FMN, and bound CAM. Neither of the purified fragments generated NO from L-arginine, alone or in combination, but the ~70 kDa fragment was able to catalyze an NADPH-dependent reduction of cytochrome *c* (Table 1). Purification of the ~55 kDa fragment enabled us to examine the spectral properties of the macrophage NOS heme independent of the enzyme's flavin prosthetic groups, whose absorbance overlaps with the heme (Baek et al., 1993, Abu-Soud & Stuehr, 1993). As

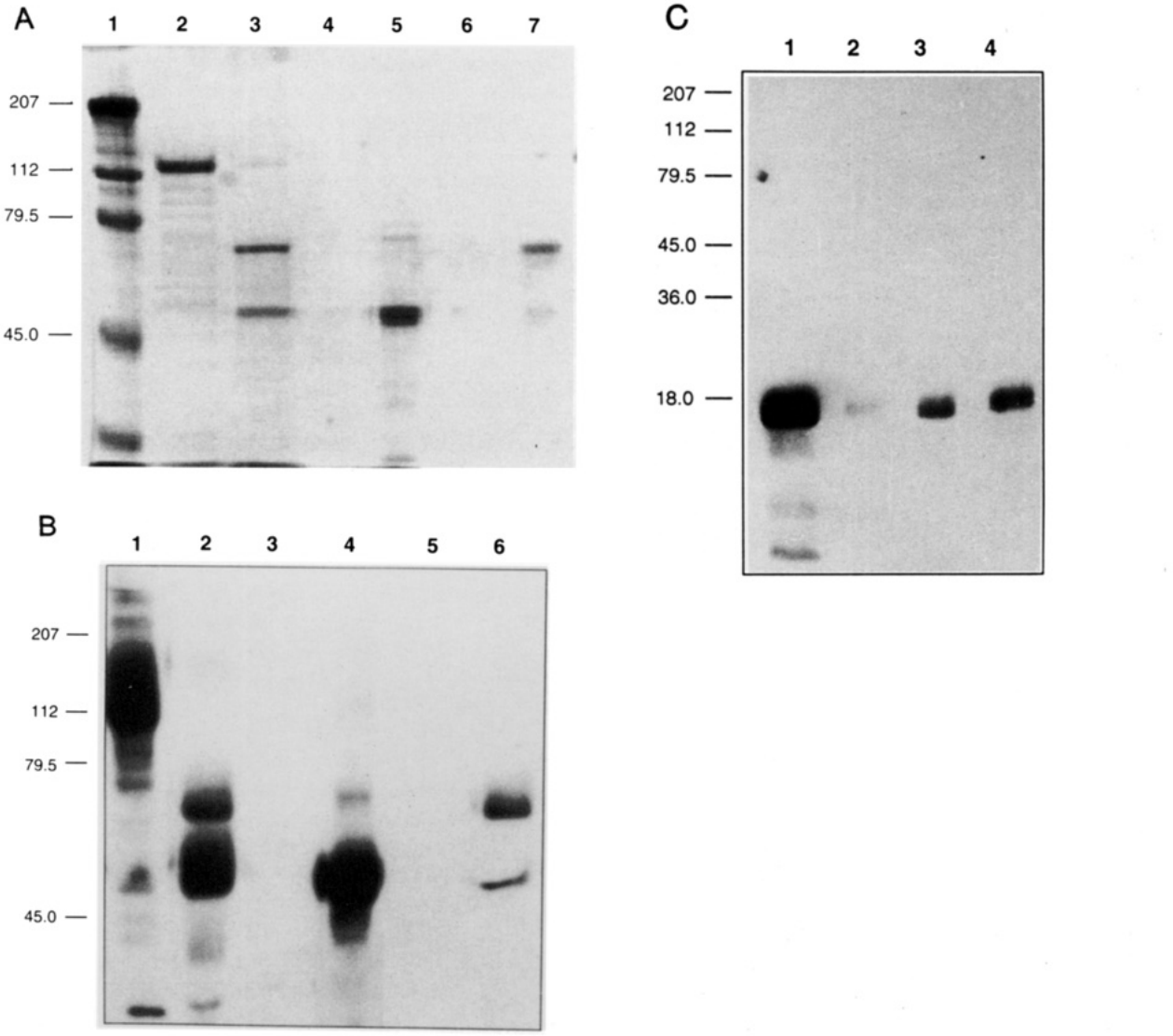

FIGURE 3: SDS−PAGE and Western blot analysis of the purified macrophage NOS fragments obtained by chromatography on 2′,5′-ADP Sepharose. Panel A: lane 1, molecular weight standards; lanes 2 and 3, 3.5 μg of dimeric macrophage NOS before and after the 150 min trypsin digestion; lane 5, 5 μg of the NOS fragment concentrated from the column flow-through; lane 7, 2.5 μg of the NOS fragment eluted from the column with NADPH. Panel B depicts Western blot analysis of a gel containing samples identical to those in panel A, using a polyclonal antibody raised against the macrophage NOS dimer. Panel C shows Western blot analysis of the purified fragments after separation on 15% SDS−PAGE, using a polyclonal anti-CAM antibody. Lane 1, 4 μg of trypsin-digested macrophage NOS dimer; lanes 2 and 3, 3 μg of the purified ~55 and ~70 kDa NOS fragments; lane 4, 4 μg of authentic CAM. In the denaturing gel system, CAM dissociates from the NOS polypeptide and runs as free CAM at 17 kDa (Cho et al., 1992).

