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Brain nitric oxide synthase is a biopterin- and flavin-containing multi-functional oxido-reductase

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Brain nitric oxide synthase is a Ca^{2+}/calmodulin-regulated enzyme which converts L-arginine into NO. Enzymatic activity of this enzyme essentially depends on NADPH and is stimulated by tetrahydrobiopterin (H_4biopterin). We found that purified NO synthase contains enzyme-bound H_4biopterin, explaining the enzymatic activity observed in the absence of added cofactor. Together with the finding that H_4biopterin was effective at substoichiometrical concentrations, these results indicate that NO synthase essentially depends on H_4biopterin as a cofactor which is recycled during enzymatic NO formation. We found that the purified enzyme also contains FAD, FMN and non-heme iron in equimolar amounts and exhibits striking activities, including a Ca^{2+}/calmodulin-dependent NADPH oxidase activity, leading to the formation of hydrogen peroxide at suboptimal concentrations of L-arginine or H_4biopterin.

L-Arginine; FAD; FMN; Nitric oxide; Tetrahydrobiopterin; Reaction mechanism

1. INTRODUCTION

Nitric oxide represents an intra- and intercellular signal molecule with soluble guanylyl cyclase as effector enzyme. It is involved in the regulation of vascular tone, acts as a messenger molecule in the central nervous system and mediates the cytotoxicity of immunologically activated cells (for review see [1–3]). Until now, at least two forms of NO-generating enzymes have been described: a Ca^{2+}-regulated enzyme which is constitutively expressed in endothelial cells [4], lung [5], adrenal gland [6], platelets [7], neutrophils [8] and brain [9–11], and a Ca^{2+}-independent, cytokine-inducible enzyme form in macrophages [12–14], Kupffer cells [15], hepatocytes [16], endothelial cells [17], and smooth muscle cells [18].

The reaction mechanism of NO formation is not yet fully understood. Both types of NO synthases similarly convert the guanidino group of L-arginine into NO and L-citrulline, essentially depend on NADPH and are stimulated by H_4biopterin [8,10,19,20]. In a first reaction step, the macrophage enzyme apparently catalyzes a monooxygenase-like hydroxylation of L-arginine into N^{ω}-hydroxy-L-arginine which apparently reacts with molecular oxygen to yield L-citrulline and NO [21,22].

In the present study we show that the observed H_4biopterin-independent activity of NO synthase is due to enzyme-bound H_4biopterin and that the cofactor is apparently recycled at the expense of NADPH during NO synthesis. The purified enzyme is further characterized as a non-heme iron-containing flavoprotein which acts as Ca^{2+}/calmodulin-dependent NADPH:oxygen oxido-reductase catalyzing the formation of hydrogen peroxide instead of L-citrulline and NO at low concentrations of L-arginine or H_4biopterin.

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2. MATERIALS AND METHODS

2.1. *Materials*

L-[2,3-^{3}H]arginine (spec. act. 40–70 Ci/mmol) was obtained from DuPont de Nemours, Dreieich, Germany and purified by HPLC using 100 mM sodium acetate buffer, pH 4.5, as eluant. The ion exchange resin AG 50W-X8 was from Sigma, Deisenhofen, Germany; 2′,5′-ADP-Sepharose was from Pharmacia-LKB, Freiburg, Germany; and (6R)-5,6,7,8-tetrahydro-L-biopterin was obtained from Dr B. Schircks Laboratories, Jona, Switzerland. NADPH was purchased from Waldhof, Düsseldorf, Germany. Other reagents, solvents and salts were of analytical grade and were obtained from Sigma, Deisenhofen, Germany, or Merck, Darmstadt, Germany.

