RESEARCH COMMUNICATION

Expression of rat brain nitric oxide synthase in baculovirus-infected insect cells and characterization of the purified enzyme

Christian HARTENECK,* Peter KLATT,† Kurt SCHMIDT† and Bernd MAYER†‡

*Institut für Pharmakologie, Freie Universität Berlin, Thielallee 67-73, D-14195 Berlin, Germany, and †Institut für Pharmakologie und Toxikologie, Karl-Franzens-Universität Graz, Universitätsplatz 2, A-8010 Graz, Austria

Rat brain nitric oxide synthase was expressed to a high level in baculovirus-infected insect cells and purified to apparent homogeneity by affinity chromatography. The enzyme had a specific activity of ~ 1 μmol of citrulline·min^{-1}·mg of protein^{-1} and contained 0.93, 0.45, 0.18 and 0.23 mol of haem, (6*R*)-5,6,7,8-tetrahydro-L-biopterin (H_4biopterin), FAD and FMN per mol of subunit respectively.

INTRODUCTION

Virtually all mammalian cells are capable of synthesizing the messenger molecule nitric oxide (NO), which regulates a variety of biological functions [1–3]. NO is produced enzymatically from the amino acid L-arginine by at least three different NO synthase (NOS) isoenzymes [4–6]. Neuronal and vascular endothelial cells contain constitutively expressed isoforms of NOS, which are dependent on Ca^{2+}/calmodulin, whereas a Ca^{2+}-independent enzyme is expressed upon stimulation with cytokines in macrophages and most other nucleated cells. Purification, characterization, and molecular cloning of these enzymes revealed that they may represent fusion proteins consisting of a cytochrome *P*-450-like oxygenase domain, which is intramolecularly coupled to an FAD- and FMN-containing cytochrome *P*-450 reductase [7–11]. Thus, according to classic *P*-450 chemistry, oxidation of L-arginine to NO and citrulline may involve reductive activation of molecular oxygen by the haem iron in the catalytic centre of the enzyme and flavin-mediated shuttle of electrons from the cofactor NADPH to the haem. In contrast to classic *P*-450 systems, all NOS isoforms described so far require the pteridine derivative (6*R*)-5,6,7,8-tetrahydro-L-biopterin (H_4biopterin) as an additional cofactor [12]. Current evidence suggests that this compound has a dual role in NO synthesis, acting as both allosteric effector and redox-active cofactor of NOS [13,14].

In the present study we have used a baculovirus/insect cell system for the high-level expression of a cDNA encoding rat brain NOS [7]. It is shown that this method allows the expression and purification of a highly active NOS in amounts sufficient for its further biochemical and biophysical characterization.

EXPERIMENTAL

Materials

Sf9 cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) (no. CRL 1711), and the culture medium TC-100 was from Sigma Chemical GmbH (Deisenhofen, Germany). Antibiotics, amphotericin B, and lipid concentrate were from GibcoBRL, Life Technologies GmbH (Vienna, Austria), fetal calf serum from SEBAK GmbH (Suben, Austria), pVL1393 from Invitrogen (San Diego, CA, U.S.A.) and BaculoGOLD DNA from Dianova (Hamburg, Germany). Porcine brain NOS was isolated as described previously [15]. L-[2,3,4,5-^{3}H]Arginine hydrochloride (57 Ci/mmol) was purchased from MedPro (Amersham) (Vienna, Austria), H_4biopterin from Dr. B. Schircks Laboratories (Jona, Switzerland), and 2',5'-ADP–Sepharose 4B, calmodulin–Sepharose 4B, and molecular-mass standards for electrophoresis from Pharmacia Biotech (Vienna, Austria). Centricon 30 microconcentrators (Amicon) were purchased through W. D. Haider (Kottingbrunn, Austria). All other materials were from Sigma (Vienna, Austria).

