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Isolation of a nitric oxide synthase from the protozoan parasite, *Leishmania donovani*

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Abstract

A soluble nitric oxide synthase (NOS) activity was purified 2800-fold from *Leishmania donovani*, the causative parasite of visceral leishmaniasis, by two-step affinity and anion-exchange chromatography. The purified enzyme ran as a prominent band of 110 kDa on SDS-PAGE whereas gel filtration experiments estimated the native molecular mass to be 230 ± 20 kDa indicating that the native enzyme exists as a dimer. The enzyme activity required NADPH and was blocked by EGTA. The enzyme kinetics, cofactor requirements, inhibition studies and Western blot analysis with brain anti-NOS antibody suggest its similarity with mammalian NOS isoform I.

Keywords: Nitric oxide synthase; *Leishmania donovani*; Enzyme purification

1. Introduction

Considerable attention has focused on nitric oxide (•NO) because of the crucial role it plays as a cell signalling agent and its function as an antileishmanial effector molecule [1]. This key molecule is formed by nitric oxide synthase (NOS; EC 1.14.13.39) which catalyzes the five-electron oxidation of L-arginine to the nitric oxide radical and citrulline [2]. The enzyme occurs in at least three distinct isoforms and is found in a variety of mammalian tissues [3]. Isoform I, initially identified in neuronal tissue, is constitutive and Ca^{2+}-regulated, whereas isoform II, mostly found in cytokine-induced macrophages, is inducible and Ca^{2+}-independent. Isoform III from endothelial cells is myristoylated, Ca^{2+}/calmodulin-regulated and found predominantly in the particulate fraction as opposed to forms I and II which are mostly soluble proteins. All the NOS enzymes characterized so far are hemoprotein dimers comprised of subunits of M_r 130 000–155 000 and require the same cofactors, NADPH, (6*R*)-5,6,7,8-tetrahydrobiopterin, FAD and FMN [4]. The enzyme has been purified from a variety of mammalian tissues [3] and very recently the enzyme has been reported to be present in the lower eukaryotic organism *Trypanosoma cruzi* [5] and in bacteria of the genus *Nocardia* [6]. The present studies provide the first demonstration for the existence of a Ca^{2+}-stimulated NOS system in the protozoan parasite *Leishmania donovani*, the causative agent of visceral leishmaniasis. Based on demonstrated cofactor

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requirements, analysis of products formed, and inhibition by N^{ω}-nitro-L-arginine, the parasite's enzyme appears to be similar to mammalian NOS isoform I.

2. Materials and methods

2.1. Materials

L-[2,3-^{3}H]Arginine (53 Ci/mmol) was obtained from DuPont/NEN, Boston, MA. (6*R*)-5,6,7,8-Tetrahydrobiopterin (H_4B) was obtained from Biochemical Research Inc., Natick, MA. L-Arginine, calmodulin (bovine), N^{ω}-nitro-L-arginine (NLA), N^{G}-methyl-L-arginine (MLA) and 2′,5′-ADP agarose were purchased from Sigma, St. Louis, MO. Electrophoresis reagents and DEAE-Bio-Gel-A were purchased from Bio-Rad.

2.2. Parasites

L. donovani strain AG83 (MHOM/IN/1983/AG83) was isolated from an Indian patient with visceral leishmaniasis [7]. Promastigotes were cultured at 22°C in medium 199 (Gibco Laboratories, Grand Island, New York) with Hanks' salts containing HEPES (12 mM), L-glutamine (20 mM), 10% (v/v) heat-inactivated fetal calf serum, 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Cell-free extract was prepared from promastigotes by freeze-thawing the cell suspension (5×10^{10} cells ml^{-1}) 3–5 times and sonicating for 5×45 s at 20 K cycles min^{-1} over ice in 0.25 M sucrose containing 5 mM KCl. The contents were centrifuged at 10 000×*g* for 20 min and the supernatant was used for subsequent enzyme purification.