2.2. *Enzyme preparation*

For the purification of Ca^{2+}-regulated NO synthase from porcine cerebellum, a method described previously [10] was scaled-up and slightly modified, resulting in a marked increase in enzyme recovery. About 3 kg of porcine cerebella were 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). After centrifugation (30 min, $10000 \times g_{av}$), solid ammonium sulfate (176 g/l) was added to the supernatant to precipitate NO synthase. The pellets were washed once with 5 l of buffer A containing 176 g/l of ammonium sulfate and then dissolved in a 20 mM triethanolamine/HCl buffer, pH 7.5, containing 0.5 mM EDTA and 10 mM 2-mercaptoethanol so that the conduc-

tivity of the suspension was not higher than about 10 mS/cm. After centrifugation (40 min, $10000 \times g_{av}$), the supernatant was mixed with 25 ml of 2',5'-ADP-Sepharose which had been pre-equilibrated with buffer A containing 10 mM 2-mercaptoethanol (buffer B). The slurry was stirred for 30 min, poured into a column, and the Sepharose was washed with 250 ml of buffer B containing 0.5 M NaCl, followed by 250 ml of buffer B and 50 ml of buffer B containing 0.5 mM NADPH. NO synthase was eluted with 80 ml of buffer B containing 10 mM NADPH, and the eluate was immediately concentrated with Centricon-30 microconcentrators (Amicon, Witten, Germany). The concentrate was washed 3 times with buffer B to reduce the concentration of NADPH, and the enzyme (0.2–0.4 mg/ml) was stored with 20% (v/v) glycerol at −70°C. Using this purification procedure, we obtained about 2 mg of NO synthase of a purity of greater than 95% as judged from the Coomassie blue-stained gels.

For some experiments, 200 μl of purified NO synthase was further chromatographed on a Superose 6 gel filtration column (1 × 30 cm; Pharmacia-LKB, Freiburg, Germany) which had been pre-equilibrated in a 50 mM triethanolamine/HCl buffer, pH 7.0, containing 0.5 mM EDTA and 0.5 M NaCl. The enzyme was eluted with the same buffer at a flow rate of 0.5 ml/min, and active fractions were pooled. Protein was determined by the method of Bradford [23] with bovine serum albumin as standard protein.

2.3. *Determination of enzyme-bound pteridines*

In 100 μl of a solution containing 0.4 mg/ml of purified NO synthase, the reduced forms of pteridines were oxidized with 0.01 M KI_3 in 5 M H_3PO_4 or in 0.1 M NaOH for 60 min in the dark. After acidification of the basic solution with 5 M H_3PO_4 and destruction of excess iodine by 0.1 M ascorbic acid, samples were applied to strong ion exchange solid phase cartridges (Varian, Palo Alto, CA, USA) and directly eluted by means of an automated device (AASP, Varian) to a 250×4 mm reversed phase column (Lichrosorb RP-18, Merck, Darmstadt, Germany) and detected by fluorescence as previously described [24].

2.4. *Determination of reduced flavins*

NO synthase purified from porcine cerebellum was chromatographed on Superose 6 as described in section 2.2., heated for 5 min in a boiling water bath in the dark, and 50 μl were subsequently injected into an HPLC system (Vista 5560, Varian). A reversed phase column (Lichrosorb RP-18, Merck) was eluted with 0.01 M potassium phosphate buffer, pH 6.0, containing 19% (v/v) methanol at a flow rate of 0.8 ml/min with a linear gradient to 57% methanol in 15 min. Flavins were detected simultaneously with UV-absorption at 447 nm, and fluorescence detection (LS4, Perkin Elmer, Beaconsfield, UK) at excitation 450 nm, emission 520 nm.

