Characterization of Neuronal Nitric Oxide Synthase and a C415H Mutant, Purified from a Baculovirus Overexpression System†

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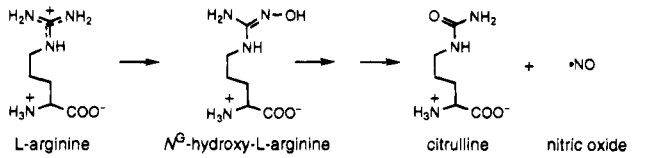
ABSTRACT: Nitric oxide synthase (NOS) catalyzes the conversion of L-arginine to citrulline and nitric oxide (•NO). A baculovirus overexpression system has been developed for a constitutive NOS isoform, cloned originally from rat cerebellum (B-NOS). Recombinant virus was used at a multiplicity of infection of 5 to infect *Spodoptera frugiperda* cells in culture, and NOS was expressed to 10% of the total soluble protein at 48 h postinfection. In order to express catalytically active enzyme, it was necessary to supplement the culture media with hemin. This increased the activity of the enzyme 7-fold. A two column affinity purification was developed for the recombinant enzyme, which gave homogeneous protein that migrated at 150 kDa on a denaturing polyacrylamide gel. A K_m for L-arginine was determined to be 2.0 ± 0.4 μM. As isolated, recombinant B-NOS exhibited a Soret maximum at 402 nm, which shifted to 394 nm in the presence of L-arginine. The Soret maximum of the reduced enzyme in the presence of CO was 444 nm. Initial rate steady-state kinetic analysis of the recombinant B-NOS showed evidence of substrate inhibition by L-arginine, which could also be seen in a partially purified preparation of B-NOS from rat cerebella. This substrate inhibition was not observed with the inducible isoform of NOS, purified from immunostimulated murine macrophages. A C415H mutant was overexpressed and purified using the same conditions established for the wild-type recombinant B-NOS. This C415H mutant exhibited no activity and did not bind heme, providing the first experimental evidence to support previously reported primary amino acid comparisons which suggest that C415 provides the coordinating thiolate to the heme moiety in B-NOS.

As studies continue with regard to the significant role nitric oxide (•NO)[1] plays in mammalian physiology, it is evident that a more detailed understanding of the enzyme responsible for its synthesis is necessary. Nitric oxide synthase (NOS) (EC 1.14.13.39) catalyzes the oxidation of L-arginine to citrulline and •NO (Scheme 1). Several isoforms of NOS have been described, which can be divided into three categories, each with a prototypic example. The isoform characterized in nervous tissue is cytosolic and constitutive, has a M_r ranging from 150 000 to 160 000, and was purified from rat (Bredt & Snyder, 1990; Schmidt et al., 1991) and porcine cerebellum (Mayer et al., 1990). NOS in endothelial cells is a constitutive isoform that is membrane bound, has a M_r = 135 000, and was purified from bovine aortic endothelial cells (Pollock et al., 1991). The best characterized inducible isoform is cytosolic, has a M_r = 130 000, and was first purified from immunostimulated murine macrophage RAW 264.7 cells (Hevel et al., 1991; Stuehr et al., 1991). The constitutive isoforms show an absolute requirement for exogenous Ca^{2+} and calmodulin, whereas the inducible isoforms in general do not show an absolute requirement.

Scheme 1: Reaction Catalyzed by NOS



The role •NO plays in physiological processes depends on the cellular site of synthesis, which corresponds to one of the previously mentioned distinct isoforms (Bredt & Snyder, 1992; Marletta, 1993). In the central nervous system, the activation of postsynaptic *N*-methyl-D-aspartate receptors causes an influx of Ca^{2+}; the Ca^{2+}-induced binding of calmodulin to NOS initiates the synthesis of •NO, which has been identified as a novel neurotransmitter. Peripheral neurons that stimulate the relaxation of gut and penile smooth muscle have been shown to mediate their effects via •NO (Burnett et al., 1992). In the circulatory system, •NO is released by endothelial cells and diffuses into the adjacent smooth muscle, where it activates guanylate cyclase. The resulting increase of intracellular cGMP leads to a transient relaxation of the vessel. Lastly, immunostimulated phagocytes in the immune system, mainly macrophages, synthesize

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[1] Abbreviations: •NO, nitric oxide; NOS, nitric oxide synthase; B-NOS, neuronal nitric oxide synthase; M-NOS, macrophage nitric oxide synthase; H_4B, 6(*R*)-5,6,7,8-tetrahydro-L-biopterin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; DTT, dithiothreitol; Sf9, *Spodoptera frugiperda*; PCR, polymerase chain reaction; MOI, multiplicity of infection; BSA, bovine serum albumin; PMSF, phenylmethanesulfonyl fluoride; P450, cytochrome P450; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride.

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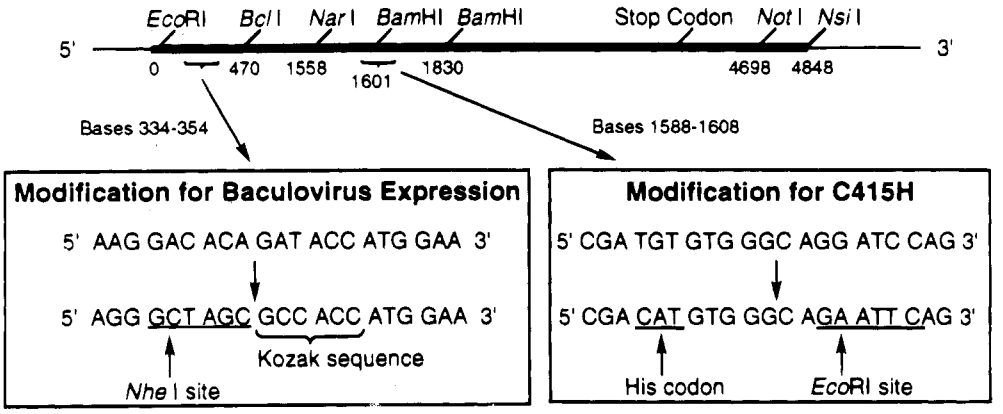
Scheme 2: Expression Modifications to B-NOS cDNA



•NO. In this setting, •NO acts as a component of the host response to infection.

Given the wide variety of processes in which •NO is involved, it should be no surprise that altered levels of •NO have been implicated in several pathological conditions. Abnormal •NO levels seem to be involved in the hypotension associated with endotoxic shock (Kilbourn et al., 1990), hypertension and atherosclerosis (Luscher, 1990), and inflammatory response-induced tissue injury (Mulligan et al., 1991). Recently, both adults and children with pulmonary hypertension have shown improvement when •NO was included in their ventilator gas (Kinsella et al., 1992; Roberts et al., 1992; Rossaint et al., 1993). In addition, inhibitors of NOS have been shown to relieve the severe hypotension in some patients suffering from septic shock (Petros et al., 1991).