Plasmid construction

The cDNA encoding rat brain NOS (GenBank accession no. X59949), a kind gift from Drs. D. S. Bredt and S. H. Snyder, was cloned in the baculovirus expression vector pVL1393 as follows. To reduce the 5' non-coding region, an Sse8387I fragment of NOS (bp 308–4241) was cloned in the *Pst*I site of pBluescriptSK(−). A clone with the C-terminal part next to the *Not*I site of the multiple cloning site of pBluescript was selected and cut with *Ehe*I (position 1560) and *Not*I (multiple cloning site of pBluescript). The 3139 bp *Ehe*I (position 1560) and *Not*I (position 4699) fragment of the original cDNA encoding the C-terminal part was then ligated in the cloning intermediate. The resulting cDNA was then cloned in the transfervector pVL1393 using *Eco*RI/*Not*I.

Sf9 cell culture, cloning of recombinant baculovirus and expression of recombinant rat brain NOS

Fall armyworm ovary cells (*Spodoptera frugiperda*; Sf9) were cultured in spinner flasks in TC-100 medium supplemented with 10% (v/v) fetal calf serum, amphotericin B (1.25 mg/l), penicillin (100000 i.u./l), streptomycin (100 mg/l), and 0.5% (v/v) lipid concentrate at 27 °C. Optimal growth (doubling time of ~ 24 h) was achieved at cell densities of $(0.5–2.0) \times 10^6$ cells/ml. The recombinant virus was generated by cotransfection of Sf9 cells with the expression vector described above and with BaculoGOLD baculovirus DNA by the lipofection method [16]. Positive viral clones were isolated by plaque assay and identified by their ability to direct the expression of the appropriate protein as

Abbreviations used: H_4biopterin, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; NO, nitric oxide; NOS, NO synthase.

‡ To whom correspondence should be addressed.

revealed by immunoblotting of cell extracts. The purified virus was amplified and titre estimated by plaque assay. For expression of NOS, Sf9 cells (6×10^8cells/400 ml) were infected with the recombinant baculovirus at a ratio of 5 p.f.u./cell in the presence of 4 mg/l haemin. After 48 h, cells were harvested by centrifugation for 3 min at 1000 *g* and washed with 100 ml of serum-free TC-100 medium. Cells were resuspended in 15 ml of buffer A (20 mM triethanolamine/HCl, pH 7.4, containing 0.5 mM EDTA and 10 mM 2-mercaptoethanol) and frozen in liquid nitrogen.

Purification of recombinant rat brain NOS

All purification steps were performed at 4 °C. Sf9 cells (6×10^8 cells in 15 ml of buffer A) were homogenized by ultrasonication followed by centrifugation at 30000 *g* for 10 min. The obtained pellet was resuspended in 15 ml of buffer A by ultrasonication and centrifuged at 30000 *g* for 10 min. The combined supernatants were added to 2',5'-ADP–Sepharose (10 ml bed volume) equilibrated with buffer A in a chromatography column (inner diameter 2.5 cm) and incubated for 30 min while the Sepharose was gently stirred. The affinity column was then sequentially washed with: (a) 100 ml of buffer B (50 mM triethanolamine/HCl, pH 7.4, containing 0.5 mM EDTA and 10 mM 2-mercaptoethanol) supplemented with 0.5 M NaCl; (b) 100 ml of buffer B; (c) 25 ml of 1 mM NADPH in buffer B; and (d) 4 ml of 10 mM NADPH in buffer B. Subsequently, bound NOS was eluted with 27 ml of 10 mM NADPH in buffer B. The eluate was adjusted to 2 mM $CaCl_2$ and 150 mM NaCl by addition of 10-fold stock solutions and added to calmodulin–Sepharose (5 ml bed volume) equilibrated with 2 mM $CaCl_2$ and 150 mM NaCl in buffer A in a chromatography column (inner diameter 2.5 cm). The mixture was gently stirred for 15 min, and subsequently the column was washed with 100 ml of buffer A containing 2 mM $CaCl_2$ and 150 mM NaCl, followed by 4 ml of buffer A containing 2 mM EGTA and 150 mM NaCl. Bound NOS was eluted with 24 ml of buffer A containing 2 mM EGTA and 150 mM NaCl. The eluate was concentrated to a protein concentration of 0.7–2.0 mg/ml using Centricon-30 microconcentrators and stored at −70 °C.

