# Overexpression of neuronal nitric oxide synthase in insect cells reveals requirement of haem for tetrahydrobiopterin binding

Barbara M. LIST*, Peter KLATT*, Ernst R. WERNER†, Kurt SCHMIDT* and Bernd MAYER*‡

*Institut für Pharmakologie und Toxikologie, Karl-Franzens-Universität Graz, Universitätsplatz 2, A-8010 Graz, Austria, and †Institut für Medizinische Chemie und Biochemie, Universität Innsbruck, Fritz-Pregl-Strasse 3, A-6020 Innsbruck, Austria

Nitric oxide synthase (NOS) catalyses the conversion of L-arginine into L-citrulline and nitric oxide. Recently we have developed a method for expression of recombinant rat brain NOS in baculovirus-infected Sf9 cells and purification of the enzymically active enzyme [Harteneck, Klatt, Schmidt and Mayer (1994) Biochem. J. **304**, 683–686]. To study how biosynthetic manipulation of the NOS cofactors haem, FAD/FMN, and tetrahydrobiopterin ($H_4$biopterin) affects the properties of the isolated enzyme, Sf9 cells were infected in the absence and presence of haemin chloride (4 $\mu$g/ml), riboflavin (0.1 mM), and the inhibitor of $H_4$biopterin biosynthesis 2,4-diamino-6-hydroxypyrimidine (10 mM). In the absence of haemin, NOS was expressed to a very high level but remained predominantly insoluble. Purification of the soluble fraction of the expressed protein showed that it had poor activity (0.35 $\mu$mol of citrulline $\cdot$ mg$^{-1}$ $\cdot$ min$^{-1}$) and was haem-deficient (0.37 equiv. per monomer). Supplementing the culture medium with haemin resulted in pronounced solubilization of the expressed enzyme, which had a specific activity of $\sim$1 $\mu$mol of citrulline $\cdot$ mg$^{-1}$ $\cdot$ min$^{-1}$ and contained 0.95 equiv. of haem per monomer under these conditions. Unexpectedly, the amount of $H_4$biopterin endogenously present in the different NOS preparations positively correlated with the amount of enzyme-bound haem ($y = 0.066 + 0.430x$; $r = 0.998$). Radioligand binding experiments demonstrated that haem-deficient enzyme preparations containing 30–40 % of the holoenzyme bound only $\sim$40 % of $H_4$biopterin as compared with haem-saturated controls. These results suggest that the prosthetic haem group is essentially involved in the correct folding of NOS that is a requisite for solubilization of the protein and tight binding of $H_4$biopterin.

## INTRODUCTION

Nitric oxide synthase (EC 1.14.13.39; NOS) catalyses oxidation of L-arginine to L-citrulline and nitric oxide (NO). Three distinct forms of NOS have been characterized so far. The isoenzymes constitutively expressed in neuronal and vascular endothelial cells require $Ca^{2+}$/calmodulin for activity, whereas macrophages and most other mammalian cells express a $Ca^{2+}$-independent, inducible NOS upon activation by various cytokines [1–4]. Neuronal NOS (nNOS) was purified from rat and pig cerebellum as a 150–160 kDa protein [5,6] which appears to exist as a homodimer in its native state [7]. Biochemical characterization of nNOS showed that it contains tightly bound *P*-450-like haem [8–11] as well as the flavins FAD and FMN [12–14] as prosthetic groups. Together with the sequence similarities to cytochrome *P*-450 reductase [15], these results suggest that NOSs may be fusion proteins of a cytochrome *P*-450 oxygenase and a flavin-containing *P*-450 reductase, the latter shuttling reducing equivalents from the co-substrate NADPH to the haem [16–19].

The pteridine derivative (6*R*)-5,6,7,8-tetrahydro-L-biopterin ($H_4$biopterin) has been identified as a cofactor of NOS [20,21], but its role in NO biosynthesis is not well understood [22]. Recent results indicate that $H_4$biopterin may be required for dimerization of the haem-containing oxygenase domain of inducible macrophage NOS [23,24] and may trigger a change in conformation of the substrate site of the neuronal enzyme [25]. Furthermore, binding of $H_4$biopterin affects the spectral properties of the haem moiety [19,26] and is competitive with 7-nitroindazole, a putative haem-site inhibitor of NOS [27]. These results indicate that the pteridine-binding site is sterically located in close proximity to the catalytic centre of NOS allowing a functionally important interaction of $H_4$biopterin with the prosthetic haem group.

We have recently described the purification of highly active nNOS from Sf9 cells infected with a recombinant baculovirus [28]. In agreement with an earlier study using the baculovirus/insect cell system for overexpression of cytochrome *P*-450 2A1 [29] and more recent work on baculovirus-mediated expression of neuronal and endothelial NOS [30–32], supplementation of the cell culture medium with haemin was essential for expression of a functionally active, haem-containing enzyme. The present work was carried out to study the characteristics of baculovirus-mediated NOS expression. In order to modulate the amount of prosthetic groups bound to the isolated recombinant enzyme, cells were infected with the baculovirus in the absence or presence of compounds known to affect the intracellular availability of haem, flavins and $H_4$biopterin. Most importantly, we found that supplementing the culture medium with haemin was essential for binding of $H_4$biopterin, pointing to an as yet unrecognized role of haem in regulating the post-translational processing of NOS.

## EXPERIMENTAL

### Materials

Sf9 cells were obtained from A. T. C. C., Rockville, MD, U.S.A. (#CRL 1711), and the culture medium TC-100 from Sigma (Vienna, Austria). 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).