Clearly the structure/function relationships among the various isoforms will be physiologically important. The development of an overexpression system will supply larger quantities of enzyme, which will facilitate continued mechanistic and other physical studies of NOS. In addition, it will allow for specific site-directed mutants to be made and expressed. Here we present the development of a baculovirus overexpression system for the constitutive rat brain NOS (B-NOS), cloned originally from rat cerebellum (Bredt et al., 1991). NOS obtained from this system has been purified and characterized. We have identified a previously unreported phenomenon in which this constitutive isoform is subject to inhibition by L-arginine. The inducible isoform purified from immunostimulated macrophages does not show this type of inhibition. In addition, we report here on the expression and purification of a C415H mutant. Through sequence analysis (McMillan et al., 1992; White & Marletta, 1993) and modeling (Renaud et al., 1993), C415 has been suggested to be the thiolate ligand to the heme iron.

MATERIALS AND METHODS

Materials. The complete cDNA for B-NOS (GenBank accession no. X52949) was kindly provided in pBluescript SK(−) by Dr. Solomon Snyder (Johns Hopkins University). DNA oligomers were synthesized by the DNA Core Facility at the University of Michigan Medical Center. All restriction enzymes were obtained from either Gibco BRL or Boehringer Mannheim. *Taq* polymerase was purified for use in the polymerase chain reaction as previously reported (Engelke et al., 1990). dNTP's were obtained from Boehringer Mannheim. Tissue culture flasks were obtained from Falcon and Costar. Electrophoresis reagents and the columns used in the purification were purchased from Bio-Rad. Nitrocellulose membranes were purchased from Schleicher and Schuell. H_4B was purchased from Dr. B. Schircks Laboratories (Jona, Switzerland) and was prepared in 50 mM Hepes (pH 7.4) containing 100 mM DTT. Sprague-Dawley rat brains were purchased from Harlan Bioproducts for Science. W-7 was purchased from Seikagaku America, Inc. L-[U-^{14}C]Arginine monohydrochloride (specific activity = 319 mCi/mmol) was purchased from Amersham Corp. Research-grade CO (99.5%) was obtained from Matheson. Ecolume scintillation fluid was purchased from ICN-Flow. Polyclonal antibody against B-NOS was a generous gift from Abbott Laboratories (Abbott Park, IL). Unless otherwise noted, all other chemicals and reagents were obtained from Sigma.

General Methods. The *Escherichia coli* strains XL1-Blue (Stratagene), GM119, and DH5α F′ (Ausubel et al., 1994) were used for all DNA manipulations. Standard growth conditions were used as previously described (Ausubel et al., 1994; Sambrook et al., 1989). Plasmid preparations were carried out using the alkaline lysis or boiling methods, and when necessary, Qiagen-tip 100 columns (Qiagen, Inc.) were used to obtain purified plasmid DNA.

Tissue Culture. Spodoptera frugiperda (Sf9) cells were a generous gift of Dr. Claudia Kent (University of Michigan). The cells were maintained at 28 °C either in spinner flasks or in monolayers. Grace's insect cell culture medium was supplemented with yeastolate, lactalbumin hydrosylate (all from Gibco BRL), 10% fetal bovine serum (Whittaker), and 1% of a 100× antibiotic/antimycotic solution (Sigma #A-9909) (Summers & Smith, 1987). Cells were subcultured approximately every 3 days. Cell density and viability of suspension cultures were determined using a hemocytometer and trypan blue exclusion.

Preparation of B-NOS cDNA for Expression. A 1600 base pair *Eco*RI/*Bam*HI fragment containing the translation start site was subcloned into RF M13mp18 (Pharmacia) so that single-stranded DNA could be prepared (Scheme 2). A mutagenic oligo (5′ CCC AAA CGT GTT CTC TTC CAT GGT GGC GCT AGC CTT CAG AAG ACT AAG C 3′) was used to introduce a complete Kozak sequence (Kozak, 1987) and a *Nhe*I site by *in vitro* mutagenesis, using the method of Eckstein (Amersham kit, version 2.1). A 470 base pair *Eco*RI/*Bcl*I fragment, which contained the mutagenized region, was excised from within the 1600 base pair *Eco*RI/*Bam*HI fragment and subcloned into pBluescript SK(−). This 470 base pair "cassette" was sequenced using the dideoxy

chain termination method (Sequenase Version 2.0 kit, United States Biochemical) to verify the correct DNA sequence. The 470 bp cassette was subcloned back into the wild-type B-NOS cDNA. A 4360 bp *NheI/NotI* fragment, containing the entire open reading frame, was subcloned into the transfer vector pVL1393 (Pharmingen) using its compatible *XbaI* and *NotI* sites in the multiple cloning site. A Qiagen-tip 100 column was used to prepare pure plasmid DNA for transfection purposes.

The technique of overlap extension using the polymerase chain reaction (PCR) (Ho et al., 1989) was used to modify the B-NOS cDNA to make the C415H point mutation. Oligonucleotide primers were designed to amplify the 272 base pair *NarI/Bam*HI fragment and to make the following mutations: the codon at position 1591 was changed from TGT to CAT, and the *Bam*HI site at position 1601 was changed to an *Eco*RI site to allow subsequent screening for the mutant (Scheme 2). A GeneAmp 9600 PCR System (Perkin Elmer Cetus) was used for the amplification of 2.5 μg of template cDNA in a 100-μL reaction volume containing 10 mM Tris (pH 9.1), 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM dGTP, dATP, dTTP, and dCTP, and 50 pmol of each primer. The samples were subjected to 94 °C for 5 min and 40 °C for 5 min, followed by 20 cycles of extension (1.5 min, 72 °C), denaturation (1 min, 94 °C), and annealing (1.5 min, 40 °C), and finally 5 min of extension at 72 °C. The 272 base pair "cassette" was also sequenced as explained above to verify the correct DNA sequence. The 272 base pair cassette was subcloned back into the wild-type B-NOS cDNA, which contained the *NheI* site and Kozak sequence at the five prime terminus of the open reading frame. The 4360 base pair *NheI/NotI* fragment, containing the open reading frame and altered codon for H415, was subcloned into the transfer vector pVL1393 using its compatible *XbaI* and *NotI* sites in the multiple cloning site. A Qiagen-tip 100 column was used to prepare pure plasmid DNA for transfection purposes.

Production of Recombinant Baculovirus. Materials needed to make recombinant virus were purchased from Pharmingen, and published methods (Summers & Smith, 1987) were followed for all the baculoviral manipulations. Briefly, Sf9 cells were cotransfected with 0.5 μg of linearized Baculogold viral DNA and 2.0 μg of either the wild-type or the mutant C415H B-NOS construct in pVL1393 using the calcium phosphate method. After 4 days, the media covering the transfected cells was collected. Cellular debris was removed with a low speed spin at 1500 rpm.

