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Purification and Characterization of Nitric Oxide Synthase (NOS_{Noc}) from a *Nocardia* Species

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We previously reported on the occurrence, partial purification, and preliminary characterization of the first reported bacterial nitric oxide synthase. The soluble *Nocardia* enzyme, designated NOS_{Noc}, has now been purified 1,353-fold by a combination of 2′,5′-ADP–agarose affinity chromatography and hydroxylapatite chromatography. NOS_{Noc} runs as a band of M_r 51,900 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The molecular mass was estimated to be 110.6 ± 0.5 kDa by gel filtration, indicating that the native enzyme exists as a homodimer in solution. An N-terminal 15-amino-acid sequence was determined for NOS_{Noc}, showing it to be different from known mammalian NOSs. N^G-Hydroxy-L-arginine was confirmed to be an intermediate in the enzymatic reaction by stoichiometric determinations of oxygen uptake, NADPH oxidation, NO formation as measured by nitrite determinations, citrulline formation, and kinetic studies. NOS_{Noc} was competitively inhibited by N^G-methyl- and N^G-nitro-L-arginine with either L-arginine or N^G-hydroxyl-L-arginine as the substrate. Furthermore, the stability and pH and temperature optima of NOS_{Noc} have been established.

Nitric oxide synthase (NOS; EC 4.14.23) converts L-arginine to L-citrulline and nitric oxide (NO). To date, NOS has been identified in only one microbial species (8). While nothing is known of the role of NOS in microorganisms, NO is thought to play a pivotal role in a wide variety of physiological and pathological processes in mammals. These processes include vasodilation and regulation of normal vascular tone, inhibition of platelet aggregation, neuronal transmission, cytostasis, hypotension associated with endotoxic shock, inflammatory response-induced tissue injury, mutagenesis, and formation of carcinogenic *N*-nitrosamines (21, 39). NOS has been intensively studied because of its important physiological and pathological functions in mammals (24, 33). Several isoforms of NOSs from different mammalian tissues and cells have been purified and characterized (7, 10, 12, 15, 16, 34, 41, 49–51, 57, 58). The catalytic mechanisms of NOSs have also been broadly studied (33). Stable-isotope studies have shown that NO derives from one of the two equivalent guanidino nitrogens of L-arginine (18, 44) and that dioxygen is the source of the oxygen atoms incorporated into citrulline and NO (27, 30). Various purified NOSs have been identified as hemeproteins (37, 52, 55) and flavoproteins (4, 15). Mammalian NOSs have NADPH, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and calmodulin binding sites, as shown by comparison of cDNA and amino acid sequences (5, 29, 31, 32, 56). In addition, N^G-hydroxyl-L-arginine (L-NOHA) has been demonstrated to be an oxidative intermediate in the catalytic process (23, 48, 53, 54). Most mechanistic details about the activation of molecular oxygen, the stoichiometry of the reaction, and the explanation of differences in various types of NOSs remain to be experimentally determined.

In a preliminary communication, we recently presented the first report of the occurrence in and partial purification of NOS from a bacterial species (8). Reflecting its origin in a *Nocardia* species, this enzyme was designated NOS_{Noc}. The soluble, partially purified NOS_{Noc} permitted initial studies to confirm the formation of NO and citrulline from arginine as a substrate, the establishment of cofactor requirements, and the demonstration of NOS_{Noc} inhibition by N^G-nitro-L-arginine (L-NNA). We now report details of the isolation, purification, and properties of NOS_{Noc}.

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MATERIALS AND METHODS

Materials. L-[2,3-^{3}H]arginine (53 Ci/mmol; 1 Ci = 37 GBq) was purchased from DuPont NEN (Boston, Mass.). (6*R*)-5,6,7,8-Tetrahydrobiopterin (H_4B) was obtained from Biochemical Research Inc. (Natick, Mass.). Hydroxylapatite was from Bio-Rad (Hercules, Calif.). L-NOHA was from Alexis Corp. (San Diego, Calif.). L-Arginine, N^G-methyl-L-arginine (L-NMA), L-NNA, 2′,5′-ADP–agarose, and other reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Preparation of crude extracts. The *Nocardia* species (strain NRRL 5646) is kept in the University of Iowa College of Pharmacy culture collection and is grown and maintained on slants of Sabouraud-dextrose agar or sporulation agar (ATCC no. 5 medium). Shaken flask cultures were grown by a standard two-stage incubation protocol (2) in 200 ml of sterile soybean meal-glucose medium held in stainless steel-capped 1-liter DeLong culture flasks. The medium contained (wt/vol) 2% glucose, 0.5% yeast extract, 0.5% soybean meal, 0.5% NaCl, and 0.5% K_2HPO_4 in distilled water and was adjusted to pH 7.0 with 6 N HCl and then autoclaved at 121°C for 20 min. Cultures were incubated with shaking at 250 rpm at 28°C on New Brunswick Scientific G25 Gyrotory shakers. A 10% inoculum derived from a 72-h-old first-stage culture was used to initiate the second-stage culture, which was incubated as before. The second-stage culture was harvested after 48 h and filtered through cheesecloth to remove remaining soybean meal solids, and the filtrate was centrifuged at 8,000 × *g* for 10 min. Typical wet weight cell yields by this cultivation process are approximately 25 g/liter.