Abbreviations used: NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal NOS; DAHP, 2,4-diamino-6-hydroxypyrimidine; $H_4$biopterin, (6*R*)-5,6,7,8-tetrahydro-L-biopterin.

‡ To whom correspondence should be addressed.

Stock solutions of haemin chloride (2 mg/ml) were prepared in 0.4 M NaOH/EtOH (1:1, v/v) [33]. [$^3$H]$H_4$Biopterin was synthesized as described previously [34]. L-[2,3,4,5-$^3$H]Arginine hydrochloride (57 Ci/mmol) was purchased from MedPro (Amersham International, Vienna, Austria), unlabelled $H_4$biopterin was from Dr. B. Schircks Laboratories (Jona, Switzerland), 2′,5′-ADP–Sepharose 4B, calmodulin–Sepharose 4B, and molecular-mass standards for SDS/PAGE (myosin, 200 kDa ; phosphorylase b, 94 kDa ; BSA, 67 kDa ; ovalbumin, 43 kDa ; carboanhydrase, 30 kDa ) from Pharmacia Biotech (Vienna, Austria). All other materials were from Sigma (Vienna, Austria).

### Expression of NOS

Infection of Sf9 cell suspensions with rat brain NOS recombinant baculovirus was performed as described previously [28]. Briefly, Sf9 cells ($\sim 3 \times 10^8$ cells/200 ml) were infected with the recombinant baculovirus at a ratio of 5 plaque-forming units (p.f.u.)/cell. Where indicated, haemin chloride (up to 10 $\mu$g/ml; usually 4 $\mu$g/ml) and riboflavin (0.1 mM) were added to the culture medium at the time of infection. For inhibition of $H_4$biopterin biosynthesis, cells were preincubated for 24 h with 10 mM 2,4-diamino-6-hydroxypyrimidine (DAHP), and the drug remained present during cell infection. At the indicated time points, usually after 48 h , cells were harvested by centrifugation for 3 min at 1000 ***g***, washed with 50 ml of serum-free TC-100 medium, and resuspended in 2 ml of chilled 50 mM triethanolamine/HCl buffer (pH 7.4), containing 0.5 mM EDTA and 10 mM 2-mercaptoethanol (buffer A). Cell homogenates were prepared by sonicating the cells on ice (3 × 10 s, 150 W) and centrifuged at 30000 ***g*** for 15 min to separate soluble and particulate fractions. Pellets were resuspended in 2 ml of buffer A by brief sonication and centrifuged at 30000 ***g*** for 15 min, and the combined supernatants were used for enzyme purification. SDS/PAGE (8 % slab gels) was performed as described previously [35]. An antiserum raised in rabbits against purified pig brain NOS [6] was used for specific detection of nNOS subsequent to immunoblotting as described [35].

### Purification of NOS

Soluble fractions of the infected Sf9 cells (up to 10 parallel preparations) were sequentially chromatographed over 2′,5′-ADP- and calmodulin–Sepharose columns (0.5 ml bed volume each). The 2′,5′-ADP–Sepharose was washed with 5 ml of buffer A, containing 0.5 M NaCl (buffer B), and NOS was eluted with 2 ml of buffer B, containing 20 mM 2′-AMP (Sigma, # A-1627; contains ~50 % of 3′ -AMP) [36]. 2′-AMP was used for enzyme elution, since commercially available NADPH was found to contain variable amounts of $H_4$biopterin (P. Klatt and B. Mayer, unpublished work). The eluate was adjusted to 2 mM $CaCl_2$ and applied to the calmodulin–Sepharose column which was washed with 5 ml of 20 mM Tris/HCl buffer (pH 7.4) containing 10 mM 2-mercaptoethanol and 2 mM $CaCl_2$, followed by elution of NOS with 1 ml of 20 mM Tris/HCl buffer (pH 7.4) containing 10 mM 2-mercaptoethanol and 4 mM EGTA. Final eluates were stored at −70 °C.

### Determination of enzyme activity and cofactors

NOS activity was measured as formation of [$^3$H]citrulline from [$^3$H]arginine as described previously [27]. The amounts of bound flavins and $H_4$biopterin were determined by HPLC and fluorescence detection as described previously [12]. For determination of enzyme-bound haem, a published HPLC method [37,38] was modified as follows. Samples containing 3–15 $\mu$M haem were diluted 1 to 4 in acetonitrile/water/trifluoroacetic acid (60:40:0.1, by vol.) prior to injection of 30 $\mu$l aliquots on to a 250 mm × 4 mm $C_{18}$ reversed-phase column fitted with a 4 mm × 4 mm $C_{18}$ guard column (LiChrospher 100 RP-18, 5 $\mu$m particle size; Merck). Samples were eluted isocratically (LiChroGraph L-6200, Merck) with acetonitrile/water/trifluoroacetic acid (60:40:0.1, by vol.) at a flow rate of 1 ml/min, and haem (retention time = 5.4 min) was detected by its absorbance at 398 nm (L-4250, Merck). The method was calibrated with myoglobin and gave essentially the same results as the pyridine/haemochrome method [39].

### Binding of [$^3$H]$H_4$biopterin to rat brain NOS

Binding experiments were performed as described previously [25,40]. Briefly, purified rat brain NOS (~3 $\mu$g) was incubated for 10 min at 37 °C in the presence of 20 nM (~18 nCi) [$^3$H]$H_4$biopterin and increasing concentrations of unlabelled $H_4$biopterin. Separation of bound from free radioligand was performed by poly(ethylene glycol) precipitation and vacuum filtration. Recovery of radioactivity retained on filters was determined by liquid scintillation counting. $K_D$ and $B_{max}$ values were calculated from individual plots using the GIPMAX non-linear least-squares regression curve-fitting program [41] and are given as means ± S.E.M.