One round of plaque purification led to the isolation of 12 plaques for the wild-type B-NOS recombinant virus and 14 plaques for the C415H mutant B-NOS recombinant virus. Virus was eluted from each agar plug overnight in 500 μL of media. The media from one agar plug, which contained recombinant virus at low titer, was used to infect a 25 cm^2 flask of Sf9 cells to further amplify the virus to approximately 1×10^8 plaque forming units (pfu)/mL. Viral DNA was prepared from infected Sf9 cells for use in the PCR to ensure that the B-NOS cDNA was properly inserted in the viral genome.

Amplified recombinant virus (1.0 mL) derived from one of the recombinant plaques was used to infect a 250-mL spinner flask containing 1×10^6 cells/mL. The culture was allowed to grow for 5−7 days to amplify large quantities of the recombinant virus. Final titer of this second passage recombinant viral stock was determined by plaque assay to be about 5×10^7 pfu/mL.

Expression of Recombinant B-NOS. Infection of Sf9 cells to produce B-NOS was carried out both in suspension cultures and in monolayers. In both cases, published methods (Summers & Smith, 1987) were followed with minor modifications. For suspension cultures, Sf9 cells were first grown to a density of $(0.7-1.0) \times 10^6$ cells/mL in a 250-mL spinner flask. Low-speed centrifugation was used to pellet the cells prior to the addition of virus. After the cells were incubated with virus for 1 h at 28 °C, fresh media was added to bring the volume of the culture back up to 250 mL for the duration of the infection. Monolayer infections were carried out in 75 cm^2 flasks, seeded with 1×10^7 cells per flask. A multiplicity of infection (MOI) of 5 was used to infect the cells, and a final media volume of 10 mL was used for each flask. All infections were allowed to progress for 24−72 h before harvesting the cells. Hemin, prepared as described below, was added to the media 2−4 h postinfection.

A fresh hemin solution was utilized as a media supplement and prepared in one of two ways. A 500 μM hemin solution prepared in 0.01 M NaOH was added dropwise to both suspension and monolayer cultures to give the desired final concentration of 7 μM (Asseffa et al., 1989; Hartmann & Ortiz de Montellano, 1992). A hemin−albumin complex (Tenhunen et al., 1968) was also utilized as a supplement to the Sf9 cells. The complex was prepared by modifying a procedure described by Grogan (personal communication). Briefly, 0.430 g of bovine serum albunim (BSA) was dissolved in 5.0 mL of H_2O. In a second vial, 0.375 g of Na_2HPO_4 was dissolved in 10 mL of 0.1 M NaOH, followed by 11 mg of hemin. Slowly, 2.0 mL of the hemin solution was added to the BSA solution. The pH was adjusted with HCl to 7.1−7.2. This solution has an albumin/hemin ratio of approximately 2:1. The hemin/albumin solution was then added dropwise directly to an infected culture to the desired final concentration of hemin. Cultures were kept in the dark to minimize heme degradation.

At the desired time, infected cultures were harvested by collecting the cell pellet at 1500 rpm. All the following manipulations were done at 4 °C. The cells were washed once in phosphate-buffered saline (pH 7.4) (PBS) and then resuspended in sonication buffer (50 mM Hepes, pH 7.4, 10% glycerol, 100 μM DTT, 5 μg of leupeptin/mL, 10 μg of benzamidine/mL, 1 μg of chymostatin/mL, 1 μg of pepstatin/mL, 1 μg of antipain/mL, 200 μM PMSF). Cells were sonicated, and the 100000*g* supernatant was prepared as previously reported (Hevel et al., 1991). The 100000*g* supernatant was assayed for B-NOS activity and frozen at −80 °C.

Purification of B-NOS. The purification of recombinant B-NOS reported here is a modification of two previously reported procedures (Hevel et al., 1991; Schmidt et al., 1991). The 100000*g* supernatant was applied directly to a 2',5'-ADP-Sepharose 4B (Pharmacia) column (3 cm × 1.5 cm) equilibrated with buffer A (10 mM K_2HPO_4, 100 mM NaCl, 0.5 mM L-arginine, 10% glycerol, 5 μM H_4B at pH 7.4). The column was washed with 10 mL of buffer A containing 0.1 mM EDTA and 0.1 mM EGTA. A 20-mL wash followed, consisting of the same components as the previous wash but containing 300 mM NaCl instead of 100 mM NaCl. The column was then rinsed with 20 mL of buffer B (10

mM K_2HPO_4, 100 mM NaCl, 0.5 mM L-arginine, 5 μM H_4B at pH 7.4). B-NOS was eluted with 10 mL of buffer B containing 10 mM NADPH and 2 mM $CaCl_2$. The ADP eluate was applied directly to a calmodulin affinity column (3 cm × 1 cm) equilibrated with buffer B containing 2 mM $CaCl_2$ and prepared as follows. One part Calmodulin Sepharose 4B resin (Pharmacia) was mixed with 1 part Sepharose 4B (Sigma). This gives a column with approximately 500 μg of calmodulin/mL of resin. The column was washed with 30 mL of buffer B containing 2 mM $CaCl_2$. B-NOS was eluted in 10 mL of buffer A containing 5 mM EGTA. The eluate was directly applied to an ultrafiltration cell (Amicon), equipped with a 50-kDa cutoff membrane (Filtron), and concentrated 10-fold, exchanging buffer A with three 10 mL volumes of 50 mM Hepes (pH 7.4) containing 100 mM NaCl, 50 μM L-arginine, 5 μM H_4B, and 20% glycerol. Arginine was left out of all buffers beginning with the calmodulin column if K_m determinations were to be made. The pure enzyme can be frozen at −80 °C in the presence of 5 μM H_4B and 20% glycerol. Glycerol can be left out of the final concentration step if the enzyme is used directly. The same purification procedure was followed for the C415H mutant enzyme.

Nonrecombinant B-NOS was partially purified from 35 rat cerebella. The frozen rat brains were thawed just prior to dissection. The cerebella were dissected from the complete brains and homogenized with a Potter-Elvehjem tissue grinder in 40 mL of a Hepes buffer with protease inhibitors, described above as sonication buffer. The 100000*g* supernatant was prepared as described for the recombinant B-NOS. The supernatant was applied to a 2',5'-ADP-Sepharose 4B column (3 cm × 1.5 cm) equilibrated with buffer C (buffer A containing no arginine). The column was washed with 20 mL of buffer C containing 0.1 mM EDTA and 0.1 mM EGTA. A 30-mL wash followed, consisting of the same components as the previous wash but containing 300 mM NaCl instead of 100 mM NaCl. The column was then rinsed with 10 mL of buffer C. B-NOS was eluted with 10 mL of buffer C containing 10 mM NADPH. The partially purified, nonrecombinant B-NOS was concentrated in an ultrafiltration cell as described for the recombinant B-NOS. The Hepes concentration buffer had no arginine and contained 10% glycerol.

