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Nitric Oxide Synthase Is a Cytochrome P-450 Type Hemoprotein†

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ABSTRACT: Nitric oxide has emerged as an important mammalian metabolic intermediate involved in critical physiological functions such as vasodilation, neuronal transmission, and cytostasis. Nitric oxide synthase (NOS) catalyzes the five-electron oxidation of L-arginine to citrulline and nitric oxide. Cosubstrates for the reaction include molecular oxygen and NADPH. In addition, there is a requirement for tetrahydrobiopterin. NOS also contains the coenzymes FAD and FMN and demonstrates significant amino acid sequence homology to NADPH–cytochrome P-450 reductase. Herein we report the identification of the inducible macrophage NOS as a cytochrome P-450 type hemoprotein. The pyridine hemochrome assay showed that the NOS contained a bound protoporphyrin IX heme. The reduced carbon monoxide binding spectrum shows an absorption maximum at 447 nm indicative of a cytochrome P-450 hemoprotein. A mixture of carbon monoxide and oxygen (80%/20%) potently inhibited the reaction (73–79%), showing that the heme functions directly in the oxidative conversion of L-arginine to nitric oxide and citrulline. Additionally, partially purified NOS from rat cerebellum was inhibited by CO, suggesting that this isoform may also contain a P-450-type heme. NOS is the first example of a soluble cytochrome P-450 in eukaroytes. In addition, the presence of FAD and FMN indicates that this is the first catalytically self-sufficient mammalian P-450 enzyme, containing both a reductase and a heme domain on the same polypeptide.

The formation of nitric oxide (•NO)[1] from L-arginine in mammalian cells is catalyzed by the enzyme •NO synthase (NOS; EC 1.14.23). This enzymatic activity has been associated with a number of important physiological functions including maintenance of vascular tone, neuronal signaling, and host response to infection (Ignarro, 1990; Bredt & Snyder, 1992; Marletta et al., 1990). In addition, sustained synthesis of •NO can be deleterious and may be important in the etiology of endotoxic shock (Kilbourn et al., 1990), inflammation-related tissue damage (Mulligan et al., 1991), and neuronal pathology (Bredt & Snyder, 1992). The NOSs purified to date fall into two general categories: (i) a constitutive form regulated by calcium and calmodulin and (ii) a cytokine-inducible form that is not known to be regulated posttranscriptionally (Fostermann et al., 1991).

Though the different forms of NOS vary slightly in their expression and regulation, the overall reaction catalyzed is the same, a five-electron oxidation of L-arginine to form •NO and citrulline. However, very little is known about the actual chemistry of catalysis. Both enzymatic forms contain conserved amino acid sequences for the binding of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) that show a high degree of sequence homology to NADPH–cytochrome P-450 reductase (Bredt et al., 1991; Lyons et al., 1992). As the sequence suggests, both forms of the purified NOSs contain a tightly bound FAD and FMN (Hevel et al., 1991; Mayer et al., 1991). The NOSs also require NADPH (Marletta et al., 1988; Mayer et al., 1991) and 6(*R*)-tetrahydro-L-biopterin (H_4B) (Tayeh & Marletta, 1989; Kwon et al., 1989; Mayer et al., 1991). These cofactor similarities

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[1] Abbreviations: •NO, nitric oxide; CO, carbon monoxide; NOS, nitric oxide synthase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; H_4B, 6(*R*)-tetrahydro-L-biopterin; P-450, cytochrome P-450; $P\text{-}450_{BM\text{-}3}$, cytochrome $P\text{-}450_3$ isolated from *Bacillus megaterium*.

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and the sequence homology indicate that all the NOSs purified to date appear to use a similar mechanism in the conversion of L-arginine to •NO and citrulline.