2.3. NOS assay

NOS was determined by monitoring the formation of L-citrulline from L-arginine according to Bredt and Snyder [8]. Enzymatic reactions were conducted in 50 mM Tris-HCl, pH 7.5, 10 μM L-[^{3}H]arginine (0.2 μCi per assay), 100 μM NADPH, 10 μM FAD, 2 mM $CaCl_2$, 1 μg of calmodulin, 10 μM H_4B and 0.1–1 μg of enzyme protein in a final incubation volume of 100 μl. Incubations were performed for 10 min at 24°C and stopped by the addition of 2 ml of ice-cold 20 mM HEPES, pH 5.5 containing 1 mM EDTA. Samples were immediately applied to a Dowex 50W-X8 column that had been pre-equilibrated with 20 mM HEPES, pH 5.5. L-[^{3}H]Citrulline was eluted with 2 ml of deionized water, and radioactivity was quantitated by scintillation counting. The authenticity of the radioactive citrulline formed was checked by comigration with a citrulline standard on a silica gel 60 plate developed with $CHCl_3$:MeOH:NH_4OH:H_2O (1:4:2:1, v/v) according to Iyengar et al. [9].

NOS activity was also determined by measuring the decrease in absorbance at 340 nm for 3 min continuously as NADPH was consumed during the conversion of L-arginine to L-citrulline by NOS according to Sherman et al. [10].

2.4. Purification of NOS

The crude soluble extract was adjusted to 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 25 unit ml^{-1} aprotinin, 0.01% (w/v) leupeptin, 0.2 mg ml^{-1} soybean trypsin inhibitor and 1 μg/ml pepstatin A and centrifuged at 100 000×*g* for 60 min at 4°C. The soluble supernatant (4 ml, 5–6 mg protein) was applied to a 2′,5′-ADP agarose column (8.5×1.5 cm) equilibrated with 10 mM Tris-HCl, pH 7.5 containing 1 mM DTT, 1 mM EDTA, 0.5 mM PMSF, 25 units ml^{-1} aprotinin, and 0.5 mM L-arginine (buffer A). The column was successively washed with 20 ml of buffer A, 20 ml of buffer A containing 0.5 M NaCl, 20 ml of buffer A containing 0.5 mM NADH, 20 ml of buffer A containing 0.5 mM NADP and 20 ml of buffer A. NOS activity was eluted with 10 ml of buffer A containing 10 mM NADPH, 3 μM H_4B and 10% (v/v) glycerol. NOS-containing fractions were pooled and immediately applied to a DEAE-Bio-Gel-A column (4.5×1 cm) equilibrated with buffer A. The column was washed with 10 ml of buffer A followed by 10 ml of buffer A containing 80 mM NaCl. NOS activity was eluted with buffer A containing 150 mM NaCl and 3 μM H_4B. During determination of K_m for arginine, buffers for the last two steps did not contain arginine.

2.5. Determination of nitrite

Nitrite was measured according to Green et al. [11]. Briefly, 500 μl of NOS incubation mixture was mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% *N*-1-naphthylethylenediamine dihydrochloride in 50% H_3PO_4) and incubated at room temperature for 30 min. Absorbance at 540 nm was then measured. Amount of nitrite released was quantified by comparison with sodium nitrite as standard.

2.6. Electrophoresis and immunoblotting

Electrophoresis was performed using a Bio-Rad Mini-Protean II dual slab gel according to the manufacturer's instructions, a discontinuous buffer system and a 7.5% separating gel. Gels were silver-stained using the silver-staining kit from Bio-Rad. Western blot analysis was performed by standard procedures using the rabbit antiserum to the rat brain NOS enzyme (Transduction Laboratories, Lexington, KY) and an alkaline phosphatase-conjugated secondary antibody (Sigma).

2.7. Gel filtration

Analytical gel filtration chromatography was carried out on a Superose 6 gel filtration column (30×1 cm; Pharmacia Biotech) equilibrated with buffer A containing 150 mM NaCl. Standards and purified NOS were applied to the column in 100 μl aliquots. Standards were monitored at 280 nm, and NOS was detected enzymatically as described earlier. The standards used were thyroglobulin, 669 000 kDa; apoferritin, 443 000 kDa; β-amylase, 200 000 kDa; bovine serum albumin, 66 000 kDa and carbonic anhydrase, 29 000 kDa.

