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Purification of a Ca^{2+}/calmodulin-dependent nitric oxide synthase from porcine cerebellum

Cofactor-role of tetrahydrobiopterin

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L-Arginine-derived nitric oxide acts as an inter- and intracellular signal molecule with cytosolic guanylyl cyclase as the effector system. Two NO synthase isoenzymes are postulated: a cytokine-inducible enzyme in macrophages and a constitutive, Ca^{2+}-regulated enzyme in various other cells. An NO synthase was isolated from porcine cerebellum by ammonium sulfate precipitation and affinity chromatography on 2',5'-ADP-Sepharose. The enzyme was identified as an NO synthase with a specific NO-chemiluminescence method and with purified cytosolic guanylyl cyclase as an NO-sensitive detection system. The purified NO synthase was, besides Ca^{2+}/calmodulin and NADPH, largely dependent on tetrahydrobiopterin as a cofactor.

Nitric oxide synthase; Ca^{2+}; Calmodulin; Tetrahydrobiopterin; Guanylyl cyclase; NADPH

1. INTRODUCTION

Nitric oxide (NO) or a labile NO-containing compound accounts for the biological activity of the endothelium-derived relaxing factor (EDRF), which activates cytosolic guanylyl cyclase [1,2]. In addition to smooth muscle relaxation, NO mediates cytotoxic effects of macrophages [3,4] and acts as a messenger molecule in the brain [5–7] as well as in blood platelets [8]. Many of the effects of NO are mediated by cGMP, and it appears that NO represents a widespread inter- and intracellular signal molecule with cytosolic guanylyl cyclase as the effector enzyme.

NO or labile NO-containing compound is formed from L-arginine by a postulated NO synthase which appears to exist in two enzyme forms, as a constitutive cytosolic protein which is dependent on NADPH and Ca^{2+} in endothelial cells [9], lung [10], adrenal gland [11], brain [12–14] and platelets [8], as well as an interferon-γ- and lipopolysaccharide-inducible cytosolic enzyme or enzyme system in macrophages [15] which is independent of Ca^{2+} but needs, in addition to NADPH, tetrahydrobiopterin (H_4biopterin) as a cofactor [16,17].

Recently, a Ca^{2+}/calmodulin-dependent enzyme which converts L-arginine into L-citrulline and which appears to be identical with an NO synthase was purified from rat cerebellum [12]. In the course of the present study, a procedure suitable for the preparative purification of an NO synthase from porcine cerebellum was worked out, and the isolated enzyme was identified as an NO synthase with a specific NO-chemiluminescence method. The enzyme activity was dependent on Ca^{2+}/calmodulin and required H_4biopterin as a cofactor. Additionally, a Ca^{2+}-dependent formation of cGMP was demonstrated in vitro by reconstitution of the isolated NO synthase with purified cytosolic guanylyl cyclase.

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2. MATERIALS AND METHODS

2.1. *Materials*

[α-^{32}P]GTP (spec. act. 800 Ci/mmol) and L-[2,3-^{3}H]arginine (spec. act. 40–70 Ci/mmol) were obtained from DuPont de Nemours, Dreieich, Germany. [^{3}H]Arginine was purified by thin layer chromatography using $CHCl_3/CH_3OH/NH_4OH$ (25%), 2:2:1 (v/v) as solvent system, 2'5'-ADP-Sepharose was from Pharmacia-LKB (Freiburg, Germany), the ion exchange resin AG 50W-X8 was from Bio-Rad (München, Germany). (6R)-5,6,7,8-Tetrahydro-L-biopterin and 7,8-dihydro-L-biopterin were from Dr. B. Schircks Laboratories (Jona, Switzerland). Other reagents, solvents and salts were of analytical grade and were obtained from Sigma (Deisenhofen, Germany), or Merck (Darmstadt, Germany).