Determination of enzyme activities and cofactors

All enzyme assays were performed in the presence of 2 mg/ml BSA to increase stability of NOS. Formation of citrulline and reduction of cytochrome *c* were determined as described previously [17,18]. Haem was determined by the pyridine/haemochrome method as described previously [19] using an extinction coefficient of 22.1 $mM^{-1} \cdot cm^{-1}$ [20]. Enzyme-bound H_4biopterin, FAD and FMN were determined by h.p.l.c. and fluorescence detection as described [21]. Calculation of molar cofactor/NOS ratios are based on a subunit molecular mass of 160 kDa as calculated from the deduced amino acid sequence of the enzyme [7] and protein determinations using the Bradford method [22] with BSA as standard.

RESULTS

A previous study on expression of rat brain NOS using the baculovirus/insect cell system pointed to high-level expression of a predominantly inactive enzyme [23]. In initial experiments, we also observed that recombinant rat brain NOS isolated from baculovirus-infected Sf9 cells exhibited poor specific activity (~200 $nmol \cdot min^{-1} \cdot mg^{-1}$), presumably due to a lack of haem (0.2–0.3 mol per mol of 160 kDa subunit; results not shown). However, it has been shown previously that addition of haemin to the culture medium during infection of Sf9 cells with mammalian microsomal cytochrome *P*-450-recombinant baculovirus markedly enhanced haem content and specific activity of the expressed enzyme [24,25]. Similarly, supplementation of the medium with 4 μg/ml haemin allowed the purification of highly active, haem-saturated rat brain NOS from Sf9 cells. As shown in Table 1, the enzyme was purified ~25-fold from 30000 *g* supernatants of the recombinant baculovirus-infected cells by affinity chromatography on 2',5'-ADP–Sepharose. The specific enzyme activity of ~1 μmol of citrulline $\cdot min^{-1} \cdot mg^{-1}$ was not further increased by chromatography of the 2',5'-ADP–Sepharose eluate over a calmodulin affinity column, but this second step allowed the convenient and efficient removal of NADPH. After concentration of the final eluate, the overall recovery of NOS was 30–40% of the activity present in the supernatants. In contrast to the porcine enzyme, rat brain NOS was unstable even when kept on ice. However, the enzyme was virtually stable in the presence of 2 mg/ml BSA (results not shown).

Table 1 Purification of recombinant rat brain NOS

NOS was purified from 6×10^8 Sf9 cells, which had been infected with rat brain NOS-recombinant baculovirus for 48 h in 400 ml of culture medium supplemented with 4 μg/ml haemin as described in the Experimental section. Enzyme activity was determined as citrulline formation. Data are representative for three similar preparations.

Fraction	Amount of protein (mg)	Total activity (μmol · min^{-1})	Specific activity (μmol · $min^{-1} \cdot mg^{-1}$)	Yield (%)
30000 *g* supernatant	224	9.63	0.043	100
2',5'-ADP–Sepharose	3.9	4.37	1.12	45
Calmodulin–Sepharose	2.9	3.28	1.13	34

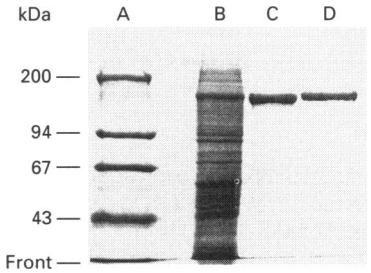

Figure 1 SDS/PAGE of recombinant rat brain NOS

The Figure shows a Coomassie Blue-stained 8% (w/v) polyacrylamide gel. Lane A, marker proteins (myosin, 200; phosphorylase *b*, 94; BSA, 67; and ovalbumin, 43 kDa); lane B, 30 μg of 30000 *g* supernatant from recombinant-baculovirus-infected Sf9 cells; lane C, 3 μg of purified recombinant rat brain NOS; lane D, 3 μg of NOS purified from porcine brain.

