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RESEARCH COMMUNICATION

# Brain nitric oxide synthase is a haemoprotein

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Brain nitric oxide (NO) synthase showed pyridine haemochrome spectra typical of ferroprotoporphyrin IX-containing enzymes. The haem content of purified NO synthase was in the range 0.7–0.9 mol/mol of 160 kDa subunit. In the presence of CO, NO, KCN and miconazole, the L-citrulline-forming activity of NO synthase was markedly diminished, demonstrating that enzyme-bound haem is involved in enzymic NO synthesis.

## INTRODUCTION

Nitric oxide (NO) is a messenger molecule in vascular, nervous and immune systems [1,2]. It is produced together with L-citrulline from L-arginine by different NO synthase isoenzymes [3]. A $Ca^{2+}$/calmodulin-regulated 160 kDa isoenzyme was purified from rat and porcine cerebella [4–6]. We have previously characterized the purified enzyme as a tetrahydrobiopterin- and iron-containing flavoprotein [7], which is able to reduce the haem iron of cytochrome *c* and cytochrome *P*-450 in an NADPH-dependent manner [8]. Furthermore, cloning of rat brain NO synthase revealed sequence similarities to cytochrome *P*-450 reductase [9]. We speculated, therefore, that an intramolecular haem moiety may be attached to this reductase domain of NO synthase and addressed this question using the pyridine haemochrome method for the determination of enzyme-bound haem. Here we present evidence that purified brain NO synthase contains stoichiometric amounts of ferroprotoporphyrin IX. Inhibition of citrulline formation by CO, NO, KCN and miconazole indicates an involvement of haem in the catalytic function of brain NO synthase.

## EXPERIMENTAL

### Materials

NO synthase was purified from porcine cerebellum as previously described [5,7]. Stock solutions of NO were prepared by dissolving NO in oxygen-free distilled water at 25 °C [10]. Haemoglobin from bovine blood and miconazole were purchased from Sigma, Deisenhofen, Germany; all other reagents were obtained from sources described previously [5,7].

### Absorption spectroscopy

Spectra were recorded using a Shimadzu UV-160A double-beam spectrophotometer. Pyridine haemochrome spectra were obtained as described in [11,12]. Briefly, 0.1 ml of 50% (v/v) pyridine in 0.2 M-NaOH was added to 0.1 ml of NO synthase preparations (1.6–2.7 μM), followed by addition of a few grains of solid sodium dithionite. Spectra were recorded against blanks containing buffer and pyridine/NaOH. The concentrations of haem were calculated from the differences in absorbance at 556 nm versus 540 nm. Absorption coefficients were determined from calibration spectra recorded with haemoglobin in the haem concentration range 1–3 μM. A molecular mass of 160 kDa per NO synthase subunit [5,9] was used for calculations of haem/protein ratios. Protein concentrations were determined by the method of Bradford [13], with BSA as standard protein.

### Determination of NO synthase activity

NO synthase activity was determined as formation of L-[2,3-$^3$H]citrulline from L-[2,3-$^3$H]arginine as described previously [14]. Purified NO synthase (0.5 μg) was incubated for 10 min at 37 °C in a total volume of 0.1 ml of a triethanolamine/HCl buffer (50 mM, pH 7.0) containing 0.1 mM-L-[2,3-$^3$H]arginine (50000–70000 c.p.m.), 0.1 mM-NADPH, 10 μg of calmodulin/ml, 3 μM free $Ca^{2+}$ [15], 5 μM-FAD, 5 μM-FMN and 10 μM-(6*R*)-5,6,7,8-tetrahydrobiopterin. For the determination of enzyme inhibition by CO, NO synthase (3 μg in 0.35 ml of the same reaction mixture, but without calmodulin) was placed in 1.5 ml reaction vials, which were then sealed and equilibrated with $CO/O_2$ (4:1) by gassing and continuously shaking the vial for 5 min at room temperature. Reactions were started by the addition of 3 μl of an aqueous solution of calmodulin (1 mg/ml). Samples were incubated for 5 min at 37 °C, and aliquots (0.1 ml) were assayed for L-citrulline-forming activity as described above.