For preparation of crude cell extracts, 5 g (wet weight) of the cell pellet was suspended in 20 ml of cold 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM dithiothreitol (DTT), 1 mM EDTA, and 2 μM H_4B and disrupted for 5 min over ice with a Sonifier Cell Disrupter 350 (Branson Sonic Power Co.). Cell debris was removed by centrifugation at 100,000 × *g* for 60 min at 4°C in a Beckman L8-55 ultracentrifuge. The 100,000 × *g* supernatant was used directly for subsequent enzyme purification steps, which were conducted at 4°C.

Enzyme assay. NOS_{Noc} activity was determined spectrophotometrically from the rapid and quantitative oxidation of oxyhemoglobin to methemoglobin by NO (11, 43). Assays for NOS activity were done with a Shimadzu 160 spectrophotometer by observing the increase in absorbance at 401 nm at 25°C. A typical sample assay contained 50 mM potassium phosphate buffer (pH 7.5), 4 μM oxyhemoglobin, 100 μM L-arginine or L-NOHA (for kinetic experiments), 100 μM NADPH, 1 mM $CaCl_2$, 10 μM FAD, 10 μM FMN, 80 μM H_4B, 150 μM

DTT, and 0.1 to 2 μg of enzyme in a final volume of 1 ml. For assays, the reference cuvette contained all components except L-arginine or L-NOHA. All assays were initiated by adding enzyme. NOS_{Noc} activity is expressed as nitric oxide produced in nanomoles per minute per milligram of protein.

Enzyme purification. Crude 100,000 × *g* supernatants (15 to 20 ml, 80 to 90 mg of protein) were applied to 4-ml 2′,5′-ADP–agarose columns equilibrated with 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM DTT, 1 mM EDTA, and 2 μM H_4B. The columns were subsequently washed with 20 ml of buffer, 20 ml of buffer containing 0.5 M NaCl, 20 ml of buffer containing 0.5 mM NADH, 20 ml of buffer containing 0.5 mM $NADP^+$, and 20 ml of buffer. Enzyme activity was then eluted with 10 ml of buffer containing 10 mM NADPH. The enzyme active fraction was concentrated with an Amicon concentrator (PM-10 membrane), washed with additional buffer to remove residual NADPH, and finally concentrated to approximately 1 ml before being applied to a 5-ml hydroxylapatite column (1.5 by 8 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT. The hydroxylapatite column was subsequently washed with 50 ml of the same buffer. The enzyme was then eluted with 100 ml of phosphate buffer with a linear gradient (10 to 100 mM), and 2-ml fractions were collected. Tubes 19, 20, and 21 (6 ml; see Fig. 1), corresponding to the peak of enzyme activity, were combined and concentrated to approximately 0.2 ml for subsequent analysis.

Determination of NADPH consumption. NADPH consumption was determined by measuring the decrease in absorbance at 340 nm as NADPH was consumed during the conversion of L-arginine to L-citrulline by NOS (13).

Citrulline determination. Citrulline produced by NOS_{Noc} was determined by monitoring the formation of [^{3}H]citrulline from [^{3}H]arginine (6). Reaction samples (50 μl containing 100 μM arginine and 1 μCi of radioactivity) in 2 ml of stop buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 2 mM EDTA [pH 5.5]) were applied to a fresh 1-ml Dowex AG50W-X8 (Na^+ form, 100-200 mesh) column. Labeled citrulline was eluted with 2 ml of distilled water, and the eluates were collected. Aliquots of 0.1 ml of eluate were dissolved in 5 ml of Budget Solve cocktail in 10-ml scintillation vials, and radioactivity was measured in a Beckman LS 3801 liquid scintillation system.

Nitrite determination. Nitrite was measured spectrophotometrically with Griess reagent (40). Griess reagent (0.5 ml of a solution of 1% sulfanilamide and 0.1% naphthylethylenediamine in 2% phosphoric acid) was added to 0.5 ml of NOS_{Noc} reaction mixtures. After standing for 15 to 30 min at room temperature, the absorbance at 543 nm was measured. Nitrite concentrations were estimated by comparison with a standard curve at the range of 0 to 1.0 μg/ml prepared with sodium nitrite in water.