The representative Coomassie Blue-stained SDS gel shown in Figure 1 demonstrates that the purified preparations of rat brain NOS (lane C) contained one single protein with an apparent molecular mass of 150 ± 2 kDa (mean ± S.E.M., $n = 3$). In good accordance with the 25-fold purification of NOS activity, densitometric analysis of the gels suggested that the enzyme accounted for ~6% of total soluble proteins (results not shown).

Enzyme kinetic characterization of three preparations of purified rat brain NOS revealed a maximal enzyme activity of 1.2 ± 0.04 μmol · $min^{-1} \cdot mg^{-1}$ and a K_m for L-arginine of 5 ± 1 μM. Calmodulin-dependent reduction of cytochrome *c* was 4.8 ± 1.02 μmol · $min^{-1} \cdot mg^{-1}$, and the K_m for the cytochrome was

Table 2 Effects of added cofactors on recombinant rat brain NOS activity

Purified NOS was assayed for rates of citrulline formation in the absence or presence of calmodulin (10 μg/ml), NADPH (0.2 mM), FAD (5 μM), FMN (5 μM) and H_4biopterin (10 μM) as described in [17]. Data are means ± S.E.M. from two independent determinations performed with three different preparations. n.d., Not detectable.

Compound omitted	NOS activity (μmol of citrulline · min^{-1} · mg^{-1})
None	0.99 ± 0.021
Calmodulin	n.d.
NADPH	n.d.
FAD	0.97 ± 0.038
FMN	0.89 ± 0.048
FAD + FMN	0.21 ± 0.048
H_4biopterin	0.37 ± 0.033

15 ± 1 μM. Dependence of the isolated enzyme on cofactors is shown in Table 2. Expectedly, formation of citrulline was not detectable in the absence of added calmodulin or NADPH. In the absence of exogenous H_4biopterin, enzyme activity was ~40% of controls. NOS activity was decreased to ~20% of controls when the enzyme was incubated without any added flavins, but omission of either FAD or FMN alone had no significant effect on citrulline formation, suggesting that FMN may be present as an impurity in the FAD and *vice versa*.

Table 3 summarizes the quantitative determination of prosthetic groups bound to rat brain NOS. The enzyme contained 1 mol of haem, 0.45 mol of H_4biopterin and ~0.2 mol of each of FAD and FMN per mol of 160 kDa subunit. The sub-stoichiometric amounts of bound biopterin and flavins are in good accordance with the effects of the added compounds on NOS activity (see Table 2). Furthermore, the presence of equimolar amounts of bound haem is demonstrated by the light absorbance spectrum of the native enzyme shown in Figure 2. The Soret band at 396 nm indicates that NOS was isolated with its haem iron being predominantly in the high spin state. Based on an equimolar haem/protein ratio, the extinction coefficient of the Soret band was 101 $mM^{-1} \cdot cm^{-1}$.

Table 3 Prosthetic groups bound to recombinant rat brain NOS

Purified NOS was analysed for bound haem, H_4biopterin, FAD and FMN as described in [18,21]. Data are means ± S.E.M. from duplicate determinations of three preparations.