Table 1: Prosthetic Group Content and Catalytic Activities of Macrophage NOS Tryptic Fragments and Native Dimeric NOS[a]

	prosthetic groups					catalytic activity	
sample	H_4biopterin	heme	FAD	FMN	CAM	NO synthesis	cyt *c* reduction
macrophage NOS dimer	0.2−0.9	1	1.0	1.0	present	1100 ± 150	3.1 ± 0.4
55 kDa fragment	0.1−0.5	1	0	0	absent	0	0
70 kDa fragment	0	0	1.1	1.0	present	0	2.8 ± 0.3

[a] Purified ~55 and ~70 kDa NOS fragments and the dimeric macrophage NOS from which they originated were analyzed for bound FAD, FMN, H_4biopterin, heme, and CAM, and their NO synthesis and cytochrome *c* reductase activities were determined, as described in Materials and Methods. CAM binding was not quantitated, but was determined qualitatively with an anti-CAM antibody. Binding values are moles bound per mole of polypeptide and represent results from at least two determinations. NO synthesis activity is expressed as nanomoles of NO/minute/milligram of protein, while cytochrome *c* reduction is expressed as moles of cytochrome *c* reduced/minute/millimoles of bound FAD, and both are the mean ± SD of three determinations.

shown in Figure 5, the reduction of heme iron shifted the ~55 kDa fragment's Soret absorbance from 397 to 414 nm and decreased its extinction relative to the ferric form, as occurs in several cytochromes P-450 (Narhi & Fulco, 1987; Appleby, 1978; Yu et al., 1974). CO binding to the ferrous fragment shifted its Soret absorbance to 444 nm, indicating that its heme iron was bound to a cysteine thiolate, as is the case for native dimeric macrophage NOS (Stuehr & Ikeda-Saito, 1992).