2.2. *Enzyme preparation*

Porcine cerebella (1–1.2 kg) were obtained from a local slaughterhouse. The enzyme purification was performed at 4°C. The arachnoidea of the cerebella was removed, the tissue was washed with isotonic NaCl solution and homogenized with an Ultra-Turrax in 3 vols of a 50 mM triethanolamine-HCl buffer, pH 7.5, containing 0.5 mM EDTA (buffer A). The homogenate was centrifuged for 30 min at 10 000 × *g*, and solid ammonium sulfate (176 g/l corresponding to 30% saturation) was added to the supernatant followed by centrifuga-

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tion for 15 min at 10000×g. The pellet was washed once with 1.5 liters of buffer A containing 176 g/l ammonium sulfate and was resuspended in 1.5 liters of a 20 mM triethanol-amine-HCl buffer, pH 7.5, containing 0.5 mM EDTA and 5 mM 2-mercaptoethanol. The protein suspension was centrifuged for 40 min at 10000×g, and the supernatant was carefully decanted and mixed with 4 ml of 2',5'-ADP-Sepharose which had been preequilibrated with buffer A containing 5 mM 2-mercaptoethanol (buffer B). The slurry was stirred for 30 min and subsequently poured into a column, and the 2',5'-ADP-Sepharose was washed with 100 ml of buffer B containing 0.5 M NaCl, followed by 100 ml of buffer B and 8 ml of buffer B containing 1 mM NADPH. NO synthase was eluted with 8 ml of buffer B containing 10 mM NADPH, and the eluate was concentrated using Centricon-30 microconcentrators (Amicon, Witten, Germany). The concentrate was washed twice in the same tubes with buffer B to reduce the concentration of NADPH which was determined spectrophotometrically. The enzyme (0.1–0.25 mg/ml) was unstable at 4°C, but was stored with 20% (v/v) of glycerol at −70°C for at least 4 weeks with only marginal losses of enzyme activity.

Protein was determined by the method of Bradford [18] with bovine serum albumin as standard protein.

2.3. *Determination of nitric oxide*

NO synthase (3 μg) was incubated for 5 min at 37°C in 1 ml of a triethanolamine/HCl buffer, pH 7.5, containing 0.1 mM L-arginine, 30 μM NADPH, 1 μM H_4biopterin, 10 μg/ml calmodulin, 3 μM free Ca^{2+} and 30 U/ml superoxide dismutase unless otherwise indicated. The samples were injected into 15 ml of distilled water, and NO was removed from the solution under a continuous flow of nitrogen (0.6 l/min) and determined with an NO-chemiluminescence detector (Ansyco, Karlsruhe, Germany). Peak heights were used for a semiquantitative evaluation of the data which are given as ppb NO.

2.4. *Reconstitution of NO synthase with purified cytosolic guanylyl cylase*

Cytosolic guanylyl cyclase was purified to an apparent homogeneity from bovine lung by immunoaffinity chromatography [19], and the formation of [^{32}P]cGMP from [α-^{32}P]GTP was measured as previously described [20] in a 50 mM triethanolamine-HCl buffer, pH 7.5, containing 100–200 ng of purified cytosolic guanylyl cyclase, 0.1 mM [α-^{32}P]GTP (200000–400000 cpm), 1 mM free Mg^{2+}, 1 mM isobutylmethylxanthine, 1 mM cGMP and 2 mM L-cysteine. Unless otherwise indicated, incubations were performed in a total volume of 100 μl at 37°C for 10 min the presence of 0.1 mM L-arginine, 10 μg/ml calmodulin, 3 μM free Ca^{2+}, 0.3 μM H_4biopterin and 100 ng of purified NO synthase which had been diluted with a 50 mM triethanolamine-HCl-buffer, pH 7.5, containing 0.5 mM EDTA. NADPH was adjusted to give a final concentration of 30 μM.

2.5. *Determination of L-citrulline*

Enzymatic formation of L-citrulline from L-arginine was determined as previously described [14] in a final volume of 100 μl in triethanolamine-HCl-buffer (50 mM, pH 7.5) containing 50 μM L-[2,3-^{3}H]arginine (150000–200000 cpm), 1 mM free Mg^{2+}, 10 μg/ml of calmodulin, 3 μM free Ca^{2+}, 0.1 mM NADPH, 5 μM FAD and 10 μM H_4biopterin unless otherwise indicated, together with 50 μl of enzyme solution which had been diluted as required with a 50 mM triethanolamine/HCl buffer, pH 7.5, containing 0.5 mM EDTA.

Blank values were determined in the absence of added enzyme. The concentrations of free Ca^{2+} and Mg^{2+} were adjusted as described previously [9].