## RESULTS AND DISCUSSION

In a previous study we demonstrated that cerebellar NO synthase contains stoichiometric amounts of enzyme-bound iron [7]. We used the pyridine haemochrome method to find out

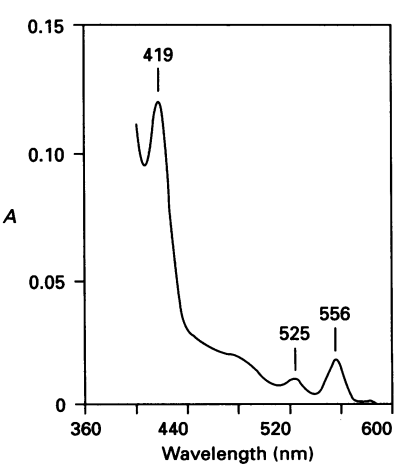


**Fig. 1. Spectrum of the reduced pyridine haemochrome of NO synthase**

The spectrum of NO synthase (0.21 mg/ml) was recorded in the presence of 0.1 M-NaOH and 25% (v/v) pyridine after addition of solid sodium dithionite as described in the Experimental section.

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**Table 1. Haem content of cerebellar NO synthase**

The haem content of four different preparations of NO synthase was determined spectrophotometrically by the pyridine haemochrome method as described in the Experimental section. NO synthase activities of these preparations were determined as formation of L-[$^3$H]citrulline from L-[$^3$H]arginine (see the Experimental section). The molar ratio haem/protein refers to the 160 kDa subunit of NO synthase.

| Preparation no. | Protein (mg/ml) | $\Delta A_{556-540}$ | [Haem] ($\mu$M) | Haem/protein molar ratio | L-Citrulline formation (nmol·mg$^{-1}$·min$^{-1}$) |
|---|---|---|---|---|---|
| 1 | 0.25 | 0.009 | 1.3 | 0.83 | 392 |
| 2 | 0.33 | 0.011 | 1.6 | 0.77 | 320 |
| 3 | 0.37 | 0.014 | 2.0 | 0.88 | 386 |
| 4 | 0.34 | 0.010 | 1.4 | 0.68 | 293 |

**Table 2. Effects of haemoprotein inhibitors on NO synthase-catalysed formation of L-citrulline**

Purified NO synthase (0.5 $\mu$g) was incubated in the presence of 10 mM-KCN, 0.1 mM-miconazole or ~0.3 mM-NO. Relative activities are expressed as percentage of the activity in the absence of inhibitors. The effects of CO were studied by equilibrating the incubation mixture with $CO/O_2$ (4:1) as described in the Experimental section. Relative activity refers to control samples equilibrated with ambient air. All rates of L-citrulline formation are given as means ± S.E.M. for three or four different experiments.

| Conditions | L-Citrulline formation (nmol·mg$^{-1}$·min$^{-1}$) | Relative activity (%) |
|---|---|---|
| Control | 360±15 | 100 |
| +KCN (10 mM) | 66±5 | 18 |
| +NO (~0.3 mM) | 144±13 | 40 |
| +Miconazole (0.1 mM) | 173±5 | 48 |
| Equilibration with: | | |
| Ambient air | 320±25 | 100 |
| $CO/O_2$ (4:1) | 94±21 | 29 |

whether the enzyme represents a haemoprotein. As shown in Fig. 1, we obtained spectra with absorption maxima at 419, 525 and 556 nm. The positions and relative intensities of these $\alpha$-, $\beta$- and $\gamma$-bands were similar to those reported for pyridine haemochrome spectra of ferroprotoporphyrin IX-containing proteins, e.g. soluble guanylate cyclase [16] and thromboxane synthase [17]. Thus NO synthase apparently contains ferroprotoporphyrin IX (haem *b* according to [11]).

