Identification and characterization of a calmodulin-dependent nitric oxide synthase from GH₃ pituitary cells

Donald J. WOLFF* and George A. DATTO

Department of Pharmacology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, NJ 08854, U.S.A.

A nitric oxide synthase activity stimulated more than 30-fold by the concurrent presence of Ca²⁺ and calmodulin (CaM), and inhibited by trifluoperazine (50 μ M), has been identified in extracts of GH₃ pituitary cells. The CaM-dependent nitric oxide synthase of the crude extract was stimulated more than 9-fold by (6*R*)-5,6,7,8-tetrahydro-L-biopterin with half-maximal stimulation occurring at a concentration of 300 nM. Fractionation of the extract on DEAE-cellulose enhanced nitric oxide synthase specific activity up to 300-fold and provided a preparation which on Western blot analysis possessed a 152 kDa protein which cross-reacted with antibodies to homogeneous bovine brain nitric oxide synthase. The DEAE-cellulose-purified enzyme exhibited apparent K_m values of 4.3 μ M, 0.4 μ M, 0.3 μ M and 4 nM for L-arginine, NADPH, Ca²⁺ and CaM respectively. The CaM-dependent nitric oxide synthase of GH₃ extract bound to 2',5'-ADP-agarose and was eluted by NADPH with a 500-fold increased specific activity. Citrulline formation by the ADP-agarose-purified enzyme was inhibited by *N*^G-nitro-L-arginine, *N*^G-methyl-L-arginine and Nitro Blue Tetrazolium with apparent K_i values of 0.2, 1.8 and 7 μ M respectively. The ADP-agarose-purified enzyme displayed cytochrome *c* reductase activity which was stimulated more than 18-fold by the concurrent presence of Ca²⁺ and CaM and inhibited by trifluoperazine. *N*^G-Nitro-L-arginine and *N*^G-methyl-L-arginine did not inhibit the cytochrome *c* reductase activity.

INTRODUCTION

Nitric oxide has been identified as a major regulatory substance exerting effects both inter- and intra-cellularly [1]. It has been implicated in modulation of platelet function [2], regulation of blood flow [3], macrophage cytotoxicity [4] and in the effects [5] and toxicity [6] of the excitatory neurotransmitter glutamate. Nitric oxide is cosynthesized with citrulline from L-arginine and molecular oxygen by nitric oxide synthases which require NADPH [1]. The effects of nitric oxide are believed to be mediated by stimulation of a soluble form of guanylate cyclase [7] which synthesizes cyclic 3',5'-GMP.

Two major forms of nitric oxide synthase have been described. One form, characterized primarily from macrophages [8,9], is induced by cytokines [10], requires tetrahydrobiopterin (THB) for activity and is independent of regulation by Ca²⁺ and calmodulin (CaM). The second form, described primarily in endothelial cells [11] and brain [12,13], is constitutive, Ca²⁺- and CaM-dependent, and does not require exogenous THB for activity. Recent reports [14,15] have indicated that a CaM-dependent nitric oxide synthase from pig and rat cerebellum, although not requiring THB, is stimulated 2–4-fold in its presence.

GH₃ cells are a particularly appropriate system for studying Ca²⁺-regulated processes. They are known to possess voltage-regulated Ca²⁺ channels [16] and to mobilize Ca²⁺ from intracellular stores in response to thyrotropin-releasing hormone by an inositol trisphosphate-dependent mechanism [17]. The regulation of Ca²⁺ mobilization in GH₃ cells has been described in detail [18,19]. Further, regulation of protein phosphorylation in these cells by cyclic AMP [20], Ca²⁺ [21] and diacylglycerol [22] has been examined extensively. Accordingly, GH₃ cells appear to be an ideal system in which to study regulation of nitric oxide synthase by Ca²⁺ and its potential modulation by signal-dependent protein phosphorylation.

We report in this paper the presence in GH₃ cells of a calmodulin-dependent nitric oxide synthase of high specific activity. This enzyme has been characterized in detail and found to be kinetically and immunologically similar to the bovine brain enzyme. The GH₃ CaM-dependent nitric oxide synthase appears unusual in its 9-fold stimulation by exogenous THB.

MATERIALS AND METHODS

Materials

Aprotinin, CHAPS, Mops, EGTA, dithiothreitol (DTT), diisopropyl fluorophosphate, 2',5'-ADP-agarose, cytochrome *c*, Nitro Blue Tetrazolium (NBT), NADPH, *N*^G-nitro-L-arginine and *N*^G-methyl-L-arginine were obtained from Sigma. L-[2,3-³H]Arginine (40–70 Ci/mmol) and ¹²⁵I-labelled protein A (2–10 μ Ci/ μ g) were obtained from NEN Research Products. Whatman DEAE-cellulose was obtained from Fisher Scientific. Bovine brain CaM was prepared by a modification of the procedure of Gopalakrishna & Anderson [23]. THB was obtained from Hoffman–LaRoche Pharmaceuticals. Trifluoperazine was obtained from Smith, Kline and French Pharmaceuticals.

