Identification and Characterization of Nitric Oxide Synthase in Salmonella typhimurium

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The presence of the nitric oxide synthase (NOS) enzyme from Salmonella typhimurium (S. typhimurium) was identified by measuring radiolabeled L-[3H]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 amionoguanidine and N6,N6-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 monoxygenase 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 Nocardia 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

[2,3,4,5-3H]-L-arginine HCl (57 Ci/mmol) was purchased
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 trypsin 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 x 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 BH₄, 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 x g for 60 min under 4, was used as the enzyme source.

Determination of nitrogen oxides

Nitrogen 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 (H₄B), 1.5 mM Ca²⁺, 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-[³H]arginine to L-[³H]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-[2,3,4,5-³H]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 x 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; phospholase 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 x g, was applied to the Mono Q anion