Enzyme Analysis. The protein concentration of the 100000*g* supernatant and throughout the purification process was determined by the Bradford protein assay or Bradford microassay (Bio-Rad) using BSA as the standard.

SDS−PAGE was carried out with a Bio-Rad Mini-PROTEAN II dual slab cell according to the manufacturer's instructions. Proteins were separated using a discontinuous buffer system (Laemmli, 1970) on a 7.5% separating gel and stained either by Coomassie Blue R-250 or by silver stain using the Bio-Rad kit. For Western blot analysis, proteins were transferred to nitrocellulose for 1 h at 250 mA in a Bio-Rad Mini Trans-Blot cell. The blots were blocked overnight with 1% nonfat dry milk (NFDM) in PBS. Primary antibody was diluted 1:4000 in 1% NFDM in PBS and incubated with the blot at room temperature for 2 h. Four washes in PBS of 10 min each were done before incubation of the blot with a 1:200 dilution of goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (Gibco-BRL) for 1 h. The chromogenic substrate 4-chloro-1-naphthol was used to detect B-NOS.

Enzyme activity was assayed throughout the purification by following the conversion of [^{14}C]arginine to [^{14}C]citrulline as previously described (Hevel & Marletta, 1994). Briefly, all assays were initiated with B-NOS (1.5 μg) and contained 100 μM [^{14}C]arginine (3 μCi/μmol), 1 mM $CaCl_2$, 10 μg of calmodulin/mL, 200 μM NADPH, 20 μM H_4B, and 267 μM DTT. All assays were carried out at 37 °C in a final volume of 300 μL in 50 mM Hepes (pH 7.4). After 3 min, the assays were terminated with 10 μL of 6 M trichloroacetic acid.

The activity of B-NOS was also measured with an initial rate assay, which follows the reaction of •NO with oxyhemoglobin to yield methemoglobin using an extinction of 60 000 M^{-1} cm^{-1} for the change in absorbance at 401 nm (Hevel & Marletta, 1994). Assays contained 5 μM oxyhemoglobin, 50 μM L-arginine, 1 mM $CaCl_2$, 20 μg of calmodulin/mL, 120 μM NADPH, 12 μM H_4B, and 167 μM DTT. Reactions were initiated with enzyme and carried out at 37 °C.

Spectral Characterization and Hemochromagen Assay. UV−visible spectral characterization was carried out on a Cary 3E spectrophotometer equipped with a Neslab RTE-100 temperature controller set a 25 °C. The ferrous-CO binding spectrum of B-NOS was obtained as follows. The head-space in a quartz cuvette containing a B-NOS sample at 4 °C was flushed with CO gas for 15 min, followed by reduction with sodium dithionite. Heme content was determined by the hemochromagen assay as described previously (White & Marletta, 1992). To accurately determine the concentration of the myoglobin standards (horse heart myoglobin, Sigma #M-1882), a ferrous deoxy spectrum was taken using an extinction of 121 mM^{-1} cm^{-1} at 434 nm (Adar, 1978).

RESULTS

Mutagenesis of cDNA and Production of Recombinant Virus. To optimize the expression of B-NOS, site-directed mutagenesis was utilized to introduce a complete Kozak sequence immediately 5' of the ATG (Scheme 2). In addition, a *Nhe*I site was added for subcloning purposes. Sequencing of the cDNA fragments which contained the site-directed mutations ensured that additional random mutations had not occurred. The reconstructed cDNA of B-NOS was subcloned into the pVL1393 transfer vector. The utilization of PCR and the overlap extension method of mutagenesis made the production of single-stranded DNA unnecessary and thus sped up the process of making the C415H mutant

The linearized Baculogold baculovirus DNA available from Pharmingen greatly reduces the time necessary to isolate a pure recombinant plaque. This linearized virus contains a lethal mutation [similar to that reported elsewhere (Kitts & Possee, 1993)] which is only suppressed upon homologous recombination with the cotransfected transfer vector, containing the B-NOS cDNA. Thus, greater than 90% of the virus present in the media surrounding the transfected Sf9 cells is recombinant virus. After cotransfection of the Sf9 cells with this modified baculoviral DNA and the pVL1393 vector, only one round of plaque purification was necessary to obtain a pure recombinant plaque. To confirm that the recombinant virus indeed contained the B-NOS cDNA in the correct orientation, PCR primers were made to generate DNA products that overlapped the cDNA/

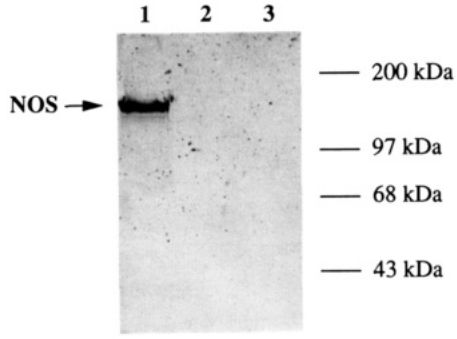


FIGURE 1: Western blot of Sf9 cell extracts. (Lane 1) Sf9 cells infected with recombinant B-NOS virus. (Lane 2) Sf9 cells infected with wild-type baculovirus. (Lane 3) Noninfected Sf9 cells. B-NOS migrates at a monomeric molecular mass of 150 kDa.

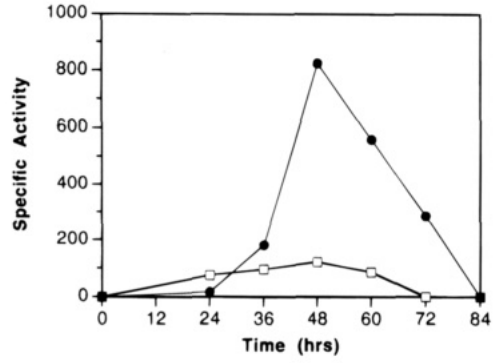


FIGURE 2: Time course of B-NOS enzymatic activity. The hemoglobin assay was used to determine the activity of samples from each culture at the time points designated: hemin supplemented (●) and control supernatant (□). Specific activity is in units of nmol/(mg·h).

viral junctions (O'Reilly et al., 1992). The correct fragment sizes were verified on agarose gels (data not shown). In addition, total cell extracts from infected cells were subjected to Western blot analysis to verify the production of B-NOS relative to control extracts (Figure 1). The only band observable was at the expected 150-kDa position.