Assay of nitric oxide synthase by measuring citrulline formation

Nitric oxide synthase activity was measured by a modification of the procedure of Bredt & Snyder [13]. Standard incubation mixtures contained 30 mM-Hepes, pH 7.5, 1 mM-EGTA, 0.85 mM-Ca²⁺ (when present), 2 μ g of CaM/ml (when present), 200 000 c.p.m. of carrier-labelled ³H-arginine and when present the indicated concentrations of NADPH and THB in a final reaction volume of 150 μ l. Reactions were conducted in 5 ml glass scintillation vials and were initiated by the addition of nitric oxide synthase appropriately diluted in 1 mg of BSA/ml. Reactions were conducted at 30 °C for 30 min. Reactions were terminated by the addition of 1 ml of AG 50WX8 resin in 20 mM-Mes, pH 5.5, containing 2 mM-EDTA. Ecolite scintillant (4 ml)

Abbreviations used: CaM, calmodulin; DTT, dithiothreitol; THB, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; NBT, Nitro Blue Tetrazolium.

* To whom correspondence should be addressed.

was added, the vials were vortexed, the resin allowed to settle and counted for radioactivity in a liquid-scintillation spectrometer. Blank samples containing all components save nitric oxide synthase source routinely provided values which contained 2–2.5% of total added radioactivity regardless of the arginine specific radioactivity (radioactivity of [^3H]arginine bound to resin is virtually completely quenched). These values were subtracted from values obtained from incubation mixtures containing enzyme. Citrulline (which does not bind to the resin under these conditions) formation was calculated from the known specific radioactivity of arginine. All incubations were conducted in duplicate and the mean values calculated. Variability of values about the mean routinely averaged $\pm 3\%$ of the mean.

A unit of enzyme activity is the amount of enzyme that catalyses formation of 1 nmol of product/min at the specified conditions of assay. Citrulline formation was found to be linear with time in incubations conducted for up to 30 min regardless of the presence of THB.

Purification of nitric oxide synthase from bovine brain and preparation of an antiserum

CaM-dependent nitric oxide synthase was purified from bovine brain extract to homogeneity by successive chromatography on columns of DEAE-cellulose, ADP-agarose and Affi-Gel CaM (D. J. Wolff & G. A. Datto, unpublished work; details available on application to the authors). Homogeneous bovine brain nitric oxide synthase (100 μg) emulsified in Freund's complete adjuvant was injected subcutaneously into three New Zealand White rabbits. After 3 weeks, each rabbit was re-injected subcutaneously with nitric oxide synthase (50 μg) dissolved in 10 mM-Tris/HCl (pH 7.5)/140 mM-NaCl (Tris-buffered saline). Rabbits were similarly boosted at weekly intervals. Blood obtained from the marginal ear vein was allowed to clot overnight and a serum was prepared by centrifugation at 2000 g for 5 min. Samples of serum were frozen at -70°C . Formation of antibodies was noted by determination of the ability of the serum to precipitate ^{125}I -labelled bovine brain nitric oxide synthase prepared by a Bolton-Hunter procedure [24] in the presence of insoluble protein A. Within 5 weeks of the initial injection, antibodies were readily detected in sera at a 1:1000 dilution.

Western blot analyses

Analyses were performed by a modification of the procedure of Burnette [25]. Briefly, samples were fractionated on a 6–16% acrylamide gradient slab gel using the procedure of Laemmli [26]. The sample was transferred to nitrocellulose sheets for 3 h at 90 V. The nitrocellulose sheet was blocked by incubation with 5% non-fat dry milk for 1 h, incubated with antiserum diluted 1:100 in 5% non-fat dry milk in Tris-buffered saline, washed with 0.05% Tween 20 in Tris-buffered saline, incubated with 20 μCi of ^{125}I -labelled protein A in 5% non-fat dry milk in Tris-buffered saline, washed three times in 0.05% Tween 20 in Tris-buffered saline, allowed to dry and subjected to autoradiography.

Preparation of GH_3 -cell nitric oxide synthase purified by adsorption to ADP-agarose

GH_3 cells were grown and harvested as described in detail previously [27]. Cells were homogenized in 5 vol. of 50 mM-Mops(pH 7.5)/1 mM-EGTA/1 mM-DTT/0.2 mM-di-isopropyl fluorophosphate/10 μg of aprotinin/ml, and a 12000 g (30 min) supernatant was prepared. To 12 ml of supernatant, 300 μl of settled ADP-agarose was added in a 15 ml plastic centrifuge tube. The slurry was rocked for 30 min at 4°C and centrifuged at 500 g for 5 min; the supernatant was discarded and the pellet was washed with homogenization buffer containing 0.5 M-NaCl, then with homogenization buffer containing 0.5 M-NaCl and

0.1% CHAPS, and finally with 1.5 ml of homogenization buffer containing 0.1% CHAPS and 5 mM-NADPH.

