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# High-level expression of functional rat neuronal nitric oxide synthase in *Escherichia coli*

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**ABSTRACT** **The neuronal nitric oxide synthase (nNOS) has been successfully overexpressed in *Escherichia coli*, with average yields of 125–150 nmol (20–24 mg) of enzyme per liter of cells. The cDNA for nNOS was subcloned into the pCW vector under the control of the *tac* promotor and was coexpressed with the chaperonins groEL and groES in the protease-deficient BL21 strain of *E. coli*. The enzyme produced is replete with heme and flavins and, after overnight incubation with tetrahydrobiopterin, contains 0.7 pmol of tetrahydrobiopterin per pmol of nNOS. nNOS is isolated as a predominantly high-spin heme protein and demonstrates spectral properties that are identical to those of nNOS isolated from stably transfected human kidney 293 cells. It binds $N^{\omega}$-nitroarginine dependent on the presence of bound tetrahydrobiopterin and exhibits a $K_d$ of 45 nM. The enzyme is completely functional; the specific activity is 450 nmol/min per mg. This overexpression system will be extremely useful for rapid, inexpensive preparation of large amounts of active nNOS for use in mechanistic and structure/function studies, as well as for drug design and development.**

Nitric oxide synthase (NOS) catalyzes the formation of NO· and L-citrulline from L-arginine through a series of oxidations using molecular oxygen (1). There are three separate genes known to encode the NOS family of proteins, including the constitutively expressed neuronal (nNOS) (2) and endothelial cell (ecNOS) (3, 4) isoforms and the inducible isoform (iNOS) (5, 6). The product of the NADPH-mediated reaction, NO·, has been implicated in neurotransmission in the brain and in neuromuscular junctions (nNOS), hemodynamic regulation (ecNOS), and cytotoxicity (iNOS). The effects of NO· produced by the nNOS and ecNOS are thought to be mediated through stimulation of guanylate cyclase activity, whereas the NO· produced by iNOS appears to act directly or via peroxynitrite on foreign cells (for reviews, see refs. 7 and 8).

The three isoforms differ in primary sequence, having only 50–60% sequence identity (9), size, intracellular location, and regulation. nNOS (160 kDa) and iNOS (130 kDa) were purified from the cytosol (8, 10–12), whereas the ecNOS (135 kDa) was found to be membrane-bound (13). The nNOS and ecNOS are constitutively expressed but modulated by intracellular $Ca^{2+}$ levels (1, 14), unlike iNOS, which is induced by bacterial endotoxin and is $Ca^{2+}$-independent (15). All three isoforms bind calmodulin and tetrahydrobiopterin ($BH_4$), as well as molar ratios of heme, FMN, and FAD (11–13, 16–20). These members of the NOS family are the only mammalian enzymes that catalyze both hydroxylation and NADPH reduction of flavins within the same protein, an attribute shared only by the *Bacillus megaterium* enzyme, cytochrome $P450_{BM-3}$ (21).

Mechanistic and structure/function studies of nNOS had initially been very difficult due to the minute amounts of protein that can be purified from cerebellar tissue. Bredt and Snyder (1), who initially isolated nNOS, reported a yield of 9 μg of pure protein from 18 rat brains. Bredt *et al.* (2) subsequently cloned and expressed nNOS in human kidney 293 cells, providing a 10-fold enrichment of nNOS in cultured cells over rat brain. Twelve liters of these cultured cells consistently yield 8–10 mg of nNOS (K. McMillan and B.S.S.M., unpublished observation). Although this is a significant improvement over initial yields, the expense and time involved in mammalian cell culture are extensive. Other laboratories have expressed nNOS using baculovirus overexpression systems. Charles *et al.* (22) report successful expression, but the majority of their recombinant nNOS is insoluble and inactive; the recombinant enzyme has a specific activity that is 100-fold lower than that of native nNOS isolated from rat cerebellar tissue. Richards and Marletta (23) improved the yield of active enzyme from the baculovirus system by adding hemin to the medium but still can isolate only ≈1 mg of pure protein from 7–10 75-$cm^2$ monolayer cultures, only about half of which contains heme.

