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Nitric oxide synthase activity from a hematophagous insect salivary gland

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The salivary glands of the hematophagous insect, *Rhodnius prolixus*, contain a nitrosylhemeprotein that dissociates its ligand, NO, to the host tissues while the insect is searching for a blood meal. We now report a salivary nitric oxide synthase activity in this insect. The activity is dependent on NADPH, FAD, tetrahydrobiopterin, calmodulin, Ca^{2+}, and converts arginine to citrulline while producing vasorelaxing activity. Molecular sieving indicates a molecular weight of 185 kDa, coeluting with a diaphorase activity. Results indicate similarity of this insect activity to the vertebrate constitutive NO synthase, suggesting NO synthesis is an evolutionary old biological pathway.

Nitric oxide; Nitric oxide synthase; Salivary gland; *Rhodnius prolixus*; Invertebrate

1. INTRODUCTION

Nitric oxide has recently been identified as an intercellular messenger with a variety of important physiological functions in vertebrates [1]. The enzyme that synthesizes NO, NO synthase, has been purified, characterized and cloned from several mammalian tissues [2]. However, evidence for production of NO in invertebrates is limited to the demonstration of NO production by horseshoe crab (*Limulus polyphemus*) hemocytes [3], and by a NO-containing hemeprotein in the salivary glands of the blood sucking bug, *Rhodnius prolixus* [4]. This nitrosylhemeprotein acts as a vasodilator and platelet antiaggregating substance during blood feeding [5]. The presence of NO in the salivary glands of *Rhodnius* suggests an interesting case of convergent evolution, since vertebrates use the same molecule in the regulation of vascular tone and platelet aggregation [1].

Vertebrate NO synthases are complex enzymes, requiring L-arginine, O_2, and NADPH as substrates, and FAD and BH4, as well as a reducing compound such as dithiotreitol, as cofactors [6,7]. The formation of NO from L-arginine is catalyzed by inducible or constitutive isoforms of NOS. The constitutive enzyme of nervous and endothelial tissues needs Ca^{2+} and calmodulin for activity; the induced enzyme from macrophages does not require these cofactors [8]. NO and citrulline are products of the reaction [6,7]. The enzyme also displays a number of activities in addition to synthesis of NO, including the transfer of two electrons from NADPH to tetrazolium dyes, a diaphorase reaction [6]. In this paper we characterized the existence of a NOS activity in an invertebrate, *Rhodnius prolixus*.

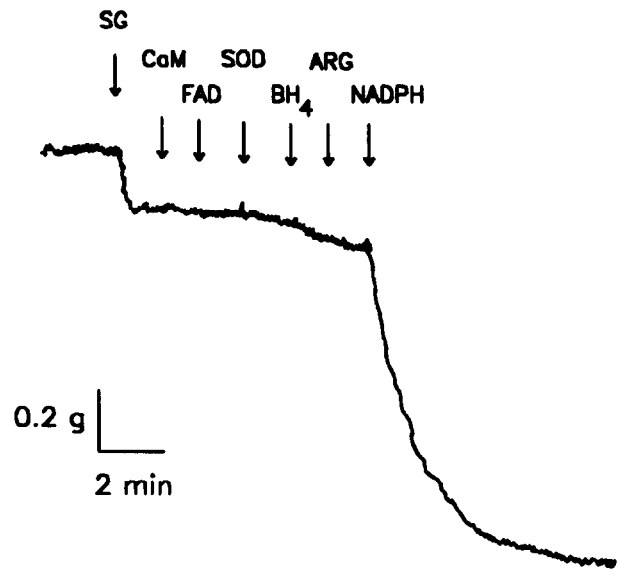

Fig. 1. *Rhodnius prolixus* salivary gland homogenates (devoid of stored secretion) from fifth instar nymphs (5 pairs of glands in 2.5 ml bath), were added to a rabbit aorta ring immersed in Krebs solution and pre-constricted with 1 μM noradrenaline. Aortic rings, whose endothelia had been scraped off with a wooden stick, had a resting tension of 2.5 g. Cofactors for NOS were added to a final concentration of: calmodulin (CaM) 10 $\mu g \cdot ml^{-1}$, FAD 6 μM, superoxide dismutase (SOD) 50 U ml^{-1}, tetrahydrobiopterin (BH4) 10 μM, L-arginine (ARG) 0.1 mM and NADPH 0.1 mM. An interval of 1 minute was given between the addition of each cofactor. Control rings were from the same rabbit and were run simultaneously with the ring containing all cofactors.