2.5. *Enzyme assays*

Routinely, NO synthase activity was determined as formation of [^{3}H]citrulline from [^{3}H]arginine as previously described [25]. For all experiments evaluating the stoichiometry of the reaction, 2-mercaptoethanol and NADPH were removed from enzyme preparations by gel filtration (see 2.2.). The ratio of NADPH utilized to L-citrulline formed was determined by incubation of the thiol- and NADPH-free enzyme (30–60 ng) at 37°C for 1 h with 0.05–1.0 nmol of NADPH in a volume of 0.1 ml in the presence of 100 μM [^{3}H]arginine (100000 cpm), 3 μM free Ca^{2+}, 10 μg/ml calmodulin and 10 μM H_4 biopterin, followed by the determination of [^{3}H]citrulline formed. Similarly, the enzyme was incubated with defined, suboptimal amounts of H_4biopterin (0.05–1 nmol in 0.1 ml) at saturating (100 μM) concentrations of NADPH. For the determination of the specific NADPH-consuming activity, NO synthase (2 μg) was incubated in a volume of 0.2 ml in the presence of 100 μM NADPH and 3 μM free Ca^{2+} in the presence and absence of 10 μg/ml calmodulin at ambient temperature, and the decrease in absorbance at 340 nm was continuously monitored against blank samples containing buffer instead of enzyme. For the continuous monitoring of oxygen-uptake using an oxygen-sensitive electrode (Braun, Melsungen, Germany), 50 μg of NO synthase were incubated at ambient temperature in 2.5 ml volumes with 100 μM NADPH and 3 μM free Ca^{2+} in the presence and absence of 10 μg/ml calmodulin.

3. RESULTS AND DISCUSSION

In previous studies it was found that the Ca^{2+}-regulated brain NO synthase shows activity without added H_4biopterin, and it had been concluded that the brain enzyme does not depend on H_4biopterin [9,26]. This contrasted to findings with the inducible macrophage NO synthase and, thus, was taken as a feature distinguishing two forms of NO synthase in mechanistic properties. Recent studies, however, revealed that the purified, Ca^{2+}-regulated enzyme exhibits a basal activity which is enhanced in the presence of added H_4biopterin [8,10]. We examined NO synthase purified from porcine cerebellum for enzyme-bound pteridines by HPLC (Fig. 1) and found that the purified protein contains 0.035–0.090 mol of biopterin per mol of 160 kDa [10] subunit, with more than 95% present in the tetrahydro form. Together with the pronounced stimulatory effect of H_4biopterin on the activity of the enzyme [10], these results clearly suggest that brain NO synthase does essentially depend on H_4biopterin as a cofactor.

NO synthase purified from activated macrophages is a flavoprotein containing FAD and FMN [27]. Supernatants of heat-denaturated purified brain NO synthase

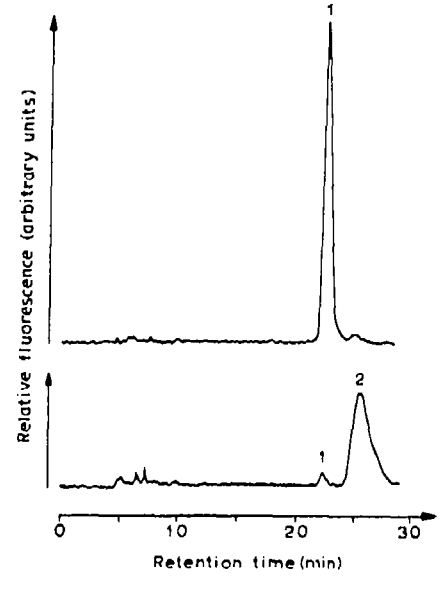

Fig. 1. HPLC analysis of enzyme-bound pteridines in NO synthase purified from porcine cerebellum. In preparations of NO synthase purified from porcine cerebellum, the reduced forms of pteridines were oxidized at acidic (*upper panel*) or alkaline (*lower panel*) conditions and analyzed by reverse phase HPLC with fluorescence detection as described in section 2. Peak 1 corresponds to biopterin, which is formed from dihydro- and tetrahydrobiopterin by oxidation in acid (*upper panel*) or from dihydrobiopterin by oxidation in alkaline medium (*lower panel*). Peak 2 corresponds to pterin, the product of tetrahydrobiopterin oxidation in alkaline media (*lower panel*).