The question of the ultimate electron acceptor from NADPH remains at the mechanistic fulcrum of our investigations. We set out to answer this question by first improving the yield of our previously reported purification of the inducible form of the NOS from murine macrophages (Hevel et al., 1991). NOS isolated by this modified procedure allowed for a more in-depth investigation of the enzyme. In work presented here, we have spectrally and enzymatically characterized the inducible murine macrophage NOS as a P-450-type hemoprotein. The presence of FAD and FMN and the striking sequence homology to NADPH–cytochrome P-450 reductase indicate that NOS is the first example of a catalytically self-sufficient mammalian P-450 enzyme, containing both a reductase and a heme domain, similar to the fatty acid monooxygenase P-450$_{BM-3}$ isolated from *Bacillus megaterium* (Nahri & Fulco, 1986). In addition, preliminary evidence is presented that suggests the rat cerebellar enzyme may also be a P-450 hemoprotein.

MATERIALS AND METHODS

Materials. Glycerol (molecular biology grade), Hepes, NADPH, NADP$^+$, L-malic acid, L-arginine, myoglobin (from horse heart), calmodulin, DEAE-cellulose, and oxyhemoglobin (human A_o, ferrous) were purchased from Sigma Chemical Co. 6(*R*)-5,6,7,8-Tetrahydro-L-biopterin was purchased from Dr. B. Schircks' laboratories. The 2′,5′-ADP Sepharose 4B resin was purchased from Pharmacia-LKB Biotechnology Inc. DEAE-Bio-Gel A, AG 50W-X8, cation-exchange resin (analytical grade), Bradford protein dye reagent, and electrophoretic reagents were purchased from Bio-Rad. L-[U-^{14}C]-arginine was purchased from Amersham Corp. (specific activity = 391 mCi/mmol). Ecolume scintillation cocktail was purchased from ICN-flow. Sprague-Dawley male rat brains were purchased from BioProducts for Science, Inc.

Purification of Murine Macrophage NOS. Murine macrophage NOS was purified by a modified method of Hevel et al. (1991). Briefly, approximately 50 000 units (unit = NOS required to produce 1 nmol of •NO/h) of 100000*g* supernatant was preincubated with 2 μM H_4B and loaded onto a 2′,5′-ADP column (1 g) preequilibrated with 10 mM K_2HPO_4, 0.5 mM L-arginine, and 10% glycerol at pH 7.5 (buffer A). The column was washed with 7 mL of buffer A, followed by 30 mL of buffer A containing 0.5 M NaCl, 3 mM L-malic acid, 0.2 mM NADP$^+$, and 2 μM H_4B, and then washed again with 15 mL of buffer A containing 2 μM H_4B. NOS activity was eluted with 25 mL of buffer A containing 3 mM NADPH, 0.75 mM NADP$^+$, 15 mM NaCl, and 2 μM H_4B directly onto a 1.5-mL DEAE-Bio-Gel A column preequilibrated with 10 mM K_2HPO_4 and 10% glycerol at pH 7.5 (buffer B). The DEAE-Bio-Gel A column was washed successively with 15 mL of buffer B containing 2 μM H_4B and 5 mL of buffer B containing 80 mM NaCl and 2 μM H_4B. Activity was eluted with 25 mL of buffer B containing 120 mM NaCl and 2 μM H_4B and then concentrated by ultrafiltration. Protein concentration was determined using the Bradford microassay with bovine serum albumin as a standard. NOS activity was determined as previously described by Olken et al. (1991). NOS purified in this manner was judged 98% pure by SDS–PAGE stained with Coomassie Blue R-250.

Partial Purification of Rat Cerebellar NOS. Partial purification of the rat cerebellar NOS was performed as previously described (Bredt & Snyder, 1990) with the following modifications. Thirty cerebella were homogenized in 200 mL of ice-cold buffer containing 10 mM K_2HPO_4 and 10% glycerol, pH 7.5 (buffer C). The homogenate was centrifuged at 20000*g* for 15 min at 4 °C. The supernatant was then loaded onto a 20-mL DEAE-cellulose column equilibrated with buffer C. The column was washed with 50 mL of buffer C containing 2 μM H_4B. Activity was eluted with a 100-mL linear gradient from 0 to 400 mM NaCl in buffer C containing 2 μM H_4B. NOS activity was determined as described above with the addition of 1 μg/mL calmodulin and 1 mM $CaCl_2$.