In this paper, we report the overexpression of active nNOS in *Escherichia coli*. Expression was directed under the *tac* promoter of the $pCW_{ori+}$ vector, a system that has been instrumental in the expression of cytochromes P450 (24, 25). This vector was chosen to promote the proper insertion of heme, as occurs for *E. coli*-expressed cytochromes P450, in an effort to abate a major drawback of the baculoviral system, poor heme incorporation. Because initial experiments were plagued by highly proteolyzed and dysfunctional protein, an expression plasmid for the chaperonins groEL and groES (26) was also included.

## MATERIALS AND METHODS

**Chemicals.** L-[2,3-$^3$H]Arginine was obtained from DuPont/NEN, and $BH_4$ was from Research Biochemicals (Natick, MA). All other chemicals were obtained from Sigma and were of the highest grade available.

**Enzymes.** *Taq* polymerase, ligase, and restriction enzymes were purchased from either Promega or New England Biolabs. Shrimp alkaline phosphatase was from United States Biochemical.

**Plasmids.** pNOS (2), containing the rat nNOS cDNA in pBluescript SK(−), was provided by Solomon Snyder and David Bredt at Johns Hopkins Medical School, Baltimore. pGroESL (27), containing groEL and groES cDNAs, was from

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Abbreviations: NOS, nitric oxide synthase; nNOS, neuronal isoform of NOS; ecNOS, endothelial cell isoform of NOS; iNOS, inducible macrophage isoform of NOS; $BH_4$, tetrahydrobiopterin; NNA, $N^{\omega}$-nitro-L-arginine; NMA, *N*-methyl-L-arginine.

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Paul Horowitz at The University of Texas Health Science Center at San Antonio. $pCW_{ori+}$ (28) was provided by Michael Waterman at Vanderbilt University, Nashville, TN.

**Recombinant DNA Manipulations.** nNOSpCW, the plasmid for the expression of nNOS in *E. coli*, was constructed as follows. The initial 1210 nt of pNOS (from the ATG start codon to the *Nar* I restriction site) were amplified by PCR to incorporate the recognition sequence for *Nde* I. Primer 1 (upstream primer, with *Nde* I site) was 5′-TCATCATCATATGGCTGAAGAGAACACGTT-3′, and primer 2 (return primer) was 5′-CATGCTTGGCGCCAT-3′. Primers were synthesized by the Center for Advanced DNA Technologies at The University of Texas Health Science Center at San Antonio. Reaction mixtures included 50 pmol of each primer, 20 ng of pNOS template, 200 μM dNTPs, 1.5 mM $MgCl_2$, 1× *Taq* polymerase buffer (50 mM KCl/10 mM Tris·HCl, pH 9.0/0.1% Triton X-100), and 2.5 units of *Taq* polymerase in 100-μl total volume. The mixture was preincubated for 3 min at 94°C before the addition of *Taq* polymerase, followed by amplification for 30 cycles: 94°C for 30 s, 55°C for 60 s, and 72°C for 90 s. The PCR product was gel-purified using the GeneClean II kit (Bio 101) and digested with *Nde* I and *Nar* I. pNOS DNA was then restricted with *Nar* I and *Xba* I to generate the remaining 3529 nt of the NOS cDNA sequence, which was also gel-purified. $pCW_{ori+}$ DNA was digested with *Nde* I and *Xba* I, and the ends were dephosphorylated. The three pieces were ligated, and the resultant products were used to transform *E. coli.* JM109 competent cells were purchased from Stratagene and transformed by using the manufacturer's instructions.

The transformation mixture was plated on LB agar containing ampicillin at 50 μg/ml, and nine colonies were screened by *Bam*HI restriction digest of alkaline lysis plasmid minipreparations. Five positive clones were further screened for isopropyl β-D-thiogalactoside-induced (0.5 mM, added at $OD_{600}$ = 0.8, along with 225 μM δ-aminolevulinic acid) expression of nNOS at 37°C by immunoblot analysis of whole cells using rabbit anti-rat nNOS IgG. All five clones exhibited bands that comigrated with that of nNOS isolated from kidney 293 cells.

