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Cloned, expressed rat cerebellar nitric oxide synthase contains stoichiometric amounts of heme, which binds carbon monoxide

(cytochrome P-450/NADPH–cytochrome P-450 oxidoreductase/oxygenation/arginine/citrulline)

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ABSTRACT **The endogenous formation of nitric oxide (NO) has become an area of intense interest as evidence for its biological functions has been obtained in three distinct tissues: circulating macrophages, in which it exerts cytotoxic effects; blood vessels, in which it has been identified as endothelium-derived relaxing factor; and neuronal cells, in which it functions as a neurotransmitter. The formation of NO in brain extracts has been shown to be catalyzed by an enzyme, termed NO synthase, which generates the NO responsible for stimulation of cGMP formation, the highest levels of which occur in the cerebellum. NO synthase catalyzes the formation of citrulline from arginine with the coincident production of NO and has been shown to be a flavoprotein, containing 1 mol each of FAD and FMN, tetrahydrobiopterin, and iron. It is also reported to contain an α-helical, calmodulin-binding consensus sequence consistent with its stimulation by calmodulin in the presence of Ca^{2+}. The formation of NO requires incorporation of one of the atoms of molecular oxygen into one of the guanidinium nitrogen atoms of arginine with the coincident formation of citrulline. This communication reports that rat cerebellar NO synthase, cloned and stably expressed in human kidney 293 cells, contains heme in amounts stoichiometric with the flavins FAD and FMN as evidenced by the appearance of a pyridine hemochrome and a reduced CO difference spectrum with an absorbance maximum at ≈445 nm. The finding of a CO-binding heme moiety explains the presence of iron in the enzyme and suggests a role for prosthetic heme as an oxygenase reaction center. This report also presents evidence for incorporation of δ-[^{14}C]aminolevulinate specifically into immunoprecipitable NO synthase in stably transfected human kidney 293 cells but not in nontransfected cells. Simultaneously, K. A. White and M. A. Marletta [(1992) *Biochemistry* 31, 6627–6631] have demonstrated a CO-binding heme prosthetic group in purified murine macrophage NO synthase and have suggested the identity of these reaction centers in both the constitutive (cerebellar) and inducible (macrophage) forms of NO synthase.**

Nitric oxide (NO) appears to fulfill multiple roles depending on the tissue in which it is formed. It has been identified as the endothelium-derived relaxing factor, a labile substance formed by endothelial cells, which mediates dilation of blood vessels (1–3). In addition to being involved in cytotoxic events in macrophages and neutrophils (4), NO also has been implicated in signal transduction events both in circulating macrophages (5) and in glutamate-mediated excitation of cGMP formation in the cerebellum, coincident with the formation of citrulline and NO from arginine (6). The availability of pure NO synthase permitted production of antisera for immunohistochemical localization (7) and molecular cloning has permitted localization by *in situ* hybridization (8). Interestingly, antiserum to the brain NO synthase crossreacts with both brain and endothelial forms but does not recognize the macrophage NO synthase. The macrophage enzyme is induced by immunological stimuli, is Ca^{2+} independent, and contributes to the cytotoxic activity of macrophages against tumor cells and microorganisms (9, 10). The constitutive, Ca^{2+}/calmodulin-dependent NO synthases are found in vascular endothelium (1, 11), brain (6–8, 12), and adrenal gland (13) and similar, but Ca^{2+} independent, activities are found in activated macrophages (14), Kupffer cells (15), hepatocytes (16, 17), and neutrophils (18, 19).

Purification and characterization of cerebellar NO synthase has been hampered by its low abundance in neural tissue, but recent success in producing large amounts of enzyme from suspension cultures of human kidney 293 cells stably transfected with NO synthase cDNA (8) has permitted spectral determination of a CO-binding heme prosthetic group on this enzyme. The existence of a heme moiety is indicated by formation of pyridine hemochromogen as well as a reduced CO-binding species, the molar contents of which are stoichiometric with the flavins FAD and FMN. The incorporation of δ-[^{14}C]aminolevulinate (δ-ALA) into NO synthase purified from stably transfected human kidney 293 cells, but not into nontransfected cells, also demonstrated the formation of heme bound to the enzyme. This finding illustrates that flavin- and heme-containing moieties exist in a single protein in mammalian tissues, comparable to that of the *Escherichia coli* and *Salmonella typhimurium* sulfite reductases (20, 21) and *Bacillus megaterium* BM-3 fatty acid hydroxylase (22, 23). It is suggested that this heme prosthetic group acts as a molecular oxygen binding and oxygenation center of NO synthase. A preliminary report of these data has been presented (ref. 24; B.S.S.M., unpublished observations).