exhibited fluorescence spectra typical for flavins (Fig. 2). The intensity of the fluorescence was not affected by acidification, indicating that a mixture of FAD and FMN was present. Subsequent to separation by HPLC, the flavins were quantitatively determined using UV and fluorescence detection with authentic FAD and FMN as reference compounds. Between 0.6 and 0.9 mol of each, FAD and FMN, were found per mol of 160 kDa subunit of NO synthase, and the flavins were present in equimolar amounts (see Fig. 2). Thus, whereas one mol of FAD and only 0.5 mol of FMN were reported to be present per mol of subunit of the purified macrophage enzyme [27], brain NO synthase apparently contains equimolar amounts of the non-covalently bound reduced flavins.

Atomic absorption spectroscopy of 2 enzyme preparations showed 0.72 and 1.11 mol of iron per mol of 160 kDa subunit. The purified enzyme lacked the typical UV/VIS absorption spectrum of heme (not shown), so that brain NO synthase, like other pteridine-dependent hydroxylating enzymes [28], represents a non-heme iron protein. For experiments investigating the stoichiometry of the reaction, we removed 2-mercaptoethanol and NADPH from the enzyme preparations by gel filtration chromatography on Superose 6. Incubations with defined, suboptimal amounts of NADPH in the presence of 10 μM H_4 biopterin showed a consumption of 1.52 ± 0.028 nmol (mean $\pm$ SE; $n = 12$) per nmol of L-citrulline formed. At saturating conditions of NADPH, 0.05 nmol of H_4biopterin was sufficient for the formation of 1.3 nmol of L-citrulline. Thus, H_4biopterin may be recycled in an NADPH-dependent reaction like in other pteridine-dependent hydroxylating enzyme systems [29].

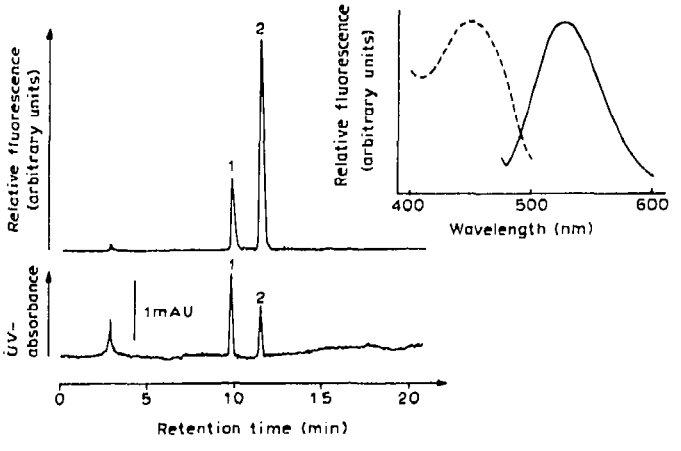

Fig. 2. HPLC profiles and fluorescence spectra (*inset*) of flavins in supernatants of heat-denatured brain nitric oxide synthase. NO synthase purified from porcine cerebellum was heated for 5 min in a boiling water bath in the dark to release non-covalently bound flavins, and the inset shows the fluorescence excitation and emission spectra of the supernatant. The excitation spectrum (dashed line) was monitored at an emission wavelength of 525 nm, the emission spectrum (solid line) was recorded at an excitation wavelength of 450 nm. Flavins were separated by reversed phase HPLC as described in section 2 and were detected simultaneously with UV-absorption at 447 nm (*lower panel*), and fluorescence detection (LS4, Perkin Elmer, Beaconsfield, UK) at excitation 450 nm, emission 520 nm (*upper panel*). Peak 1 was identified as FAD, peak 2 as FMN.