### Experimental protocol and data evaluation

Unless otherwise indicated, data represent mean values ± S.E.M. of 3–4 independent experiments. To modulate cofactor contents, NOS was expressed under four different conditions: (i) no additions to the culture medium, (ii) addition of haemin chloride (4 $\mu$g/ml), (iii) haemin plus riboflavin (0.1 mM), and (iv) haemin plus DAHP (10 mM). Four independent cell infections were carried out under each of these conditions, followed by purification of the expressed NOS, yielding a total of 16 preparations of the purified enzyme. NOS activity of these preparations was measured as formation of [$^3$H]citrulline from [$^3$H]arginine in two independent enzyme assays. Haem, FAD, FMN and $H_4$biopterin were determined twice in each of the preparations and are expressed as means ± S.E.M. of the calculated molar ratios, i.e. as mol of cofactor per mol of NOS subunit (equiv. per monomer), based on a subunit molecular mass of 160 kDa [15]. Protein content was determined with the Bradford method [42] using BSA as standard protein. Concentrations of the purified protein were corrected according to amino acid analysis data which showed that the Bradford method overestimated the NOS protein by a factor of 1.12 ± 0.016 ($n$ = 5) (P. Klatt and H. P. Bächinger, unpublished work).

## RESULTS

### Characteristics of NOS expression in recombinant-baculovirus-infected Sf9 cells

Infection of Sf9 cells for 48 h with recombinant rat brain NOS baculovirus resulted in high-level expression of a 160 kDa protein that was recognized by a rabbit antiserum against nNOS, whereas infection of the cells with the wild-type virus did not result in detectable NOS expression. The expressed protein was almost exclusively insoluble, unless the culture medium was supplemented with haemin chloride during infection. As recently shown by others [32], haemin induced a pronounced solubilization of NOS, with a maximal effect at 4 $\mu$g of haemin per ml (results not shown). In agreement with our initial study on NOS overexpression using the baculovirus system [28], the expressed

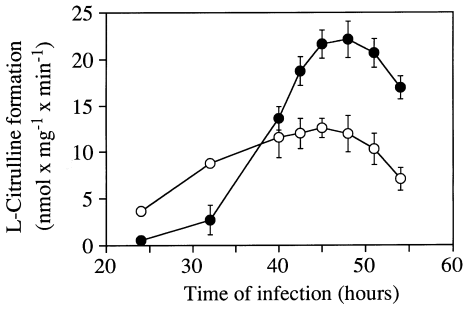


**Figure 1 Time course of expression of NOS activity in soluble fractions of Sf9 cells infected in the absence and presence of haemin**

Sf9 cells infected with rat brain NOS recombinant baculovirus in the absence (open) and presence (closed) of 4 $\mu$g/ml haemin chloride were harvested at the indicated time points. NOS activity (nmol of citrulline $\cdot$ mg$^{-1}$ $\cdot$ min$^{-1}$) was determined in supernatants of three different cell preparations and is given as mean $\pm$ S.E.M.

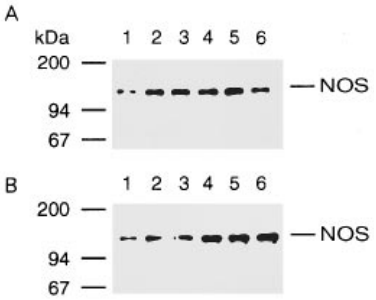


**Figure 2 Time course of expression of the NOS protein in soluble fractions of Sf9 cells infected in the absence and presence of haemin**

Sf9 cells infected with rat brain NOS recombinant baculovirus in the absence (**A**) and presence (**B**) of 4 $\mu$g/ml haemin chloride were harvested after 24 (lane 1), 32 (lane 2), 40 (lane 3), 45 (lane 4), 47.5 (lane 5), and 50 (lane 6) h. Soluble fractions (5 $\mu$g of protein) were subjected to SDS/PAGE and immunoblotting.

enzyme accounted for approximately 5 % of total soluble protein under these conditions.

Infection time turned out to be critical for expression of NOS activity in Sf9 cells. As shown in Figure 1, NOS activity appeared in cell supernatants with a rather sharp maximum 48 h after the cells had been infected with the NOS recombinant baculovirus in the presence of 4 $\mu$g/ml of haemin chloride (filled symbols). Omission of haemin during infection (Figure 1, open symbols) resulted in a much broader range of optimal infection times and reduced NOS activities in the cell supernatants. Figure 2 shows the time-course of expression of the NOS protein, which confirmed the delayed NOS expression in the presence of haemin as observed in activity measurements. Figure 3 shows that the specific NOS activities in crude Sf9 cell supernatants decreased from 48 $\pm$ 2.4 ($n = 3$) to 16 $\pm$ 0.3 nmol of L-citrulline $\cdot$ mg$^{-1}$ $\cdot$ min$^{-1}$ when the protein concentration was increased from 1 to 40 $\mu$g/tube, (0.1 ml) indicating that the insect cell extracts may contain an endogenous inhibitor of nNOS.