Assay of cytochrome *c* reductase activity

The assay of cytochrome *c* reductase activity was performed by using nitric oxide synthase purified through the ADP-agarose step as described above with the single modification that DTT was removed from all wash buffers. DTT, if present, reduces cytochrome *c* chemically without nitric oxide synthase and hence must be removed to assay activity. Reactions were conducted in disposable polystyrene cuvettes in a reaction volume of 1 ml containing 100 mM-Hepes, pH 7.5, 1 mM-EGTA, 0.85 mM- Ca^{2+} (when present), 10 μg of CaM/ml (when present) and the indicated concentrations of NADPH and cytochrome *c*. Incubations were initiated by addition of the nitric oxide synthase source, and absorbance changes at 550 nm were followed using a Shimadzu 160U spectrophotometer. Calculation of product formation was performed using the published absorption coefficient for reduced cytochrome *c* [28].

Miscellaneous procedures

Protein concentrations were determined either as absorbance at 280 nm or according to the method of Bradford [29] with BSA as the reference standard. Solutions of known Ca^{2+} concentration buffered with EGTA were constructed and calculated as described by Whitaker & Baker [30]. THB was stored with desiccant in a -20°C freezer, and solutions were prepared from the solid state by reconstitution immediately before use.

RESULTS

Properties of the nitric oxide synthase activity of GH_3 pituitary cell extract

An extract of GH_3 cells prepared as described in the legend to Table 1 was examined for nitric oxide synthase activity in the presence of EGTA, 8 μM - Ca^{2+} , and 8 μM - Ca^{2+} with exogenously added CaM. Nitric oxide synthase activity stimulated over 11-fold by micromolar concentrations of Ca^{2+} was observed (Table 1). Addition of exogenous CaM produced a barely detectable enhancement of activity consistent with the presence of near-saturating concentrations of CaM in the extract. To confirm that the effect of Ca^{2+} was mediated by endogenous CaM, the effect of trifluoperazine, a known CaM inhibitor, on the Ca^{2+} -dependence was determined. At 50 μM -trifluoperazine, the Ca^{2+} -dependent stimulation of activity was eliminated, but could be restored partially by the addition of exogenous CaM. Since THB has been shown to stimulate the activity of pig cerebellum [14] CaM-dependent nitric oxide synthase and to be required for expression of the nitric oxide synthase activity of macrophages [9], we examined its effect (33 μM) on the nitric oxide synthase activity of the GH_3 extract. At this concentration, it was found to stimulate the CaM-dependent nitric oxide synthase activity more than 6-fold. Trifluoperazine was also able to inhibit the activity stimulated by THB. As measured in the presence of THB, the nitric oxide synthase activity of GH_3 extract was stimulated more than 33-fold by the concurrent presence of Ca^{2+} and CaM; thus apparently all of the activity seen in the extract under the conditions of measurement is the CaM-stimulated form of activity.

The THB concentration-dependence of nitric oxide synthase activity of GH_3 extract was determined (Fig. 1). Stimulation of activity by THB occurred over a broad range of concentrations from 10^{-9} to 10^{-4} M, with half-maximal stimulation occurring at a concentration of approx. 300 nM. THB at saturating concentrations stimulated activity more than 9-fold relative to incubations without THB. The THB concentration-dependence

Table 1. Effects of Ca²⁺, CaM, THB and TFP on the nitric oxide synthase activity of GH₃ cell crude extract

GH₃ cells were homogenized in 5 vol. of 50 mM-Mops (pH7.5)/1mM-EGTA/1mM-DTT/0.2mM-di-isopropyl fluoro-phosphate/10 µg of aprotinin/ml, and a 12000 g (30 min) supernatant was prepared. Nitric oxide synthase activity was assayed by measuring citrulline formation in standard incubation mixtures containing 70 nM-[³H]arginine as described in the Materials and methods section under three conditions: 1 mM-EGTA, 8 µM-Ca²⁺ or 8 µM-Ca²⁺ + 2 µg of CaM/ml. Incubations were carried out without further additives containing THB (33 µM), trifluoperazine (50 µM) or both THB (33 µM) and trifluoperazine (50 µM). The data shown are the means of duplicate determinations and are from a typical experiment representative of three experiments with similar results.

