Arch Pharm Res Vol 23, No 4, 407-412, 2000

Identification and Characterization of Nitric Oxide Synthase in *Salmonella typhimurium*

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(Received March 31, 2000)

The presence of the nitric oxide synthase (NOS) enzyme from *Salmonella typhimurium* (*S. typhimurium*) was identified by measuring radiolabeled L-[^{3}H]citrulline and NO, and Western blot analysis. NOS was partially purified by both Mono Q ion exchange and Superose 12HR size exclusion column chromatography, sequentially. The molecular weight of NOS was estimated to be 93.3 kDa by Western blot analysis. The enzyme showed a significant dependency on the typical NOS cofactors; an apparent *Km* for L-arginine of 34.7 mM and maximum activity between 37°C and 43°C. The activity was inhibited by NOS inhibitors such as aminoguanidine and N^G,N^G-dimethyl-L-arginine. Taken together, partially purified NOS in *S. typhimurium* is assumed to be a different isoform of mammalian NOSs.

Key words: *Salmonella typhimurium*, Nitric oxide, Nitric oxide synthase

INTRODUCTION

Nitric oxide (NO) is a ubiquitous multifunctional secretion product of mammalian cells that is important in regulating the basic physiological and pathological processes such as vasodilation (Kiechle and Malinski, 1993), platelet function (Palmer *et al.*, 1988), neurotransmission (Nathan, 1992), and host-defence mechanisms (Moncada *et al.*, 1991). The monooxygenase enzyme, NO synthase (NOS), catalyzes the five electron oxidation of L-arginine with oxygen and NADPH as a co-substrate to ultimately yield L-citrulline and NO.

Advances in the study of mammalian NOSs by enzyme purification and characterization, gene cloning, and heterologous expression have led to greatly increased understanding of their roles. Furthermore, NO has been appreciated as an obligatory electron acceptor for energy conservation during the denitrification pathway in bacteria (Kalkowski and Conrad, 1991). In addition, several reports have recently suggested the possibility of the existence of a NOS-like system in microorganisms. NOS from *Nocardia* sp. was partially purified and demonstrated a similar cofactor dependency to NOSs in mammals (Chen and Rosazza, 1994; 1995). Morita *et al.* (1997) reported that *Lactobacillus fermentum* IFO 3956 synthesized NO from two equivalent guanidino nitrogens of L-arginine, and suggested it might possess a bacterial type of NOS. In our previous reports (Choi *et al.* 1997; 1998), we identified NOS in *Staphylococcus aureus* ATCC 6538P and established its NOS-like characteristics. Although the possible involvement of *Norcardia* NOS in pathogenesis has been suggested, the biological functions of NOS-like systems in microorganisms remain to be elucidated.

In the present study, an attempt to identify the presence of bacterial NOS from *Salmonella typhimurium*, which is gram-negative, facultatively anaerobic, non-endospore forming, and motile bacteria, causing some life-threatening illnesses such as localized gastroenteritis, septicemia, and typhoid fever (Cohen *et al.*, 1987), was made. The presence of NOS in *S. typhimurium*; the formation of NO and L-citrulline from L-arginine by a partially purified enzyme, the requirements for mammalian NOS cofactors, and a decrease in enzyme activity by NOS inhibitors was demonstrated.

MATERIAL AND METHODS

Reagents

[2,3,4,5-^{3}H]-L-arginine HCl (57 Ci/mmol) was purchased

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from Amersham (U.K.). Flavine adenine dinucleotide (FAD), flavine mononucleotide (FMN), DL-dithiothreitol (DTT), β-nicotinamide adenosine dinucleotide phosphate (reduced form) (NADPH), calmodulin (CaM), and Dowex 50W (H^+ form) were obtained from the Sigma Chemical Co. (U.S.A.), iNOS antibody from Transduction Lab. (U.S.A.) and tryptic soy broth was obtained from Difco Lab. (U.S.A.). The remainder of the chemicals used in this study were obtained from various commercial sources and were of the highest grade available.