MATERIALS AND METHODS

Materials. L-[2,3-^{3}H]Arginine, L-[*ureido*-^{14}C]citrulline, and [4-^{14}C]-δ-ALA were obtained from DuPont/NEN, and (6*R*)-5,6,7,8-tetrahydro-L-biopterin was obtained from Research Biochemicals (Natick, MA). All other chemicals were research grade.

Cell Culture and Enzyme Purification. Rat brain NO synthase was overexpressed in stably transfected human kidney 293 cells (8) utilizing a neomycin-resistant (neo^{r}) factor to permit drug selection with G418. Suspension cultures were grown in roller bottles containing 500 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum/100 units of penicillin per ml, 100 μg of streptomycin per ml, and 10 mM Hepes (pH 7.2) at 37°C. Cells were

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Abbreviations: δ-ALA, δ-aminolevulinate; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol.

harvested from 6–8 liters of 7- to 8-day cultures (>90% viability) by centrifugation and washed twice with phosphate-buffered saline containing 1 mM EDTA (pH 7.3). Between 8 and 12 g (wet weight) of cells was obtained from these cultures.

NO synthase was purified by a modification of the method of Bredt and Snyder (12). The washed cells were homogenized in 3 vol of buffer A [50 mM Tris·HCl/1 mM EDTA/0.1 mM phenylmethylsulfonyl fluoride (PMSF)/0.1 mM dithiothreitol (DTT)/0.5 μM leupeptin/0.5 μM pepstatin/10 μg of antipain per ml, pH 7.5] using a Dounce homogenizer. The homogenate was centrifuged for 30 min at 100,000 × g at 4°C. The cytosolic extract was purified by a biospecific affinity-chromatography method developed for NADPH–cytochrome P-450 oxidoreductase by Yasukochi and Masters (25). The extract was applied to a 2-ml column of 2′,5′-ADP-Sepharose 4B equilibrated with buffer B [10 mM Tris·HCl/10% (vol/vol) glycerol/0.1 mM EDTA/0.1 mM PMSF/0.1 mM DTT/0.5 μM leupeptin/0.5 μM pepstatin, pH 7.5]. The affinity medium was washed with 10 vol of buffer B, 5 vol each of 0.5 M NaCl in buffer B, 1 mM 2′-AMP in buffer B, and buffer B. The enzyme was eluted with 5 ml of 10 mM NADPH in buffer B. Fractions of 1 ml were collected and pooled on the basis of cytochrome *c* reductase activity or absorbance at 400 nm. The pooled fractions were concentrated to <1 ml by ultrafiltration using an Amicon YM-30 membrane. The concentrated 2′,5′-ADP-Sepharose fraction was chromatographed on a Sephacryl 200HR column (0.9 × 50 cm) equilibrated with buffer C (50 mM Tris·HCl/10% glycerol/0.1 M NaCl/0.1 mM EDTA/0.1 mM PMSF/0.1 mM DTT/0.5 μM leupeptin/0.5 μM pepstatin, pH 7.5). Fractions of 1 ml were collected and frozen as aliquots at −80°C.