Additive	Nitric oxide synthase activity (pmol of citrulline formed/min per mg of protein)		
	EGTA	Ca ²⁺	CaM
None	0.69	7.98	8.15
THB (33 µM)	1.48	49.4	50.8
Trifluoperazine (50 µM)	0.78	0.80	2.41
THB (33 µM)+TFP (50 µM)	1.23	1.23	1.83

Fig. 1. Effect of THB on the CaM-dependent nitric oxide synthase activity of GH₃ cell crude extract

A GH₃ cell extract, prepared as described in the legend to Table 1, was used as the source of nitric oxide synthase activity. It was measured by assaying citrulline formation in standard incubation mixtures containing 70 nM-[³H]arginine, 8 µM-Ca²⁺, 2 µg of added CaM/ml and the indicated concentration of THB by the procedure described in the Materials and methods section. Incubation mixtures containing no added THB formed 8.9 pmol of citrulline/min per mg of protein, and incubation mixtures containing 333 µM-THB formed 82 pmol/min per mg of protein (maximal activity). Results are expressed as the percentage of this maximal activity. Each point is the mean of duplicate determinations, and the data shown are from a typical experiment representative of three experiments with similar results.

of nitric oxide synthase activity was also examined by using enzyme purified on ADP-agarose (results not shown). Enzyme activity was stimulated 8-fold by THB over the same broad range of concentration, with half-maximal stimulation occurring at

Fig. 2. DEAE-cellulose chromatography of GH₃ cell extract

A GH₃ cell extract, prepared from 3 ml of wet packed cells as described in the legend of Table 1, was adjusted with deionized water to a conductivity equal to that of homogenization buffer and was applied to a column (0.9 cm × 10 cm) of DEAE-cellulose which had been pre-equilibrated with homogenization buffer. The column was washed with 50 ml of homogenization buffer before a gradient of NaCl (—) in homogenization buffer was initiated. The limit buffer contained 0.4 M-NaCl in a total gradient volume of 60 ml. Fractions (2 ml) were collected and were assayed for protein as absorbance at 280 nm (●) or nitric oxide synthase activity (△) in standard incubation mixtures containing 70 nM-[³H]arginine, 8 µM-Ca²⁺, 2 µg of CaM/ml, 1 mM-NADPH and 300 µM-THB.

600 nM-THB. The specific activity of the CaM-dependent nitric oxide synthase of GH₃ crude extract (82 pmol of citrulline formed/min per mg of protein) was 20-fold higher than that of a bovine brain crude extract prepared and assayed identically (results not shown).

Properties of the CaM-dependent nitric oxide synthase partially purified from GH₃ extracts by DEAE-cellulose chromatography

In order to allow a more detailed examination of the properties of the GH₃-cell nitric oxide synthase and their comparison with those described for the CaM-dependent nitric oxide synthase prepared from rat [12,13], pig [14] and bovine brain (D. J. Wolff & G. A. Datto, unpublished work), we subjected a crude extract to fractionation on a column of DEAE-cellulose as described in the legend to Fig. 2. The CaM-dependent nitric oxide synthase activity bound to DEAE-cellulose and was eluted as a major and a minor form by NaCl. The major form of activity, which was eluted at the leading edge of the NaCl gradient, represented a 60% recovery of applied activity, and exhibited a 318-fold increased specific activity relative to the sample applied to the column. The minor peak of activity which was eluted later in the NaCl gradient represented a 15% recovery of applied activity. Unlike the major form of activity, the minor form was highly unstable, which prevented its further kinetic characterization.

To ascertain the relationship of the GH₃-cell nitric oxide synthase (major form) to an activity previously purified to homogeneity from bovine brain, selected DEAE-cellulose fractions were examined in Western blots for cross-reactivity with antibodies prepared from the bovine brain enzyme as described in the Materials and methods section. The unfractionated GH₃ cell extract (applied sample) contained (Fig. 3, lane D) a major protein of 152 kDa which cross-reacted with the antibody to bovine brain nitric oxide synthase. The cross-

Fig. 3. Autoradiogram of ^{125}I -labelled protein A binding to SDS/PAGE-fractionated samples from DEAE-cellulose chromatography of GH_3 cell extract probed by Western blot analysis using a rabbit antiserum to purified bovine brain nitric oxide synthase

The samples were: lane A, 150 ng of homogeneous bovine brain enzyme; lane B, 50 ng of enzyme; lane C, 15 ng of enzyme; lane D, GH_3 cell extract (225 μg of protein); lane E, DEAE-cellulose fraction 30 (5 μg); lane F, DEAE-cellulose fraction 7 (6.3 μg); lane G, DEAE-cellulose fraction 36 (11 μg); lane H, DEAE-cellulose fraction 38 (16 μg); lane I, rat brain cerebellar extract (150 μg). Samples as indicated above were subjected to SDS/PAGE in the presence of prestained molecular-mass markers (kDa) on a 6–16% acrylamide gel using the system of Laemmli [26]. Gel proteins were transferred to nitrocellulose, and Western blot analysis was performed as described in the Materials and methods section.