3. Results and discussion

The results provide evidence that a NOS system similar to that found in mammals exists in the protozoan parasite *L. donovani*. The purification protocol, which included affinity chromatography on ADP-agarose and ion-exchange chromatography on DEAE-Biogel-A, was a modification of that employed for the purification of brain NOS [3] and a constitutive PMN-derived NOS [12]. The results of a typical purification are shown in Table 1. The final specific activity was 3116 nmol of citrulline produced per mg of enzyme per min, with an overall recovery of 22%. The specific activity of the purified NOS remained unchanged with dilution over the protein concentration range (0.05–2.0 μg ml^{-1}). However, the specific activity decreased slowly, falling to 75% of its original value after 6 h, when NOS was incubated at 22°C under standard assay conditions. Increased stability was observed with 10% glycerol and the enzyme could be stored overnight in 50% glycerol at −70°C without any apparent loss of activity, suggesting that the specific activity and yield of the purified enzyme detailed in Table 1 are probably less than those obtained with glycerol. In order to ascertain whether equimolar quantities of NO and citrulline are formed from L-arginine, NOS was also assayed in terms of NO produced, quantified by the accumulation of nitrite with Griess reagent [11]. Two microgram of the purified enzyme (specific activity 3100 nmol min^{-1} mg $protein^{-1}$) produced equimolar amounts of nitrite (171 ± 8 nmol) and citrulline (195 ± 9 nmol) (mean ± S.D., $n = 3$) while 372 ± 12 nmol ($n = 3$) of NADPH was consumed during the 30 min incubation. The representative silver-stained SDS gel shown in Fig. 1A demonstrates that the purified preparations of *L. donovani* NOS (lane 3) contained one single protein with an apparent mo-

Table 1
Purification of NO synthase from *L. donovani* promastigotes[a]

Purification step	Protein (μg ml^{-1})	Specific activity (nmol min^{-1} mg $protein^{-1}$)[b]	Fold of purification
100 000 × *g* supernatant	5240	1.1	1
2′,5′-ADP agarose	728	998	907
DEAE-Bio-Gel-A	3.6	3116	2832

[a]NOS activity was assayed in triplicate samples by conversion of L-arginine to citrulline.

[b]A unit is defined as the amount of enzyme required to produce 1 nmol of citrulline per min.

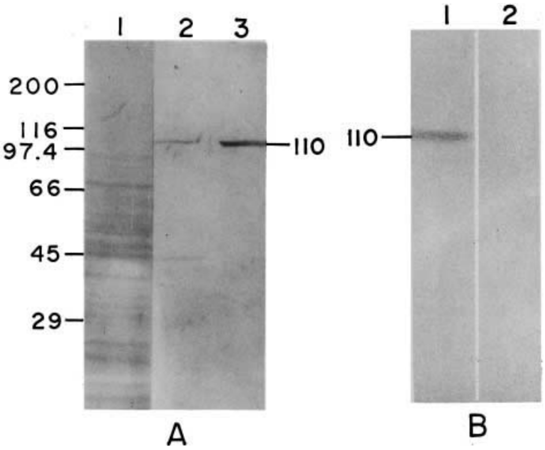

Fig. 1. SDS-PAGE and Western blot analysis of fractions from purification of *L. donovani* NOS. A: The polyacrylamide gel (7.5%) was silver-stained. Lane 1, 100 000×*g* supernatant, 1 μg of protein. Lane 2, peak fraction from ADP agarose column, 0.2 μg of protein. Lane 3, peak fraction from DEAE-Bio-Gel-A column, 0.2 μg of protein. B: Proteins were detected using an antiserum to the rat brain NOS. Lane 1, peak fraction from DEAE-Bio-Gel-A column, 0.2 μg of protein. Lane 2, same as lane 1, but treated with irrelevant immune serum. Molecular masses in kDa are indicated.

lecular mass of 110±5 kDa (mean±S.D., $n=3$). This was about half of that estimated for active NOS (230±20 kDa) using Superose gel filtration column. The identity of the 110 kDa protein was further confirmed as NOS by Western blot analysis. An antiserum to the rat brain NOS reacted with the NOS purified from *L. donovani* (Fig. 1B). The polyclonal anti-brain NOS antiserum did not cross-react with mac NOS (iNOS) by immunoblot analysis. However, it recognized both the brain and endothelium Ca^{2+}-dependent NOS from human and mouse.