Determination of oxygen consumption. Oxygen consumption during NOS_{Noc} incubations was measured with a YSI model 53 oxygen monitor equipped with a 3-ml water-jacketed reaction vessel kept at 25°C. Oxygen concentrations were calculated from the deflection of the chart recorder versus 206 μM oxygen-total oxygen content in the reaction vessel (25).

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a Bio-Rad Mini-Protein II dual-slab cell with a discontinuous buffer system (28) and a 12% separating gel. Gels were stained with Coomassie blue.

Native molecular weight determination. Analytical gel filtration chromatography was carried out with an Alltech Macrosphere 150 column (7 μm, 4.6 by 25 cm). The mobile phase of 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM DTT and 0.2 M NaCl was used to equilibrate the column and to elute protein samples at a flow rate of 0.5 ml/min. Eluting protein peaks (retention volumes, R_v) were monitored at 280 nm. The standard proteins (M_r) used were bovine thyroglobulin (669,000; R_v, 1.82 ml), horse spleen apoferritin (443,000; R_v, 1.97 ml), sweet potato β-amylase (200,000; R_v, 2.27 ml), yeast alcohol dehydrogenase (150,000; R_v, 2.47 ml), bovine serum albumin (66,000; R_v, 2.76 ml), and bovine carbonic anhydrase (29,000; R_v, 3.16 ml). The native molecular weight was estimated by plotting log M_r versus R_v. The peak corresponding to the eluted enzyme (M_r 106,000) was collected and assayed for NOS activity.

Analysis of the N-terminal amino acid sequence. Purified NOS_{Noc} (6 μg of protein) was centrifuged onto a Prospin membrane until dry to remove phosphate salts and DTT. The protein on the membrane was washed and removed with 1 ml of methanol-water (1:1), and the N-terminal amino acid sequence was determined by automated microsequencing with Edman degradation reactions on a 475A Sequencer (Applied Biosystems, Inc.) in the Protein Structure Facility at the University of Iowa, Iowa City. The N-terminal amino acid sequence was determined twice with separately purified samples of NOS_{Noc}.

Determination of pH and temperature optima and NOS_{Noc} stability. Purified NOS_{Noc} (see Table 1) was used in the determination of enzyme activity versus incubation temperature, pH, and enzyme stability. For temperature optimum experiments, the buffer was 50 mM potassium phosphate (pH 7.5). The samples were incubated at various temperatures for 5 min before each determination. For pH optimum determinations, three different buffers were used over appropriate pH ranges. These included 50 mM sodium acetate, 50 mM potassium phosphate, and 50 mM Tris-HCl.

Enzyme stability experiments were conducted by incubating NOS_{Noc} in 50 mM potassium phosphate buffers (pH 7.5) held at 4, 25, and 37°C. For these experiments, each determination was done in triplicate.

Other determinations. The concentrations of protein at all purification steps were measured by the Bradford protein microassay, with bovine serum albumin as the standard (43). The UV-visible absorption spectrum of NOS_{Noc} (10 μg in 100 μl of 20 mM phosphate buffer, pH 7.0) was determined with a Sim-Aminco model DW2000 UV-visible spectrophotometer with 100-μl cuvettes. The spectrum was scanned over the range of 200 to 600 nm.

TABLE 1. Purification of NOS_{Noc}[a]

Step	Total protein (μg)	Total activity (nmol/min)	Sp act (nmol/min/mg of protein)	Yield (%)	Purification (fold)
Crude extract	82,720	12.31	0.15	100	1
ADP-agarose	348	10.92	31.4	88.7	209
Hydroxylapatite	24	4.83	203	39.2	1,353

[a] The data are representative of two experiments.

RESULTS

Enzyme purification. The results of purification of *Nocardia* NOS_{Noc} are summarized in Table 1. NOS_{Noc} was purified 1,353-fold by two chromatographic steps. The first step of purification involved affinity chromatography over 2′,5′-ADP–agarose, to which the enzyme binds. Enzyme activity was selectively eluted from the column with 10 mM NADPH. Although NOS_{Noc} was purified some 209-fold by this step, a number of contaminating proteins were also eluted from the column by 10 mM NADPH. Final purification of NOS_{Noc} was achieved by hydroxylapatite chromatography. The elution profiles of NOS_{Noc} activity and other proteins from the hydroxylapatite column are shown in Fig. 1. Three major protein peaks were observed in the elution profile. The relatively small protein peak in the middle of the chromatogram corresponded to NOS_{Noc} enzyme activity. NOS_{Noc} activity from the hydroxylapatite column was eluted in 16 fractions (32 ml) with 40 to 50 mM phosphate buffer. The enzyme was determined to be greater than 98% pure by SDS-PAGE (Fig. 2).