Determination of Citrulline Formation and Cytochrome *c* Reductase Activity. NO synthase activity was assayed by measuring the stoichiometric formation of L-[^{3}H]citrulline from L-[^{3}H]arginine (12). The reaction mixtures contained 20 mM NaHepes (pH 7.5), 0.1 mM EDTA, 0.1 mM DTT, 25 μM L-arginine, 10 μg of calmodulin per ml, 2 mM $CaCl_2$, 10 μM tetrahydrobiopterin, 100 μg of bovine serum albumin per ml, and 500,000 cpm of L-[2,3-^{3}H]arginine in a 200-μl vol. Reactions were started by the addition of NADPH to a final concentration of 50 μM and mixtures were incubated at 25°C. Three 20-μl aliquots were withdrawn at 2-min intervals and the reaction was stopped by dilution with 80 μl of ice-cold 0.1 M Hepes/5 mM EGTA, pH 5.5. The samples were heated for 1 min in a boiling water bath and stored at −20°C until analyzed by ion-exchange HPLC. Separation of citrulline and residual arginine was obtained with a Mono S column (Pharmacia) by a modification of the method of Chenais *et al.* (26). Radioactivity was monitored by a Beckman model 171 flow-through detector, which was connected on-line with a Beckman System Gold HPLC.

NADPH–cytochrome *c* oxidoreductase activity (27) was assayed at 25°C with a Beckman DU 7400 diode array spectrophotometer with a Peltier temperature control accessory. Reaction mixtures contained 1–2 μg of protein, 50 μM cytochrome *c*, 50 mM NaP_i, 0.1 mM EDTA (pH 7.5) in a final vol of 1.0 ml. Reactions were started by addition of NADPH to a final concentration of 50 μM and were monitored at 550 nm ($\varepsilon = 0.021\ \mu M^{-1}$) for 1 min. Protein was estimated by the Bradford micromethod using human serum albumin as a standard (28).

Determination of Flavin and Heme Content. The flavin content of purified enzyme fractions was determined fluorometrically (29) with FAD and FMN purified by reverse-phase HPLC used as standards (30). Measurement of heme content was by pyridine hemochromogen (31) and reduced CO-binding spectra (32). Spectral analyses were performed with a Beckman DU 7400 diode array spectrophotometer and 100-μl microcuvettes. All spectra were normalized to 0 absorbance at 700 nm by Sigma Plot software.

Incorporation of [^{14}C]-δ-ALA into NO Synthase. Incorporation of metabolically labeled heme (33) into NO synthase was obtained by growth of stably transfected kidney 293 cells in the presence of [^{14}C]-δ-ALA. To confluent 150-cm^2 culture flasks containing 10 ml of 10% fetal bovine serum/DMEM, 0.625 μCi (1 Ci = 37 GBq) of [^{14}C]-δ-ALA was added. After 24 hr, the cells were harvested and homogenized. NO synthase was partially purified by 2′,5′-ADP-Sepharose chromatography. Recovery of radiolabeled NO synthase from the 2′,5′-ADP-Sepharose 4B fraction was by immunoprecipitation using rabbit anti-rat brain NO synthase antiserum in the presence of bovine serum albumin (5 mg/ml). Protein A-Sepharose was then added to bind the NO synthase–antibody complex. After 2 hr, the samples were centrifuged and radioactivity remaining in the supernatant was measured by liquid scintillation spectrometry. Appropriate control experiments were performed with preimmune serum and non-transfected kidney 293 cells.

RESULTS

For purification of cloned rat brain NO synthase from human kidney 293 cells, up to 1.5 g (wet weight) of cells per liter was obtained using the described culture conditions. Table 1 shows a typical purification from 10 g of cells, which yielded ≈1.2 mg of purified NO synthase in the peak fraction from Sephacryl 200HR chromatography. The total yield from gel filtration was generally 3–4 mg of NO synthase. Activities shown in Table 1 were obtained by measuring formation of [^{3}H]citrulline from [^{3}H]arginine. Values obtained for the NADPH-dependent reduction of cytochrome *c* were ≈3-fold higher. Cytochrome *c* reduction is a convenient assay for following NO synthase purification.

Enzyme bound to 2′,5′-ADP-Sepharose was found not to be eluted by up to 10 mM 2′-AMP, the specific nucleotide used to elute NADPH–cytochrome P-450 reductase, perhaps due to binding of both the nicotinamide and adenine moieties of pyridine nucleotides by the enzyme. Only partial recovery (<50%) was obtained by elution with 10 mM $NADP^+$, in comparison with complete elution by 10 mM NADPH, demonstrating a significant difference in relative ligand affinities. The addition of a wash step with 2′-AMP before elution by NADPH was found to result in further purification by 2′,5′-ADP-Sepharose chromatography. The relatively low recovery of NO synthase after affinity chromatography is attributable to loss during concentration by ultrafiltration.