Prosthetic group	Molar ratio of prosthetic group/NOS subunit
Haem	0.93 ± 0.050
H_4biopterin	0.45 ± 0.032
FAD	0.18 ± 0.032
FMN	0.23 ± 0.021

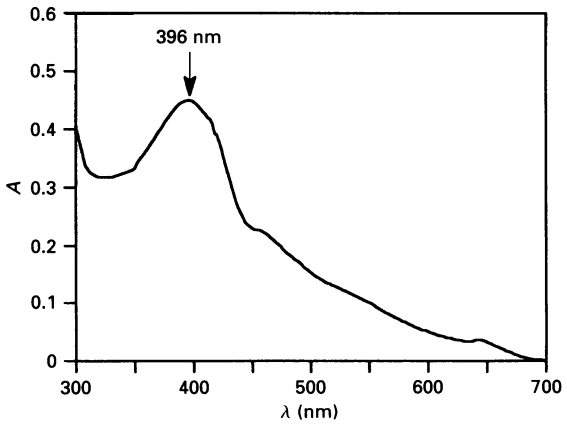

Figure 2 Light absorbance spectrum of recombinant rat brain NOS

Absorbance spectrum of purified NOS (0.7 mg/ml) recorded at 20 °C and normalized to zero absorbance at 700 nm.

DISCUSSION

Infection of haemin-treated Sf9 cells with rat brain NOS-recombinant baculovirus resulted in high-level expression of active NOS (4–6% of total soluble protein), which was conveniently purified to apparent homogeneity by affinity chromatography on 2',5'-ADP–Sepharose. From 400 ml suspensions of the cells we obtained 3–4 mg of the purified enzyme with a $V_{max.}$ of 1.2 μmol · min^{-1} · mg^{-1} and a K_m for L-arginine of 5 μM; these values are similar to those published previously for brain and macrophage NOS obtained from different sources [10,15,26–28]. Reduction of cytochrome *c* by porcine and rat brain NOS has been shown to occur at rates ~20-fold higher than for formation of citrulline [13,18]. Thus, the cytochrome *c* reductase activity of NOS expressed in Sf9 cells was comparably low (~5 μmol · min^{-1} · mg^{-1}), presumably due to the low amount of FAD and FMN bound to the purified enzyme.

As determined by the pyridine/haemochrome method and revealed from the native spectrum, the purified enzyme contained haem in a 1:1 stoichiometry, confirming that haemin added to the culture medium is incorporated by the insect cells and utilized for synthesis of expressed haemoproteins. Recombinant brain NOS contained significantly lower amounts of FAD and FMN than the enzyme obtained from tissue sources [21,29], suggesting that Sf9 cells have a limited capacity for flavin biosynthesis. Interestingly, the amount of reduced biopterin bound to the recombinant enzyme (0.45 mol per mol of 160 kDa subunit) was virtually the same as reported for NOS isolated from rat cerebellum [29]. Further studies are underway to see whether the flavin and biopterin content of purified NOS can be increased by supplementation of the Sf9 cell culture medium with precursors of their biosynthesis.

As expected, rat brain NOS exhibited close biochemical and immunological similarities to NOS isolated from porcine brain. However, there was a striking difference apparent in the stability of the two enzymes. Porcine brain NOS is stable for at least 24 h at cold room temperature (results not shown), whereas the rat enzyme was highly unstable at 0 °C unless fairly high amounts of albumin were added. The reason for this instability is not known and is currently being investigated in our laboratory.

The present study suggests that baculovirus-mediated expression of NOS may provide a useful tool for the efficient large-scale purification of active wild-type as well as mutated NOS isoforms and, thus, for biochemical and biophysical characterization of these enzymes, which should lead to a better understanding of NOS function and regulation.

We thank Drs. D. S. Bredt and S. H. Snyder (Johns Hopkins University, Baltimore, MD, U.S.A.) for the generous gift of rat brain NO synthase cDNA, and Dr. E. R. Werner (University of Innsbruck, Austria) for his invaluable help in setting up the methods for h.p.l.c. determination of biopterins and flavins. The excellent technical assistance of Eva Leopold is gratefully acknowledged. This work was supported by grants P 10030 (K.S.) and P 10098 (B.M.) of the Fonds zur Förderung der Wissenschaftlichen Forschung in Austria and the Deutsche Forschungsgemeinschaft and Sonderforschungsbereich 366 in Germany.