Spectral Characterization of Heme. An absolute absorbance spectrum of purified murine macrophage NOS was recorded on a Hewlett-Packard 8452A UV/visible spectrophotometer from 350 to 600 nm. To eliminate any interference from the unbound H_4B and oxidation products derived from H_4B, the buffer of the sample was exchanged via ultrafiltration at 4 °C three times with 45 mM Hepes (pH 7.5) containing 10% glycerol. Reduced CO binding difference spectra of NOS were obtained in the following manner. A sample of NOS was first bubbled gently with CO for 30 s, the spectrum was recorded from 350 to 600 nm, followed by reduction of the sample with sodium dithionite, and then the spectrum was rerecorded.

Heme Content Determination. Heme content was determined by a modification (Pernecky, 1988) of methods previously described by Fuhrhop and Smith (1975). Briefly, a 366-μL sample of NOS in 10 mM K_2HPO_4 (pH 7.4) was transferred to a cuvette to which 130 μL of pyridine and 3.5 μL of 10 N NaOH were added. The sample was bubbled for 4 min with argon and reduced with sodium dithionite, the spectrum was recorded from 500 to 600 nm, and the absorbance difference between 540 and 556 nm was determined. Concentration of heme was calculated using myoglobin as a standard.

Inhibition of Citrulline Formation by CO. The involvement of the heme in catalytic turnover was examined by following the inhibition of the conversion of [^{14}C]-L-arginine to [^{14}C]-citrulline in the presence of CO by using a modifiction of a previously described method (Bredt & Snyder, 1989). The samples (450 μL) contained 100 μM L-arginine (specific activity = 3.3 μCi/μmol), 20 μM H_4B, 40–60 μL (2.5–4 μg) of purified murine macrophage NOS, and 200 mM Hepes (pH 7.5). Reaction vials were then sealed with septa and equilibrated with either 80% CO/20% O_2 or ambient air (control) for 7 min at room temperature. The reaction was initiated with 100 μM NADPH, incubated for 5 min at 37 °C, and terminated with TCA (15 μL of 6 N). An aliquot (300 μL) of each reaction was applied to a 1-mL AG 50W-X8 cation-exchange column (sodium form) and washed with 3 mL of water directly into 15 mL of Ecolume scintillant. Each sample was then counted and corrected for the small amount of cirtulline formed (1.2%) in the absence of NADPH. Similar experiments were carried out using a partially purified preparation of NOS from the rat cerebellum. The peak fraction of the DEAE-cellulose column was used (50 μL of partially purified NOS/sample), with the addition of 1 μg/mL calmodulin and 1 mM $CaCl_2$ to each reaction mixture.

RESULTS

Spectral Characterization. Murine macrophage NOS gave the visible spectrum shown in Figure 1, which displays a rather broad peak with a λ_{max} at 406 nm. From the estimated protein concentration of the sample it was concluded that the absorbance was derived from a moiety with a relatively large extinction coefficient. The presumed extinction coefficient

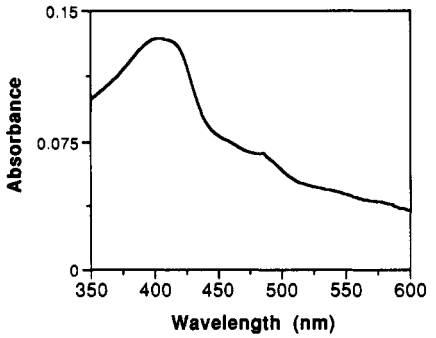

FIGURE 1: Absolute absorbance spectrum of murine macrophage NOS. An absolute absorbance spectrum of purified murine macrophage NOS was recorded on a Hewlett-Packard 8452A UV/visible spectrophotometer from 350 to 600 nm. To eliminate any interference from the unbound H_4B and oxidation products derived from H_4B, the buffer of the sample was exchanged three times with 45 mM Hepes (pH 7.5) containing 10% glycerol via ultrafiltration at 4 °C.