In subsequent manipulations, when pGroELS was cotransformed with nNOSpCW, transformants were plated on LB agar containing ampicillin at 50 μg/ml and chloramphenicol at 35 μg/ml. Due to severe proteolysis of nNOS when JM109 cells were lysed, both plasmids were also cotransformed into the protease-deficient *E. coli* strain BL21. Transformation of BL21 was via electroporation using an Invitrogen electroporator II according to manufacturer's instructions.

**Protein Expression.** Fernbach flasks containing 1 liter of modified Terrific Broth (20 g of yeast extract, 10 g of bactotryptone, 2.65 g of $KH_2PO_4$, 4.33 g of $Na_2HPO_4$, and 4 ml of glycerol) and ampicillin at 50 μg/ml and, when pGroELS was present, chloramphenicol at 35 μg/ml were inoculated with 1 ml of an overnight culture (grown in LB plus antibiotics) and shaken at 250 rpm at 37°C. Protein expression was induced at $OD_{600}$ = 1.0–1.4 with the addition of isopropyl β-D-thiogalactoside to 0.5 mM. The heme and flavin precursors, δ-aminolevulinic acid and riboflavin, were also added to final concentrations of 450 μM and 3 μM, respectively. When pGroELS was present, the culture medium also contained 1 mM ATP. The flasks were moved to room temperature (25°C) and shaken in the dark at 250 rpm. The cells were harvested at ≈40 hr after induction, and the cell paste was frozen at −80°C until purification.

JM109 cells containing pNOSpCW grown after induction at 37°C exhibit no detectable peak at 445 nm in CO difference spectra; all protein is present as a 420-nm species. JM109 cells containing pNOSpCW grown at 25°C for 36–40 hr after induction have both 445- and 420-nm species; this protein is heavily proteolyzed upon cell lysis, as judged by immunoblot analysis. The absolute amount of protein production in JM109 cells is enhanced 5- to 10-fold upon coexpression of nNOS with groEL and groES, but this protein is still heavily proteolyzed upon cell lysis. A small amount of activity (1% that of nNOS purified from 293 cells, data not shown) can be detected. BL21 cells containing pNOSpCW do not appear to express nNOS as detectable by CO difference spectra. BL21 cells containing both pNOSpCW and pGroELS, however, produce 125–150 nmol of nNOS (20–24 mg) per liter of culture, as quantitated by CO difference spectra. All data presented in this paper are derived using protein purified from BL21 that has been cotransformed with both pNOSpCW and pGroELS.

**Protein Purification.** Harvested cells were resuspended in 30 ml of resuspension buffer (100 mM Tris·HCl, pH 7.4/1 mM EDTA/1 mM dithiothreitol/10% (vol/vol) glycerol/1 mM phenylmethylsulfonyl fluoride/leupeptin at 5 μg/ml/pepstatin at 5 μg/ml per liter of initial culture and were lysed by pulsed sonication (4 min, 80% power, large probe, Fisher Scientific model 550). Cell debris was removed by centrifugation at 150,000 × *g* for 70 min. The supernatant was applied to a 2′,5′-ADP-Sepharose 4B column (6 ml, Pharmacia) equilibrated in buffer B (50 mM Tris·HCl, pH 7.4/0.1 mM EDTA/0.1 mM dithiothreitol/10% glycerol/100 mM NaCl). The column was extensively washed with at least 10 column volumes of buffer B and then washed again with buffer B/500 mM NaCl. The protein was eluted with buffer B/500 mM NaCl/5 mM 2′-AMP. The colored fractions were pooled and concentrated (Centriprep 30, Amicon), and L-arginine and $BH_4$ were added to final concentrations of 2 mM and 1 mM, respectively. This fraction was incubated overnight at 4°C and applied to a S-200 gel filtration column (480 ml, 2.5-cm diameter, Pharmacia) equilibrated in buffer B. The nNOS-containing fractions were pooled, concentrated, and stored at −80°C. All manipulations were done at 4°C. The cytosolic extract contains 125–150 nmol of nNOS (100% yield), as determined by CO difference spectra, the 2′,5′-ADP-Sepharose 4B column pool contains 50–100 nmol of enzyme (≈55% yield), and the S-200 column pool contains 25–45 nmol of enzyme (≈30%). Enzymatic activity, as measured by the conversion of L-arginine to L-citrulline (see below), parallels the heme content. Optimization of this purification procedure may further increase these yields. The ratio of heme to FMN content was determined to be 1:1. The concentrations of the flavins were determined by using the method of Faeder and Siegel (29).