Overexpression of B-NOS. In order to obtain consistent levels of expression, a MOI of 5 was necessary. Even though large quantities of B-NOS protein could be detected by Coomassie stain or by Western blot analysis, the enzyme had very little activity. An earlier report indicated that insect cells were deficient in heme biosynthesis and that hemin supplementation was necessary to produce active P450 2A1 (Asseffa et al., 1989). To test the effectiveness of adding hemin to the B-NOS infections, a time course was carried out with two suspension cultures, one with added free hemin at a final concentration of 7 μM and the other with media added as a control. The hemin and media supplements were added to the cultures at 1 h postinfection. Aliquots (25 mL) were removed from each culture every 12 h starting at 24 h, and 100000*g* supernatant was prepared for each sample. The specific activity of each B-NOS aliquot was determined using the hemoglobin assay and plotted as shown in Figure 2. Both cultures, with and without added hemin, showed similar levels of expression by Coomassie stain (data not shown), but the hemin supplemented culture had 7-fold greater activity. The maximum activity over the time course was at 48 h postinfection. After 48 h the insect cells began to lyse; therefore, cytosolic proteins were lost in the media. This loss of B-NOS provides an explanation for theprecipitous drop in the specific activity shown in Figure 2. By using

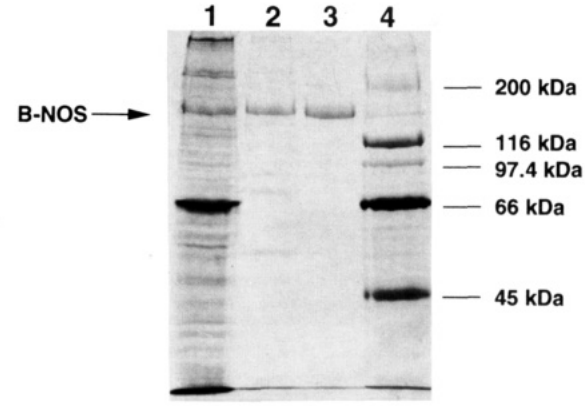


FIGURE 3: Purification of recombinant B-NOS. Shown is a 7.5% SDS−PAGE gel that was silver stained. (Lane 1) 100000*g* supernatant prior to affinity chromatography. (Lane 2) NADPH eluate from the 2',5'-ADP-Sepharose 4B column. (Lane 3) concentrate from the calmodulin Sepharose 4B column. (Lane 4) Molecular weight standards as marked. Recombinant B-NOS migrates at 150 kDa.

the hemin−albumin complex as a carrier of hemin, up to 24 μM hemin could be added without any noticeable toxic effect on the Sf9 cells (data not shown).

While it is easier to grow larger numbers of cells in spinner flasks, the yield of recombinant B-NOS was 6−10-fold higher per cell when the cells were infected in monolayers. The time course of expression in monolayers was similar to that observed in suspension cultures, with peak B-NOS levels occurring between 48 and 60 h. In monolayers, however, cellular lysis did not occur to the extent at which it did in suspension culture. Hemin supplementation had the same effects on enzyme activity as it did in suspension cultures (data not shown).

Purification of Recombinant B-NOS. Most purifications published for either the constitutive or inducible forms of NOS use both an ADP affinity resin and an anion exchange resin. With recombinant B-NOS, the enzyme was essentially pure after elution from the ADP column, as shown in lane 2 of Figure 3. Pure B-NOS was obtained by further chromatography on calmodulin affinity resin (lane 3, Figure 3). To ensure that the B-NOS in the ADP eluate had no calmodulin bound, which would interfere with binding to the calmodulin resin, EGTA and EDTA were added in the first rinse and wash. The ADP elution buffer contained $CaCl_2$ to facilitate the binding of B-NOS to the calmodulin resin. In order to remove the EGTA used in the calmodulin column elution buffer, the buffer was exchanged with 50 mM Hepes (pH 7.4) as the protein was concentrated. Added H_4B and arginine help stabilize the protein and improve the purification yield. Table 1 shows the purification levels achieved with activities reported based on the citrulline assay. At 48−60 h, B-NOS makes up about 10% of the cytosolic fraction, and 1 mg of pure protein can be obtained from seven to ten 75 cm^2 monolayer cultures.

Requirements for NOS Activity. The purified recombinant B-NOS has an absolute requirement for L-arginine, NADPH, Ca^{2+}, and calmodulin (data not shown). When purified in the presence of L-arginine and H_4B, the enzyme shows a small variable dependence on exogenous H_4B in the assay mixture which results in a 10−15% increase in activity. The use of H_4B-free buffer, while concentrating the enzyme after

Table 1: Purification of Recombinant B-NOS

purification step	total units[a]	protein (mg/mL)	specific activity[b] (nmol/(mg·h))	yield (%)
100000*g* supernatant	46800	4.5	1600	100
2′,5′-ADP-Sepharose 4B	32300	0.29	11700	69
concentrate from calmodulin Sepharose 4B	21400	1.2	16200	46

[a] A unit is defined as that amount of enzyme required to produce 1 nmol of •NO per hour. [b] Activity was measured in duplicate by the citrulline assay and represents values obtained from a typical purification.

Table 2: Dependence of B-NOS Activity on H_4B

enzyme preparation	specific activity[a] (nmol/(mg·h))	increase in activity (%)
H_4B-deficient enzyme (no H_4B in assay)	10300	
H_4B-deficient enzyme (with H_4B in assay)[b]	15000	46%
(1 h later)		
H_4B-deficient enzyme (no H_4B in assay)	8700	
H_4B-deficient enzyme (with H_4B in assay)[b]	12600	45%

[a] Activity was measured in duplicate by the citrulline assay and represents a typical enzyme preparation. [b] H_4B was added at a concentration of 20 μM.

the final purification step, generates a pterin-deficient enzyme which shows about a 50% activity increase upon addition of H_4B (Table 2). The instability of pterin-deficient enzyme with time was demonstrated by assaying B-NOS again, 1 h later. The protein was kept at 4 °C between assays. If either FAD or FMN are added back to the assay, an activity increase is observed. This is consistent with fluorescence spectroscopy that showed a 10−20% loss of both FAD and FMN in purified B-NOS (data not shown). When EGTA (5 mM) and calmodulin antagonists [calmidazolium (10 μM), W-7 (50 μM), trifluoperazine (50 μM), and chlorpromazine (50 μM)] were each added to the citrulline assay, a complete loss of enzymatic activity was observed; thus, the dependence of arginine oxidation on Ca^{2+}/calmodulin was confirmed.