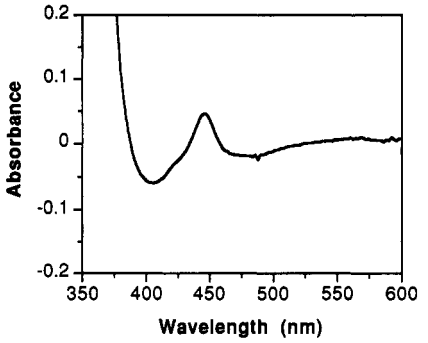

FIGURE 2: Reduced CO binding difference spectrum of murine macrophage NOS. The sample was prepared as described in Figure 1. The spectrum was obtained by first bubbling a sample of NOS gently with CO for 30 s, recording the spectrum from 350 to 600 nm, followed by reduction of the sample with sodium dithionite, and then recording the spectrum. Shown is the difference spectrum after dithionite reduction.

coupled with the observed λ_{max} suggested the presence of a heme. The putative Soret peak shape implies that the NOS was isolated in a mixture of high- and low-spin states (Jefcoate, 1978). The coalescence of a high-spin peak with a typical λ_{max} at 389 nm and a low-spin peak with a typical λ_{max} at 416 nm would produce a peak as observed, with an apparent λ_{max} at 406 nm (Peterson, 1971). An unusual feature of the NOS spectrum is the prominent shoulder in the 450–475-nm region. This shoulder has been previously observed in P-450_{BM-3} isolated from *B. megaterium* (Nahri & Fulco, 1986) and was attributed by Narhi and Fulco to the presence of FAD and FMN. Since NOS also contains both one FAD and one FMN per 130-kDa subunit (Hevel et al., 1991), it can be postulated that the presence of the shoulder in the NOS spectrum is also related to a similar flavin–heme interaction. The dithionite-reduced CO difference spectrum (Figure 2) confirms the presence of the heme and defines NOS as a P-450-type hemoprotein. The resulting peak has an absorbance maximum of 447 nm, and a corresponding broad trough is formed with a minimum at 406 nm. Using the reduced CO difference spectrum (Figure 2), the concentration of holoenzyme used in the spectral characterization was estimated. This was accomplished by calculating the absorbance difference between 447 and 490 nm and converting the absorbance change to cytochrome P-450 concentration using an extinction coefficient of 91 000 M^{-1} cm^{-1} (Omura & Sato, 1964). The concentration of cytochrome P-450 was determined to be 0.69 μM.

Table I: Inhibition of NOS Citrulline Formation by CO

NOS source	expt	nmol of citrulline/h[a] control[b]	nmol of citrulline/h[a] CO/O_2[b]	% inhibition
macrophage	1	48.2	11.5	76
	2	48.7	10.9	78
	3	51.3	10.7	79
	4	46.9	12.8	73
cerebellum	1	8.0	3.0	62
	2	9.4	3.0	68

[a] Assays were performed in 200 mM Hepes (pH 7.5) as described under Materials and Methods and contained either 2.5–4.0 μg of purified macrophage NOS or 50 μL of the partially purified rat cerebellar NOS. [b] Control samples were ambient air, and CO/O_2 samples were equilibrated with a mixture of 80% CO/20% O_2 as described under Materials and Methods.

Heme Content. The specific heme content of the macrophage NOS was determined using the reduced pyridine hemochrome assay as described under Materials and Methods, and the content ranged from 3.3 to 6.5 nmol of heme/mg of NOS (theoretical value = 7.69 nmol of heme/mg of protein). This specific content was similar to that of other P-450-type hemoproteins after purification (Guengerich, 1979) and indicates that some of the NOS could have been isolated as the apoenzyme. The porphyrin identified in the NOS is a protoporphyrin IX by comparison to myoglobin in the hemochrome assay. The hemochromes for both NOS and myoglobin show absorbance maxima at 540 and 556 nm, which is characteristic for a protoporphyrin IX in this assay (Fuhrhop & Smith, 1975).

