Expression of human inducible nitric oxide synthase in *Escherichia coli*

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Abstract We have expressed active full-length human inducible nitric oxide synthase (iNOS) in *E. coli*. Expression required co-expression with calmodulin, a particularly tight-binding cofactor. The extracts also required tetrahydrobiopterin to display activity. Specific activity of the purified recombinant iNOS was similar to iNOS purified from murine macrophages. This result indicates that no special processing events unique to eucaryotic cells are necessary for iNOS activity.

Key words: Inducible nitric oxide synthase; Calmodulin co-expression; Recombinant expression; *Escherichia coli*; Heterologous protein expression

1. Introduction

Nitric oxide (NO) is an important mediator of many biological responses [1,2]. Production of NO from arginine and O_2 is catalyzed by NO synthase (NOS). There are three known isoforms of NOS, named for their initial cloning source: endothelial (eNOS), brain (bNOS), and macrophage or inducible (iNOS). These three proteins, homodimers with subunit molecular masses of 125–150 kDa, share significant homology [3,4]. All contain heme, FAD, FMN and tetrahydrobiopterin (THB). eNOS and bNOS, constitutive enzymes involved in signaling, are regulated by calcium/calmodulin. In contrast, expression of iNOS requires cytokine induction. The iNOS enzyme activity is not regulated by calcium even though it contains tightly bound calmodulin. The level of NO production by iNOS is considerably greater than that of eNOS and bNOS. High levels of NO are cytotoxic, leading to the physiologic role of iNOS in immunological defense, and its pathologic role in inflammatory disease.

Natural sources of the NOS isoforms generally yield low amounts of protein. High level expression of bNOS has been achieved in human 293 embryonic kidney cells [5] and *Spodoptera frugiperda* insect cells after infection with baculovirus containing the bNOS cDNA [6]. In this paper, we describe the high level expression of human iNOS in *Escherichia coli*. Expression of iNOS is dependent on the co-expression of calmodulin, and its activity is dependent on exogenous addition of THB.

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Abbreviations: THB, tetrahydrobiopterin; CHAPS, [3-[3-cholamidopropyl]-dimethylammonio]-1-propane-sulfonate; NMMA, N^G-methyl-L-arginine; IPTG, isopropylthiogalactopyranoside.

2. Materials and methods

2.1. Cloning of human iNOS

Human iNOS was cloned from DLD cells [7] by reverse transcription of total RNA using a cDNA Cycle kit (Invitrogen) and an oligo dT primer followed by PCR amplification using four sets of oligonucleotide primers. A full-length iNOS cDNA was assembled from the products of the four PCR reactions to make piNOS17–10.

2.2. Bacterial expression vector for iNOS

The iNOS cDNA was cloned into pJF123, which contains a *tac* promoter-*lac* operator, a *lacI*^{q} gene, and a pUC19 ampicillin gene and origin. To accept the iNOS cDNA, pJF123 was modified to add appropriately placed *NcoI*/*XbaI* sites making pJF402. Vectors piNOS17–10 and pJF402 were digested with *NcoI*/*XbaI* (New England Biolabs), appropriate fragments isolated, and ligated to make the iNOS expression vector piNOS48–16.

2.3. Isolation of the human calmodulin cDNA and expression in E. coli

1 μg of brain polyA$^+$ RNA (Clontech) was reverse transcribed with an oligo dT primer using a cDNA Cycle kit (Invitrogen) followed by polymerase chain reaction amplification with 5′ primer GACGCCATGGCTGA-CCAACTGACTGAAGAG and 3′ primer CCATGGATCCTCACTTTGCTGTCATTTGT-AC, which added an *NcoI* site at the ATG start and a *BamHI* site downstream of the stop codon. The PCR product was cut with *NcoI*/*BamHI* and ligated into pSL1190 (Pharmacia), yielding pSL1190cam-12.

A calmodulin expression vector was created by ligating the calmodulin cDNA (from pSL1190cam-12 cut with *NcoI*/*BamHI*) with pJF402, making pCam5–2. The *tac* promoter/*lac* operator + calmodulin + *lacI*^{q} fragment (from pCam5–2 cut with *XmnI*/*AvaI*) was ligated into pACYC184 [8] to make pACYC:Cam2–1. Transforming *E. coli* JS5 (BioRad) with this vector yielded IPTG-inducible expression of calmodulin, shown immunologically using an anticalmodulin monoclonal antibody (Upstate Biotechnology) and by Coomassie staining after SDS-PAGE.