3. RESULTS

An enzyme purified from porcine cerebellum was identified as an NO synthase by NO-chemiluminescence and reconstitution with purified cytosolic guanylyl cyclase. NO synthase was labile in the crude supernatant which was obtained from porcine cerebellum, and this inactivation of the enzyme was not prevented by various commercially available protease inhibitors. To achieve a rapid removal of the enzyme from the supernatant, it was precipitated with ammonium sulfate and subsequently isolated by affinity chromatography on 2',5'-ADP-Sepharose. Sodium dodecyl sulfate-polyacrylamid gel electrophoresis (SDS-PAGE) of the isolated protein showed one stained band corresponding to a molecular mass of about 160 kDa. The purity of the enzyme was greater than 95% (Fig. 1). A native molecular mass of about 200 kDa was calculated from gel permeation chromatography experiments (not shown).

As NO synthase activity correlates with the Ca^{2+}/calmodulin-dependent formation of L-citrulline [14], this parameter was monitored during protein purification, and the results of one representative of five preparations are summarized in Table I. From 20 g of protein in the supernatant, 0.23 mg of purified NO synthase were obtained. The specific enzyme activity was 0.73 μmol L-citrulline·mg^{-1}·min^{-1}, and the enzyme was purified 4500-fold from the supernatant with 5% recovery.

As cytosolic guanylyl cyclase is the physiological effector system of L-arginine-derived nitric oxide, reconstitution experiments with purified guanylyl cyclase and NO synthase were performed. As shown in Fig. 2, Ca^{2+} enhanced the formation of cGMP about 300-fold in a concentration-dependent manner when NO synthase was incubated together with purified cytosolic guanylyl cyclase in the presence of L-arginine

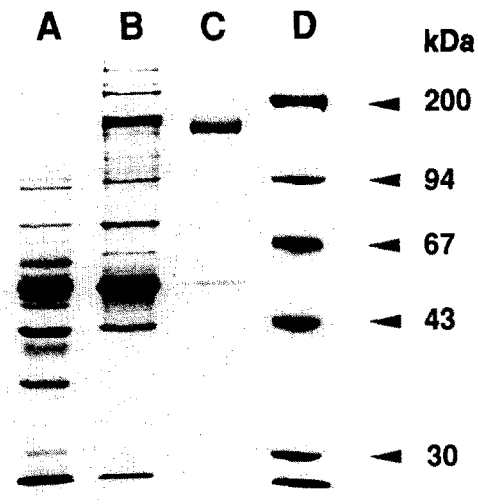

Fig. 1. SDS/PAGE analysis of fractions of NO synthase purification. SDS/PAGE was performed according to the method of Laemmli [21] in 10% slab gels which were stained with Coomassie blue. Lanes: (A) 20 μg of 10000×g supernatant; (B) 20 μg of ammonium sulfate precipitate; (C) 3 μg of 2',5'-ADP-Sepharose eluate; (D) molecular mass markers: myosin, 200 kDa; phosphorylase *b*, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 KDa.

Table I

Purification of NO synthase from porcine cerebellum supernatant (10000×g) by ammonium sulfate precipitation and affinity chromatography on 2',5'-ADP-Sepharose

Fraction	Protein (mg)	Specific activity (nmol·ml^{-1} ·min^{-1}	Recovery (%)	Purification factor
10000×g-supernatant	20000	0.16	100	1
Ammonium sulfate precipitate	2900	0.83	50	5
2',5'-ADP-Sepharose eluate	0.23	730	5	4500

Each fraction was assayed for L-citrulline formation as described in section 2. The 2'-5'-ADP-Sepharose eluate was assayed after concentration and washing with Centricon-30 microconcentrators. The data are representative for 5 preparations.

and GTP·Mg^{2+} as enzyme substrates and with required cofactors of NO synthase. Half-maximal effects of Ca^{2+} were observed at about 0.4 μM, and the maximally active concentration was 1 μM. Similar concentrations of Ca^{2+} were required for the formation of L-citrulline (not shown). This effect of Ca^{2+} was completely dependent on calmodulin, which exerted half-maximal effects on the L-arginine-dependent activation of cytosolic guanylyl cyclase and on the formation of L-citrulline at about 35 and 70 nM, respectively. In the absence of NO synthase or L-arginine Ca^{2+} in a concentration up to 10^{-5} M did not alter significantly cGMP formation (not shown).