Some physical and kinetic parameters of purified *L. donovani* NOS are listed in Table 2. The K_m for L-arginine as substrate (4.9±0.3 μM) is somewhat lower than that for mammalian enzyme [3]. NLA was found to be a competitive inhibitor of *Leishmania* NOS with an apparent K_i of 6.8±0.4 μM. Similar observations for NLA have been made with mammalian NOS systems in which the range of K_i is usually 0.5–10 μM [13]. Dependence of the isolated enzyme on cofactors is shown in Table 3. *L. donovani* NOS requires the addition of Ca^{2+}, calmodulin and NADPH for activity after purification and is inhibited by EGTA and trifluoperazine, a calmodulin antagonist. NOS activity was decreased to 55% of the control value when H_4B was omitted from the incubation mixture. In the absence of flavins, the enzyme activity was 25% of the control, but omission of either FAD or FMN had no significant effect on citrulline formation, suggesting that either of the flavins is sufficient for coenzyme function or one may be present as an impurity in the other. Kinetic characteristics, cofactor requirements, inhibitor studies and immunoblot analysis of *Leishmania* NOS exhibited close biochemical and immunological similarities to constitutive isoforms of mammalian NOS, including the enzymes from brain [3] and vascular endothelium [14]. However, *Leishmania* NOS subunits (110 kDa) are somewhat smaller than the SDS-denatured constitutive NOS from mammals, the subunits of which range from 150 to 160 kDa [3,12–14].

The significance of the occurrence of NOS in

Table 2
Physical and kinetic characteristics of *L. donovani* NO synthase

Molecular mass	
Native	230 kDa
Denatured	110 kDa
K_m, L-arginine	4.9 μM
K_m, NADPH	0.7 μM
V_{max}	3.5 μmol of citrulline formed min^{-1} mg $protein^{-1}$
K_i, N^{ω}-nitro-L-arginine	6.8 μM

Table 3
Effect of added cofactors on *L. donovani* NO synthase

Addition or omission	NOS activity[a] (μmol min^{-1} mg $protein^{-1}$)	Inhibition (%)
None	3.21±0.11	0
+2 mM EGTA	0.16±0.01	95
+50 μM trifluoperazine	0.29±0.01	91
−Ca^{2+}	0.09±0.01	97
−Calmodulin	1.55±0.06	52
−(Ca^{2+}+calmodulin)	0.03±0.01	99
−NADPH	0.03±0.01	99
−Tetrahydrobiopterin	1.77±0.09	45
−FAD	2.82±0.13	12
−FMN	2.99±0.12	7
−(FAD+FMN)	0.80±0.04	75

Assay mixture contained 50 mM Tris-HCl, pH 7.5, 2 mM $CaCl_2$, 0.1 mM NADPH, 10 μM FAD, 10 μM FMN, 1 μg calmodulin, 10 μM tetrahydrobiopterin and 0.5 μg of enzyme protein in a final volume of 100 μl.

[a]Data represent mean±S.D. of three independent determinations.

Leishmania is not known at present. It is, however, known that NO generated by macrophages is cytotoxic for a variety of microorganisms including *L. donovani* [1] and *L. major* [15]. Moreover, *Leishmania* infection induced increased NO synthesis in vivo and cytokine-inducible synthesis of NO from L-arginine is an important effector mechanism in expression of natural resistance to *Leishmania* [16]. The relationship between the two NO-generating systems in the parasite and in their host cell warrants further investigation. It may, however, be mentioned that Ca^{2+} plays a crucial role during establishment of *Leishmania* infection as there is direct evidence for defective regulation of Ca^{2+}- and calcium-dependent signalling in *Leishmania*-infected macrophages [17]. It is also known that in higher eukaryotes, Ca^{2+} release from internal stores activates a Ca^{2+}-dependent constitutive NOS to generate NO and citrulline [18]. The NO activates guanylyl cyclase to generate cGMP which in turn modulates Ca^{2+} entry. The presence of constitutive NOS raises the possibility of a similar type of cross-talk between the Ca^{2+} and NO signalling systems in a lower eukaryote like *Leishmania*.

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