Because mixing of the heme- and flavin-containing fragments did not reconstitute NO synthesis, we wondered whether substrate binding was lost due to trypsin lysis. To check this possibility, the ~55 kDa heme-containing fragment was treated with the NOS heme-binding agent imida-

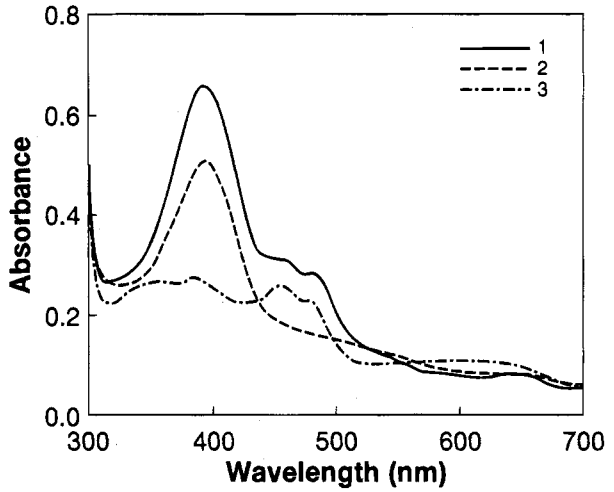

FIGURE 4: Light absorption spectra of the trypsin-digested dimeric macrophage NOS (9 μM, trace 1), the purified ~55 kDa NOS fragment (7 μM, trace 2), and the purified ~70 kDa NOS fragment (10 μM, trace 3).

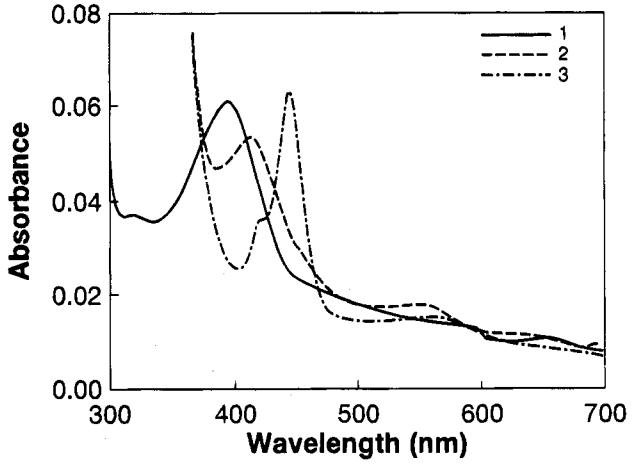

FIGURE 5: Spectral analysis of the isolated heme-containing fragment of dimeric macrophage NOS. The cuvette contained the macrophage NOS heme-containing fragment at 1 μM in 40 mM BTP (pH 7.5) containing 1 mM L-arginine, 4 μM H_4biopterin, and 0.5 mM DTT under an argon atmosphere. Trace 1, purified heme domain in its resting, ferric state; trace 2, dithionite-reduced deoxy ferrous form; trace 3, CO-bound ferrous form. The results are representative of two identical experiments.

zole (McMillan & Masters, 1993; Wolf & Gribin, 1994), which converted the fragment's heme iron from a predominantly high-spin form (absorbance maximum at 395 nm) to a low-spin form (maximum, 420 nm) (Figure 6). Addition of L-arginine to the imidazole-bound heme fragment converted its heme iron back to a high-spin form (Figure 6). This indicated that the L-arginine binding site was intact in the fragment, and the loss of NO synthesis activity was not due to an impairment in substrate binding.

Quaternary Structure of the Fragments. Because macrophage NOS is a homodimer in its active form, we wished to determine whether either trypsin-generated fragment had maintained a dimeric structure. The native molecular masses of the purified fragments were estimated by size exclusion chromatography. As shown in Figure 7, the heme-containing fragment that migrated as a ~55 kDa peptide in SDS−PAGE had an estimated native molecular mass of 112 kDa, indicating that it was dimeric. In contrast, the flavin-containing fragment that had an estimated molecular mass of ~70 kDa by SDS−PAGE had an estimated native molecular mass of 74 kDa, indicating that this fragment was monomeric in its native form.