Although a similar procedure used to isolate nNOS from kidney 293 cells yields enzyme that is ≥90% pure, the enzyme isolated from *E. coli* is ≈70% pure, with only one other major contaminant (Fig. 1, lane 5) which, based on immunoblot analysis, is not a proteolytic fragment of nNOS. The spectra and activities presented in this paper were generated using this preparation of enzyme. If, instead of the S-200 gel filtration column, the enzyme is applied to a Mono Q ion-exchange fast protein liquid chromatography column (Pharmacia) in buffer

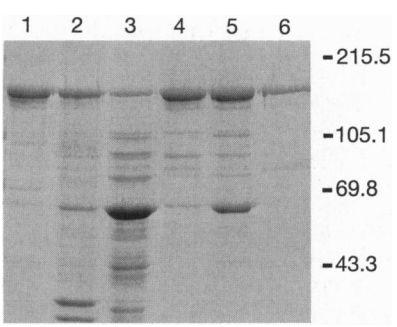


FIG. 1. SDS/PAGE analysis of nNOS during purification. A Coomassie G-250-stained 7.5% polyacrylamide gel is shown. Lanes: 1, 3.5 ng of human kidney 293 cell-expressed nNOS; 2, 15 ng of resuspended membrane fraction; 3, 25 ng of cytosolic extract; 4, 5 ng of 2′,5′-ADP-Sepharose fraction; 5, 5 ng of S-200 column fraction; and 6, 2.5 ng of Mono Q column fraction.

B and eluted at ≈200 mM NaCl in a 0–500 mM NaCl gradient, this major contaminant is eliminated (Fig. 1, lane 6).

**Spectrophotometric Methods.** Absolute spectra and CO difference spectra were done essentially as described by McMillan and Masters (30) except that all measurements were done in buffer B, and the CO difference spectra were obtained by reducing the protein and then bubbling the sample cuvette with CO. Substrate perturbation difference spectra were done as described (30), but in the presence of 1 mM imidazole to shift the entire population of nNOS to the low-spin heme state. The molar protein concentration was determined based on heme content and $\Delta\varepsilon_{444-475} = 75\ mM^{-1}$ (18, 31). All spectral analyses were performed by using a Shimadzu model 2101 UV/visible dual-beam spectrophotometer.

**Measurement of NO· Formation.** Nitric oxide formation was measured by using both the hemoglobin capture assay (32), done at 25°C as described by Sheta *et al.* (33) and the method of Bredt and Snyder (1), which monitors the formation of L-[$^3$H]citrulline from L-[$^3$H]arginine, as described (19). Each reaction mixture, containing 0.5 μg of enzyme, was incubated at 25°C for 2 min (over which time the reaction is linear). For $K_m$ analysis, the concentration of L-[$^3$H]arginine in the reaction mixture was varied over the range of 2.0–10.0 μM.

**Pterin Analysis.** Determination of pterin content was done as described by Gross and Levi (34), based on the method of Fukushima and Nixon (35), by acid hydrolysis of a 10-ng protein sample followed by quantitation of pterin by $C_{18}$ reverse-phase HPLC.

**Determination of $N^{\omega}$-nitro-L-arginine (NNA)-Binding Constant.** The NNA-binding constant was determined by direct titration of purified nNOS with [$^3$H]NNA. In these experiments, done in 96-well poly(vinylidene difluoride) plates in 100-μl total volume, 10 pmol of nNOS and radiolabeled NNA (specific activity ≈23,000 dpm/pmol) were incubated at room temperature for 15 min in 50 mM Tris·HCl, pH 7.6/1 mM dithiothreitol, in the presence or absence of 10 μM $BH_4$. Assays were also done in the presence or absence of 100 μM *N*-methyl-L-arginine (NMA), a potent inhibitor of L-arginine binding. The incubation was stopped by aspiration of the sample through the poly(vinylidene difluoride) membrane. The wells were washed twice with 200 μl of 50 mM Tris, pH 7.6, and air-dried for 10 min; 25 μl of scintillation cocktail was added, and radioactivity of samples was counted.