2.4. Purification of iNOS in E. coli

E. coli JS5 containing piNOS48–16 + pACYC:Cam2–1 was grown at 30°C in 20 l of Luria broth to an $O.D._{660}$ of 1.0. IPTG (100 μM) was added and the culture was grown overnight. Cells were harvested, resuspended in 20 mM HEPES pH 7.5, 1 mM DTT, 1 mM PMSF, 1 mM EDTA, 2 μM THB (Biomol Research Labs), 2 μM hemin (Sigma), and 10 μM each of FAD and FMN (Buffer A) to $O.D._{660}$=100, then broken by sonication. A cell-free supernatant was made 20% (w/v) ammonium sulfate. Following centrifugation, the supernatant was adjusted to 40% ammonium sulfate. The resulting protein pellet was resuspended in buffer A minus EDTA and flavins (Buffer B) to 1–2 mg/ml and conductivity <6 mS. CHAPS (Sigma) was then added to 0.5% (w/v) and the sample was loaded onto a 300-ml Q Sepharose column (Pharmacia) equilibrated in buffer B + 50 mM NaCl and 0.1% CHAPS. The charged column was washed, then eluted using a 3-l gradient in buffer B and 0.1% CHAPS from 50 to 300 mM NaCl. The iNOS activity peak was pooled and concentrated with 50% saturated ammonium sulfate. The pellet was redissolved in 40 ml of buffer B + 100 mM NaCl and was loaded onto a 1000-ml Sephacryl 300 [Pharmacia] column. iNOS containing fractions (>90% pure) were pooled.

2.5. iNOS assay

iNOS activity was measured by the conversion of radioactive arginine to citrulline [9]. Reactions (50 μl) contained 20 mM HEPES pH 7.5,

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0.1 mM DTT, 20 μM FAD, 20 μM FMN, 10 μM THB, 20 μM hemin, 1 mM NADPH, 2 mg/ml BSA, 50 μM cold arginine and 1 μM [^{3}H]arginine (60 Ci/mmol; Amersham). After incubation at 37°C for 30–60 min, reactions were stopped by adding 50 μl of 0.1 M sodium citrate pH 5.5, 100 μM N^G-methyl-L-arginine (NMMA). The reaction mix was then passed through 100 μl of Dowex resin (AG50-X8) in a 96-well filtration (Millipore) plate to separate citrulline (unbound) from arginine (bound). The filtrate was counted in 1450 Microbeta Plus liquid scintillation counter (Wallac). All assays contained sufficient enzyme to convert 10–20% of the tritiated arginine to product. Activities are reported in U/ml, with U defined as pmol of citrulline formed/min. To remove any cold arginine, samples of *E. coli* cell lysates expressing iNOS activity were desalted on a G-25 column prior to assay.

3. Results

3.1. Expression of iNOS in E. coli

Expression of human iNOS from vector piNOS48–16 in *E. coli* resulted in very low NOS activity (see Fig. 1). SDS-PAGE and Western blotting showed similarly low expression of soluble iNOS protein. Calmodulin has been shown to be very tightly associated with murine iNOS [10]; it is not removed by EGTA or purification. We reasoned that co-expression of human iNOS and calmodulin might be required for proper iNOS folding. We cloned human calmodulin, assembled an expression vector compatible with co-transfection with piNOS48–16, and compared NOS activity in cells expressing iNOS with and without calmodulin co-expression. Fig. 1 shows that the iNOS activity with calmodulin co-expression was approximately 100-fold higher than without co-expression, reaching 3000–5000 U/mg in the crude extract. Assuming a specific activity of 1×10^6 U/mg [11], the expression level is approximately 0.3–0.5% of total soluble protein. This was confirmed by SDS-PAGE of the active extract (Fig. 2), showing a 125-kDa iNOS band not seen in a negative control.

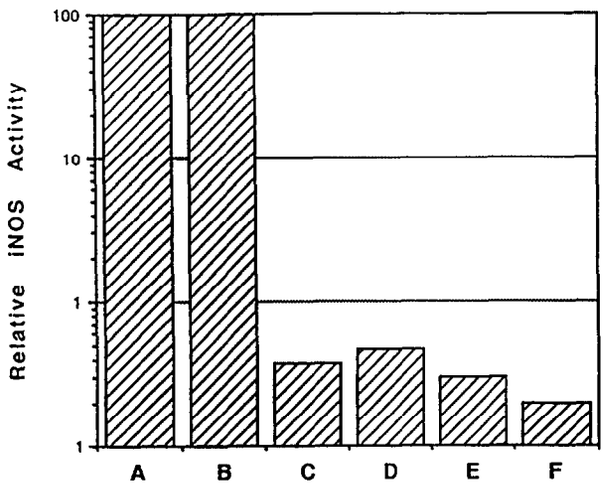

Fig. 1. Comparison of iNOS expression in *E. coli* with and without co-expression of calmodulin. The assays were run on crude extracts matched to the same protein concentration and assayed as described in section 2 except 1 μM undiluted labeled arginine was used. Column A, iNOS + calmodulin co-expression extract; B, same as A but with 10 μg/ml calmodulin added; C, iNOS alone extract; D, same as C but with 10 μg/ml calmodulin added; E, negative control extract; F, same as E but with 10 μg/ml calmodulin added. Results were normalized to columns A and B (which gave identical activities of 27,000 pmol/min.-mg.) and are the average of three assays.