Fig. 4. Effects of N^G -methyl-L-arginine, N^G -nitro-L-arginine and NBT on citrulline formation by GH_3 -cell nitric oxide synthase

Standard incubation mixtures were constructed containing 70 nM [^3H]arginine, 1 mM-NADPH, 8 μM - Ca^{2+} , 2 μg of CaM/ml and the indicated concentrations of N^G -nitro-L-arginine (Δ), N^G -methyl-L-arginine ($\square$) or NBT ($\bullet$). Incubations were initiated with 0.34 unit of nitric oxide synthase purified from GH_3 cell extract by ADP-agarose chromatography as described in the Materials and methods section. Values are expressed as the percentage of control values of incubations containing no added inhibitor. Each point is the mean of duplicate determinations and the data shown are from a typical experiment representative of three experiments with similar results.

Table 2. Kinetic characteristics of the nitric oxide synthase of GH_3 cells purified through the DEAE-cellulose chromatographic step

K_m (L-arginine) (μM)	4.3
K_m (NADPH) (μM)	0.4
K_m (calmodulin) (nM)	4.0
$K_{0.5}$ (Ca^{2+}) (μM)	0.3*
V_{max} (μmol of citrulline/min per mg)	0.27

* As measured in incubation mixtures containing 4 μM -CaM.

reacting protein migrated on SDS/PAGE identically with authentic bovine brain nitric oxide synthase (Fig. 3, lanes A, B and C) and also apparently identically with a protein from unfractionated rat cerebellar extract (Fig. 3, lane I). Examination of fraction 30 from the DEAE-cellulose chromatography, where CaM-dependent nitric oxide synthase activity peaked, revealed a marked enrichment for the cross-reacting protein (Fig. 3, lane E). Fractions drawn from other areas of the NaCl gradient, including the minor peak of activity, showed no significant cross-reactivity (Fig. 3, lanes F, G and H). Proteins other than the 150 kDa protein in a GH_3 cell extract showed some cross-reactivity with the antibody preparation. We suspect that these represent breakdown products of the parent molecule since fractionation on DEAE-cellulose did not affect their resolution from the major cross-reacting protomer (Fig. 3, lanes D and E).

The nitric oxide synthase activity of the DEAE-cellulose-fractionated enzyme (fraction 30) was examined in detail kinetically. After DEAE-cellulose chromatography which resolved nitric oxide synthase from endogenous CaM, expression of activity required the concurrent presence of Ca^{2+} and CaM (results not shown). The L-arginine concentration-dependence of citrulline formation was determined as described in the Materials and methods section in incubation mixtures containing saturating concentrations of NADPH (1 mM), Ca^{2+} (8 μM), CaM (4 μM) and

Fig. 5. Cytochrome *c* concentration-dependence of cytochrome *c* reduction

Standard incubation mixtures were constructed as described in the Materials and methods section containing 1 mM-NADPH, 8 μM - Ca^{2+} , 2 μg of CaM/ml and the indicated concentration of cytochrome *c*. Incubations were initiated with 0.54 unit of GH_3 -cell nitric oxide synthase purified through the ADP-agarose step, and the absorbance changes at 550 nm were monitored. The rate of formation of reduced cytochrome *c* was calculated from the known absorption coefficient of reduced cytochrome *c* [28]. Each point is the mean of duplicate determinations and the data shown are from a typical experiment representative of two experiments with similar results.

THB (300 μ M). Under these conditions, citrulline formation followed Michaelis–Menten kinetics and exhibited a K_m for L-arginine of 4.3 μ M (Table 2). The NADPH concentration-dependence of citrulline formation was measured in incubation mixtures containing 70 nM-L-arginine. In incubation mixtures containing 70 nM-L-arginine and saturating concentrations of Ca²⁺ (8 μ M), CaM (4 μ M) and THB (300 μ M), the NADPH concentration-dependence of activity obeyed Michaelis–Menten kinetics with a K_m value of 0.4 μ M (Table 2). The CaM concentration-dependence of citrulline formation was measured in incubation mixtures containing 70 nM-L-arginine, and saturating concentrations of NADPH (1 mM), Ca²⁺ (8 μ M) and THB (300 μ M). A K_m value of 4 nM for CaM was determined (Table 2). The Ca²⁺ concentration-dependence of citrulline formation was measured in incubation mixtures containing 70 nM-L-arginine and saturating concentrations of NADPH (1 mM), CaM (4 μ M) and THB (300 μ M) and was found to exhibit half-maximal activity at 0.3 μ M-Ca²⁺. At saturating concentrations of all components, the maximal velocity of nitric oxide synthase activity was 0.27 μ mol of citrulline formed/min per mg of protein, a value approximately one-tenth of that for homogeneous enzyme from bovine brain.