**Determination of Heme Content.** The heme content of the purified protein preparation was measured by CO difference spectra (described above) and by the pyridine hemochromogen method (36). A 30-μl aliquot of pyridine was added to 70 μl of purified protein, along with 1.5 μl of 10 M NaOH. The sample was reduced with two grains of dithionite, and the spectrum was read after 2 min. Heme concentration was determined by the absorbance at 556 nm, assuming $\varepsilon = 34\ mM^{-1}$.

## RESULTS

**Spectral Characteristics of Purified *E. coli*-Expressed Enzyme.** Fig. 2 shows the absolute spectrum of nNOS isolated from *E. coli*. It exhibits a broad peak at 400 nm and secondary maxima at 550 and 650 nm, indicative of a predominantly high-spin heme, although some low-spin form is present, as evidenced by the shoulder at 410 nm. Shoulders are also apparent at 450 and 475 nm and are due to flavin absorbance; this spectrum is identical to that of nNOS isolated from human kidney 293 cells (19). As shown in Fig. 2, the maximum heme absorbance at 400 nm can be shifted to the low-spin form (peak at 428 nm) by the addition of imidazole to 1 mM or completely to the high-spin form (peak at 395 nm) by the addition of arginine to 2 μM.

Fig. 2 *Inset* also shows the characteristic cytochrome P450-like CO difference spectrum of nNOS with a peak at 444 nm. The molar concentration of heme-containing enzyme, calculated from the peak at 444 nm, assuming $\Delta\varepsilon_{444-475} = 75\ mM^{-1}$, is 1.9 μM. For comparison, a pyridine hemochromogen was also done to determine the heme concentration (data not shown); this method yielded a concentration of 2.1 μM, in reasonable agreement with that calculated from the CO difference spectrum.

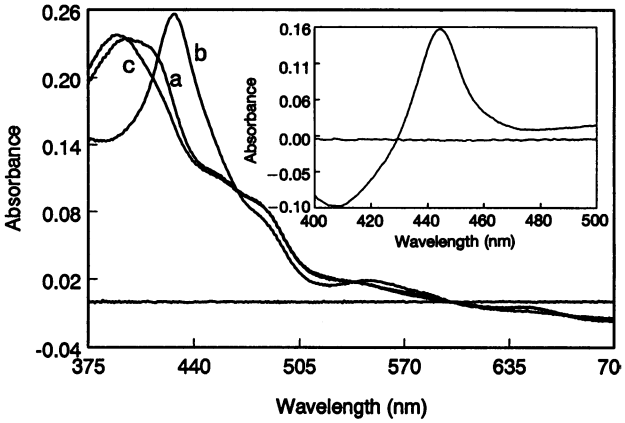


FIG. 2. Absolute absorbance and CO difference spectra of nNOS as purified from *E. coli*. Experiments were done as described using 3.2 μM nNOS. Curve: a, unperturbed spectrum of purified nNOS; b, spectrum after addition of 1 mM imidazole to nNOS; and c, spectrum after addition of 2 μM L-arginine to nNOS. (*Inset*) CO difference spectra, using 1.9 μM nNOS.

With the technique of difference spectrophotometry, the perturbation of the heme spectrum by increments of L-arginine, in the presence of 1 mM imidazole, was measured (Fig. 3). A type I spectrum, characterized by a maximum at ≈390 nm and a minimum at ≈430 nm, was observed. A spectral binding constant ($K_s$) was calculated from the apparent $K_s$, derived from a plot of 1/absorbance change vs. 1/[L-arginine] (Fig. 3 *Inset*), using the following equation:

$$\text{apparent } K_s = K_s(1 + [\text{imidazole}]/K_{d\ \text{imidazole}}).$$

Assuming $K_{d\ \text{imidazole}}$ to be 160 μM (30, 37), the value of $K_s$ for arginine binding to nNOS is 717 nM.