Purified NOS_{Noc} had a specific activity of 203 nmol of NO

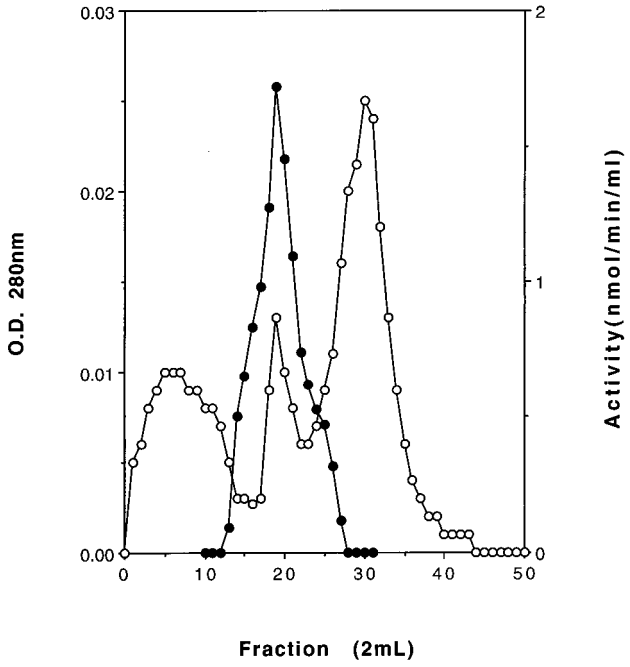

FIG. 1. Elution profile of NOS_{Noc} by hydroxylapatite chromatography. Elution was achieved by using a linear gradient of potassium phosphate buffer (pH 7.0) from 10 to 100 mM. ○, optical density at 280 nm; ●, enzyme activity.

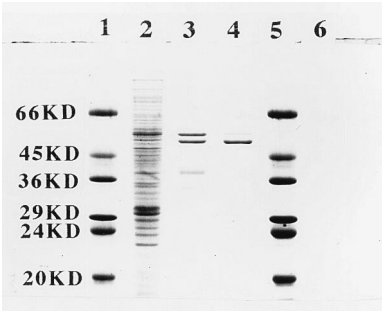

FIG. 2. SDS-PAGE of NOS_{Noc}. Shown is an SDS gel prepared by diluting 30 ml of 40% acrylamide stock solution (Fisher) containing acrylamide-bisacrylamide (37.5:1) to 100 ml. The developed gel was Coomassie blue stained. Lanes 1 and 5, molecular weight markers: bovine serum albumin (66,000), egg albumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), and trypsin inhibitor (20,000); lane 2, crude extracts (10 μg of protein); lane 3, NADPH eluate from 2′,5′-ADP–agarose column (2 μg of protein); lane 4, eluate from hydroxylapatite column containing purified enzyme (1 μg of protein); lane 6, control with sample buffer.

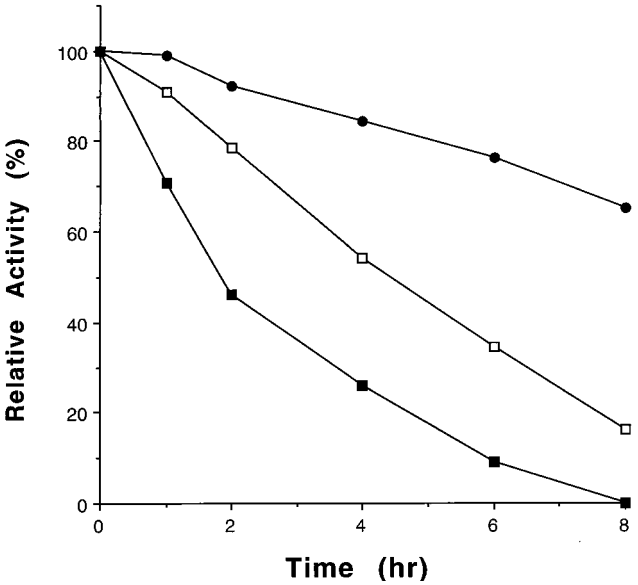

FIG. 3. Stability of NOS_{Noc} at 4°C (●), 25°C (□), and 37°C (■). The values are the means of three determinations, and the experiment shown is representative of two.

produced per mg of protein per min. This represented a 1,353-fold purification of NOS_{Noc} from crude extracts, with 39% recovery of total enzyme activity. From this purification, soluble NOS represents approximately 0.03% of the crude supernatant proteins of the microorganism.