## Purification of NOS and biosynthetic manipulation of cofactor availability in Sf9 cells

As described previously, active recombinant rat brain NOS was conveniently purified to homogeneity from supernatants of

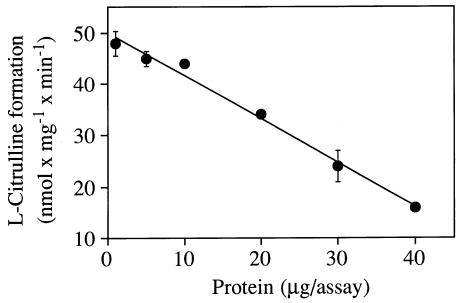


**Figure 3 Effect of the total protein concentration on NOS activity in Sf9 cell supernatants**

Sf9 cells were infected for 48 h with rat brain NOS recombinant baculovirus in the presence of 4 $\mu$g/ml haemin chloride. NOS activity was determined in the presence of the indicated concentrations of soluble protein and is given as nmol of L-citrulline $\cdot$ mg$^{-1}$ $\cdot$ min$^{-1}$ (mean $\pm$ S.E.M., $n = 3$).

infected Sf9 cells by sequential affinity chromatography on 2′,5′-ADP–Sepharose and calmodulin–Sepharose [28]. For the present study, we have down-scaled this method to allow us simultaneous handling of up to 10 preparations. From $\sim$100 mg of soluble protein (200 ml of Sf9 cell suspension; $3 \times 10^8$ cells), we obtained 1–1.3 mg of NOS with a specific activity of approx. 1 $\mu$mol of L-citrulline $\cdot$ mg$^{-1}$ $\cdot$ min$^{-1}$. The amount of recovered NOS and the specific activities of the isolated enzyme (0.9–1.1 $\mu$mol $\cdot$ mg$^{-1}$ $\cdot$ min$^{-1}$) were highly reproducible within different preparations.

To modulate the amount of NOS-bound haem, flavins and $H_4$biopterin, we expressed the enzyme under four different conditions of infection: (i) no additions to the culture medium; (ii) addition of haemin chloride (4 $\mu$g/ml); (iii) addition of haemin chloride plus 0.1 mM riboflavin, a precursor of flavin biosynthesis; and (iv) addition of haemin chloride plus 10 mM DAHP, an inhibitor of GTP cyclohydrolase I, the rate-limiting enzyme in $H_4$biopterin biosynthesis [43]. DAHP was added to the cells 24 h before infection with the baculovirus and remained present during the 48 h period of infection. The various treatments had no effect on cell viability as judged by visual inspection of the infected cells and determined as release of lactate dehydrogenase to the culture medium (results not shown). The representative SDS gel shown in Figure 4 demonstrates that

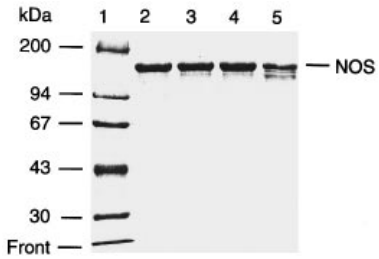


**Figure 4 SDS/PAGE analysis of different NOS preparations**

NOS was purified from Sf9 cells infected under different conditions with rat brain NOS recombinant baculovirus and analysed by SDS/PAGE. Lane 1, molecular mass standards; lane 2, infection in the absence of added haemin; lane 3, infection in the presence of 4 $\mu$g/ml haemin chloride; lane 4, infection in the presence of haemin chloride and 0.1 mM riboflavin; lane 5, infection in the presence of haemin chloride and 10 mM DAHP. Shown is a Coomassie Blue-stained 9% gel representative of four.

**Table 1 Cofactor content of rat brain NOS expressed in Sf9 cells under different conditions of infection**

Haemin and riboflavin were added to the cell culture medium at the time of infection, DAHP was added 24 h prior to infection and remained present during protein expression. Data are mean values ± S.E.M. of four different preparations in which the various cofactors were determined twice.

| Additions | Haem | FAD | FMN | $H_4$biopterin |
|---|---|---|---|---|
| | (mol of cofactor per mol of 160 kDa subunit) | | | |
| None | 0.37 ± 0.08 | 0.39 ± 0.02 | 0.40 ± 0.03 | 0.22 ± 0.04 |
| Haemin (4 μg/ml) | 0.95 ± 0.09 | 0.40 ± 0.09 | 0.46 ± 0.11 | 0.47 ± 0.06 |
| Haemin + riboflavin (0.1 mM) | 0.64 ± 0.04 | 0.79 ± 0.04 | 1.09 ± 0.11 | 0.35 ± 0.02 |
| Haemin + DAHP (10 mM) | 0.91 ± 0.08 | 0.40 ± 0.08 | 0.46 ± 0.12 | 0.059 ± 0.002 |

treatment of the cells with the different drugs had no great effect on subsequent enzyme purification, although we consistently observed a lower intensity of the 155 kDa band and an additional appearance of a few minor bands at 120–150 kDa upon expression in the presence of DAHP (lane 5). The minor bands were recognized by a specific NOS antiserum (results not shown), indicating that pteridine-deficient NOS may be more susceptible to proteolysis than the enzyme obtained from control infections.

### Cofactor content of the different NOS preparations

The various preparations of purified NOS were analysed for enzyme-bound haem, FAD, FMN and $H_4$biopterin (Table 1). If expressed in the absence of added haemin, purified NOS contained 0.37 ± 0.08 equiv. haem per monomer. Preincubation of this haem-deficient enzyme for 20 min at ambient temperature in the presence of a 2-fold molar excess of haemin chloride (28 μM) resulted in stoichiometrical incorporation of haem (1.05 equiv. per monomer) as determined subsequent to removal of free haem by gel-permeation chromatography, but did not restore the enzymic activity of the protein (results not shown). The haem-deficient enzyme contained equimolar amounts of FAD and FMN (molar ratio ~0.40), but only 0.22 equiv. of $H_4$biopterin per monomer. As expected, infection in the presence of haemin chloride induced a marked increase in the haem content of the enzyme to a molar ratio of 0.95 without significant effect on the amount of bound flavins. Surprisingly, however, presence of haemin resulted in a 2-fold increase of bound $H_4$biopterin to a molar ratio of 0.47. The pteridine content of the enzyme was not further increased by supplementing the cell culture medium, which contained 4 mM L-arginine, with $H_4$biopterin or sepiapterin, a precursor of $H_4$biopterin biosynthesis [43]. Moreover, the stoichiometry of cofactor binding was not improved by including the flavins or $H_4$biopterin/L-arginine in the purification buffers (results not shown).