Properties of the CaM-dependent nitric oxide synthase purified from GH₃ cell extract by adsorption to ADP-agarose

Investigators from a number of laboratories have found that both the CaM-dependent [12,13] and the CaM-independent forms [8] of nitric oxide synthase bind to ADP-agarose and are eluted with NADPH. This technique has been the major contributor to the successful purification of nitric oxide synthase from these systems. Accordingly, we have examined whether similar behaviour extends to the enzyme from the GH₃ cell extract. Nitric oxide synthase was completely extracted from GH₃ cell extracts by ADP-agarose and recovered in eluates containing 0.1% CHAPS and NADPH as detailed in the Materials and methods section. The enzyme was recovered (43 %) successfully and exhibited a specific activity when measured at saturation concentrations of L-arginine, Ca²⁺, CaM and THB of 0.51 μ mol/min per mg of protein, approximately one-sixth of that seen for the homogeneous bovine brain enzyme. Examination of the NADPH-containing eluate by SDS/PAGE with Coomassie Blue staining revealed two major bands (not shown), one of 152 kDa and a more prominent band of 40 kDa. Only the 152 kDa protomer cross-reacted with antibodies to the bovine brain nitric oxide synthase in Western blots (results not shown).

Both CaM-dependent [30] and CaM-independent [10] nitric oxide synthase activities from a variety of sources have been shown to be inhibited competitively with respect to the L-arginine substrate by the L-arginine analogues, N^G-nitro-L-arginine and N^G-methyl-L-arginine. Accordingly, we examined the effect of these L-arginine analogues on the CaM-dependent nitric oxide synthase activity of the GH₃-cell enzyme using the highly purified ADP-agarose eluate. Measurements were conducted in incubation mixtures containing 70 nM-L-arginine, a concentration more than 60-fold below the K_m for the substrate. Under these conditions, the apparent K_i would virtually equal the K_i values, regardless of a competitive pattern of inhibition, since for competitive inhibition $K_{iapp.} = K_i[1 + (S/K_m)]$ [31]. An apparent K_i value of 0.22 μ M for N^G-nitro-L-arginine and an apparent K_i value of 1.8 μ M for N^G-methyl-L-arginine were determined for the enzyme (Fig. 4).

Hope *et al.* [32] have reported that NADPH diaphorase activity from brain exhibits nitric oxide synthase activity and that the dye NBT inhibits competitively the conversion of L-arginine into citrulline. To determine whether the GH₃-cell enzyme displayed similar properties, we examined the effect of NBT on

citrulline formation by the ADP-agarose-purified enzyme. NBT inhibited activity with an apparent K_i value of 7 μ M (Fig. 4). Since NADPH diaphorase activities from brain exhibit the ability to reduce a variety of substances including cytochrome *c* [27,32], we examined whether the ADP-agarose-purified GH₃-cell enzyme displayed this property. The NADPH-containing eluate from ADP-agarose catalysed an NADPH-dependent reduction of cytochrome *c* which was stimulated 18-fold by the concurrent presence of Ca²⁺ and CaM (results not shown). This activity was diminished to undetectable values by the addition of 100 μ M-trifluoperazine (results not shown). Examination of the cytochrome *c* concentration-dependence of this activity (Fig. 5) revealed that half-maximal activity was expressed at 15 μ M-cytochrome *c*. The maximal activity expressed at saturating concentrations of cytochrome *c*, NADPH, Ca²⁺ and CaM was 0.2 μ mol of cytochrome *c* reduced/min per mg of protein. THB was not added to these incubation mixtures since it was found to reduce cytochrome *c* chemically in the absence of added enzyme. This maximal velocity is approx. 5 % of that seen with the bovine brain enzyme (D. J. Wolff & G. A. Datto, unpublished work). Addition of L-arginine, N^G-nitro-L-arginine or N^G-methyl-L-arginine to incubations containing 15 μ M-cytochrome *c* produced no detectable alteration of cytochrome *c* reduction by the enzyme.