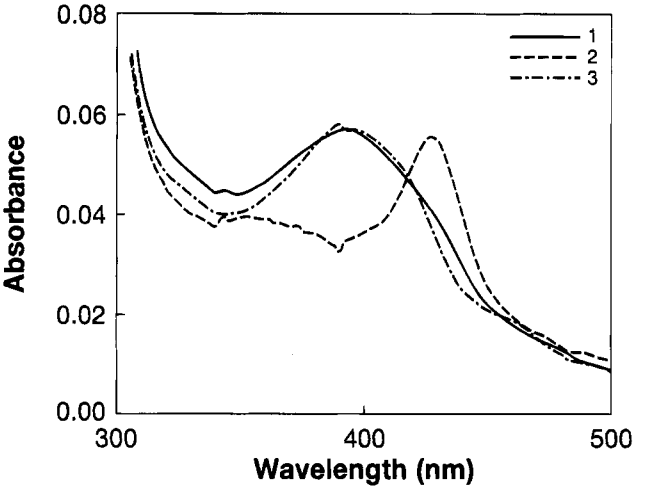

FIGURE 6: Spectral analysis of substrate binding by the purified heme-containing fragment. For this study, the ~55 kDa heme-containing fragment (0.3 mL) was first dialyzed against 2 × 250 mL of 40 mM BTP (pH 7.9) containing 0.5 mM DTT and 2 μM H_4biopterin to remove L-arginine that was present in the protein sample from the purification. Trace 1, spectrum of the substrate-free heme-containing fragment (0.8 μM) after dialysis; trace 2, sample spectrum after the addition of 1 mM imidazole, which converted the heme iron to its low-spin ferric form; trace 3, imidazole-treated sample after the addition of 1 mM L-arginine. Results shown are one of two similar experiments.

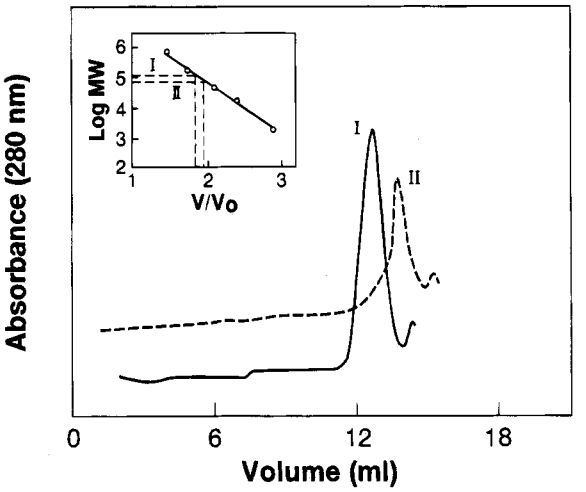

FIGURE 7: Size exclusion chromatography of the purified heme- and flavin-containing fragments of macrophage NOS. Elution profiles of the purified heme-containing fragment (I) or flavin-containing fragment (II), following their separate injection onto a Superdex 200HR gel filtration column under nondenaturing conditions, as described in Materials and Methods. The inset compares the relative retention of the fragment protein peaks with proteins of known molecular weight (○), giving estimated values of 112 000 for the heme-containing fragment and 74 000 for the flavin-containing fragment. Molecular weight standards shown are bovine thyroglobulin (670 000), bovine γ-globulin (158 000), ovalbumin (44 000), horse myoglobulin (17 000), and vitamin B_{12} (1350). The data are from one to three separate determinations.

DISCUSSION

Macrophage NOS contains heme, FAD, FMN, H_4-biopterin, and CAM prosthetic groups and is homodimeric in its active form. In this report, we have cleaved dimeric macrophage NOS into two fragments to examine the distribution of prosthetic groups and to determine which domains are involved in forming subunit dimeric structure.