In order to quantify the amount of heme bound to the recombinant B-NOS, an alkaline pyridine hemochrome derivative was formed, and the absorbance difference between 556 and 540 nm was used to calculate heme content. The heme-supplemented baculovirus expression system, in conjunction with our reported purification, consistently yields protein containing 0.4−0.6 equiv of heme per monomer, as measured by the pyridine chromophore. To verify that the heme environment of the recombinant B-NOS showed the same optical properties as a P450-type heme, a reduced CO-binding spectra was recorded (Figure 4). The spectrum of B-NOS as isolated has a Soret maximum at 402 nm (spectrum not shown), which shifts to 394 nm in the presence of L-arginine. Finally, the Soret shifts to a wavelength of 444 nm, when the heme iron is reduced in the presence of CO, as is typical of a P450-type heme. This particular preparation of enzyme contained 0.48 equiv of heme per monomer.

An unusual observation was made when experiments were performed to determine the K_m for L-arginine. When the arginine concentration was varied from 1.0 μM to 1 mM, a decrease in the initial velocity of •NO formation by recombinant B-NOS was observed above arginine concentrations of 30 μM (panel A, Figure 5). This inhibition became quite

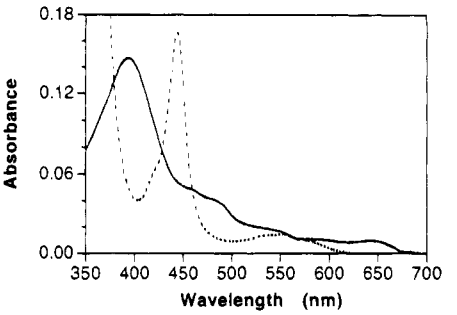


FIGURE 4: Ferric and ferrous-CO spectra of B-NOS. A 500-μL sample of B-NOS contained 240 μg of purified protein in the presence of 50 μM L-arginine and 5 μM H_4B (—). The heme content as measured by the hemochromagen assay was 0.48 equiv of heme per 150-kDa monomer of B-NOS. The reduced CO spectrum (- - -) was obtained as described under Materials and Methods.

substantial between the concentrations of 100 μM and 1 mM arginine. When the data are plotted as the double reciprocal (panel B, Figure 5), a hyperbolic curve is observed, indicating kinetics characteristic of substrate inhibition by L-arginine (Cleland, 1979). Because of this inhibition at higher arginine concentrations, the K_m was calculated using a range of arginine concentrations from 1 to 31 μM. A least-squares analysis using the hyperbolic Michaelis−Menton equation gave a K_m value of 2.0 ± 0.4 μM. A similar pattern of inhibition is also observed with the reaction intermediate N^G-hydroxy-L-arginine (data not shown). A partially purified preparation of B-NOS from rat cerebella was obtained to verify this phenomenon with nonrecombinant B-NOS. This preparation shows the same pattern of substrate inhibition by L-arginine (panels C and D, Figure 5). This type of inhibition seen with B-NOS does not occur with the inducible isoform (M-NOS) isolated from immunostimulated murine macrophages (panel E, Figure 5). The double-reciprocal plot for M-NOS is linear (panel F, Figure 5), confirming previous observations (Hevel et al., 1991).

C415H Mutant B-NOS. The wild-type B-NOS was used to establish the appropriate conditions for overexpression and purification, and those same conditions were used to obtain the C415H mutant. When assayed for •NO production using the initial rate hemoglobin assay, the enzyme exhibited no activity. As can be seen in Figure 6, the absorbance spectrum suggests that no heme is bound to the purified enzyme. No Soret peak is visible in the expected 400-nm region of the spectrum. The absence of heme was verified by using the hemochromagen assay, which was unable to detect any bound heme. In the wild-type spectrum, the Soret has a significant absorbance shoulder from 450 to 500 nm which is due to FAD and FMN in the reductase domain of the enzyme. While the C415H mutant does not exhibit absorbance in the Soret region, it does contain the same absorbance from 450 to 500 nm, representing a similar flavin content as compared with the wild-type enzyme. As is noted in the figure legend, similar concentrations of both wild-type and C415H mutant were used to obtain each spectrum.

DISCUSSION

Given the size and complex redox nature of NOS, it seemed doubtful that *E. coli* would be a productive system to express the holoenzyme. In addition, many bacterial organisms, including *E. coli*, do not utilize or contain the necessary enzymes to synthesize H_4B either *de novo* from