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Abbreviations: NOS, nitric oxide synthase; CaMn calmodulin; BH4, tetrahydrobiopterin.

2. MATERIALS AND METHODS

Organic chemicals were obtained from Sigma Chem. Co. (USA), except for tetrahydrobiopterin, which was obtained from ICN Biochemicals (USA). Tritiated arginine was obtained from New England Nuclear (USA). Inorganic reagents were of P.A. or A.C.S. grade.

Insects were reared at the Department of Entomology, from insects kindly provided by Dr. Robert Tesh, Yale Arbovirus Research Unit, New Haven, CT. Fifth instar nymphs of *Rhodnius prolixus* were used as a source of salivary glands. These insects had been fed as 4th instar nymphs at least 30 days before. Glands were dissected out from the insects immersed in phosphate-buffered saline (0.15 M NaCl and 10 mM $NaPO_4$, pH 7.0), and the glands subsequently torn open with fine forceps (Dumont #5) to remove the secreted contents. These gland ghosts where washed in PBS (20 pairs in 100 μl, 10,000 $\times$ *g* for 5 s), resuspended in 100 μl PBS and sonicated for 30 seconds for cellular disruption. In some experiments glands were resuspended to a higher concentration (1 pair per μl).

Rabbit aortic rings smooth muscle bioassays were performed as in [5], using oxygenated (95% O_2 and 5% CO_2) Krebs solution.

NOS activity was determined in a reaction mixture, final volume 0.5 ml, containing 50 mM triethanolamine pH 7.5, 0.1 M sodium chloride, 1 mM calcium chloride, 1 mM dithiothreitol, 10 μM tetrahydrobiopterin, 3 μM FAD, 10 $\mu g \cdot ml^{-1}$ calmodulin, 10 μM [^{3}H]L-arginine (2×10^6 cpm·ml^{-1}) and 2.5 pairs of gland homogenate (0.1 mg protein per pair). 50 μl aliquots were mixed at different times with 0.5 ml of 20 mM HEPES buffer pH 5.5, 2 mM EGTA and 1 mM citrulline, and were applied to 1 ml Alltech (USA) cation extract columns (Na^+ form)[9]. [^{3}H]Citrulline was quantified by liquid scintillation spectroscopy of the flow-through. To identify reaction products such as cit-