Properties of NOS_{Noc}. From comparison of the NOS_{Noc} R_v of 2.48 ml with the R_vs of proteins of known molecular weight by Macrosphere 150 gel filtration chromatography, the eluted and still active enzyme was estimated to possess a mass of 110.6 ± 0.5 kDa. By SDS-PAGE, the denatured molecular weight of the enzyme was estimated to be 51,900 (Fig. 2). Thus, NOS_{Noc} appears to be a homodimeric protein.

NOS_{Noc} is colorless, and an absorption maximum was observed at 280 nm in the UV-visible spectrum. A shoulder at 390 to 430 nm, which corresponds to the characteristics of a hemeprotein, was barely evident in the UV-visible spectrum. Absorption in the range of 450 to 550 nm, typical for flavins, was not observed.

Fifteen amino acid residues from the N-terminal end of the protein were determined by Edman degradation in duplicate analyses of two separately purified NOS_{Noc} samples. The N-terminal amino acid sequence is H_2N-Thr-Leu-Leu-Asp-Ser-Lys-Ile-Trp-Pro-Asp-Arg-Val-Phe-Ile-Asp-. The BLASTP and TBLASTN programs (1) were used to search the updated SwissProt, GenPept, GenBank, and EMBL databases, but no matching amino acid sequences were found.

The stability of NOS_{Noc} was measured at 4, 25, and 37°C. The enzyme lost about 30% of its activity after 8 h at 4°C, whereas it exhibited half-lives of 4 and 2 h at 25 and 37°C, respectively (Fig. 3).

The optimum pH of NOS_{Noc} occurs between 7.0 and 7.5 with potassium phosphate buffer, and enzyme activity is maximum at 30°C.

Confirmation of L-NOHA as an intermediate. Determinations of the stoichiometry of oxygen consumed, NADPH oxidized, and NO and citrulline formed and kinetic experiments were carried out to confirm that L-NOHA is a substrate for NOS_{Noc} and a likely intermediate in the biosynthesis of nitric oxide from L-arginine. Table 2 shows the stoichiometric relationships between product formation and cofactor utilization with either L-arginine or L-NOHA as the substrate. With L-arginine as the substrate, NOS_{Noc} consumed 2 mol equivalents each of NADPH and O_2, while 1 mol each of L-citrulline and nitrite were formed. With L-NOHA as the substrate, NOS_{Noc} consumed 1 mol equivalent each of NADPH and O_2, while 1 mol each of L-citrulline and nitrite were formed.

A comparison between L-NOHA and L-arginine as substrates for NOS_{Noc} was made. The K_m measured for L-NOHA was nearly threefold greater (15.8 μM) than that measured for L-arginine (5.7 μM). The V_{max} values determined for L-NOHA and L-arginine, 303 and 274 nmol/min/mg of protein, respectively, were similar. By fitting the experimental data to the EZ-FIT program developed by Perrella (45), NO synthesis from either L-NOHA or L-arginine was found to be competitively inhibited by L-NMA and L-NNA. However, the inhibition constants (K_i) of different inhibitors for different substrates were not the same. The K_i of L-NMA for L-NOHA was 60% higher than that for L-arginine, while the K_i of L-NNA for L-NOHA was approximately 120% greater than that for L-arginine (Table 3). Both L-NMA and L-NNA show typical competitive inhibition for both L-NOHA and L-arginine (Fig. 4 and 5).

DISCUSSION

Nocardia sp. strain NRRL 5646 produces a 100,000 × *g* soluble form of NOS that has been designated NOS_{Noc} (8). In our previous work, the partially purified enzyme was used to

TABLE 2. Stoichiometry of L-arginine and L-NOHA as substrates for NOS_{Noc}[a]

Substrate	Mean amt (nmol) ± SD			
	Citrulline formed	Nitrite formed	NADPH consumed	Oxygen consumed
L-Arginine	4.21 ± 0.17	4.63 ± 0.28	8.77 ± 0.32	8.89 ± 0.33
L-NOHA	4.54 ± 0.21	4.57 ± 0.20	4.38 ± 0.24	4.46 ± 0.16

[a] Enzyme reactions were carried out in the standard manner for 120 min as described in Materials and Methods; 0.18 μg of purified NOS_{Noc} (specific activity, 200 nmol of NO formed per min per mg of protein) was used for each reaction. Values are the means of four reactions. Both L-arginine and L-NOHA were used at 100 μM.