DISCUSSION

A Ca²⁺-, CaM- and NADPH-dependent nitric oxide synthase has been identified in extracts of GH₃ pituitary cells. Nitric oxide synthase activity fractionated on DEAE-cellulose is co-eluted with a 152 kDa protomer which cross-reacts with antibodies to bovine brain CaM-dependent nitric oxide synthase. Nitric oxide synthase from brain is known to reside on a protein whose denatured monomeric mass is approx. 150 kDa. The Ca²⁺, CaM, NADPH and L-arginine concentration-dependences of citrulline formation for the GH₃-cell nitric oxide synthase are virtually identical with those found for the homogeneous enzyme from bovine brain (D. J. Wolff & G. A. Datto, unpublished work), and are very similar to those reported for enzyme from rat [12,13] and pig [14] cerebellum. Citrulline formation by the GH₃-cell nitric oxide synthase is inhibited by N^G-nitro-L-arginine, N^G-methyl-L-arginine and NBT with apparent K_i values virtually identical with those observed for the bovine brain enzyme (D. J. Wolff & G. A. Datto, unpublished work) and very similar to those observed for CaM-dependent nitric oxide synthase(s) from endothelium [11] and rat cerebellum [13]. Further, the GH₃-cell enzyme is eluted from DEAE-cellulose and ADP-agarose under conditions found applicable previously in diverse systems. Clearly, the GH₃-cell CaM-dependent nitric oxide synthase resembles physically, kinetically and immunologically CaM-dependent nitric oxide synthase described from other tissue sources.

NADPH diaphorase activity from rat brain has been reported to exhibit nitric oxide synthase activity [32]. NADPH-dependent diaphorases catalyse the NADPH-dependent reduction of a variety of electron acceptors such as NBT, cytochrome *c* and 2,6-dichlorophenol-indophenol [33]. Preparations of the GH₃-cell nitric oxide synthase purified by elution from ADP-agarose with NADPH were found to catalyse cytochrome *c* reduction (Fig. 5). That reduction of cytochrome *c* and formation of citrulline from L-arginine are catalysed by the same enzyme in this preparation is supported by the observation that, in this highly purified preparation (only two bands on SDS/PAGE, one of 152 kDa), both activities depended on the concurrent presence of Ca²⁺, CaM and NADPH for expression of activity, and both activities were completely inhibited by trifluoperazine. However, both activities were not susceptible to inhibition by analogues of L-

arginine. Although *N*^G-nitro-L-arginine and *N*^G-methyl-L-arginine inhibited citrulline formation with *K_i* values of 0.2 and 1.8 μ M respectively, even at concentrations of more than 100–1000-fold above these values no effect on cytochrome *c* reduction measured at its *K_m* value was observed. This behaviour is identical with that observed for the bovine brain enzyme and is consistent with the proposal that cytochrome *c* and L-arginine interact at different sites on the enzyme or with different enzyme forms.

The most novel property of the GH₃-cell CaM-dependent nitric oxide synthase was its marked (7–9-fold in different preparations) stimulation by exogenous THB. THB exerted its stimulatory activity over an unusually broad range of concentrations. Similar behaviour was observed regardless of whether the enzyme source was an unfractionated crude extract or a highly purified ADP-agarose eluate. The basis for this broad concentration-dependence is uncertain, but it is possible that multiple sites with different affinities exist on the enzyme for THB. In support of this possibility it should be noted that, although a single stylized curve has been drawn through the data provided in Fig. 1, an apparent point of inflection occurs at a concentration of 1 μ M. This point of inflection was noted in each of three separate experiments, regardless of whether a crude extract or ADP-agarose-purified enzyme source was used. It is thus possible that interaction with two classes of sites of low and high affinity are necessary to effect full activation of the enzyme by THB.

A requirement for THB by the CaM-independent nitric oxide synthase of macrophages has been noted by several laboratories [1,8–11,34]; however, the CaM-dependent form expresses activity without added THB. Only recently has the stimulation of the CaM-dependent form by THB been emphasized, with up to a 4-fold stimulation of citrulline formation being noted for the pig cerebellar enzyme. Our observation for the GH₃-cell CaM-dependent nitric oxide synthase suggests that the THB-dependence of activity may vary dramatically for enzymes derived from different tissue sources, and that THB should be included when examining a biological tissue source for the presence of this enzyme. It is possible that the CaM-dependent nitric oxide synthase from some sources retains a tightly bound form of THB, which supports activity in the absence of added THB, whereas enzyme prepared from other sources with a lower affinity for bound THB may contain variable fractions of enzyme in a THB-depleted state, allowing stimulation by exogenously added THB. The marked stimulation of activity by THB encourages us to consider the possibility that bound THB is an obligatory component of the electron-transport sequence of this flavoprotein [35]. However, Giovanelli *et al.* [15] have reported that for the rat cerebellar enzyme THB does not function as a reactant in the oxygenation of arginine since product formation exceeded the stoichiometric availability of THB. Further, the regeneration of THB from the dihydro form would be expected to be inhibited by methotrexate, which had no effect on enzyme activity. These workers reported that citrulline formation was non-linear with time in incubations without THB. The GH₃-cell enzyme which is stimulated markedly by THB did not exhibit similar behaviour but provided linear product formation in incubations conducted for 30 min regardless of the presence of THB. We therefore believe that THB does not exert its effects by enzyme stabilization. It seems reasonable to believe that the effects of THB are mediated by an allosteric stimulation of enzyme activity. Additional studies will need to be performed to evaluate this mechanistic proposal.