Culture and preparation of enzyme source

S. typhimurium ATCC 13311 was purchased from the American Type Culture Collection (U.S.A.) and maintained on slants of tryptic soy agar and grown by a two-stage incubation method. One colony derived from the agar plate was inoculated in 10 ml of tryptic soy broth (pH 7.3) and cultured for 18 h on a rotary shaker at 160 rpm at 37°C under aerobic conditions. A 10% inoculate derived from a first stage culture was used to initiate the second culture, which was diluted 250-fold with freshly prepared tryptic soy broth in a 1 L culture flask then again incubated under the same conditions. After culturing for 18 h, the bacterial cells were harvested by centrifugation at 25,000 × g for 10 min, and washed immediately with phosphate buffered saline (pH 7.4) twice. To prepare crude cell extracts, the cell pellet was suspended in 5 volumes of 20 mM bis-Tris buffer (pH 6.5) containing 1 mM DTT, 2 μM NADPH, 2 μM BH_4, 10 μg/ml leupeptin, 0.1 mg/ml AEBSF, and 10 μg/ml trypsin inhibitor. The suspension was then disrupted for 30 min with a ultrasonic cell disrupter (Vibra Cell, Sonics & Materials Inc., U.S.A.) in ice-bath. The supernatant, obtained by centrifugation at 105,000 × g for 60 min under 4, was used as the enzyme source.

Determination of nitrogen oxides

Nitrgen oxides (NOx) produced by NOS in *S. typhimurium* were measured directly by a NO Analyzer (Model 7020, Antek Instruments, U.S.A.). Briefly, a 50 μL of reaction mixture containing the appropriate amounts of enzyme sources, 10 μM FAD/FMN, 100 μM NADPH, 100 μM tetrahydrobiopterin (H_4B), 1.5 mM Ca^{2+}, 2 μg CaM, and 200 μM L-arginine with a total final volume of 200 μl was incubated for 10 min at 37°C then injected directly into a hot vanadium (III) reduction solution. The NO chemiluminescence reduced from the nitrogen oxides in the reaction mixture was measured and a quantitative evaluation on NO production was determined by a standard calibration curve method using a sodium nitrite standard.

Assay of NOS activity

The NOS enzyme activities were determined using a minor modification of a method based on the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline as described previously (Bredt and Snyder, 1989). The reaction was carried out under the same conditions as those described in Determination of nitrogen oxides, except that 25 mM L-[2,3,4,5-^{3}H]arginine (approximately 200,000 dpm) was used as a substrate. The reaction was terminated by the addition of a 1 ml of ice-cold Dowex-50W (Na^+ form) suspension, pre-equilibrated in a 20 mM sodium acetate buffer (pH 5.5) containing 1 mM citrulline, 2 mM EDTA, and 0.2 mM EGTA. The reaction mixture was then left for 10 min and centrifuged at 11,000 × g for 5 min. Subsequently, the supernatant was placed on a column, and filtered into 10 ml of a water-miscible scintillation cocktail solution and counted. The enzyme specific activity was expressed as the amount of enzymes that catalyze the formation of product per min per mg of protein.

Western blot analysis

For Western blot analysis, the enzyme NOS (approx. 5.0 μg protein) was electrophoresed on a 10% polyacrylamide slab gel at pH 8.3. The proteins were transferred onto a PVDF membrane (Millipore, USA) for 1 h at 60 mA with a Trans-Blot Semi-Dry Transfer cell (BIO-RAD, USA). The nonspecific binding on the PVDF membrane was blocked by incubation with a 5% skim milk in tris-buffered saline solution (TBS), pH 7.4, for 1 h at room temperature with gentle shaking. After washing with a 0.1% Tween 20 solution and a 0.2% sodium azide in TBS (TTBS) solution, the membrane was incubated overnight with rabbit anti-mouse iNOS antibodies diluted 1:1000 in TBS. The membrane was then incubated with alkaline phosphatase-conjugated goat anti-rabbit antibodies for 2 hr at room temperature. After washing with TTBS, the membrane was developed in a 10 ml solution containing 5-bromo-4-chloro-3-indolyl phosphate, nitroblue tetrazolium, and a color development reagent. The molecular weights of the standard proteins were: β-galactosidase, 116,000; phospholylase *b*, 97,400; bovine serum albumin, 66,000.