TABLE 3. Kinetic properties of NOS_{Noc}[a]

Substrate	Mean value ± SD			
	K_m (μM)	V_{max} (nmol/min/mg of protein)	K_i	
			L-NMA	L-NNA
L-Arginine	5.7 ± 0.3	274 ± 13	6.2 ± 0.4	9.8 ± 0.6
L-NOHA	15.8 ± 0.8	303 ± 15	10.2 ± 0.8	21.4 ± 1.2

[a] The data are the means of three determinations. The kinetic constants were obtained by fitting experimental data to the EZ-FIT program. The values of K_i were obtained by fitting to the model of competitive inhibition.

confirm its catalytic nature and to permit preliminary determinations of substrate, products, and cofactors required in the conversion of L-arginine to L-citrulline and NO (Fig. 6). Citrulline produced by NOS_{Noc} was identified by high-pressure liquid chromatography (HPLC) analysis of a citrulline derivative, thin-layer chromatography (TLC) analysis of radioactive citrulline, and Dowex AG50W-X8 filtration, which resolves arginine and citrulline. We also showed that NOS_{Noc} enzyme activity was dependent on the presence of NADPH, Ca^{2+}, FAD, FMN, and H_4B, demonstrating the cofactor requirement similarity of the *Nocardia* enzyme with NOS from polymorphonuclear neutrophils. Omission of any of these cofactors from incubation mixtures results in loss of all enzyme activity, and addition of calmodulin to the reaction mixtures does not increase enzyme activity (8). NOS_{Noc} activity can be measured directly in crude cell-free preparations. This suggests that the flavins and pterins required for enzyme activity are lost as a consequence of protein purification. Flavins are common components in bacteria, whereas pterins are not. However, while pterins do occur as cofactors in bacteria (19, 20), the identification of the natural pterin of *Nocardia* sp. strain NRRL 5646 remains to be determined.

Column affinity chromatography over 2′,5′-ADP–agarose has been used in the purification of every reported mammalian NOS obtained from various tissues and cells (7, 10, 12, 15, 16, 34, 41, 49–51, 57, 58). In our experiments, NOS_{Noc} was tightly bound to 2′,5′-ADP–agarose and, after several washes with NaCl, NADH, and $NADP^+$, was best eluted with 10 mM NADPH to yield maximum activity. Lower concentrations of NADPH resulted in only partial elution of NOS_{Noc} from 2′,5′-ADP–agarose and substantial peak broadening. Anion-exchange and gel filtration chromatographies have also been widely used for mammalian NOS purifications. However, in our hands, hydroxylapatite chromatography was more successful as a final step for subsequent purification of NOS_{Noc}.

By SDS-PAGE, the purified protein ran as a prominent major band indicating a molecular mass of 51.9 kDa, considerably smaller than mammalian NOSs, which give denatured molecular masses by SDS-PAGE in the range of 115 to 150 kDa (7, 10, 12, 15, 16, 34, 41, 49–51, 57, 58). Different types of mammalian NOSs have been characterized as hemeproteins (37, 52, 55). Limitations in the amount of pure enzyme precluded conclusive UV-visible spectral analysis of NOS_{Noc}. However, the appearance of a shoulder at 390 to 430 nm suggests the presence of heme in the *Nocardia* enzyme, while lack of absorption in the range of 450 to 550 nm suggests that FAD and FMN are not bound to the purified enzyme. The lack of bound FAD and FMN in purified NOS_{Noc} was attributed to dissociation of these cofactors during enzyme purification. A similar result was reported by Yui et al., who observed no characteristic absorptions in the UV-visible spectrum for purified rat macrophage NOS (57). Spectral characterizations of