NO synthase activity was enhanced in the presence of H_4biopterin. As shown in Fig. 3A, H_4biopterin stimulated the formation of L-citrulline with half-maximal effects at 0.6 μM and maximally active concentrations of about 10 μM. This activation of the enzyme was not observed when H_4biopterin was substituted with up to 10 μM 7,8-dihydro-L-biopterin (not shown).

In addition, we performed large scale incubations of NO synthase for a semi-quantitative determination of NO by chemiluminescence. In the absence of H_4biopterin, a conversion of 0.1 mM L-arginine into NO was observed at a signal-to-noise ratio of about 2:1 (10 ppb), whereas no signal was obtained in the absence of L-arginine or Ca^{2+}/calmodulin (not shown). As shown

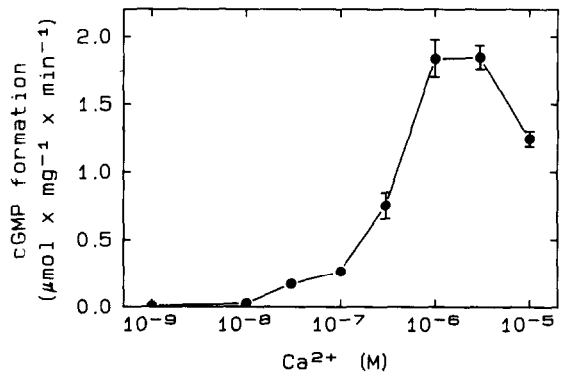

Fig. 2. Effect of Ca^{2+} on the formation of cGMP in the presence of isolated NO synthase and purified cytosolic guanylyl cyclase. Purified NO synthase (100 ng) was incubated in the presence of cytosolic guanylyl cyclase (200 ng) for 10 min at 37°C with increasing concentrations of Ca^{2+} in the presence of L-arginine, NADPH, calmodulin and H_4biopterin as described in section 2. Guanylyl cyclase activity was determined in triplicates, and the results of four separate experiments are shown as mean values ± SE.

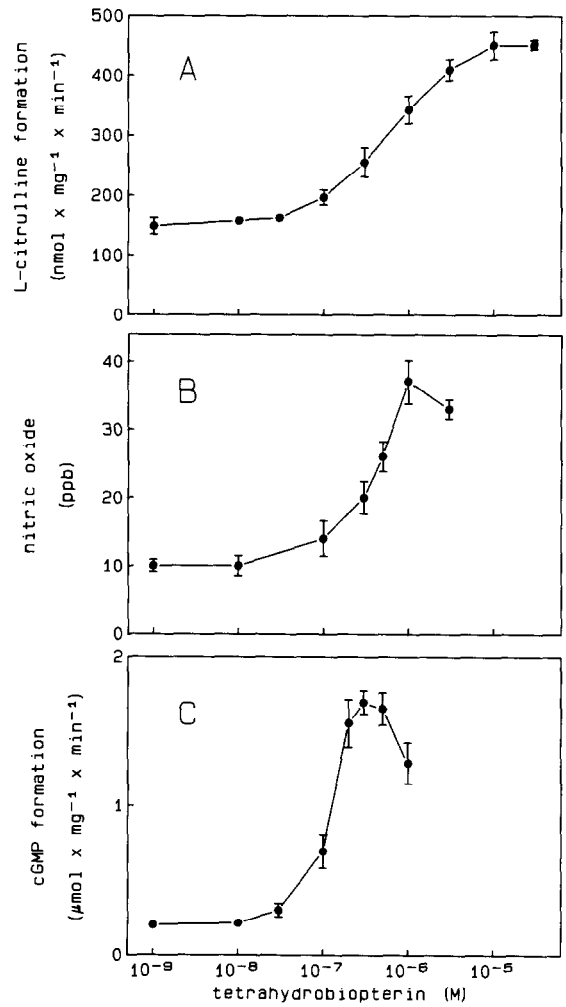

Fig. 3. Effect of H_4biopterin on the activity of purified NO synthase. (A) Purified NO synthase (100 ng) was incubated with increasing concentrations of H_4biopterin, and the formation of [^{3}H]citrulline from 0.1 mM [^{3}H]arginine was determined as described in section 2. Each value was determined in duplicates, and the results of three separate experiments are shown as mean values ± SE. (B) Purified NO synthase (3 μg) was incubated with L-arginine and cofactors for 5 min at 37°C, and the formation of NO was determined by chemiluminescence as described in section 2. Mean values ± SE of 3 separate experiments are given as ppb. (C) Purified NO synthase (100 ng) was incubated in the presence of 200 ng of cytosolic guanylyl cyclase in the presence of L-arginine, NADPH, calmodulin, and Ca^{2+} as described in section 2. Guanylyl cyclase activity was determined in triplicates, and the results of 5 separate experiments are shown as mean values ± SE.