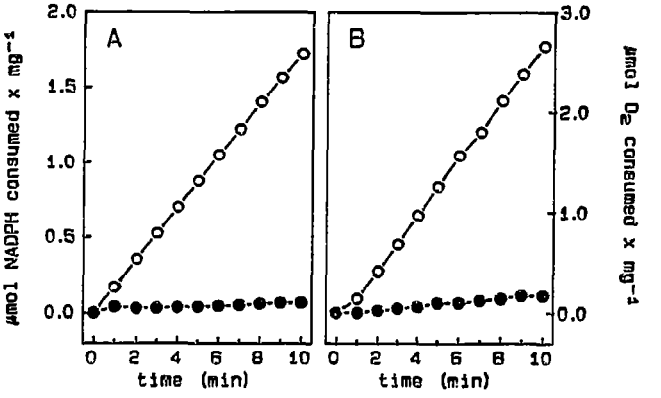

Fig. 3. NADPH consumption and uptake of molecular oxygen catalyzed by purified brain NO synthase in the absence of L-arginine and tetrahydrobiopterin. (A) NO synthase purified from porcine cerebellum (2 μg) was incubated at ambient temperature in 0.2 ml of a 50 mM triethanolamine/HCl buffer, pH 7.0, containing 100 μM NADPH and 3 μM free Ca^{2+} in the absence (filled circles) or presence (open circles) of 2 μg of calmodulin. The decrease in absorbance at 340 nm was continuously monitored against blank samples containing buffer instead of enzyme. The data are representative for 4 similar experiments. (B) NO synthase (50 μg) was incubated in 2.5 ml as described for (A) in the absence (filled circles) or presence (open circles) of 25 μg of calmodulin. Oxygen uptake was continuously monitored using an oxygen-sensitive electrode (Braun, Melsungen, Germany) which had been calibrated in the presence of xanthine oxidase and defined amounts of xanthine. Data are representative for 3 similar experiments.

The time-course of NADPH consumption catalyzed by purified NO synthase was studied as a decrease in absorbance at 340 nm at ambient temperature. As shown in Fig. 3A, a calmodulin-dependent NADPH-consuming activity of approximately 150 nmol·mg^{-1}·min^{-1} was found. This NADPH consumption was not dependent on the presence of L-arginine or H_4biopterin and was accompanied by a calmodulin-dependent uptake of molecular oxygen (Fig. 3B). Under these conditions, formation of hydrogen peroxide was observed, which was reduced in the presence of L-arginine and abolished when H_4biopterin was additionally present (Heinzel, B., John, M., Klatt, P., Böhme, E. and Mayer, B., unpublished). Thus, in the absence of L-arginine or H_4biopterin, the Ca^{2+}/calmodulin-activated enzyme appears to reduce molecular oxygen to hydrogen peroxide, similar to the plasma membrane-associated NADPH oxidase of neutrophils, which consists of various components including a flavoprotein [30].

The results reported here favor a hypothetical reaction scheme for the biosynthesis of NO as shown in Fig. 4. As initial step, a H_4biopterin-dependent N^{ω}-hydroxylation of L-arginine may result in the formation of N^{ω}-hydroxy-L-arginine, a compound which was