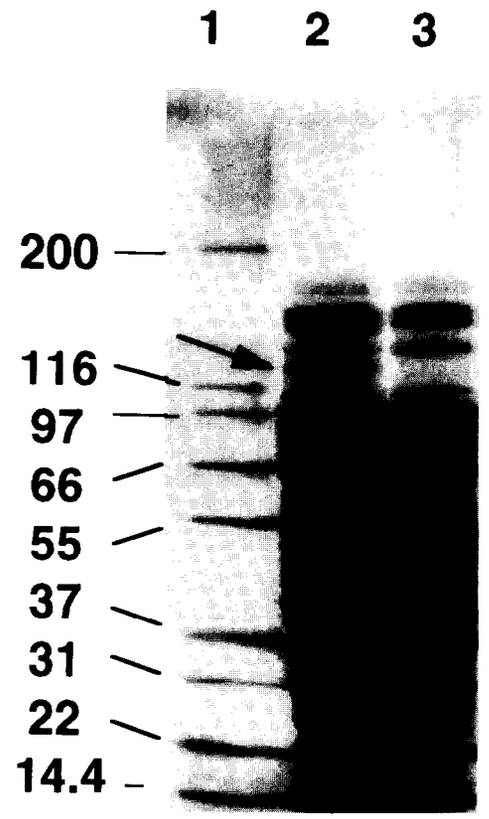

Fig. 2. SDS-PAGE of human iNOS expression in *E. coli*. Lane 1 is a molecular mass marker set (sizes in kDa on the left margin): lane 2 is piNOS48–16 + pACYC:cam2–1 co-expressed in *E. coli*; lane 3 is a negative control. The arrow indicates the position of the human iNOS polypeptide (see lane 2). The polypeptide is absent in the negative control (lane 3).

3.2. Characterization of E. coli expressed iNOS

The specific activity of iNOS in the crude cell supernatant was estimated to be 500–1000 nmol citrulline/min/mg of iNOS, based on the amount of iNOS polypeptide on an SDS-polyacrylamide gel (relative to known BSA samples). This compares favorably with 1000 nmol/min/mg determined for purified murine iNOS [11]. The iNOS activity in the crude cell extract exhibited a K_m for arginine of 8 μM, similar to that determined for murine iNOS [11]. The activity was unaffected by 1 mM EGTA or 1 mM $CaCl_2$ ± 10 μg/ml calmodulin. The iNOS activity was totally dependent on exogenously added THB (Fig. 3), with half-maximal activity seen at 120 nM THB. Activity was not dependent on the other iNOS cofactors. The effect of iNOS inhibitors (NMMA, imidazole, and aminoguanidine) on *E. coli*-derived iNOS was similar to that displayed by recombinant iNOS expressed in human 293 embryonic kidney cells (X. Fan, pers. commun.), with IC_{50} values of 2 μM, 120 μM, and 80 μM, respectively.

3.3. Purification of iNOS

iNOS was purified to >90% purity as shown in Table 1. The purification consisted of an ammonium sulfate precipitation followed by a Q-Sepharose ion-exchange chromatography and Sephacryl S300 gel filtration. Characterizing the individual iNOS pools by SDS-PAGE (Fig. 4, note lane 5) shows that calmodulin copurifies with the human iNOS protein (verified

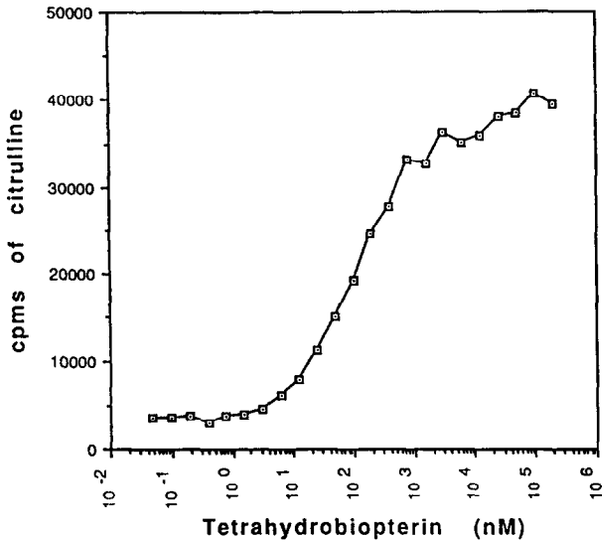

Fig. 3. The effect of THB on iNOS activity in *E. coli* extracts co-expressing iNOS and calmodulin. The cell were broken and processed as described in section 2 but without added THB. The assay was run with varying amounts of THB from 50 pM to 200 μM. Activity is given in counts/min (cpms) of citrulline.