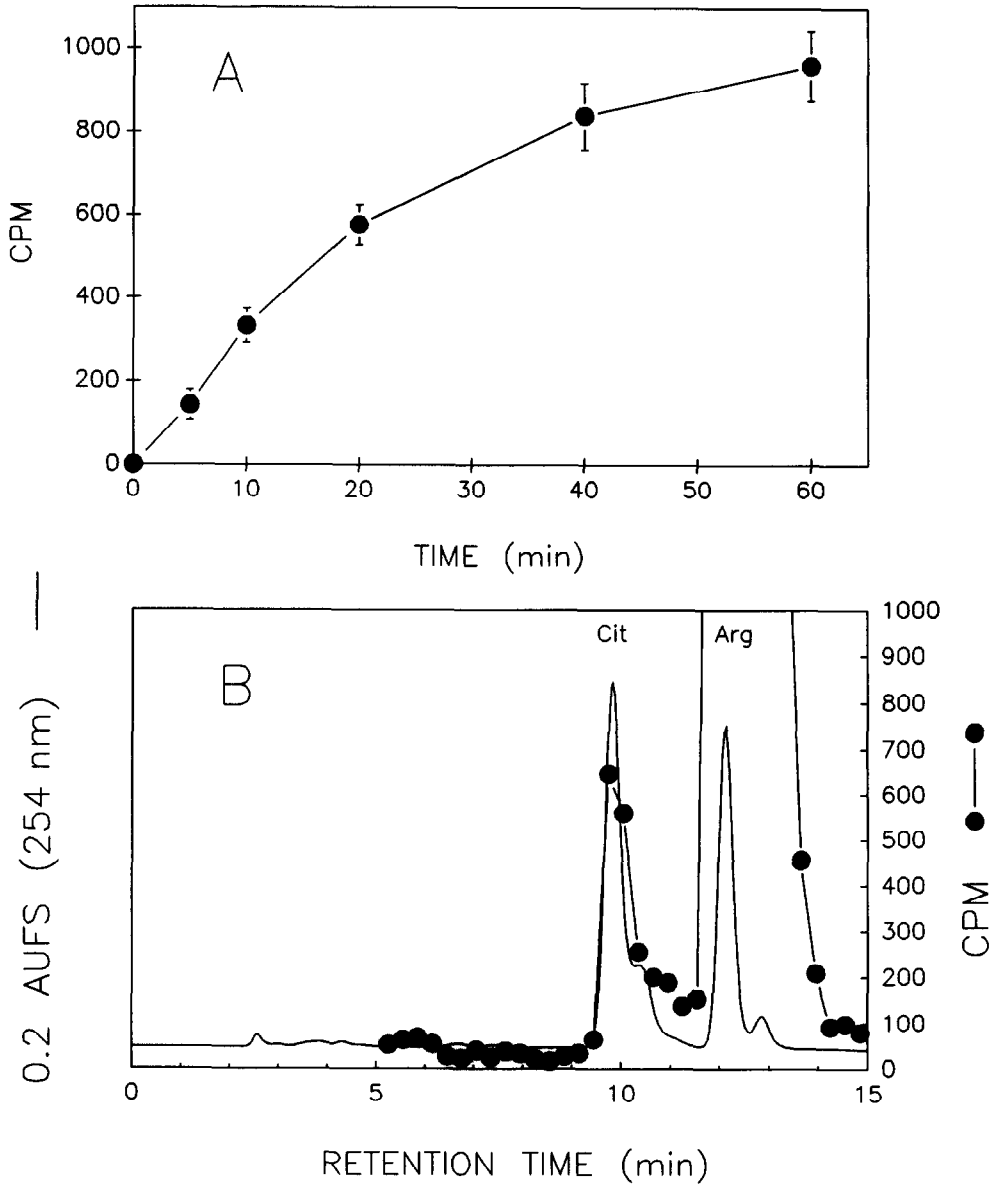

Fig. 2. Production of [^{3}H]citrulline from [^{3}H]arginine by *Rhodnius* salivary gland homogenates. (A) Time-dependent formation of citruline from arginine. The symbols and bars indicate the mean ± S.E. of 3 experiments. (B) Reverse-phase HPLC of derivatized amino acids after 15 min incubation with salivary homogenate and cofactors. The absorbance at 254 nm represents internal standards (50 pmol each) of arginine and citrulline added after stopping the reaction. Other experimental details can be found in section 2.

rulline, reaction mixture identical to those above described were used, but containing 80,000 cpm of [^{3}H]arginine μl^{-1}. After incubation for 15 min, 1 μl aliquots were mixed with 20 μl 5% trifluoroacetic acid, centrifuged, and the supernatant evaporated. Arginine and citrulline (50 pmol each) were added, and the sample processed by reverse-phase HPLC as in [10], using an Econosphere C18 column (4.3×250 mm) obtained from Alltech (USA).

Molecular sieving HPLC of *R. prolixus* salivary gland homogenates was done with twenty pairs of homogenized salivary glands applied to TSK-250 column (300 mm × 7.5 mm) and pre-column (75 mm × 7.5 mm, obtained through Bio-Rad, USA) equilibrated with PBS and running at a flow rate of 0.8 ml·min-1. Fractions were collected every 0.4 min and were assayed for NOS and diaphorase activity. Absorbance at 280 nm was monitored. NOS activity was monitored by taking 25 μl of each fraction and adding 25 μl of reaction mixture as above. The diaphorase reaction media contained 50 mM phosphate buffer pH 7.5, 0.1 M sodium chloride, 0.1% Triton X-100, 0.1 mM calcium chloride, 1 mM NADPH and 0.5 mg·ml^{-1} 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (final concentration). 50 μl of fractions were taken and added to the reaction mixture to a final volume of 100 μl, progression of the reaction, at 37°C, was followed colorimetrically at 550 nm in a 96-well microplate reader (Molecular Devices Thermomax microplate reader with a computer based kinetics measurement module). All HPLC procedures used an LDC - Milton Roy series 4,000 pump and detector.