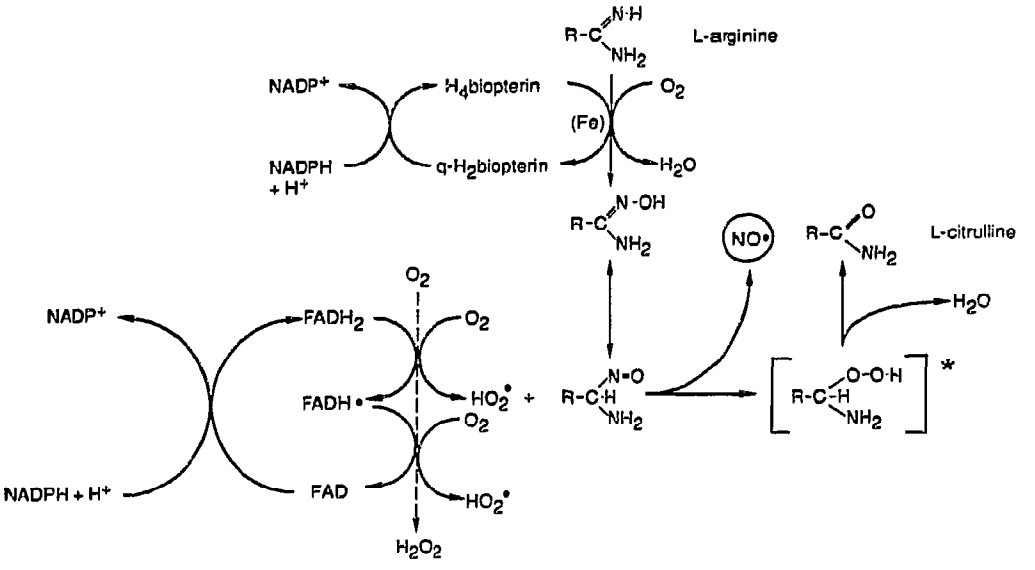

Fig. 4. Hypothetical reaction mechanism of enzymatic NO formation from L-arginine. NADPH and O_2 consumption, L-citrulline and NO formation are reactions of NO synthase induced by Ca^{2+}/calmodulin. NO synthase contains FAD and FMN in equimolar amounts. According to the functions of FAD and FMN in cytochrome P_{450} reductase [36], FAD may transfer electrons from NADPH to FMN, and $FMNH_2$ may represent the final electron-donator. (Fe) indicates the possible involvement of non-heme iron according to a reaction scheme for H_4 biopterin-dependent monooxygenases as postulated by Davies and Kaufman [28]. $HO_2^{\cdot}$ may represent hydroperoxyl radical or an as yet unidentified reduced oxygen species. * hypothetical intermediate. (---), alternative pathway used at suboptimal concentrations of L-arginine or H_4biopterin.

identified as an intermediate in NO formation by the macrophage enzyme [22]. This intermediate may react with an oxygen radical in a substitution reaction, leading to the formation of NO and of a hypothetical hydrogen peroxide derivative of L-arginine, which may be cleaved into L-citrulline and water. In a flavin-coupled reaction, 2 oxygen radicals may be formed in one-electron transfer steps at the expense of one molecule of NADPH, explaining our finding that 1.5 mol of NADPH are required for the formation of one mol of L-citrulline.

NO synthase contains H_4biopterin, FAD and FMN as coenzymes and represents a non-heme iron protein. The enzyme apparently acts as a multi-functional oxido-reductase exhibiting various enzymatic activities, including H_4biopterin-dependent monooxygenase, NADPH-dependent dihydropteridine reductase and NADPH oxidase activity. These enzyme activities may simply provide the actual NO synthase with its substrates, i.e. with N^{ω}-hydroxy-L-arginine and oxygen radicals. This proposed reaction scheme provides an explanation for the results described here and for data obtained previously with the macrophage enzyme [21,22,27]. However, besides its function as electron-donator in the hydroxylation of L-arginine, H_4biopterin may have an additional protective effect on NO synthase [22,27].

Our results may have important physiological implications, as toxic processes which have been attributed to oxygen radicals [31] may take place subsequently to activation of NO synthase by Ca^{2+} at suboptimal levels of L-arginine or H_4biopterin. The intracellular concentrations of L-arginine are between 0.1 and 2 mM [32] and do not appear to physiologically limit NO formation. Reduced levels of H_4biopterin, however, were found in the brain and cerebrospinal fluid of patients affected with various neural diseases (for review see [33]). The key enzyme in the de novo synthesis of H_4 biopterin, GTP cyclohydrolase I, was shown to be immunologically inducible [34], and inhibition of this enzyme resulted in a reduced formation of NO_2^-/NO_3^- upon stimulation of murine fibroblasts by cytokines [35]. Low levels of intracellular H_4biopterin may, therefore, limit NO formation and, according to our results, give rise to an NO synthase-catalyzed generation of oxygen radicals or hydrogen peroxide.

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