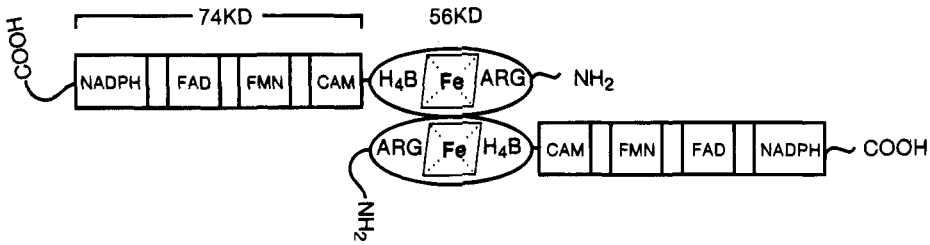

FIGURE 8: Model for domain organization and quaternary structure in macrophage NOS. The N-terminal region of the macrophage NOS polypeptide represents a catalytic domain that contains binding sites for L-arginine, heme, and H_4biopterin and is responsible for subunit dimeric structure. It is tethered to the C-terminal domain, which may exist as an independent monomeric extension. The C-terminal domain binds CAM, FAD, FMN, and NADPH and functions to transport electrons into the catalytic N-terminal domain.

Trypsin cut both subunits in dimeric NOS at the N-terminal side of their CAM binding sites, splitting the NOS into two nonidentical fragments. Biochemical and sequence analyses indicated that the cut site was within a polypeptide region containing several adjacent trypsin-sensitive residues. The N-terminal fragment so generated was dimeric, contained normal amounts of heme and H_4biopterin, and could bind added L-arginine, CO, or imidazole. In contrast, the NOS C-terminal fragment was monomeric, contained CAM, FAD, and FMN, bound NADPH, and catalyzed cytochrome *c* reduction at a rate equivalent to that of the full-length dimeric enzyme.

These data provide the first direct evidence that the heme, H_4biopterin, and substrate binding sites in macrophage NOS are each located between the N-terminus and the CAM binding site. Prior to this report, a modeling study had suggested that heme iron bound in this region at Cys_{194} (Renaud et al., 1993). That the heme, substrate, and pterin binding sites are all present within the macrophage NOS N-terminal fragment is consistent with spectral data showing that L-arginine binds near the heme (Wang et al., 1993; Matsuko et al., 1994) and kinetic data indicating that L-arginine and H_4biopterin bind to NOS in a cooperative manner (Klatt et al., 1994; Wolf & Gribin, 1994).

The prosthetic group composition of the C-terminal fragment reflected the fact that it contains consensus binding regions for flavin, NADPH, and CAM, as determined in the cloning studies (Bredt et al., 1991; Xie et al., 1992). That CAM remained bound to this fragment suggests that proteolytic cleavage near the CAM binding site did not destroy or alter its affinity. Indeed, tightly bound CAM may have protected its binding site from proteolysis in macrophage NOS. An opposite situation exists for neuronal NOS, in that CAM binding exposes additional trypsin cut sites in the N- and C-terminal regions of the polypeptide. Cleavage also occurs at these sites and leads to the loss of both cytochrome *c* reductase and NO synthase activity (Sheta et al., 1994). Thus, limited proteolysis experiments suggest that the macrophage and neuronal NOS isoforms become structurally heterogeneous with respect to susceptible trypsin cleavage sites upon binding CAM.

The macrophage NOS C-terminal fragment also maintained cytochrome *c* reductase activity equivalent to that of the native dimer. Their equivalence is consistent with CAM remaining bound to the peptide following trypsin cleavage, because both neuronal (Klatt et al., 1992) and macrophage NOSs[2] display reduced cytochrome *c* reductase activity in the absence of bound CAM. In general, an intact FAD and FMN domain is required for dual-flavin enzymes to efficiently reduce heme proteins like cytochrome *c* (Kurzban et al., 1990; Vermillon et al., 1981). Thus, the macrophage NOS C-terminal fragment may approximate the minimal structure required to catalyze cytochrome *c* reduction, in that the reaction occurs independent of dimeric structure, bound heme, H_4biopterin, or the N-terminal domain of NOS.