3. RESULTS AND DISCUSSION

When a rabbit aortic ring was incubated with *Rhodnius* salivary gland homogenates, from which stored saliva containing nitrosylhemeprotein had been removed, a small vasodilation was observed (Fig. 1). Addition of arginine and several vertebrate NOS cofactors, and superoxide dismutase (to increase the half life of NO [9]), caused no further vasodilation. When NADPH was added, 100% vasodilation was achieved within a few minutes. This vasodilation was not observed in the absence of homogenate, and could be reproduced with the omission of some cofactors, but not in the absence of arginine, NADPH or the homogenate (not shown). This experiment suggests the production of NO from arginine and NADPH.

To further investigate the fate of arginine, salivary gland homogenates were incubated with radiolabelled arginine and the reaction mixture applied to a cation-exchange column [9] to check for the production of radioactive citrulline. A radiolabelled product was generated that did not bind to the column, suggesting conversion of arginin e to citrulline (Fig. 2A). Indeed, derivatization and re verse phase chromatography of the reaction products [10] (Fig. 2B), indicated that *Rhodnius* homogenates were converting arginine to citrulline.

Utilizing this assay, a number of co-factors and inhibitors of NOS were studied (Fig. 3). As with the vertebrate constitutive NOS [11], the reaction was inhibited by the arginine analog L-NMMA. Evidence for a calmodulin role was indicated by activation of the reaction by exogenous calmodulin, and complete inhibition when EGTA was added instead of Ca^{2+}. The reaction was also activated by exogenous tetrahydrobiopterin in a similar magnitude to vertebrate enzymes [12]. FAD strongly activated the reaction, and NADH did not substitute for NADPH. However, in contrast to the vertebrate NOS, dithiotreitol had no effect on the reaction. We also tested other reducing reagents such as