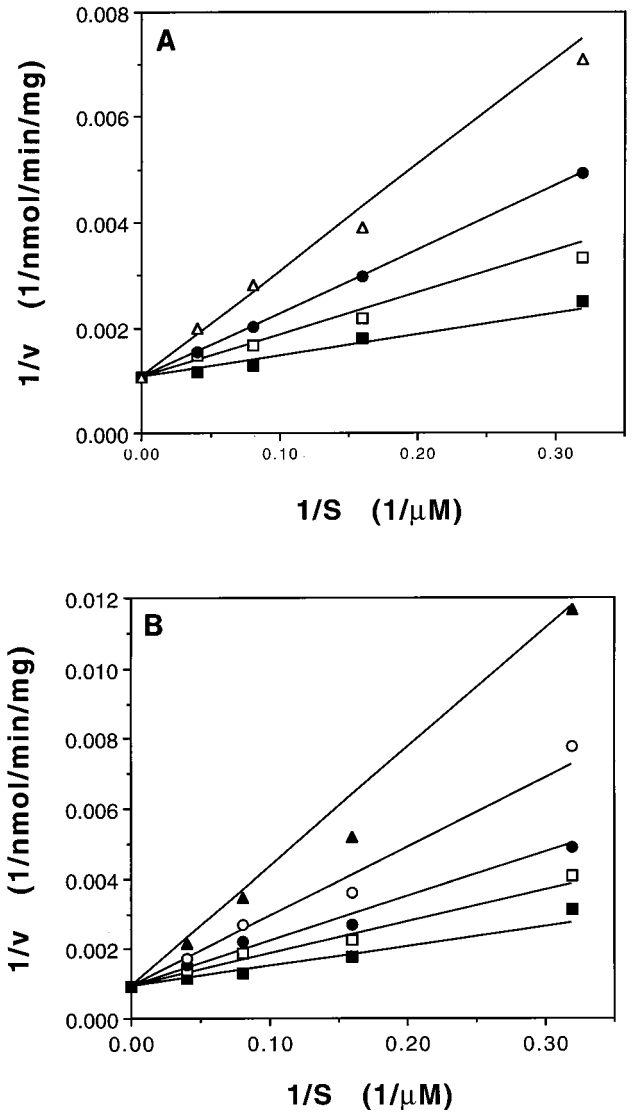

FIG. 4. Double-reciprocal plots of the inhibition of NOS_{Noc} in the presence of L-arginine. The inhibition experiments were carried out as for standard enzyme assays under initial velocity conditions. (A) Inhibition by L-NMA at 0 μM (■), 12.5 μM (□), 25 μM (●), and 50 μM (△). The values are the means of three measurements. (B) Inhibition by L-NNA at 0 μM (■), 6.125 μM (□), 12.5 μM (●), 25 μM (○), and 50 μM (▲). The values are the means of three measurements.

mammalian NOSs have usually required relatively larger amounts of cloned NOS (37, 55), presently unavailable for *Nocardia* sp. strain NRRL 5646. The 15-amino-acid N-terminal sequence screened through the BLASTP and TBLASTN programs is unlike sequences reported for purified mammalian NOSs. NOS_{Noc} is reasonably stable, losing about 30% of its activity after 8 h at 4°C. The half-lives of most mammalian NOSs under similar conditions are less than 6 h (7, 34, 51, 57).

Marletta recently reviewed aspects of the structure of NOS and the mechanism by which the enzyme is thought to catalyze the oxidation of arginine through L-NOHA to NO and citrulline (33). L-NOHA is a demonstrated intermediate in the biosynthesis of NO from L-arginine in mammalian tissues and cells (9, 26, 47, 48, 53).

We examined the possible intermediacy of L-NOHA in the synthesis of NO by NOS_{Noc} and found that the K_m of 15.8 mM

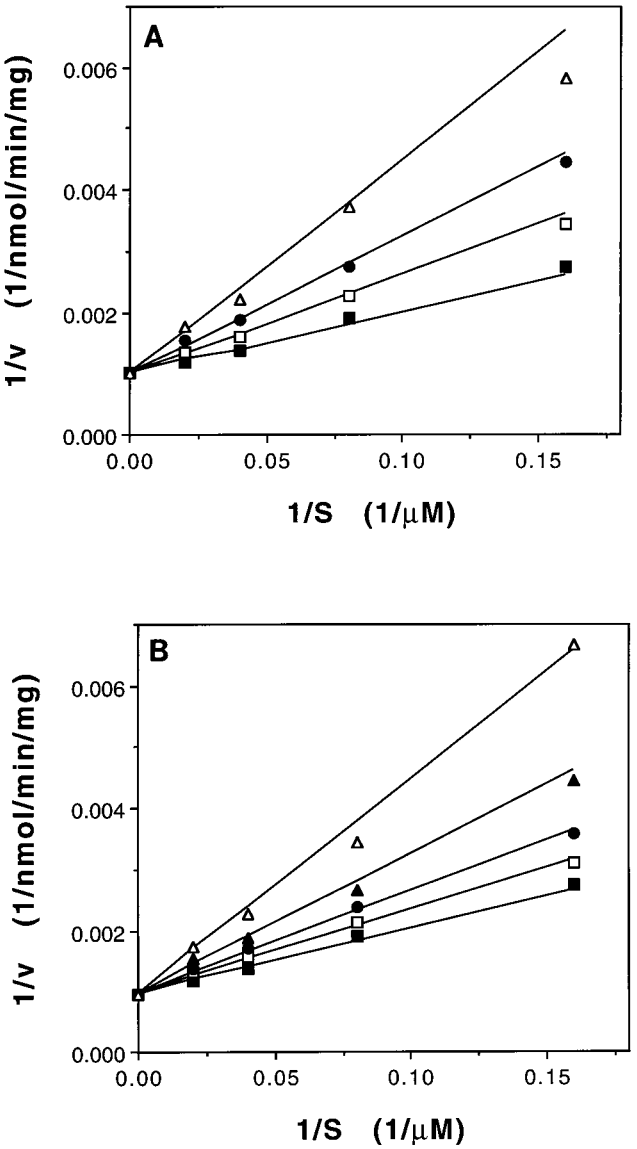

FIG. 5. Double-reciprocal plots of the inhibition of NOS_{Noc} in the presence of L-NOHA. The inhibition experiments were carried out as for standard enzyme assays under initial velocity conditions. (A) Inhibition by L-NMA at 0 μM (■), 12.5 μM (□), 25 μM (●), and 50 μM (△). The values are the means of three measurements. (B) Inhibition by L-NNA at 0 μM (■), 6.125 μM (□), 12.5 μM (●), 25 μM (▲), and 50 μM (△). The values are the means of three measurements.