Gel-filtration chromatography of the concentrated 2′,5′-ADP-Sepharose fraction produced a homogeneous product in high yield. The purified enzyme migrated as a single band (≈160 kDa) during SDS/PAGE (Fig. 1*A*, lanes 3–5) and exhibited immunoreactivity toward anti-rat brain NO synthase antiserum (Fig. 1*B*).

The described purification scheme can be completed in 8 hr. Rapid purification and inclusion of 10% glycerol in the

Table 1. Representative purification of cloned rat brain NO synthase from kidney 293 cells

	Total activity, nmol·min^{-1}	Specific activity, nmol·min^{-1}·mg^{-1}	Yield, %
Cytosolic extract	2350	5	100
2′,5′-ADP-Sepharose pool	350	48	15
Sephacryl 200HR peak fraction	270	230	11

NO synthase activity was measured as formation of [^{3}H]citrulline from [^{3}H]arginine as described.

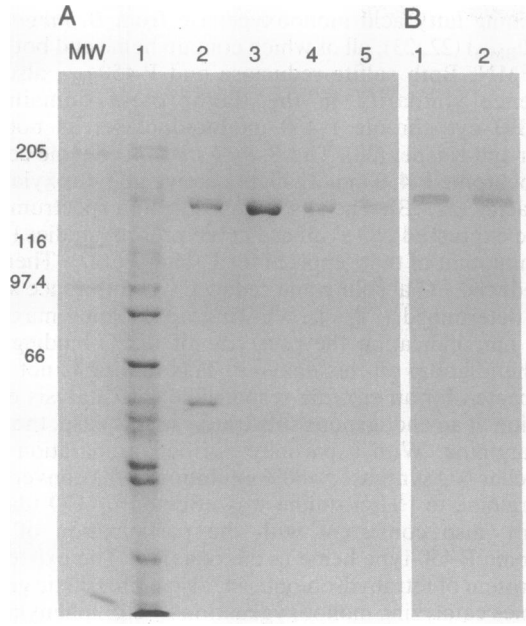

FIG. 1. SDS/PAGE and Western blot analysis of NO synthase during purification. (*A*) Coomassie G-250-stained 7.5% polyacrylamide gel. Lanes: 1, 50 μg of cytosolic extract; 2, 5 μg of concentrated 2′,5′-ADP Sepharose fraction; 3–5, 5, 2, and 1 μg of Sephacryl 200HR peak fraction (sample used in Figs. 3 and 4). (*B*) Western blot analysis was performed on samples transferred to nitrocellulose by an electroblot procedure, using rabbit anti-NO synthase and goat alkaline phosphatase-linked anti-rabbit IgG. Lanes: 1, 1 μg of Sephacryl 200HR peak fraction; 2, 50 μg of cytosolic extract. MW, size markers (kDa).

purification buffers enhance enzyme stability, with minimal loss of activity over 1 week at −80°C.

The ability to purify sufficient quantities of cerebellar NO synthase (see above) has made it possible to examine the spectral properties of the enzyme. As shown in Fig. 2, the absorbance maximum is at 400 nm with a shoulder between 450 and 475 nm. This is not characteristic of the exclusive presence of flavin chromophores, which display an absorbance maximum at 450 nm. Enzyme purified by either 2′,5′-ADP-Sepharose/calmodulin agarose chromatography (33) or the 2′,5′-ADP-Sepharose/gel filtration method described here displayed spectra identical to those shown. The wavelength absorbance maximum at 400 nm is typical of a Soret band for a high-spin heme prosthetic group. This, in conjunction with the reported measurement of stoichiometric iron (34), prompted the determination of heme content.