To increase the flavin content of the isolated NOS, haemin was added to the culture medium together with riboflavin (0.1 mM), a precursor of flavin biosynthesis. Under these conditions, the amount of enzyme-bound FAD and FMN was increased to 0.79 and 1.09 equiv. per monomer, respectively. However, for unknown reasons the presence of riboflavin decreased the haem content of the expressed enzyme to a molar ratio of 0.64. As observed with NOS expressed under haem-deficient conditions, the riboflavin-induced reduction in the haem content was accompanied by a significantly reduced amount of bound $H_4$biopterin (0.35 equiv. per monomer).

Finally, we attempted to express $H_4$biopterin-free NOS in order to enable more conclusive studies on the function of pteridines in NO synthesis and to see whether $H_4$biopterin-deficiency affected haem binding. For this purpose, cells were pretreated with 10 mM DAHP, an inhibitor of GTP cyclohydrolase I, the rate-limiting enzyme in pterin biosynthesis. The pterin-deficient cells expressed a virtually $H_4$biopterin-free NOS (0.07 equiv. per monomer) whose haem and flavin content was identical to that of the enzyme obtained from cells treated with haemin only.

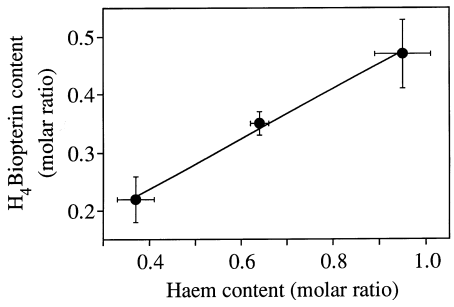


**Figure 5 Correlation between haem and $H_4$biopterin content of recombinant rat brain NOS**

Analysis of the data shown in Table 1 (control, haemin, riboflavin; mean ± S.E.M.; $n = 4$) with a standard linear regression curve-fitting program (intercept = 0.066; slope = 0.43; $r = 0.998$).

### Correlation between haem content and $H_4$biopterin binding

Figure 5 shows that there was a linear relationship between the amount of enzyme-bound haem and the $H_4$biopterin content of the different NOS preparations (control, haemin and riboflavin data were taken from Table 1). Linear regression analysis revealed an intercept of 0.066 and a slope of 0.430 ($r = 0.998$). Binding studies with $^3$H-labelled $H_4$biopterin further confirmed the essential role of haem for pteridine binding. Figure 6 shows that the haem-deficient NOS bound considerably smaller amounts of $H_4$biopterin than the enzyme expressed in the presence of haemin chloride, albeit both preparations exhibited similar affinities for the pteridine, as evident from the virtually identical slopes of the Scatchard plots shown in Figure 6(B). From individual curves we have calculated $B_{max}$ values of 0.129 ± 0.004 and 0.052 ± 0.011 (mean ± S.E.M.; $n = 3$) equiv. $H_4$biopterin bound per monomer of haem-containing and haem-deficient NOS, respectively. The $B_{max}$ value obtained with the haem-containing enzyme is similar to that reported previously for pig brain NOS [25], and the reduction of $B_{max}$ to 40 % of controls upon haem-deficiency is in good accordance with the 37 % of haem-containing NOS present in these preparations (see Table 1). The $H_4$biopterin binding

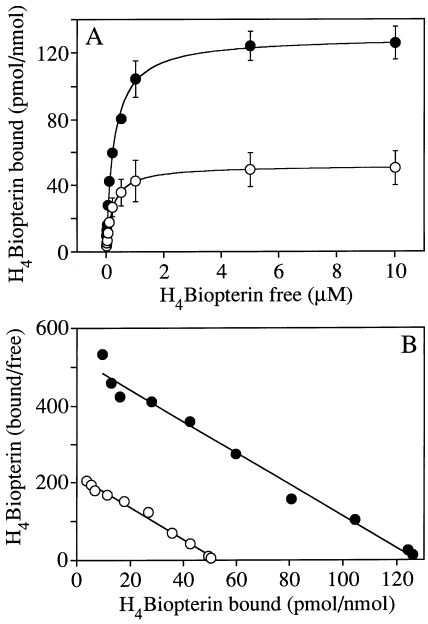


**Figure 6 [$^3$H]$H_4$biopterin binding to recombinant rat brain NOS**

Purified NOS (3 $\mu$g) obtained from Sf9 cells infected with recombinant baculovirus in the absence (unfilled) or presence (filled) of haemin chloride (4 $\mu$g/ml) was assayed for [$^3$H]$H_4$biopterin binding in the presence of increasing concentrations of the unlabelled ligand. (**A**) Saturation of [$^3$H]$H_4$biopterin binding expressed as mean values $\pm$ S.E.M. of three independent assays performed in duplicates. (**B**) Scatchard plot of the mean values.

activity of haem-deficient NOS was not restored when binding was done in the presence of a 2-fold molar excess of haemin chloride (results not shown). The $K_D$ values calculated from individual experiments were $0.26 \pm 0.013$ and $0.22 \pm 0.042$ $\mu$M (mean $\pm$ S.E.M.; $n = 3$) for haem-containing and haem-deficient NOS, respectively.