**Pterin Analysis of Purified *E. coli*-Expressed Enzyme.** Pterin content was determined in two different samples of nNOS: (*i*) partially purified enzyme before $BH_4$ incubation (fraction 1; pre-S-200 column chromatography); (*ii*) purified enzyme after $BH_4$ incubation (fraction 2; post-S-200 column chromatography). The analysis reveals that, as isolated, fraction

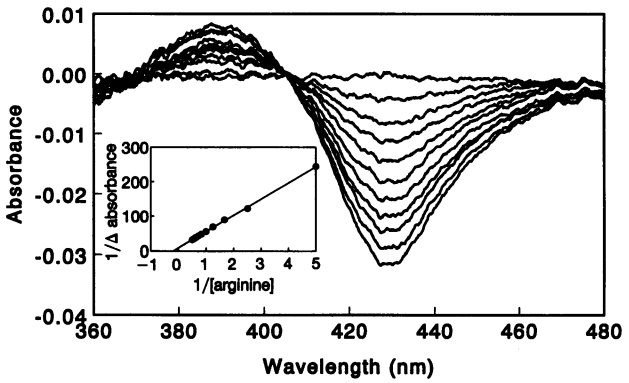


FIG. 3. Substrate perturbation difference spectra of *E. coli*-expressed nNOS. Experiments were done as described by using 1.5 μM nNOS in the presence of 1 mM imidazole. Purified enzyme was titrated with L-arginine to final concentrations of 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0 μM (baseline and sequential minima, respectively). (*Inset*) Plot from which the apparent $K_s$ is derived.

1 contains 0.096 pmol of $BH_4$ per pmol of nNOS—i.e., 10% of the expressed nNOS contains $BH_4$ (data not shown). No other pterin moiety was present. This is in contrast to the nNOS heme domain expressed in *E. coli* JM109, which contained only 1–2% $BH_4$, as well as being ≈30% saturated with non-$BH_4$ pterin (K. McMillan, S.S.G., and B.S.S.M., unpublished observation). Fraction 2 was complemented with 0.636 pmol of $BH_4$ per pmol of nNOS—i.e., 64% saturated (data not shown). Thus, if stoichiometric binding of $BH_4$ is required for activity, this preparation of nNOS contains 64% active enzyme.

**Binding of NNA to Purified *E. coli*-Expressed Enzyme.** The binding of [$^3$H]NNA as a function of ligand concentration was determined in the presence and absence of additional $BH_4$ for both fractions 1 (pre-$BH_4$) and 2 (post-$BH_4$). Fig. 4 shows that NNA binds significantly to *E. coli*-expressed nNOS fraction 1 (circles) only in the presence of added $BH_4$. The binding constant ($K_d$) and the maximum amount of NNA bound ($B_{max}$) differ greatly depending on whether or not $BH_4$ is added (*Inset*). Fraction 2 (triangles), which is 65% $BH_4$-saturated, binds NNA equally well in the presence or absence of added $BH_4$; $K_d$ and $B_{max}$ are the same, regardless of whether $BH_4$ is added or not. In the presence of 100 μM *N*-methyl-L-arginine (L-NMA), binding of NNA is essentially abolished in either fraction (data not shown). Thus, NNA binding depends on the presence of bound $BH_4$ and the $K_d$ for NNA binding is ≈45 nM. In addition, fraction 1 enzyme can be reconstituted with $BH_4$ up to 54%, and fraction 2 enzyme does not bind additional $BH_4$—i.e., it appears to be maximally complemented.

**Enzymatic Activity of Purified *E. coli*-Expressed Enzyme.** The conversion of L-arginine to L-citrulline was assayed for fractions 1 (pre-$BH_4$) and 2 (post-$BH_4$) in the presence and absence of additional $BH_4$. The turnover numbers for fraction 1 were 75 and 202 nmol/min per mg without and with $BH_4$ in the assay mixture, respectively, a stimulation of 2.7-fold. The turnover numbers for fraction 2 were 189 and 435 nmol/min per mg without and with $BH_4$ in the assay mixture, respectively, a stimulation of 2.3-fold. The enzymatic activity is inhibited by 95% by 100 μM NMA in all cases; this is consistent with the inhibition of NNA binding by NMA. The turnover numbers for both fractions in the presence of $BH_4$ were confirmed by using the hemoglobin capture assay; the activities of fractions 1 and 2 were 239 and 468 nmol/min per mg, respectively, demonstrating excellent agreement between the two methods. These turnover numbers are very similar to those obtained with nNOS purified from human kidney 293 cells in which activities between 300 and 450 nmol/min per mg are typically observed.