Although the N-terminal and C-terminal macrophage NOS fragments no longer catalyzed NO synthesis from L-arginine, they clearly could exist and function independently, as judged by their individual abilities to bind substrate, CO, or NADPH and catalyze electron transfer. This supports a bidomain structure for the macrophage NOS polypeptide in which the region catalyzing substrate oxygenation is tethered to the region responsible for electron import. CAM binding between the two domains is consistent with its functioning to trigger heme iron reduction in NOS and suggests a model whereby CAM binding changes the relative positioning of the reductase and oxygenase regions to promote interdomain electron transfer (Abu-Soud & Stuehr, 1993). Because a similar bidomain structure has recently been proposed for neuronal NOS (Sheta et al., 1994), it appears that this structural pattern may exist among all NOS isoforms.

The N-terminal macrophage NOS fragment remained dimeric even after losing its C-terminal portion, which in turn existed independently only in a monomeric form. Thus, all determinants required to maintain subunit dimeric structure appear to be located within the N-terminal fragment. Our previous work had identified roles for L-arginine, H_4-biopterin, and heme in forming and stabilizing the dimeric structure of macrophage NOS (Baek et al., 1993). In that report, dissociated macrophage NOS subunits were found to contain only FAD, FMN, and CAM and required the coincident presence of L-arginine, heme, and H_4biopterin to associate into dimers, with stoichiometric amounts of heme and H_4biopterin becoming bound in the process. When viewed in this context, it is entirely consistent that the NOS domain that contains the L-arginine, heme, and H_4biopterin binding sites remains dimeric following trypsin removal of the flavin-containing portion of NOS. On the basis of these data, we propose a model for macrophage NOS quaternary structure in which the subunits associate in a head-to-head manner, with only the N-terminal regions of each subunit interacting to form the dimeric structure (Figure 8).

Because our results did not support a dimeric interaction between the C-terminal portions of the NOS subunits, they are depicted in Figure 8 to exist as independent extensions in the native dimer. This is the most conservative interpretation of the data; however, it is still possible that the C-terminal domains may associate with each other within full-length dimeric NOS, albeit their affinity for one another apparently is inadequate to hold the isolated domains

[2] L. Yoho and D. J. Stuehr, unpublished results.

together. In either case, the C-terminal domain clearly functions to accept and transfer electrons independent of dimeric structure and in the absence of the N-terminal domain, which suggests that an interaction may not be necessary.

Our current findings, along with previous work (Baek et al., 1993), present a unifying picture regarding how prosthetic group binding, domain organization, and subunit architecture are related within the macrophage NOS isoform. In contrast, beyond its domain organization (Sheta et al., 1994), neuronal NOS structure has not been characterized, and it is unclear under what conditions dimer is formed, how the subunits might interact, and whether the binding of heme, L-arginine, and H_4biopterin is linked to subunit association, as occurs in the macrophage NOS (Baek et al., 1993).

It is intriguing that the subunit association in macrophage NOS may exclusively involve the domain thought to contain the active site. A similar arrangement has been proposed for other multidomain dimeric enzymes, such as spinach nitrate reductase (Kubo et al., 1988) and rat liver sulfite oxidase (Southerland et al., 1978). In both cases, a molybdenum-containing catalytic domain possesses the determinants that maintain dimeric structure, while associated regions in the peptide that shuttle electrons through the enzyme remain monomeric. Thus, macrophage NOS may belong to a family of multidomain redox enzymes, whose subunit interactions follow this general pattern.

A head-to-head subunit arrangement in macrophage NOS raises exciting possibilities regarding structure–function relationships. For example, do two subunits associate to form a single catalytic site, or are two completely independent catalytic sites maintained within the active dimer? Within this context reside specific questions concerning intersubunit electron transfer, heme–heme interactions, substrate docking, and concerted catalysis. It is interesting to note that the closely related flavoheme protein cytochrome $P\text{-}450_{BM3}$ is fully functional as a monomer (Narhi & Fulco, 1987). This implies that the macrophage NOS subunit could inherently be capable of catalytic self-sufficiency. However, it is not, and instead must undergo a dimerization reaction that is driven by the prosthetic group that make up its active site. The reasons for this paradox are under active investigation.

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