Amino acid analysis

The presence of L-citrulline, produced endogenously from L-arginine by NOS, was identified by an amino acid analyzer (LC8500A, Hitachi, Japan). The reaction was performed as described earlier and was terminated by ice-fixation for 30 min, then a 50 μl 10% trichloroacetic acid solution was added with vigorous stirring. 50 μl of the supernatant, obtained by centrifugation, was injected directly with an autosampler in the amino acid analyzer.

Partial purification of NOS

1 ml of the cytosolic fraction, obtained by ultracentrifugation at 105,000 × g, was applied to the Mono Q anion

exchange column (Pharmacia LKB, Sweden) pre-equilibrated with a 20 mM bis-Tris buffer (pH 6.5) containing 1 mM DTT, 2 μM NADPH, 2 μM H_4B and 1 mM EDTA. The enzyme was loaded onto the column and subsequently eluted with 40 ml of the above buffer including 0.75 M NaCl in a linear gradient manner at a flow rate of 0.5 ml/min, which was monitored at 280 nm. The NOS active fractions, identified by NO determination and a citrulline formation assay, were collected and concentrated with centrifugal filter device (Ultrafree-15, cut-off: 10 kDa, Millipore, U.S.A.). The filtrates, concentrated up to 1.0 ml, were loaded onto a Superose 12HR size exclusion column (1.6 × 60 cm, Pharmacia LKB, Sweden), which was previously pre-equilibrated with 20 mM bis-Tris buffer (pH 6.5) containing 0.15 M NaCl, 2 mM NADPH, 2 μM H_4B, 1 mM DTT, and 2 mM EDTA, and was monitored at 280 nm at a flow rate of 0.5 ml/min. The partially purified NOS from *S. typhimurium* ATCC 13311 was designated NOS_{Stm} and was used in all subsequent experiments.

Protein determination

The protein concentration was estimated using the Bradford method (Bradford, 1976) with bovine serum albumin as a standard.

RESULTS

Determination of NO and L-citrulline formed

The determination of NOS_{Stm} activity was confirmed by measuring the formation of nitrogen oxides and the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline. Because there exists a possibility that L-[^{3}H]citrulline could also be produced by a route other than NOS such as arginine deiminase, the amount of NOx should be measured by a NO analyzer. NOx, formed endogenously from L-arginine by NOS_{Stm}, is shown in Fig. 1A. It was found that the amount of L-[^{3}H] citrulline formed was in very close agreement with the amount of NO produced (data not shown). Using an amino acid analyzer, the increase of L-citrulline was also found to be consistent with the decrease in L-arginine concentration in the NOS_{Stm} reaction mixture (Fig. 1B).

To examine the relationship between cell growth and NOS activity, the changes of NOS activity and protein absorbance during culture were determined by L-[^{3}H] citrulline formation and was monitored at 280 nm, respectively. The highest NOS_{Stm} enzyme activity was shown at the mid-stationary phase (Fig. 2).

Partial purification of NOS_{Stm}

NOS_{Stm} was partially purified from *S. typhimurium* by Mono Q ion exchange column chromatography, diafiltration and Superose 12HR size exclusion column chro-