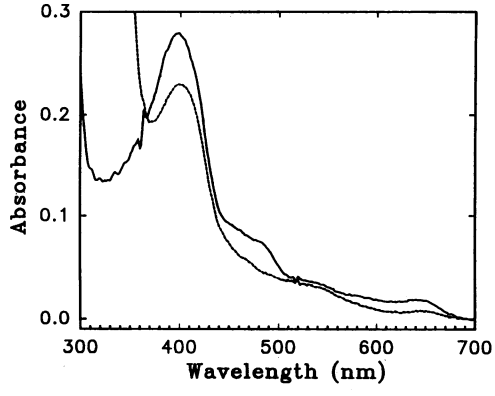

FIG. 2. Absorbance spectrum of NO synthase. Sample of NO synthase obtained from a representative fraction purified by Sephacryl 200HR chromatography as isolated (solid line). Reduction with a single grain of dithionite 2 min after addition (dashed line).

Measurement by us of total heme content of cerebellar NO synthase has repeatedly demonstrated the presence of heme in stoichiometric amounts with FAD and FMN. Total heme was determined by the formation of the pyridine hemochrome after alkali denaturation of NO synthase. As shown in Fig. 3, the pyridine hemochrome, with an absorbance maximum at 556 nm, gave a calculated heme concentration of 3.2 μM, which was equal to the FAD and FMN concentrations of 3.2 μM each.

NO synthase displays reduced CO difference spectra typical of cytochrome P-450 (32), with a wavelength absorbance maximum at 443–445 nm and with a minimum at 400 nm (Fig. 4*B*). The difference spectrum has a trough at 475 nm, attributable to the flavins. Also shown in Fig. 4*A*, secondary wavelength absorbance maxima typical of a high-spin heme were at 650 and 550 nm in the absolute CO and reduced CO spectra, respectively. The calculated heme content, using a $\Delta\varepsilon_{450-490}$ of 0.091 μM^{-1} was 3.1 μM, which is in agreement with the pyridine hemochromogen measurement (see above). However, with fractions eluting after the peak fraction, values for the heme concentration obtained from the pyridine hemochrome were as much as twice those calculated from reduced CO-binding spectra. Reduced CO binding is dependent on the native coordination state of the heme moiety within the protein, while the pyridine hemochrome measures total heme. The peak fractions were also characterized by an A_{280}/A_{400} ratio of 3.0 ± 0.1, while later-eluting fractions generally exhibited substantially higher values.

The heme moiety of certain hemoprotein enzymes can be metabolically labeled by δ-ALA (35). Incorporation of metabolically labeled heme into NO synthase (expressed and partially purified; see *Materials and Methods*) was demonstrated by a 20-fold higher recovery of radioactivity in the immunoprecipitated NO synthase in comparison with nontransfected control cells (data not shown). SDS/PAGE of the affinity product revealed a single prominent band of ≈160 kDa, corresponding to NO synthase (data not shown). Immunoprecipitation with anti-rat brain NO synthase antiserum and protein A-Sepharose resulted in 91% recovery of radioactivity from extracts of transfected cells, while no radioactivity was recovered using preimmune serum. These results, while not verifying the stoichiometry, further demonstrate the presence of a heme moiety in rat brain NO synthase.

DISCUSSION

NO synthase activity involves distinct enzyme proteins in brain, endothelium, and macrophages. Enzymes catalyzing

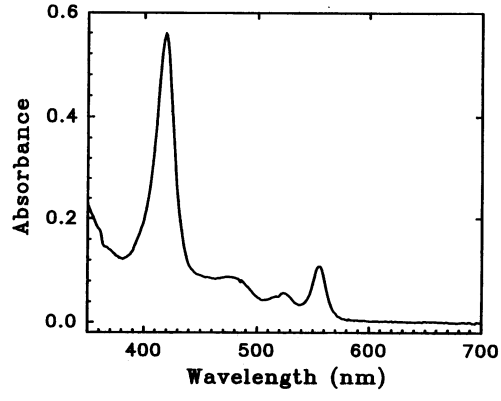

FIG. 3. Determination of heme content of purified NO synthase. Spectrum of pyridine hemochromogen performed with a 70-μl aliquot of NO synthase by addition of 2 μl of 5 M KOH and 30 μl of pyridine, followed by reduction with a single grain of dithionite. Maximal absorbance was obtained after 2 min. The chromophore was estimated to be 3.2 μM (ε_{556} = 0.034 mM^{-1}). Flavin concentrations were determined to be 3.2 μM each for FAD and FMN.