### Specific activities of the different NOS preparations and effects of added cofactors

Enzyme activities of the different NOS preparations were determined as formation of [$^3$H]citrulline from [$^3$H]arginine. The specific NOS activities shown in Table 2 correlated well with the amount of bound cofactors. The haem-deficient enzyme obtained by expression in the absence of added haemin chloride, containing haem in a molar ratio of 0.37, had a specific activity of 0.35 $\mu$mol·mg$^{-1}$·min$^{-1}$ in the presence of exogenously added FAD, FMN and $H_4$biopterin. Omission of these compounds led to a reduction of enzyme activity that was in good accordance with the amount of the endogenously present cofactors. Thus, cofactor-deficiency of NOS was overcome by including the respective compounds in the enzyme assays, showing that the expressed enzyme was functionally intact. The haem-saturated NOS prepared from cells infected in the presence of haemin chloride had a specific activity of about 1 $\mu$mol·mg$^{-1}$·min$^{-1}$ when reconstituted with added flavins and $H_4$biopterin. Omission of $H_4$biopterin resulted in about 50 % reduction of citrulline formation, and about 40 % of maximal enzyme activity was observed in the absence of added flavins.

Additional supplementation of the haemin-containing culture medium with 0.1 mM riboflavin resulted in expression of a NOS which exhibited 70 % of maximal activity in the absence of added flavins. This enzyme contained FMN in a 1:1 stoichiometry but only 0.8 equiv. of FAD per monomer, suggesting that lack of the latter flavin limits NOS activity. It is interesting that maximal activities of these enzyme preparations were only slightly lower than those of NOS obtained from cells infected in the absence of riboflavin, even though the haem content of the enzyme was reduced to 0.64 equiv. per monomer when the cells had been treated with riboflavin.

The pteridine-deficient NOS obtained from DAHP-treated Sf9 cells had a very low specific activity of 0.07 $\mu$mol·mg$^{-1}$·min$^{-1}$ if incubated in the absence of added $H_4$biopterin. In the presence of 10 $\mu$M exogenous $H_4$biopterin, the maximal activity was 0.7 $\mu$mol·mg$^{-1}$·min$^{-1}$. This value is lower than that of the $H_4$biopterin-containing enzyme (1.1 $\mu$mol·mg$^{-1}$·min$^{-1}$), presumably reflecting proteolytic degradation of the $H_4$biopterin-deficient NOS as evident from the SDS/PAGE analysis shown in Figure 3 (lane 5). Reduction of NOS activity to 40 % of that of controls upon omission of FAD and FMN was in accordance with the flavin content of these preparations (molar ratios of 0.44 and 0.46 for FAD and FMN, respectively).

## DISCUSSION

The baculovirus/Sf9 cell expression system allows the convenient and reproducible preparation of purified nNOS. We have recently described a large-scale purification method yielding about 100 mg of the highly active recombinant enzyme [44]. Although the amount of pure protein obtained with this method is certainly sufficient for further biochemical and biophysical protein characterization, deficiency in bound flavins and $H_4$biopterin indicated that the NOS preparations were not sufficiently homogeneous

**Table 2 Effects of added cofactors on the activity of rat brain NOS expressed in Sf9 cells under different conditions of infection**

NOS activity was determined as formation of [$^3$H]citrulline from [$^3$H]arginine. Purified NOS (0.1–0.3 $\mu$g) was incubated at 37 °C for 10 min at pH 7.0 in the presence of 0.1 mM [$^3$H]arginine (50 000–100 000 c.p.m.), 0.2 mM NADPH, 10 $\mu$M $H_4$biopterin ($H_4$B), 5 $\mu$M FAD, 5 $\mu$M FMN, 0.5 mM $CaCl_2$, 10 $\mu$g/ml calmodulin and 0.1 mM CHAPS. $H_4$Biopterin and/or flavins were omitted where indicated (w/o). Data are mean values $\pm$ S.E.M. of four different preparations, each assayed twice.

| Additions | Full system | w/o Flavins | w/o $H_4$B | w/o $H_4$B + flavins |
|---|---|---|---|---|
| | ($\mu$mol of citrulline·mg$^{-1}$·min$^{-1}$) | | | |
| None | $0.35 \pm 0.02$ | $0.17 \pm 0.02$ | $0.19 \pm 0.02$ | $0.10 \pm 0.01$ |
| Haemin (4 $\mu$g/ml) | $1.09 \pm 0.07$ | $0.42 \pm 0.02$ | $0.56 \pm 0.04$ | $0.19 \pm 0.02$ |
| Haemin + riboflavin (0.1 mM) | $0.91 \pm 0.07$ | $0.65 \pm 0.05$ | $0.52 \pm 0.03$ | $0.32 \pm 0.02$ |
| Haemin + DAHP (10 mM) | $0.69 \pm 0.04$ | $0.28 \pm 0.06$ | $0.07 \pm 0.01$ | $0.03 \pm 0.01$ |

for X-ray crystallography or other biophysical techniques which could resolve the tertiary structure of the protein. Therefore, we attempted to express and purify a holoenzyme containing the various prosthetic groups in close to 1:1 stoichiometry by manipulation of cofactor biosynthesis in Sf9 insect cells during infection with the NOS recombinant baculovirus.