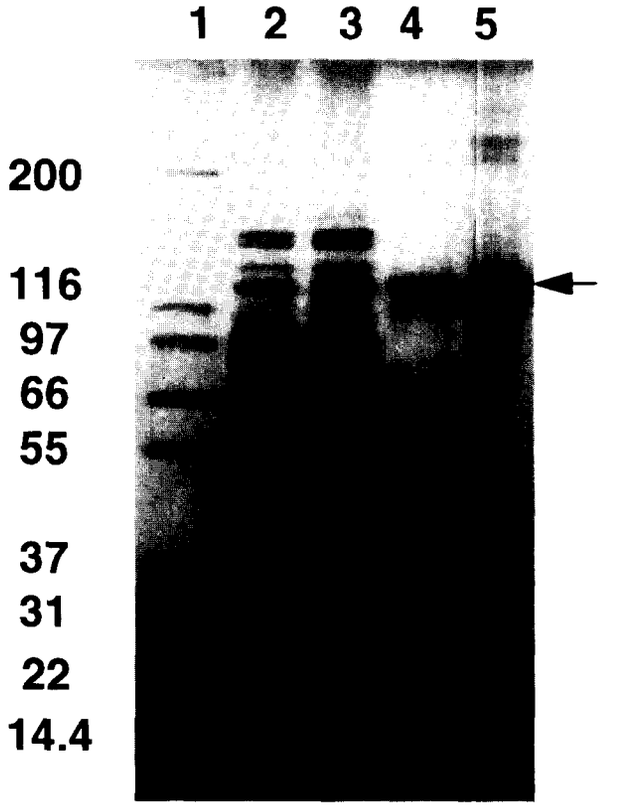

Fig. 4. SDS-PAGE of the purification of human iNOS from *E. coli*. Lane 1, molecular mass marker set (sizes in kDa in the left margin); lane 2, cell lysate; lane 3, 40% ammonium sulfate precipitate; lane 4, Q Sepharose pool; and lane 5, pool from the front half of the S300 iNOS peak. The arrow indicates iNOS protein.

immunologically). Overall yield of iNOS activity was 24%. The specific activity of pure material (fractions from the leading edge of the S300 column) was 600,000 U/mg. This compares favorably with pure murine iNOS at 1,000,000 U/mg [11] and rat bNOS at 230,000 U/mg [5]. The yield of iNOS from 20 l of cells (32 g starting protein in the extract) was 30–40 mg.

4. Discussion

In this report, we show expression of soluble, active iNOS was dependent on its co-expression with calmodulin. We postulate that the extremely tight association of calmodulin with iNOS serves a structural purpose in allowing iNOS to fold correctly. This is in contrast to eNOS and bNOS where calmodulin-binding is regulated by calcium; calmodulin is freely dissociable if calcium is removed by EGTA. *E. coli* expressed iNOS appears to have the same specific activity as murine iNOS, a similar K_m for arginine, and similar IC_{50} values for three iNOS inhibitors as does mammalian-derived iNOS. This indicates that no mammalian-specific modification of iNOS is necessary for NOS activity. The enzyme activity was completely dependent on added THB with a half-maximal concentration of 120 nM. The binding constant of THB for bNOS has been shown to be 37 nM and 250 nM in the presence and absence of 100 μM arginine, respectively [12].

iNOS produces NO in an unregulated manner. The toxic effects of NO would be expected to make its stable, unregulated expression in cells very difficult. To date, it has not been possible to make a stably transfected mammalian cell line expressing iNOS at high levels, although we have developed transient systems. Expression of iNOS has only been described in a baculovirus system [6], a transient expression system in which long-term survival of the cells expressing iNOS is not required. Expression of inactive iNOS in *E. coli* lacking THB overcomes the toxicity problems. In addition, the level of expression we report, 3000–5000 U/mg, is higher than the level reported in a baculovirus expression system [13] of 350 U/mg. In conclusion, the expression of human iNOS in *E. coli* should greatly aid future studies of the enzyme. We have demonstrated that pure iNOS can be obtained by a simple procedure and at yields compatible with spectral and structural analysis. The complete dependence of *E. coli* expressed iNOS on THB offers a system to study the role of this cofactor in the catalytic mechanism.

Table 1
Purification of human iNOS

Sample	Volume (ml)	Protein conc. (mg/ml)	Total protein (mg)	Total activity (U)	Specific act. (U/mg)
Cell lysate	2600	12.5	32475	78,600,000	2,420
40% amm. sul. ppt.	3300	1.2	3960	77,600,000	19,600
Q Sepharose pool	470	0.35	165	23,000,000	149,000
S300 pool	180	0.22	40	19,200,000	480,000

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