REFERENCES

1 Moncada, S., Palmer, R. M. J. and Higgs, E. A. (1991) Pharmacol. Rev. **43**, 109–142
2 Garthwaite, J. (1991) Trends Neurosci. **14**, 60–67
3 Snyder, S. H. and Bredt, D. S. (1992) Sci. Am. **266**, 28–35
4 Nathan, C. (1992) FASEB J. **6**, 3051–3064
5 Marletta, M. A. (1993) J. Biol. Chem. **268**, 12231–12234
6 Knowles, R. G. and Moncada, S. (1994) Biochem. J. **298**, 249–258
7 Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R. and Snyder, S. H. (1991) Nature (London) **351**, 714–718
8 White, K. A. and Marletta, M. A. (1992) Biochemistry **31**, 6627–6631
9 Stuehr, D. J. and Ikeda-Saito, M. (1992) J. Biol. Chem. **267**, 20547–20550
10 Klatt, P., Schmidt, K., Uray, G. and Mayer, B. (1993) J. Biol. Chem. **268**, 14781–14787
11 Sheta, E. A., Mcmillan, K. and Masters, B. S. S. (1994) J. Biol. Chem. **269**, 15147–15153
12 Mayer, B. (1993) Semin. Neurosci. **5**, 197–205
13 Baek, K. J., Thiel, B. A., Lucas, S. and Stuehr, D. J. (1993) J. Biol. Chem. **268**, 21120–21129
14 Klatt, P., Schmid, M., Leopold, E., Schmidt, K., Werner, E. R. and Mayer, B. (1994) J. Biol. Chem. **269**, 13861–13866
15 Mayer, B., John, M. and Böhme, E. (1990) FEBS Lett. **277**, 215–219
16 Groebe, D. R., Chung, A. E. and Ho, C. (1990) Nucleic Acids Res. **18**, 4033
17 Mayer, B., Klatt, P., Werner, E. R. and Schmidt, K. (1994) Neuropharmacology, in the press
18 Klatt, P., Heinzel, B., John, M., Kastner, M., Böhme, E. and Mayer, B. (1992) J. Biol. Chem. **267**, 11374–11378
19 Klatt, P., Schmidt, K. and Mayer, B. (1992) Biochem. J. **288**, 15–17
20 Berry, E. A. and Trumpower, B. L. (1987) Anal. Biochem. **161**, 1–15
21 Mayer, B., John, M., Heinzel, B., Werner, E. R., Wachter, H., Schultz, G. and Böhme, E. (1991) FEBS Lett. **288**, 187–191
22 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
23 Charles, I. G., Chubb, A., Gill, R., Clare, J., Lowe, P. N., Holmes, L. S., Page, M., Keeling, J. G., Moncada, S. and Riveros-Moreno, V. (1993) Biochem. Biophys. Res. Commun. **196**, 1481–1489
24 Asseffa, A., Smith, S. J., Nagata, K., Gillette, J., Gelboin, H. V. and Gonzalez, F. J. (1989) Arch. Biochem. Biophys. **274**, 481–490
25 Gonzalez, F. J., Kimura, S., Tamura, S. and Gelboin, H. V. (1991) Methods Enzymol. **206**, 93–99
26 Bredt, D. S. and Snyder, S. H. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 682–685
27 Stuehr, D. J., Cho, H. J., Kwon, N. S., Weise, M. F. and Nathan, C. F. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 7773–7777
28 Hevel, J. M., White, K. A. and Marletta, M. A. (1991) J. Biol. Chem. **266**, 22789–22791
29 Schmidt, H. H. H. W., Smith, R. M., Nakane, M. and Murad, F. (1992) Biochemistry **31**, 3243–3249

Received 9 September 1994/5 October 1994; accepted 7 October 1994