The $K_m$ value for L-arginine was determined to be 2.8 μM for *E. coli*-purified nNOS (data not shown). Concomitant measurement using human kidney 293 cell-purified nNOS yielded a $K_m$ value of 1.9 μM. These values are in excellent agreement with each other and with the $K_m$ value of 2 μM for nNOS reported by Bredt and Snyder (1) and McMillan *et al.* (19).

## DISCUSSION

We have developed a method for the overexpression of nNOS in *E. coli*, a system that offers a quick and inexpensive way to produce large quantities of active enzyme. We can produce 125–150 nmol (20–24 mg) of nNOS per liter of *E. coli* culture. The three elements composing this successful method are the vector used (pCW), the coexpression of chaperonins with nNOS, and the *E. coli* strain in which the proteins are expressed (BL21). It is intriguing that a simple prokaryotic system such as *E. coli* has the ability to overexpress as complex a mammalian enzyme as nNOS, which contains protoporphyrin IX heme, FAD, FMN, and $BH_4$ as prosthetic groups. The lack of calmodulin produced in this system is an advantage in that the nNOS is not activated to produce cytotoxic NO·.

The production of nNOS is controlled by the *tac* promoter of pCW, an expression vector chosen because it has proved to be invaluable for the expression of cytochromes P450 (24, 25), a family of enzymes to which the N-terminal domain of nNOS has been compared, as well as cytochrome P450/NADPH-cytochrome P450 reductase fusion proteins (38, 39), chimeric constructs that mimic the activities of hydroxylase and reductase domains in a single protein. The heme moiety appears to be inserted correctly into the majority of expressed nNOS protein, as judged by the absolute and CO difference spectra and the spectral perturbation by the substrate L-arginine. The lack of heme repletion, which seems to be a major drawback of the expression of NOS in a baculovirus system (22, 23), is overcome by expression in *E. coli*.

In the current report, nNOS is coexpressed with the *E. coli* groE molecular chaperonin system (groEL and groES). In the absence of these proteins, expression of nNOS is much lower in *E. coli* strain JM109 and undetectable in *E. coli* BL21. Chaperonins facilitate the proper folding of some proteins, probably by inhibiting aggregation and/or by alleviating kinetic blocks to folding, and have made possible the expression