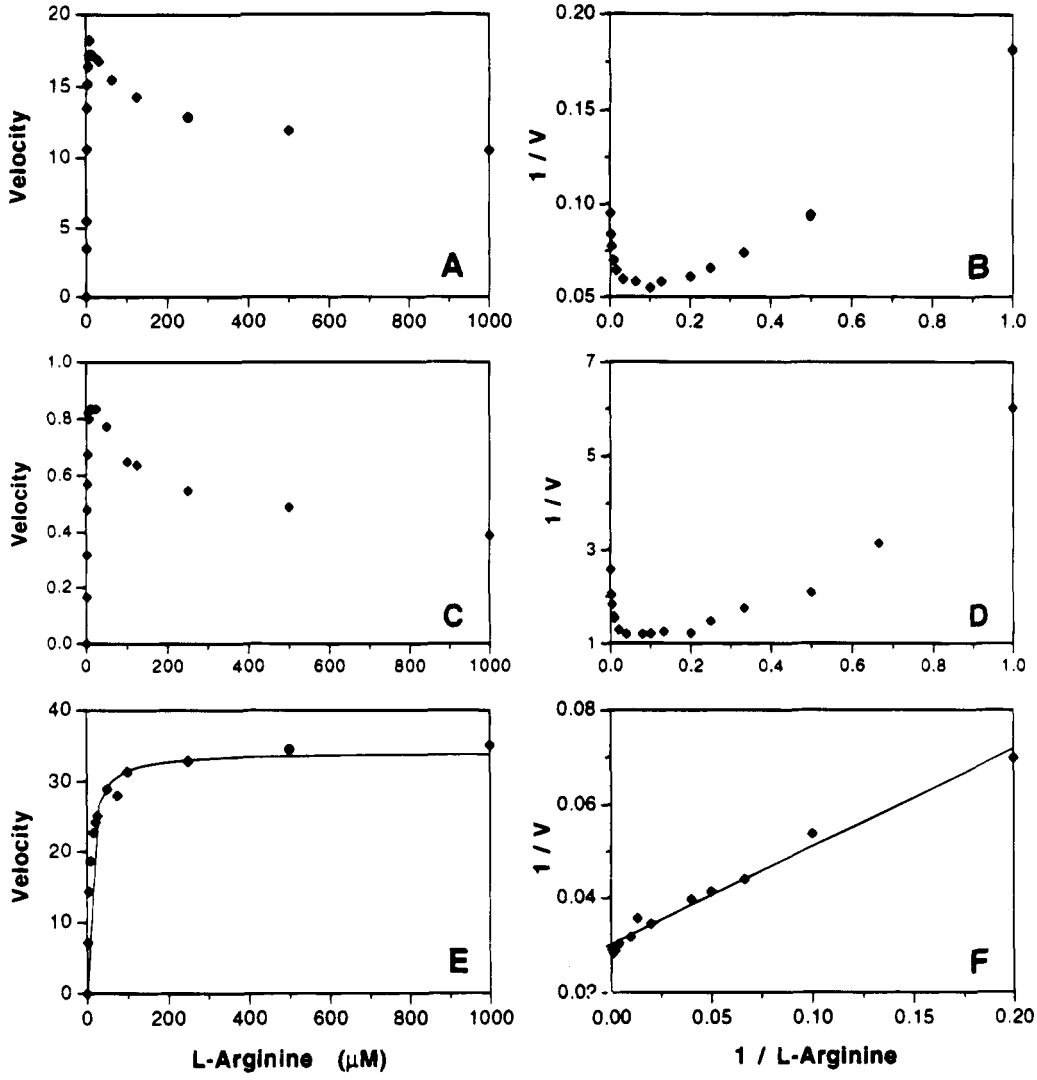


FIGURE 5: Substrate inhibition by L-arginine. Arginine concentrations were varied from 1 μM to 1 mM, and the specific activity of purified B-NOS was determined by the hemoglobin assay and plotted in one of two formats, as velocity [(nmol/(mg·h)) $\times$ 10^{-3}] versus L-arginine concentration or as the double reciprocal. Panels A and B represent data obtained from purified recombinant B-NOS, whereas panels C and D represent data obtained from paritally purified B-NOS from rat cerebella. Panels E and F contain data from purified M-NOS. Kaleidagraph Version 3.0 software was used to fit the data in panels E and F.

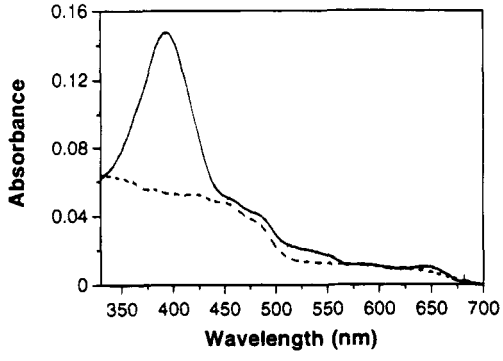


FIGURE 6: Wild-type and C415H mutant spectra of B-NOS. A 3.2 μM sample of purified wild-type B-NOS (—) is compared with a 2.5 μM sample of the purified C415H mutant B-NOS (- - -).

GTP or from precursors later in the biosynthetic pathway. Although the exact function of this relatively tightly bound cofactor has not been established, it is clear that NOS requires H_4B for maximum activity and to maintain the stability of the enzyme (Giovanelli et al., 1991; Hevel & Marletta, 1992; Kwon et al., 1989; Pollock et al., 1991; Tayeh & Marletta, 1989). A baculovirus expression system seemed to be an appropriate choice, given that it is a eucaryotic system in which protein processing and all the necessary cofactors and prosthetic groups should be available. A wide variety of other eucaryotic proteins have been successfully expressed using this system (O'Reilly et al., 1992). Since NOS has been identified in lower eucaryotes, one concern was that Sf9 cells might express an endogenous form of NOS that could potentially contaminate purified preparations of recombinant NOS. Western blot analysis of cell extracts from Sf9 cells infected with the wild-type baculovirus and from Sf9 cells which were not infected demonstrated that no endogenous protein was present which cross-reacted with the B-NOS polyclonal antibody (Figure 1).

There has been very little biochemical investigation of the various insect cell lines which are used to express proteins with baculovirus. This is particularly important in the case of expressing NOS, because of the requirement for the tightly bound prosthetic groups, iron-protoporphyrin IX, H_4B, FMN, and FAD. It has been reported for the expression of heme-requiring proteins that the insect cells were notably deficient in heme biosynthesis (Asseffa et al., 1989; Hartmann & Ortiz de Montellano, 1992). However, the insect cell hosts are

apparently able to take up heme from the media; therefore, this problem can be overcome by adding low concentrations of the desired heme analogue to the medium. The use of albumin as a carrier of heme helps to diminish the toxic effects which free heme has on cells in culture.

Heme supplementation during the infection and expression led to a 7-fold increase in B-NOS activity in the 100000*g* supernatant. Analysis of the heme content of purified recombinant B-NOS was carried out, and, as shown in the spectra in Figure 4, B-NOS displays the expected shift in the Soret to 444 nm upon reduction of the heme in the presence of CO. This unique λ_{max} has been shown to result from a ferrous CO complex with a thiolate as the axial ligand, therefore providing evidence that the heme in this expressed NOS has been incorporated correctly into its binding site. The two column affinity purification reported here consistently yields B-NOS with 0.4−0.6 equiv of heme per 150-kDa monomer of B-NOS. This apparent loss of heme during purification has been observed with several of the cytochrome P450 isoforms (Guengerich, 1979). For example, when the purification of the 119-kDa cytochrome P450 monooxygenase from *Bacillus megaterium* ($P450_{BM-3}$) was reported by Narhi and Fulco (1986), the P450 content of the purified enzyme was 0.55 equiv of heme per mole of protein. $P450_{BM-3}$ and NOS are the only P450s reported which have the reductase domain fused to the heme containing oxygenase domain.

In general, the expression of flavo proteins has not been a problem; however, the pterin-requirement of NOS presents somewhat of a unique problem. Baculovirus has been used for the expression of pterin-dependent hydroxylases such as phenylalanine hydroxylase (Gibbs et al., 1993), but the pterin-dependent hydroxylases utilize H_4B as a cosubstrate and do not require the reduced pterin for stability. HPLC analysis of Sf9 cell supernatant indicated the presence of pterins (unpublished result) and provided another reason for the baculovirus system as an appropriate choice for expression of NOS. The stability of purified recombinant B-NOS was similar to that reported for the enzyme purified from the cerebellum, and therefore suggestive that appropriate amounts of pterin were being supplied by the insect cells. A relatively small activity dependence was observed for the pure enzyme when all of the purification buffers contained H_4B. When H_4B was omitted from the final buffer while concentrating the enzyme, a pterin-deficient enzyme was obtained which lost substantial activity over a period of 1 h at 4 °C (Table 2). When H_4B was added back to the enzyme and assayed, a 50% increase in activity was observed. These observations are similar to what has been seen with other purified NOS isoforms. The pterin is tightly bound but will dissociate leading to enzyme which shows a greater dependence on H_4B for maximal activity and is dramatically less stable. The activity increase observed when adding back H_4B to pterin-deficient enzyme varies from one purification to the next. Experiments with the inducible NOS purified from immunostimulated macrophages clearly demonstrated a relationship between bound pterin and activity in individual preparations of M-NOS (Hevel & Marletta, 1992).