As expected and described previously for expression of neuronal [28,30,32] and endothelial NOS [31], supplementing the cell culture medium with haemin chloride was essential to obtain a functionally intact enzyme. These findings confirm that haematin (FeOH protoporphyrin IX), the active form of dissolved haemin chloride, is taken up by Sf9 cells and utilized for the biosynthesis of haem proteins. If expressed in haemin-treated cells, NOS contained haem at a molar ratio approaching the theoretical value of 1.0 and exhibited a normal haem spectrum with a Soret band at 396 nm (results not shown). We failed, however, to express and purify nNOS saturated with its other prosthetic groups, the flavins and $H_4$biopterin. Addition of riboflavin (or FAD plus FMN, results not shown) resulted in a pronounced increase in the amount of bound flavins, but FAD was still present in substoichiometric amounts, and, moreover, the haem content of the expressed enzyme was significantly reduced under these conditions of cell infection (Table 1). Since pteridine supplementation of the cells or purification buffers did not increase the $H_4$biopterin content of the isolated enzyme, it appears that more sophisticated techniques, e.g. folding of the insoluble enzyme in the presence of cofactors, will be necessary to obtain homogenous NOS preparations.

In line with a recent study on overexpression of nNOS in baculovirus-infected insect cells [32], haemin supplementation increased the amount of the expressed enzyme in soluble fractions. These findings suggest that haem availability regulates the post- or co-translational processing of the expressed enzyme. A few previous reports hint at similar effects of haem on solubilization of other haem proteins. It has been described that inhibition of haem biosynthesis in human myeloid leukaemic cells resulted in the accumulation of inactive haem-deficient myeloperoxidase in the endoplasmic reticulum of the cells [45]. Similarly, supplementing the growth medium of *E. coli* with precursors of haem biosynthesis was found to be essential for solubilization of recombinant *Vitreoscilla* haemoglobin [46], and the haem-deficient apoform of horseradish peroxidase C was shown to accumulate as an inactive protein in inclusion bodies of *E. coli*, with haemin being essential for *in vitro* folding and activation of the enzyme [47].

If NOS was expressed in the absence of added haemin, the purified preparations consisted of 60–70% of the haem-free enzyme (Table 1), but contained virtually the same amount of flavins as controls, showing that haem-deficiency does not affect binding of FAD or FMN. These results further confirm the bidomain structure of NOS, with a flavin-containing reductase domain being both sterically and functionally separated from the oxygenase domain [17–19,24,48]. Surprisingly, haem-deficient NOS contained less $H_4$biopterin than the native form of the enzyme. This $H_4$biopterin-deficiency appears to be a consequence of the reduced haem content and not vice versa, because the virtually $H_4$biopterin-free enzyme still contained close to stoichiometric amounts of haem (0.91 equiv. per monomer). A comparison of haem-containing and haem-deficient enzyme preparations with respect to [$^3$H]$H_4$biopterin binding (Figure 5) has provided convincing evidence that haem-free NOS does not exhibit a specific pteridine-binding site, and preliminary experiments from our laboratory indicate that haem-deficient NOS does not bind the substrate analogue $N^G$-nitro-L-arginine (P. Klatt and B. Mayer, unpublished work). Together with previous results showing that the affinity of pig brain NOS for $H_4$biopterin was markedly increased in the presence of L-arginine [25], these results indicate that pteridine binding is tightly regulated by an allosteric interaction of the substrate site and the prosthetic haem group of the enzyme. Similarly, the simultaneous presence of haem, L-arginine and $H_4$biopterin was found to be essential for association of haem- and pteridine-deficient macrophage NOS monomers [23], suggesting that the synergism between L-arginine and haem to create a high-affinity pteridine-binding site is not confined to the neuronal isoenzyme.

Extrapolation of the linear correlation between the amount of haem and $H_4$biopterin bound to NOS (intercept close to zero; see Figure 4) also indicates that the haem-free enzyme does not bind $H_4$biopterin. Interestingly, the slope of this linear correlation was close to 0.5, suggesting that haem and $H_4$biopterin are present in a ratio of 2:1. Since the haem-containing enzyme binds one haem per subunit, these results suggest that each NOS dimer contains two haem moieties but only one tightly bound $H_4$biopterin. However, NOS activity was increased $\sim$2-fold by added $H_4$biopterin (Table 2), confirming a number of previous studies which have unambigously demonstrated that the degree of NOS stimulation by exogenous $H_4$biopterin is inversely proportional to the pteridine content of the respective preparations (for review see [22]). Thus, each dimer is apparently able to bind two molecules of $H_4$biopterin, but for unknown reasons contains only one as a tightly bound prosthetic group. This is further supported by our findings that increased pteridine availability during cell infection and the presence of $H_4$biopterin during enzyme purification did not increase the amount of NOS-bound $H_4$biopterin (results not shown). Moreover, radioligand binding studies have shown that compounds which antagonize the association of exogenous $H_4$biopterin to pig brain NOS do not displace the endogenous cofactor [25]. Taken together, these rather intriguing results suggest that NOS dimers contain two different pteridine-binding sites, a high-affinity site involved in tight binding of the cofactor and a low-affinity site from which $H_4$biopterin readily dissociates. Since the dimers are composed of identical subunits, the two affinity states of the pteridine-binding domain could only result from negative cooperativity occurring upon association of NOS monomers. With respect to this peculiar stoichiometry of $H_4$biopterin binding, nNOS appears to differ from the inducible macrophage isoform, which was reported to contain one equiv. of $H_4$biopterin per monomer if reconstituted with the exogenous cofactor [49].

In summary, we have shown that the baculovirus/Sf9 cell expression system is a useful and highly reproducible tool to obtain pure and enzymically active recombinant NOS. Biosynthetic manipulation of NOS cofactors during cell infection enabled us to modulate the amount of tightly bound prosthetic groups, and these experiments revealed a novel role of haem for the co- or post-translational processing of NOS. Haem turned out to be essential for both enzyme solubilization and $H_4$biopterin binding, suggesting that the prosthetic haem group has an important allosteric function in regulating the correct folding of the expressed protein.