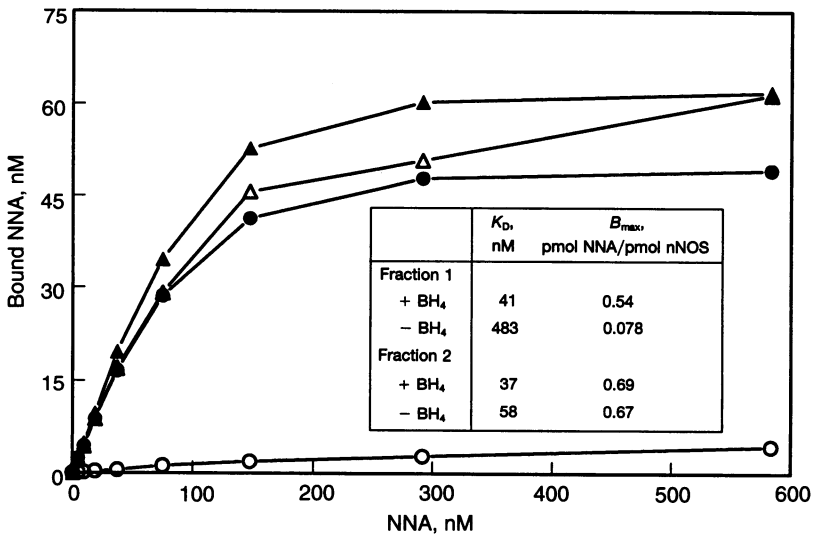


FIG. 4. Binding of [$^3$H]NNA to *E. coli*-expressed nNOS. Experiments were done as described using 10 pmol of nNOS. ○ and ●, Semipurified *E. coli*-expressed nNOS that has been passed over the 2′,5′-ADP-Sepharose 4B column but has not yet been incubated with $BH_4$ during purification (fraction 1); △ and ▲, purified *E. coli*-expressed nNOS that had been incubated with $BH_4$ during purification as described (fraction 2); ○ and △, assays done in the absence of additional $BH_4$; ● and ▲, assays done in the presence of 10 μM $BH_4$. (*Inset*) Table of binding data described by curves.

of several mammalian proteins in *E. coli* that did not express otherwise (26, 40). The observation that substantially more nNOS is produced in the presence of chaperonins further demonstrates the usefulness of this approach.

*E. coli* strain JM109 was initially chosen for the expression of nNOS but, upon lysis of the cells, the calmodulin-binding site proved extremely susceptible to proteolytic attack. As a result of this sensitivity, large amounts of the proteolytically produced domains of *E. coli*-expressed nNOS were initially purified, a problem that was alleviated by coexpressing the nNOS and chaperonins in BL21, a strain reported to be lacking both *lon* and *ompT* proteases.

The nNOS enzyme produced by *E. coli* appears indistinguishable, in all respects examined, from that produced by nNOS stably transfected human kidney 293 cells; the absolute spectrum and its perturbation by the substrate L-arginine (spectral binding constant ≈717 nM), the CO difference spectrum, and the specific activity of *E. coli*-expressed nNOS and mammalian cell-expressed nNOS are identical. The binding of [$^3$H]NNA by nNOS depends on the presence of $BH_4$, exhibits a binding constant of ≈45 nM, and saturates at ≈1 nmol of [$^3$H]NNA/1 nmol of enzyme. Klatt *et al.* (41) report a $K_d$ of 170 nM for [$^3$H]NNA binding to nNOS and indicate that binding was not dependent on $BH_4$. This small difference in $K_d$ values may depend on the specific assay used or the degree of $BH_4$ repletion, although the enzyme used by Klatt *et al.* appears to be $BH_4$-replete based on their observation that the addition of $BH_4$ had no effect on [$^3$H]NNA binding.

The *E. coli*-expressed nNOS is extremely active, with a turnover of 450 nmol/min per mg of NOS, as measured by both the hemoglobin capture assay and the conversion of L-[$^3$H]arginine to L-[$^3$H]citrulline, and this activity is 95% inhibited by 100 μM NMA, indicating that NMA is effectively competing at the substrate-binding site. The $K_m$ value of the enzyme for L-arginine is 2.8 μM, in good agreement with both that of nNOS isolated from transfected human kidney 293 cells (≈2 μM) (19) and that reported by Bredt and Snyder (1) for nNOS isolated from rat brain (≈2 μM).

The observation that fraction 2 seems to be fully complemented with $BH_4$ and yet $BH_4$ added to the assay increases activity significantly is interesting; perhaps the $BH_4$ bound to the enzyme is unstable or destroyed during catalysis. The difference between the activities exhibited by the pre- and post-$BH_4$ fractions, however, is 2-fold whether or not $BH_4$ is included in the assay; it appears that the earlier the nNOS is saturated with $BH_4$, the higher the activity—i.e., the more stable the enzyme.

The large amounts of intact, active nNOS, which has a specific activity at least as high as that of kidney 293 cell-expressed enzyme, that can be generated using this system will make possible mechanistic, kinetic, and spectroscopic studies leading to the understanding of structure/function relationships. The approach outlined here may also be useful for the overexpression of the other NOS isoforms, ecNOS and iNOS, in *E. coli*. This overexpression system also provides a source of nNOS that is essentially $BH_4$-free for analysis of $BH_4$ function in NOS catalysis. The availability of this quantity of the NOS enzyme will be extremely useful for site-directed mutagenesis and, given the important physiological roles played by the NOS isoforms, drug design and development.

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