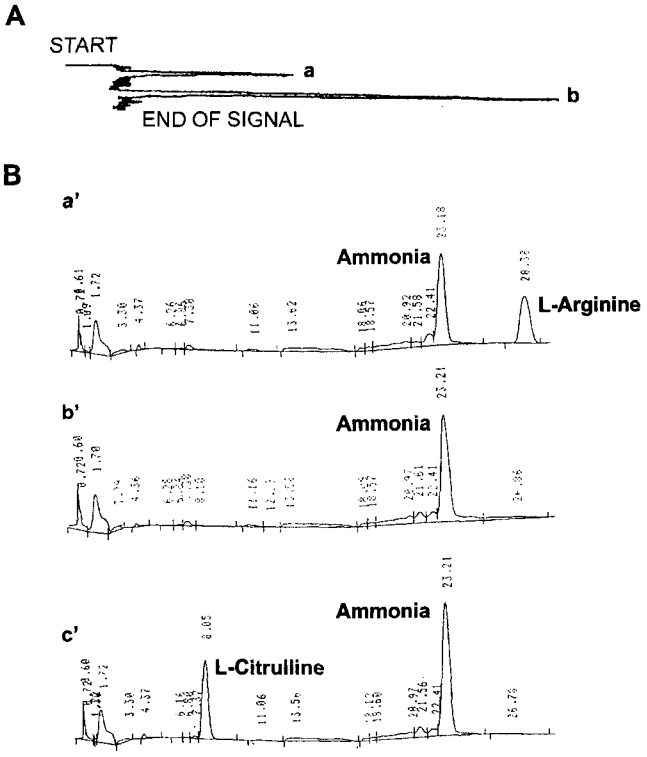

Fig. 1. Determination of NO and L-citrulline formed endogenously by NOS_{Stm}. A. The reaction mixture was injected directly into the hot vanadium (III) reduction solution, and the chemiluminescences of NO reduced from nitrogen oxides in reaction mixture was measured. a, reaction mixture without L-arginine; b, reaction mixture with L-arginine B. Analysis of L-citrulline using amino acid analyzer. NOS in *S. typhimurium* was incubated without (b') or with (c') L-arginine as substrate followed by the procedures as described under Materials and Methods. a', reaction blank without NOS_{Stm}. These experiments were repeated at least three times with similar results.

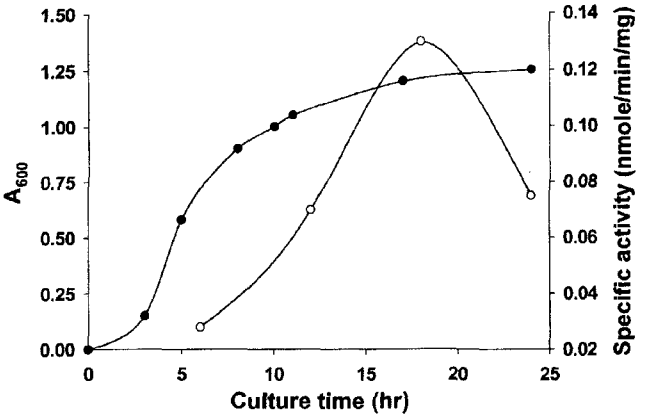

Fig. 2. Relationship between NOS_{Stm} activity and cell growth. The closed circles represent absorbance at 600 nm, and open circles show NOS_{Stm} activity measured by L-[^{3}H]citrulline formation assay. Specific activity was expressed as nmole of [^{3}H]citrulline produced for 1 min by 1 mg of NOS_{Stm}. The values are the means of three different measurements.

matography, sequentially. The active fraction (2 ml) with a specific activity of 0.56 nmole/min/mg protein was used for further characterization. Fig. 3 shows the Western blot analysis of the NOS_{Stm}. The characteristic band detected with the anti-mouse iNOS antibody, which was raised against the C-terminal (amino acid 961-1144) containing the NADPH consensus sequence of mouse iNOS, was visible in approximately the 93.3 kDa region as calcu-lated by comparison with molecular weight marker proteins. This observation indicates that the NOS_{Stm}, partially purified from *S. typhimurium*, might have similar NADPH consensus sequences to those of mammalian NOSs.

Characterization of NOS_{Stm}

There was a direct relationship between the concentration of L-arginine and NOS_{Stm} (Fig. 4). The apparent *Km* value for L-arginine was 34.7 μM. As shown in Fig. 5, the NOS_{Stm} was relatively stable from 30 to 43°C, and the optimal temperature was between 37°C and 43°C.