CO Inhibition of Citrulline Formation. CO inhibition of Fe^{II}–heme catalysis has been previously demonstrated as a signature of P-450-catalyzed reactions (Estabrook et al., 1963; Jefcoate, 1978). Therefore, CO inhibition of [^{14}C]citrulline production from [^{14}C]-L-arginine by NOS was measured. The data shown in Table I demonstrate that, in the presence of 80% CO/20% O_2, the formation of [^{14}C]citrulline by murine macrophage NOS was significantly inhibited compared to that of the ambient air control. The percent inhibition of NOS from two independent macrophage purifications assayed in duplicate ranged from 73% to 79%. The turnover numbers calculated from the macrophage data in Table I ranged from 41 to 68 min^{-1} for the controls. However, the specific activity of independent purification varies (Hevel & Marletta, 1992). This could be attributed to the variable heme content noted above. Consequently, the results in Table I are expressed as nanomoles of citrulline per hour for better comparison. A partially purified preparation of the rat cerebellar NOS showed a similar level of inhibition over the control (62–68%), suggesting that this form of the enzyme also involves a heme in catalysis.

DISCUSSION

The data presented establish murine macrophage NOS as a unique member of the P-450 hemoproteins. Like other P-450 hemoproteins, NOS has an absolute absorbance (Figure 1) maximum in the low 400-nm region (λ_{max} = 406), contains a bound protoporphyrin IX heme, and shows a reduced CO difference spectrum (Figure 2) with the characteristic 450-nm peak centered at 447 nm. In addition, the reaction catalyzed by NOS is inhibited by CO, a hallmark of P-450 reactions. However, the macrophage NOS displays some novel characteristics. Similar to P-450_{BM-3}, the absolute absorbance spectrum contains a shoulder in the 450–475-nm region (Figure 1). As mentioned above, Nahri and Fulco (1986)

have attributed this shoulder in P-450$_{BM-3}$ to the interaction of the heme with the flavins bound to the protein. The shoulder observed in the NOS spectrum (Figure 1) may also be due to a similar heme/flavin interaction since macrophage NOS possesses one FAD and FMN as well as a heme per 130-kDa monomer (Hevel et al., 1991). The presence of the flavins and heme on the same polypeptide in NOS differs dramatically from the other P-450 systems characterized. In order to observe catalytic activity, the membrane-associated inducible mammalian P-450s must typically be reconstituted with lipid in the presence of an electron shuttling protein, NADPH–cytochrome P-450 reductase, which acts as a conduit for electrons from NADPH to the ferric heme of P-450. With the flavin prosthetic groups and the sequence homology to NADPH–cytochrome P-450 reductase (Lyons et al., 1992), NOS appears uniquely capable to act as a self-sufficient P-450. In addition, NOS is the first example of a mammalian P-450 that is cytosolic.

The apparent catalytic self-sufficiency of NOS suggests that reducing equivalents from the reduced nucleotide could be shuttled through the flavins to the oxidative active site. Furthermore, the reaction sequence involves hydroxylation of L-arginine to N^G-hydroxy-L-arginine (Pufahl et al., 1992; Stuehr et al., 1991). Hydroxylation reactions are characteristic of P-450-type hemoproteins, lending further support to the postulate that the heme present in NOS is involved in the mechanism of conversion of L-arginine to citrulline and •NO.