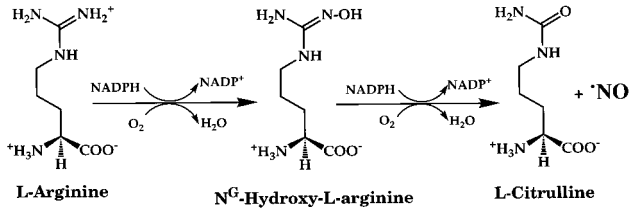

FIG. 6. Biosynthetic pathway of nitric oxide from L-arginine by NOS_{Noc}.

for L-NOHA is about threefold greater than that for L-arginine. This result is similar to that reported for macrophage NOS by Stuehr et al. (53). Stoichiometry experiments demonstrated that with NOS_{Noc}, during the enzymatic synthesis of 1 mol each of L-citrulline and NO, 2 mol each of NADPH and O_2 were consumed with L-arginine as the substrate. The oxidation of L-NOHA to citrulline and NO required 1 mol each of NADPH and O_2. The measured balance in arginine used to citrulline and NO formed precluded any need to measure nitrate in our purified preparation (17). Uncoupled NADPH oxidation in our purified NOS_{Noc} preparation was insignificant. The mechanism by which NOS_{Noc} and mammalian NOSs catalyze the oxidation of arginine to citrulline remains largely unknown. The NOSs from mammals characterized so far are exceedingly complex systems, containing a cytochrome P450-type component (4, 37, 55) as well as an FMN and FAD flavoprotein component (51). Interestingly, Bredt et al. have noted structural similarities between NOS and cytochrome P450 reductase (5). With the known differences among in the mammalian NOSs, the discovery of an NOS in bacteria with physical and catalytic properties apparently different from those discovered in mammals is unsurprising. The mechanisms of oxygen activation, sources of reducing electrons, the consequential formation of active oxygen species during the NOS reaction, and other reaction details remain to be determined (26, 33, 35, 38, 46). The availability of NOS_{Noc} and the solid stoichiometry results reported here provide the means to further address these complex questions.

L-NMA and L-NNA are typical competitive inhibitors of mammalian NOSs. In some cases, L-NMA shows irreversible inactivation with macrophage NOSs (22, 36, 42, 43). Both L-NMA and L-NNA appear to be reversible competitive inhibitors of NOS_{Noc} in the presence of either L-arginine or L-NOHA as the substrate. The results from our studies confirm L-NOHA as an intermediate in the pathway from L-arginine to L-citrulline and NO. Interestingly, the K_i of L-NNA is higher than that of L-NMA with NOS_{Noc}. However, the K_i of L-NNA is usually equal to or lower than that for L-NMA for constitutive mammalian NOSs (22, 36).

Different stoichiometric relations among products formed and cofactors utilized, differences in K_i measurements, the uniqueness of the measured N-terminal amino acid sequence, and differences in molecular weight between NOS_{Noc} and mammalian NOSs all underline possible differences in substrate and cofactor binding to the enzyme active site and in the catalytic mechanisms involved in the conversion of L-arginine to L-citrulline and NO. This work has elaborated many important features of the first reported NOS system observed in bacteria. The enzyme from *Nocardia* sp. strain NRRL 5646 clearly possesses the characteristics of an NOS. We have speculated (8) that NOS_{Noc} may be involved in mechanisms of infection and microbial virulence. We suggest that, following the pattern of discovery of other important mammalian enzyme systems in microorganisms, such as the cytochrome P450s, it is likely that other microbial NOS systems with properties resembling those of other mammalian NOSs will be discovered. In this light, Noguchi et al. recently reported the detection by Fourier transform infrared spectroscopy of NO bound to the nonheme iron center of an enzyme in intact cells of a *Rhodococcus* species (40). The observation of NO in a *Rhodococcus* sp. is interesting because of the relatively close relationship between *Rhodococcus* and *Nocardia* species. We are pursuing further experiments on the structure and mechanism of the NOS_{Noc} described in this paper and are examining the presence of other forms of NOS in *Nocardia* sp.

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