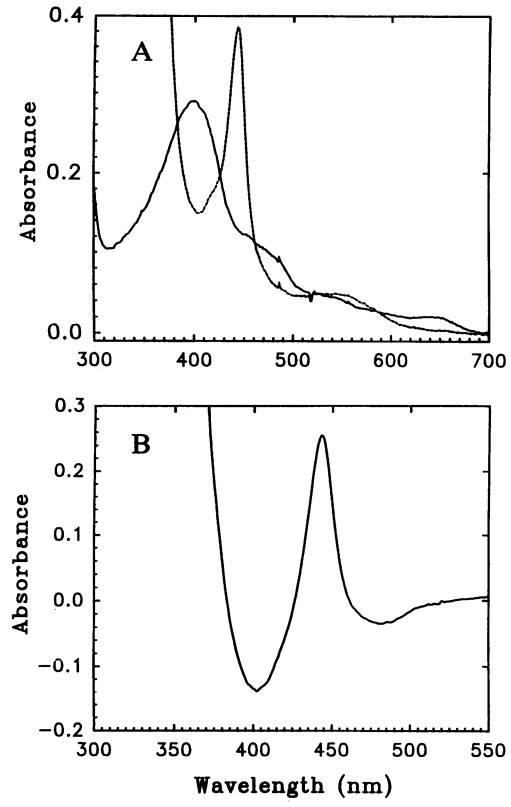

FIG. 4. Binding of CO to reduced NO synthase. (*A*) Absolute spectra. The sample was bubbled with CO for 30 sec and the spectrum was recorded (solid line) and then reduced with a single grain of dithionite (dashed line). Maximal absorbance at 443 nm was obtained after 2 min. (*B*) Reduced CO difference spectrum obtained by normalization of spectra in *A* to 0 absorbance at 700 nm and then subtraction of these normalized spectra. The chromophore was estimated to be 3.1 μM ($\Delta\varepsilon_{450-490}$ = 0.091 μM^{-1}). Flavin concentrations were determined to be 3.2 μM each for FAD and FMN.

the formation of NO in these various tissues bear strong resemblance to one another but also are differentially controlled, necessitating some differences in primary structure and prosthetic group/cofactor constituency. This manuscript describes the purification and characterization of rat cerebellar NO synthase from human kidney 293 cells stably transfected with NO synthase cDNA. Since the report of Bredt *et al.* (8) that the cloned rat brain NO synthase demonstrates 36% sequence identity and 58% homology in its 641 C-terminal residues with NADPH–cytochrome P-450 reductase, intense activity has revolved around further characterization of its structure and prosthetic group constituency. Cerebellar NO synthase is reported to contain 1 mol each of FAD and FMN (36, 37), as confirmed in our laboratory. In addition, the improvement in the expression/purification protocol reported here has permitted our laboratories to prepare sufficient quantities of NO synthase to determine its spectral properties. Examination of the optical absorbance spectrum of purified NO synthase revealed a broad, predominant peak at 390–400 nm, indicative of a high-spin heme (Fig. 2). This observation led to additional experiments in which this possibility was confirmed. The determination of a pyridine hemochromogen in more than a dozen preparations of NO synthase and the formation of a reduced CO difference spectrum provided further evidence of the presence of heme in this enzyme.