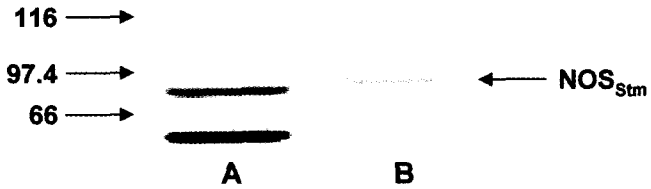

Fig. 3. Estimation of molecular weight of NOS_{Stm} by Western blot analysis. The enzyme (approx. 5.0 μg) was electrophoresed on 10% SDS-PAGE, and then was detected with anti-mouse iNOS antibody. These experiments were repeated three times with similar results. The lanes are as follows. A, crude cytosol; B, partially purified enzyme

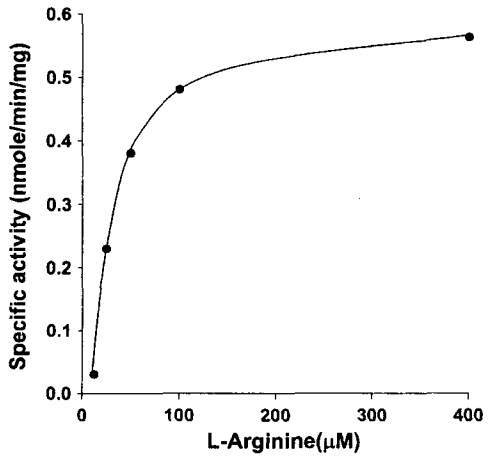

Fig. 4. Relationship between the concentration of L-arginine and NOS_{Stm}. Detailed experimental conditions are described under Materials and Methods.. Specific activity was expressed as nmole of [^{3}H]citrulline produced for 1 min by 1 mg of NOS_{Stm}. The values are the means of three different measurements.

NOS_{Stm} was inhibited by typical NOS inhibitors such as aminoguanidine and N^G,N^G-dimethyl-L-arginine in a concentration dependent manner. However, α-aminobutyric acid and *O*-methylisourea, known as specific inhi-bitors against arginine deiminase (ADI) failed to decrease NOS_{Stm} activity. Taken together, these results suggest that the NOS from *S. typhimurium* ATCC13311 is an isoform, distinct from mammalian NOS.

NOS_{Stm} showed a significant dependency from 27.3 to 64.2% on mammalian NOS cofactors such as NADPH, FAD/FMN, CaM, H_4B, and Ca^{2+}, as measured by the NO analyzer (Table I). The strict requirement for NOS_{Stm} activity was observed, when each cofactor was omitted. These cofactor requirements demonstrate that NOS_{Stm} has similar consensus sequence sites including sites for heme, L-arginine, and cofactors for NOS in mammals.

DISCUSSION

The presence of NOS in *S. typhimurium* was established

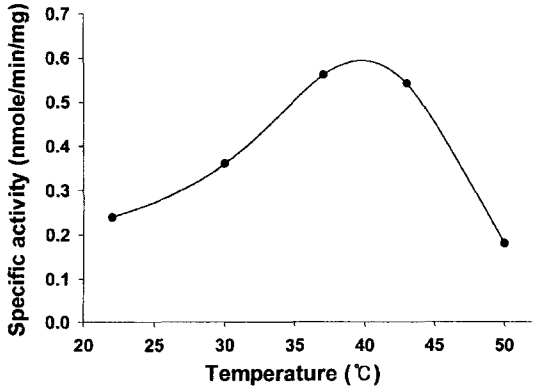

Fig. 5. Optimum temperature of NOS_{Stm}. The NOS_{Stm} activity was measured at various temperatures. Specific activity was expressed as nmole of [^{3}H]citrulline produced for 1 min by 1 mg of NOS_{Stm}. The values are the means of three different measurements.

Table I. Cofactor dependency of NOS_{Stm} activity.

Cofactor omitted	Residual activity (%)
None	100.0
All	49.7
NADPH	35.8
CaM	46.7
FAD/FMN	44.2
H_4B	69.7
Ca^{2+}	72.7

NOS_{Stm} activity was measured as described under Materials and Methods. When the designated cofactor was omitted, it was equally replaced with 20 mM bis-Tris buffer (pH 6.5) in the reaction mixture, and the results shown were expressed as the residual activity of the control enzyme activity. None, all cofactors added; All, all cofactors omitted; CaM, calmodulin

by a series of experiments including analysis of NO formed, radio-labeled L-citrulline formation assays, Western blot analysis with anti-mouse iNOS antibodies, and cofactor or inhibitor studies. The maximal NOS_{Stm} activity was shown at the mid-stationary growth phase, which has been appreciated as a harsh condition for bacterial cell survival (Fig. 2). This observation suggests that the presence of NOS_{Stm} is essential for survival.