The substrate inhibition observed during experiments to determine the K_m for arginine was completely unexpected, since this type of inhibition has not previously been reported for any NOS isoform. Instead of observing typical saturation kinetics, a substantial decrease in activity was observed with L-arginine concentrations above 50 μM. In order to rule out the possibility that this phenomenon was a peculiar characteristic of the recombinant enzyme, a partially purified preparation of B-NOS from rat cerebella was obtained. The same pattern of inhibition by L-arginine was observed with the nonrecombinant B-NOS. With the M-NOS isoform, this pattern of inhibition is not seen up to concentrations of 3 mM L-arginine (data shown only up to 1 mM).

While the mechanism of this inhibition is at present unknown, it is possible that it might have physiological significance. It has been reported that plasma levels of arginine are in the range of 50−100 μM (Altman, 1961). It is assumed that the intracellular concentration of L-arginine mirrors that found in the plasma, since arginine transport occurs by a facilitated diffusion pathway (White & Christensen, 1982). If these levels are similar in the nervous system, then the observed inhibition would suggest that B-NOS does not normally function at maximum velocity *in vivo*, whereas the inducible isoform would function at maximum velocity. The simplest mechanistic interpretation is the existence of a second arginine binding site. If higher levels of arginine somehow are involved in displacing arginine from the active site, then the substrate-induced spin state change might be influenced at these higher concentrations. Preliminary binding spectra thus far have been inconclusive. Whatever the mechanism, no substrate inhibition is observed with the inducible isoform, purified from immunostimulated murine macrophages. Different analogues of L-arginine have already been shown to inhibit B-NOS and M-NOS with varying affinities and by apparently different mechanisms. For example, N^G-nitro-L-arginine has been shown to inhibit B-NOS with characteristics of a slow-onset type inhibitor (Furfine et al., 1993; Klatt et al., 1994). The apparent irreversible inactivation appears to be in part due to a slow off rate of this arginine analogue. On the other hand, N^G-nitro-L-arginine is a freely reversible inhibitor of M-NOS (Furfine et al., 1993). The substrate inhibition seen with B-NOS further suggests that there are distinct differences by which the constitutive and inducible isoforms bind and process L-arginine and arginine analogues.

In an effort to learn more about the active site of NOS, and more specifically where the heme interacts with the enzyme, the cDNA was modified to change the cysteine at position 415 to a histidine. The heme was originally characterized (White & Marletta, 1992; McMillan et al., 1992) to be a P450-type heme, on the basis of the shift in the Soret to approximately 450 nm when the heme is reduced in the presence of CO. The relationship of NOS to the P450 supergene family is not obvious since the P450 consensus sequence for heme binding that includes the thiolate ligand to the iron (F x x G x x x $\underline{\underline{C}}$ x G) is not present in any of the reported NOS sequences (Nelson et al., 1993). Interestingly, several other proteins that show the optical properties of the P450s but lack the consensus sequence catalyze atypical P450 reactions. These enzymes include chloroperoxidase, thromboxane synthase, and allene oxide synthase. The N-terminal heme domain portion of NOS contains seven invariant cysteines which can be identified by aligning all of the NOS's that are currently in the GenBank database. Of these seven cysteines, C415 and C672 are attractive candidates based on the limited homology to the P450 consensus sequence (McMillan et al., 1992; White & Marletta, 1993) and further

speculation based on computer modeling studies (Renaud et al., 1993).

The mutation of a thiolate cysteine ligand to a histidine residue was designed to generate an NOS mutant which still bound stoichiometric quantities of heme with the ability to reversibly bind molecular oxygen. Most likely, the bound oxygen ferrous iron complex would be not be easily reduced, and therefore catalysis of L-arginine to nitric oxide and citrulline would not occur. The C415H mutant should also demonstrate a Soret λ_{max} at approximately 420 nm in the presence of CO. As is evident from the C415H mutant spectrum in Figure 6, however, no Soret peak was present even though the enzyme appears to still have the flavins bound. A pyridine hemochromagen assay confirmed that no heme was bound to the purified C415H mutant. In addition, the mutant enzyme had no detectable activity as measured by the hemoglogin assay. If C415 is indeed the thiolate ligand to iron, it is quite possible that the heme binding site cannot accommodate the sterically larger imidazole side chain of the histidine, and the heme moiety can no longer bind. Other mutants at position 415 using residues such as a serine or alanine may be useful in helping to further confirm the identification of C415 as the thiolate ligand in B-NOS. C415 corresponds to C194 in the M-NOS and to C186 in the bovine endothelial NOS (Lamas et al., 1992; Nishida et al., 1992).

Similar thiolate-ligand mutants of several P450s have also failed to bind heme properly. Attempts have been made to characterize a histidine substitution for the coordinating cysteine in cytochrome $P450_{cam}$ from *Pseudomonas putida* (Sligar et al., 1991) and in cytochrome P450 1A2 from rat liver (Shimizu et al., 1988). In the $P450_{cam}$ mutant, the enzyme was isolated as the apoprotein and reconstituted with heme. The reconstituted enzyme had no camphor hydroxylation activity, yet did have a reduced CO-dithionite difference spectrum with a $\lambda_{max} = 420$ nm. In the rat liver P450 1A2, expressed in yeast, crude extracts were reduced with dithionite in the presence of CO and the difference spectrum contained no absorbance at 450 nm and very little absorbance at 420 nm. In both cases, care must be taken in interpreting the results, because no heme stoichiometry was reported; thus, heme associated with either mutant is not necessarily bound correctly and the absorbance at 420 nm could represent nonspecific heme binding, especially in the crude yeast extracts.

In summary, we have developed an overexpression system utilizing baculovirus as a vector. Given the conditions described throughout this report, we can express B-NOS to a level of 10% of the total soluble protein in Sf9 cells infected at an MOI of 5. Hemin supplementation enables us to harvest and purify active, wild-type enzyme. All other prosthetic groups are present in adequate amounts such that additional media supplements do not appear to be necessary. This system will enable us to investigate further the differences between B-NOS and M-NOS, which are demonstrated by the previously mentioned studies with arginine analogues and by the substrate inhibition of B-NOS reported here. The C415H mutant enzyme, and its inability to bind heme, is the first experimantal evidence to suggest that cysteine 415 provides the thiolate ligand to the heme. This supports the tentative assignment made as a result of sequence comparisons done by our laboratory as well as by others. In addition to the C415H mutant, the baculovirus system will enable us to make other site-directed mutants to probe structure/function relationships of this novel enzyme and to bring biophysical methods to bear on remaining mechanistic questions.

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