Indeed, the spectral properties along with the presence of FAD and FMN in NO synthase reminded us of the bacterial enzymes, the NADPH–sulfite reductases from *S. typhimurium* (21) and *E. coli* (20, 21) and the cytochrome P-450-containing fatty acid monooxygenase from *B. megaterium* ($P\text{-}450_{BM\text{-}3}$) (22, 23), all of which contain heme and both FAD and FMN. Both sulfite reductase and $P\text{-}450_{BM\text{-}3}$ also have sequence similarity in the flavoprotein domain with NADPH–cytochrome P-450 oxidoreductase, as noted by Porter and Kasper (38). The *B. megaterium* enzyme contains a cytochrome P-450 moiety highly active in hydroxylation of fatty acids (22, 23). The absolute absorption spectrum of the cloned expressed NO synthase in the present studies (Fig. 2) is reminiscent of that reported for $P\text{-}450_{BM\text{-}3}$ (22). Therefore, the reduced CO absolute and reduced CO difference spectra were determined (Fig. 4), showing absorbance maxima at ≈445 nm, indicating the presence of a CO-binding heme prosthetic group on this enzyme. This finding is not totally unexpected for an enzyme responsible for catalysis of oxygenation of an endogenous substrate—in this case, the amino acid arginine. With a partially purified preparation of rat cerebellar NO synthase, >80% inhibition of the conversion of [^{3}H]arginine to [^{3}H]citrulline was effected by CO (data not shown), also consistent with the participation of a cytochrome P-450-type heme in the reaction. The existence in this protein of tetrahydrobiopterin, also a prosthetic group in enzymes catalyzing monooxygenations—e.g., phenylalanine hydroxylase (39)—did not necessitate the search for an alternative oxygenation reaction center. Although others had searched for sequence similarities to cytochromes P-450, particularly for the putative heme-binding cysteinyl peptide, various algorithms did not reveal obvious sequence homologies. However, examination of the sequences of three different NO synthases from three tissue sources (8, 40, 41) reveals the following sequence similarities: rat brain NO synthase (8), RNASRC^{414}VGRIQW; murine macrophage NO synthase (39), RNAPRC^{194}IGRIQW; bovine endothelial NO synthase (40), RNAPRC^{186}VGRIQW. One can only speculate at this time whether these homologous sequences represent the heme-binding peptides in these various NO synthases, but we are now in a position to perform experiments that will permit determination, at the very least, of the role of cysteine^{414} in NO synthase-catalyzed reactions. The position of this cysteine residue in rat cerebellar NO synthase is somewhat displaced, probably because of the larger size of this enzyme (≈160 kDa) relative to the macrophage and endothelium NO synthases (≈135 kDa).

Other heme thiolate enzymes have been reported, including chloroperoxidase from *Caldariomyces fumago* (42, 43) and both thromboxane synthase (44, 45) and prostacyclin synthase (44), which contain the cysteine thiolate heme ligand and exhibit similar reduced CO difference spectra. These do not share other properties of the various cytochromes P-450, such as requirements for NAD(P)H reducing equivalents, and are based instead on a peroxygenase reaction mechanism in which the peroxide donor and oxygen acceptor are in the same molecule (44).

It is important to note that cytochromes P-450 were originally identified according to the visible wavelength absorption maximum around 450 nm of the reduced CO difference spectrum. It has been possible only recently, due to accumulating knowledge of primary structures, substrate specificities, and/or reaction mechanisms, to be more specific in this definition. Furthermore, the suggested nomenclature of Nebert *et al.* (46) has permitted a logical cataloguing of the various cytochromes P-450 in this gene superfamily, now comprising >150 members. It is appropriate at this time to identify this unique and complex flavin-, pterin-, and heme-containing NO synthase from rat cerebellum only as a cysteine thiolate heme-binding protein until more is known of its structure–function relationships.

White and Marletta (47) have simultaneously demonstrated the existence of a CO-binding heme in the inducible macrophage NO synthase and the inhibition of the formation of

citrulline from arginine by CO in both purified murine macrophage and partially purified rat cerebellar NO synthase preparations. This report and the present study corroborate the involvement of a CO-binding heme in the catalysis of NO formation in these two tissues. Also noteworthy are the facts that (*i*) these studies document the presence of a NADPH–cytochrome P-450 reductase-like flavin-binding moiety and a CO-binding heme domain in the same polypeptide chain in mammalian tissue, and (*ii*) the NO synthases from macrophages and rat cerebellum are cytosolic in origin. The latter observation affords an opportunity to study a soluble system in which the NO synthase is catalytically competent—i.e., it contains its own reductase, which acts as a shuttle for electrons from NADPH to the oxygenation center(s). In addition, this soluble enzyme is more amenable to structural studies and could lend insight into the multiplicity of cytochrome P-450-mediated electron transport systems. Of course, the finding of an additional prosthetic group for NO synthase raises anew the question of enzymatic mechanism. The presence of two potential monooxygenation centers, tetrahydrobiopterin and a CO-binding heme, raises again the question of stoichiometry of this reaction and provides interesting insights into the determination of its kinetic mechanism.

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