The amount of haem bound to NO synthase was calculated from its pyridine haemochrome spectra using haemoglobin as standard. In good agreement with a previous study describing the spectral properties of soluble guanylate cyclase [16], we obtained an absorption coefficient of $13.8 \pm 0.4$ mM$^{-1}$·cm$^{-1}$ (mean ± S.E.M., $n = 15$). As shown in Table 1, the molar haem/NO synthase ratio ranged from 0.68 to 0.88. The NO synthase activities of these preparations correlated with the amount of enzyme-bound haem, indicating a variable loss of haem in the course of enzyme purification. These data fit well to the iron content of cerebellar NO synthase reported previously [7], and we conclude, therefore, that NO synthase contains 1 mol of haem/160 kDa subunit. The involvement of this haem moiety in NO synthesis was studied by measuring NO synthase activities in the presence of various compounds known to interfere with haem-catalysed reactions. As shown in Table 2, citrulline formation was reduced to 18% of the respective controls in the presence of 10 mM-KCN. Incubation of NO synthase in the presence of 10 $\mu$l of an NO-saturated solution corresponding to approx. 0.3 mM-NO [10] resulted in 60% inhibition of citrulline formation (Table 2). This inactivation, which may be due to the formation of haem–NO complexes, was only observed at very high concentrations of NO, indicating that autoinhibition of NO synthase does not occur under physiological conditions. Miconazole also markedly inhibited NO synthase activity to 48% of the respective control value, and in the presence of $CO/O_2$ (4:1) NO synthase-catalysed L-citrulline formation was reduced to 29% as compared with control samples (ambient air). These data indicate that the haem moiety bound to NO synthase is involved in the enzymic conversion of L-arginine into L-citrulline and NO.

Since CO and miconazole are known as *P*-450 inhibitors [18], we speculated whether NO synthase represents a *P*-450 enzyme. However, the native protein (up to 0.68 mg/ml; 4.25 $\mu$M) showed no absorption in the Soret region, and neither did treatment of the native enzyme with sodium dithionite in the presence of CO result in the appearance of a Soret band (results not shown), although protein concentrations were sufficiently high so that the Soret band should have been detectable. The reasons for these findings are presently unclear, but, although brain NO synthase contains stoichiometric amounts of FAD and FMN, the native protein does not exhibit a flavin spectrum [19], indicating that this enzyme has novel and unusual spectral properties.

The function of the haem in NO synthesis remains to be established. Interestingly, a soluble 119 kDa mono-oxygenase from *Bacillus megaterium* (*P*-450$_{BM-3}$) contains an FAD/FMN reductase domain which is attached to a *P*-450 haem group located on the same polypeptide [20]. Although this protein shows considerable sequence similarity to eukaryotic *P*-450 enzymes [21], it is unique with respect to the combination of FAD/FMN-mediated electron transfer and haem-catalysed mono-oxygenase activity within one single protein [22]. The equimolar presence of FAD, FMN, iron [7] and protoporphyrin IX (the present study) in cerebellar NO synthase, together with our findings that the enzyme catalyses both a KCN-insensitive cytochrome *c* reduction [8] and a KCN-sensitive L-citrulline formation (the present study), suggest that NO synthase may represent such a self-sufficient *P*-450 protein, catalysing a mono-oxygenase-like oxidation of L-arginine. Recently published data demonstrate that NO and L-citrulline are formed from $N^{\omega}$-hydroxy-L-arginine in the presence of hydrogen peroxide and various haemoproteins [23]. Accordingly, NO synthase-bound haem may alternatively catalyse the oxidation of intermediary $N^{\omega}$-hydroxy-L-arginine by hydrogen peroxide, which is a product of NO synthase-catalysed oxygen reduction [24]. Very recently it was reported that cytokine-inducible NO synthase purified from activated macrophages is a *P*-450-type haemoprotein [25]. Although this isoform markedly differs from brain NO synthase with respect to its regulation, these data, together with our findings, suggest that the mechanism of haem-dependent oxygen activation is common to both NO synthase isoenzymes and may thus represent a crucial step in NO biosynthesis.

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