in Fig. 3B, H_4biopterin increased the formation of NO in a concentration-dependent manner up to about 40 ppb with half-maximal effects of 0.4 μM, and a sharp maximum was reached at 1 μM H_4biopterin. In Fig. 3C, reconstitution experiments which were performed in the presence of purified NO synthase and cytosolic guanylyl cyclase are shown. H_4biopterin markedly increased the formation of cGMP with maximally active concentrations of 0.2–0.5 μM and half-maximal effects at about 0.15 μM. In the absence of purified NO synthase or L-arginine H_4biopterin did not increase the formation of cGMP (not shown). Rather steep, biphasic concentration/response curves were obtained when the formation of NO or cGMP was determined, whereas the formation of L-citrulline reached a plateau at 10 μM H_4biopterin.

4. DISCUSSION

NO synthase was rapidly isolated from a 10000 × *g* supernatant obtained from porcine cerebellum by ammonium sulfate precipitation and affinity chromatography on 2′,5′-ADP-Sepharose. Enzyme activity was strictly dependent on Ca^{2+} and calmodulin, confirming results published previously in crude [8,9,11,13,22], partially purified [10,14,22] and purified [12] enzyme preparations. The isolated protein represented a 160 kDa (SDS-PAGE) or 200 kDa (gel permeation chromatography) monomer and appears to be similar to an enzyme previously purified from rat cerebellum [12].

In various cell types, hormone-induced increases in intracellular free Ca^{2+} lead to an increased formation of cGMP [23–25]. In the present study, it is shown that in a defined system, consisting of purified NO synthase and purified cytosolic guanylyl cyclase, the formation of cGMP was increased about 300-fold by Ca^{2+} in the presence of required substrates and cofactors. Thus, this widespread signal transduction system, which involves Ca^{2+}-regulated NO-formation followed by an activation of cytosolic guanylyl cyclase, can be mimicked in vitro by reconstitution of the purified enzymes.

In contrast to the Ca^{2+}-regulated NO formation described previously in e.g. endothelial cells, brain and platelets, NO formation in activated macrophages is stimulated by H_4biopterin and not depedent on Ca^{2+} [16,17,26,27]. In the present study, the activity of Ca^{2+}-regulated NO synthase isolated from porcine cerebellum was largely dependent on the presence of H_4biopterin, and it appears, therefore, that both, Ca^{2+}-regulated NO synthase and the partially purified macrophage enzyme require H_4biopterin as a cofactor. About 20–30% of maximal enzyme activity were observed in the absence of added H_4biopterin. Thus, it is possible that a basal H_4biopterin-independent enzyme activity is stimulated in the presence of H_4biopterin, or, alternatively, that H_4biopterin is an obligatory coenzyme of NO synthase with some amount of holoenzyme still present in purified preparations. The function of NADPH and H_4biopterin in the biosynthesis of NO is not yet clear. The enzyme was saturated at least 10-fold with NADPH during the enzyme assays, and it is unlikely, therefore, that H_4biopterin acts as an NADPH-regenerating system. It has been known so far that H_4biopterin represents an essential cofactor in hydroxylations of aromatic amino acids by mixed-functioned oxygenases [28]. Similarly, reduced biopterins appear to be required for the monoxygenase-like hydroxylation of L-arginine by the partially purified NO forming enzyme system obtained from activated macrophages [16,17]. As H_4biopterin and 7,8-dihydro-L-biopterin are equally potent in this system, it has been suggested that NADPH may be required for the reduction of 7,8-dihydro-L-biopterin or the respective quinoid form of this compound by the action of a dihydropteridine reductase or a dihydrofolate reductase, respectively [17]. As described here, however, isolated NO synthase selectively utilized H_4biopterin and NADPH as cofactors and does not recycle these compounds. It appears, therefore, that two differently regulated isoforms of NO synthase are obtained from either activated macrophages or brain, but both enzymes seem to catalyze the conversion of L-arginine into NO in a similar manner with NADPH and H_4biopterin acting as cofactors.

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