The molecular weight of partially purified NOS_{Stm} through ion exchange and size exclusion column chromatography was estimated to be 93.3 kDa by Western blot analysis. This differs from that of the other bacterial NOSs; 51.9 kDa of *Nocardia* sp. NOS (Chen and Rosazza, 1994; 1995), 99.7 kDa of *Staphylococcus aureus* ATCC 6538P (Choi *et al.*, 1998), and mammalian NOS isoforms with a molecular weight of 130 kDa (iNOS and eNOS) (Hevel *et al.*, 1991; Stuehr *et al.*, 1991; Pollock *et al.*, 1991) and 160 kDa (nNOS) (Schmidt *et al.*, 1991; Mayer *et al.*, 1990).

As shown in Fig. 5, the optimum temperature for NOS_{Stm} was between 37 and 43. This value is significantly different from that of *Nocardia* sp. and *Staphylococcus aureus* ATCC 6538P NOS, but is very similar to the NOSs in mammals. The NOS_{Stm} activity was mostly dependent on mammalian NOS cofactors including NADPH, FAD/FMN, H_4B, Ca^{2+}, and CaM (Table I).

On the other hand, there is another enzyme arginine deiminase (ADI) that can also convert L-arginine to L-citrulline except the liberation of ammonia results rather than NO. ADI is present in a variety of microorganisms, including *Pseudomonas*, *Bacillus*, and *Streptococcus* species (Mercenier *et al.*, 1982; Broman *et al.*, 1978; Simon *et al.*, 1982). The possibility of the purified enzyme being ADI was disregarded on the basis of following results: the formation of NO simultaneously with L-citrulline (Fig. 1), detection by anti-mouse iNOS antibodies (Fig. 3), the remarkable dependency on typical NOS cofactors for its optimal activity (Table I), and its inhibition by specific NOS inhibitors (Fig. 6).

The significance and specific functions of bacterial NOS is at present unknown. Further studies into researching whether NOS_{Stm} may be involved in human pathogenic infection of *S. typhimurium* is necessary.

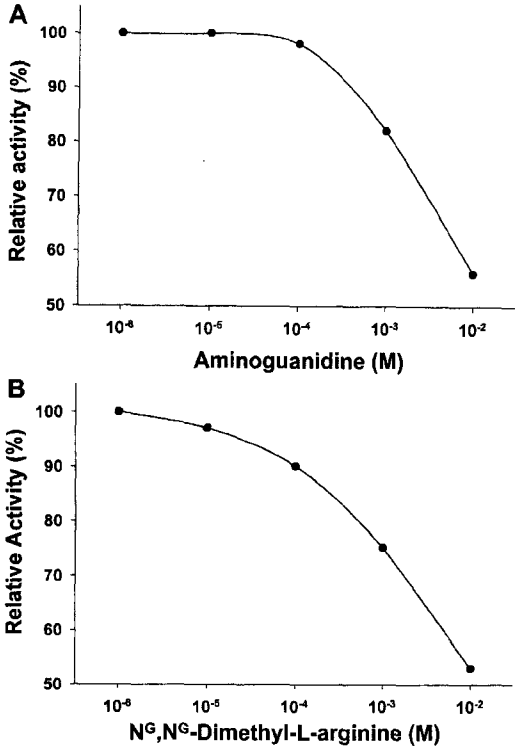

Fig. 6. Effect of NOS inhibitors on NOS_{Stm}. The effect of aminoguanidine (A) and N^G,N^G-dimethyl-L-arginine (B) on NOS_{Stm} activity were measured as described under Materials and Methods. Results are expressed as the relative activity of the control enzyme activity (without inhibitor). The values are the means of three different measurements.

ACKNOWLEDGMENTS

This work was financially supported by a grant (KOSEF-1999-2-209-012-3) from Korea Science and Engineering Foundation.

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