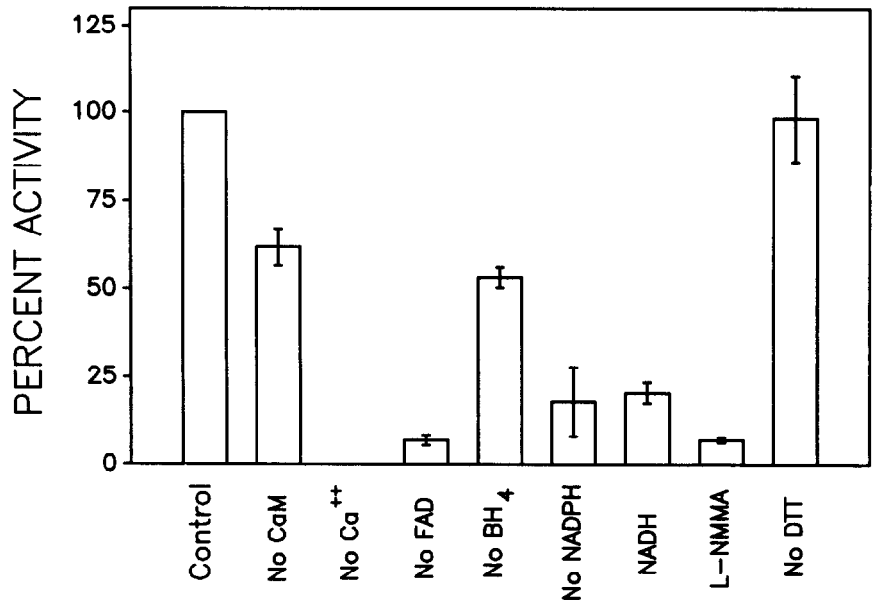

Fig. 3. Cofactor requirements of *Rhodnius* NOS. Reaction media contained 50 mM triethanolamine pH 7.5, 0.1 M sodium chloride, 0.5 pair of homogenized salivary glands and 10 μM [^{3}H]arginine (2×10^6 cpm·ml^{-1}). The various cofactors were added in order to obtain a final concentration of 1 mM for calcium chloride (Ca^{2+}), 10 μg·ml^{-1} calmodulin (CaM), 3 μM FAD, 0.1 mM NG-monomethyl-L-arginine (L-NMMA), 10 μM tetrahydrobiopterin (BH_4), 1 mM NADPH, 1 mM NADH and 1 mM dithiothreitol (DTT). The reaction was stopped using the same procedures as in Fig. 2A. The bars indicate mean ± S.E.M. of 3 experiments.

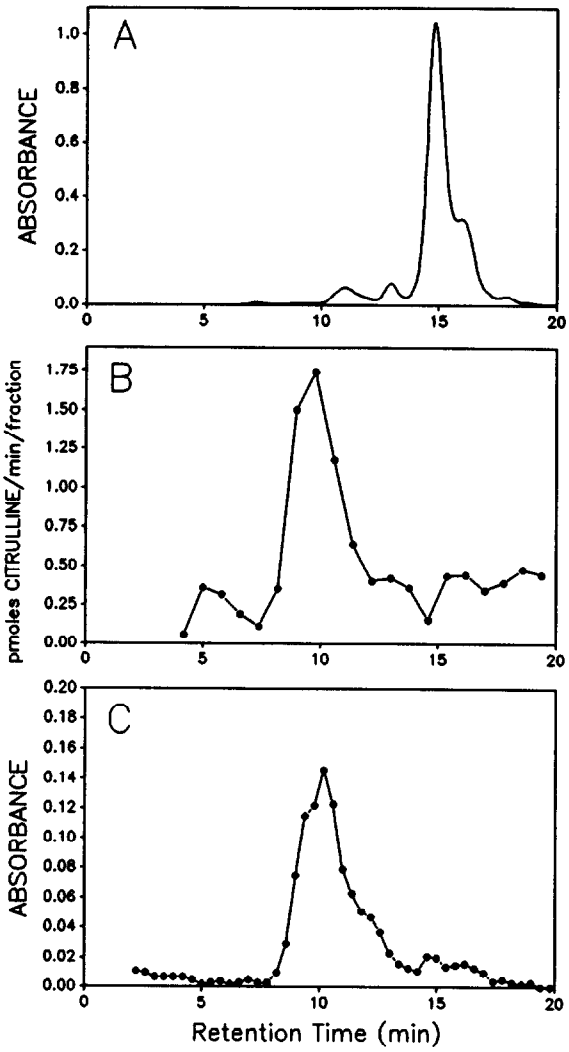

Fig. 4. Molecular sieving HPLC of *R. prolixus* salivary gland homogenates. (A) Absorbance at 280 nm. (B) NOS activity. (C) Diaphorase activity. Experimental details can be found in section 2.

2-mercaptoethanol, cysteine and glutathione, which produced no increase in activity (not shown). Perhaps endogenous regeneration of reduced biopterin is accomplished by the homogenate.

To determine the apparent molecular weight of *Rhodnius* NOS, as well as to test for diaphorase activity in the salivary glands, homogenates were chromatographed on a TSK-250 column. Arginine converting activity and diaphorase activity, tested with NADPH and the tetrazolium dye MTT, coeluted with an apparent molecular mass of 185 kDa (Fig. 4) similar to the vertebrate monomer, estimated at 160 kDa [13].

The synthesis of NO involves distinct enzymes in the nervous system, endothelium and macrophages [14]. The enzymes that catalyze the formation of NO in these tissues require different cofactors for complete activity. The remarkable similarity between our results for *Rhodnius* NOS and the vertebrate constitutive enzyme suggests that the constitutive forms of NOS are homologous enzymes in vertebrates and invertebrates, indicating that NO signal transduction must have originated at an early point in eukaryote evolution.

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