Indeed, the CO inhibition studies with macrophage NOS (Table I) clearly show that the heme functions directly in the conversion of L-arginine to •NO and citrulline. Preliminary results with partially purified rat cerebellar NOS suggest the presence of a heme functioning in an analogous fashion. With evidence for the catalytic involvement of the heme in citrulline formation, a P-450-type mechanism could be hypothesized for the formation of N^G-hydroxy-L-arginine. As illustrated in Figure 3, reducing equivalents from NADPH could be shuttled directly through the flavins in the reductase domain to the heme center. The resulting perferryl Fe–heme complex ([FeO]$^{3+}$) could catalyze the formation of N^G-hydroxy-L-arginine. We have recently demonstrated that NOS also contains tightly bound H_4B. Purified macrophage NOS of highest activity results when the H_4B:130-kDa ratio is 1:1 (Hevel & Marletta, 1992). H_4B in concert with a non-heme iron is typically involved in hydroxylation reactions, such as those catalyzed by phenylalanine hydroxylase (Dix et al., 1987; Gottschall et al., 1982; Kuhn et al., 1980), and therefore, the involvement of the H_4B in this hydroxylation step cannot be excluded.

Nothing is known about the course of the reaction after the N-hydroxylation step. Heavy isotope studies (Kwon et al., 1990; Leone et al., 1991) show that the oxygens of •NO and citrulline have both been derived from O_2. There are two reasonable mechanisms to explain these isotope results. The first involves two separate hydroxylation steps, one hydroxylation utilizing the heme and the other involving the pterin. This sequence would still require a single electron oxidation step to form •NO. The second possibility is related to the reactions catalyzed by the enzymes aromatase and lanosterol 14-demethylase. These P-450 enzymes utilize 3 equiv each of NADPH and O_2. The first 2 equiv are used to carry out successive hydroxylations on a methyl group to yield an aldehyde intermediate via classical P-450 chemistry described earlier for the formation of N^G-hydroxy-L-arginine. Considerable debate has been focused on the third step of the

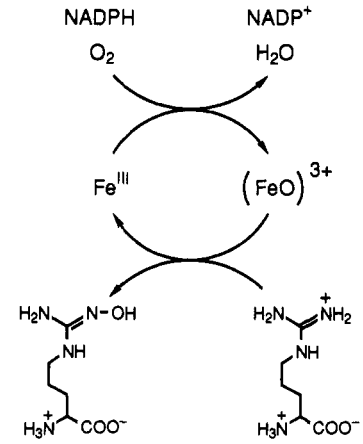

FIGURE 3: Hydroxylation of L-arginine by a P-450-type reaction.

reaction that leads to the final products. A proposal originally suggested by Akhtar and co-workers (1981) and more recently supported by enzymatic studies carried out by Vaz et al. (1991) involves the third equivalent of NADPH and O_2 to generate an Fe^{III} peroxide anion species which acts as a nucleophile in the reaction. Additional model chemistry by Cole and Robinson (1991) has further substantiated this mechanism. An analogous reaction sequence for the NOS would involve one hydroxylation followed by the Fe^{III} peroxide attack on N^G-hydroxy-L-arginine. This mechanism still requires a single electron oxidation step to yield •NO and citrulline. The heme could also play a key role in the one-electron oxidation required during the course of the reaction. For example, hydroxylamines and oximes are known to reduce ferric iron. Consequently, N^G-hydroxy-L-arginine, shown as its more stable oxime tautomer (Clement & Kampchen, 1985) (Figure 3), might reduce the Fe^{III} heme directly, thereby generating the odd-electron species that is ultimately required.

The identification of NOS as a P-450 hemoprotein has far-reaching implications. The presence of FAD and FMN substantiates this as the first example of a catalytically self-sufficient mammalian P-450 enzyme, containing both a reductase and a heme domain, similar to the fatty acid monooxygenase P-450$_{BM-3}$ (Nahri & Fulco, 1986). As such, NOS will provide a mammalian P-450 model to study the unique interaction between the reductase and the heme domains, both structurally and in the electron transport reaction. Ongoing studies continue to unravel the mechanistic function of each of the cofactors in the reaction catalyzed by NOS.

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