Finally, we believe that the possession by GH₃ cells of a CaM-

dependent nitric oxide synthase which so closely resembles the activity purified from diverse brain sources will allow this cell line to be used to study the regulation of enzyme activity in intact cells by Ca²⁺-mobilizing agents and its potential modulation by signal-dependent protein phosphorylation.

This work was supported by National Institute of Health grant USPHS NS 11252.

REFERENCES

- Moncada, S., Palmer, R. M. J. & Higgs, E. C. (1991) *Pharmacol. Rev.* **43**, 109–134
- Radomski, M. W., Palmer, R. W. J. & Moncada, S. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5193–5197
- Rees, D. D., Palmer, R. M. J. & Moncada, S. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3375–3378
- Hibbs, J. B., Vavrin, Z. & Taintor, R. R. (1987) *J. Immunol.* **138**, 550–565
- Garthwaite, J. (1991) *Trends Neur. Sci.* **14**, 60–67
- Dawson, V. L., Dawson, T. M., London, E. D., Bredt, D. S. & Snyder, S. H. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6368–6371
- Garbers, D. L. (1989) *J. Biol. Chem.* **264**, 9103–9106
- Yui, Y., Hattori, R., Kosuga, K., Eizawa, H., Hiki, K., Ohkawa, S., Ohnishi, K., Terao, Y. & Kawai, C. (1991) *J. Biol. Chem.* **266**, 3369–3371
- Stuehr, D. J., Kuon, N. S., Nathan, C. F., Griffith, O. W., Feldman, P. L. & Wiseman, J. (1991) *J. Biol. Chem.* **266**, 6259–6263
- Marletta, M. A., Yoon, P. S., Iyengar, R., Leaf, C. D. & Wishnok, J. S. (1988) *Biochemistry* **27**, 8706–8711
- Forstermann, U., Pollack, J. S., Schmidt, H. H. W., Heller, M. & Murad, F. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 1788–1792
- Schmidt, H. H. W., Pollack, J. S., Nakane, N., Gorsky, L. D., Forstermann, U. & Murad, F. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 365–369
- Bredt, D. S. & Snyder, S. H. (1990) *Proc. Natl. Acad. Sci.* **87**, 682–685
- Mayer, B., Mathias, J. & Boehme, E. (1990) *FEBS Lett.* **277**, 215–219
- Giovanelli, J., Campos, K. L. & Kaufman, S. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 7091–7095
- Tan, K.-N. & Tashjian, A. H. (1984) *J. Biol. Chem.* **259**, 418–426
- Rebecchi, M. J., Kolesnick, R. N. & Gershengorn, M. C. (1983) *J. Biol. Chem.* **258**, 227–234
- Brostrom, C. O. & Brostrom, M. A. (1990) *Annu. Rev. Physiol.* **5**, 577–590
- Tashjian, A. H. (1979) *Methods Enzymol.* **58**, 527–535
- Drust, D. S., Sutton, C. A. & Martin, T. F. J. (1982) *J. Biol. Chem.* **257**, 3306–3312
- Drust, D. S. & Martin, T. F. J. (1984) *J. Biol. Chem.* **259**, 7566–7573
- Drust, D. S. & Martin, T. F. J. (1984) *J. Biol. Chem.* **259**, 14520–14530
- Gopalakrishna, R. & Anderson, W. G. (1987) *Biochem. Biophys. Res. Commun.* **104**, 830–836
- Bolton, A. E. & Hunter, W. M. (1973) *Biochem. J.* **133**, 529–539
- Burnette, W. N. (1981) *Anal. Biochem.* **112**, 195–203
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Chin, K. V., Cade, C., Brostrom, C. O., Galuska, E. M. & Brostrom, M. A. (1987) *J. Biol. Chem.* **262**, 1644–1649
- Kemp, M. C., Kuonen, D. K., Sutton, A. & Roberts, P. J. (1988) *Biochem. Pharmacol.* **37**, 3063–3070
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Whitaker, M. J. & Baker, P. F. (1983) *Proc. R. Soc. London B* **218**, 397–413
- Segel, I. H. (1975) in *Enzyme Kinetics*, pp. 100–125, John Wiley and Sons, New York
- Hope, B. T., Michael, G. J., Knigge, K. M. & Vincent, S. R. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 2811–2814
- Kuonen, D. R., Kemp, M. C. & Roberts, P. J. (1988) *J. Neurochem.* **50**, 1017–1025
- Tayeh, M. A. & Marletta, M. A. (1989) *J. Biol. Chem.* **264**, 19654–19658
- Bredt, D. S., Huang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R. & Snyder, S. H. (1991) *Nature (London)* **351**, 714–718