We wish to thank Drs. D.S. Bredt and S.H. Snyder for the generous gift of rat brain NOS cDNA, Dr. C. Harteneck for preparing the recombinant baculovirus, and Eva Leopold for excellent technical assistance. This work was supported by grants P 10098, P 10655 (B.M.), P 10573 (K.S.), and P 9685 (E.R.W.) of the Fonds zur Förderung der Wissenschaftlichen Forschung in Austria.

## REFERENCES

1 Marletta, M. A. (1993) J. Biol. Chem. **268**, 12231–12234
2 Masters, B. S. S. (1994) Annu. Rev. Nutr. **14**, 131–145

3 Nathan, C. and Xie, Q. W. (1994) J. Biol. Chem. **269**, 13725–13728
4 Griffith, O. W. and Stuehr, D. J. (1995) Annu. Rev. Physiol. **57**, 707–736
5 Bredt, D. S. and Snyder, S. H. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 682–685
6 Mayer, B., John, M. and Böhme, E. (1990) FEBS Lett. **277**, 215–219
7 Schmidt, H. H. H. W., Pollock, J. S., Nakane, M., Gorsky, L. D., Förstermann, U. and Murad, F. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 365–369
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 McMillan, K., Bredt, D. S., Hirsch, D. J., Snyder, S. H., Clark, J. E. and Masters, B. S. S. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 11141–11145
11 Klatt, P., Schmidt, K. and Mayer, B. (1992) Biochem. J. **288**, 15–17
12 Mayer, B., John, M., Heinzel, B., Werner, E. R., Wachter, H., Schultz, G. and Böhme, E. (1991) FEBS Lett. **288**, 187–191
13 Bredt, D. S., Ferris, C. D. and Snyder, S. H. (1992) J. Biol. Chem. **267**, 10976–10981
14 Schmidt, H. H. H. W., Smith, R. M., Nakane, M. and Murad, F. (1992) Biochemistry **31**, 3243–3249
15 Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R. and Snyder, S. H. (1991) Nature (London) **351**, 714–718
16 Abu-Soud, H. M. and Stuehr, D. J. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 10769–10772
17 Klatt, P., Schmidt, K., Uray, G. and Mayer, B. (1993) J. Biol. Chem. **268**, 14781–14787
18 Sheta, E. A., McMillan, K. and Masters, B. S. S. (1994) J. Biol. Chem. **269**, 15147–15153
19 McMillan, K. and Masters, B. S. S. (1995) Biochemistry **34**, 3686–3693
20 Kwon, N. S., Nathan, C. F. and Stuehr, D. J. (1989) J. Biol. Chem. **264**, 20496–20501
21 Tayeh, M. A. and Marletta, M. A. (1989) J. Biol. Chem. **264**, 19654–19658
22 Mayer, B. and Werner, E. R. (1995) Naunyn-Schmiedeberg's Arch. Pharmacol. **351**, 453–463
23 Baek, K. J., Thiel, B. A., Lucas, S. and Stuehr, D. J. (1993) J. Biol. Chem. **268**, 21120–21129
24 Ghosh, D. K. and Stuehr, D. J. (1995) Biochemistry **34**, 801–807
25 Klatt, P., Schmid, M., Leopold, E., Schmidt, K., Werner, E. R. and Mayer, B. (1994) J. Biol. Chem. **269**, 13861–13866
26 Wang, J. L., Stuehr, D. J. and Rousseau, D. L. (1995) Biochemistry **34**, 7080–7087
27 Mayer, B., Klatt, P., Werner, E. R. and Schmidt, K. (1994) Neuropharmacology **33**, 1253–1259
28 Harteneck, C., Klatt, P., Schmidt, K. and Mayer, B. (1994) Biochem. J. **304**, 683–686
29 Asseffa, A., Smith, S. J., Nagata, K., Gillette, J., Gelboin, H. V. and Gonzalez, F. J. (1989) Arch. Biochem. Biophys. **274**, 481–490
30 Richards, M. K. and Marletta, M. A. (1994) Biochemistry **33**, 14723–14732
31 Seo, H. G., Fujii, J., Soejima, H., Niikawa, N. and Taniguchi, N. (1995) Biochem. Biophys. Res. Commun. **208**, 10–18
32 Riveros-Moreno, V., Heffernan, B., Torres, B., Chubb, A., Charles, I. and Moncada, S. (1995) Eur. J. Biochem. **230**, 52–57
33 Gonzalez, F. J., Kimura, S., Tamura, S. and Gelboin, H. V. (1991) Methods Enzymol. **206**, 93–99
34 Werner, E. R., Schmid, M., Werner-Felmayer, G., Mayer, B. and Wachter, H. (1994) Biochem. J. **304**, 189–193
35 Klatt, P., Heinzel, B., John, M., Kastner, M., Böhme, E. and Mayer, B. (1992) J. Biol. Chem. **267**, 11374–11378
36 Yasukochi, Y. and Masters, B. S. S. (1976) J. Biol. Chem. **251**, 5337–5344
37 Kindt, J. T., Woods, A., Martin, B. M., Cotter, R. J. and Osawa, Y. (1992) J. Biol. Chem. **267**, 8739–8743
38 Olken, N. M., Osawa, Y. and Marletta, M. A. (1994) Biochemistry **33**, 14784–14791
39 Berry, E. A. and Trumpower, B. L. (1987) Anal. Biochem. **161**, 1–15
40 Klatt, P., Schmidt, K., Brunner, F. and Mayer, B. (1994) J. Biol. Chem. **269**, 1674–1680
41 Brunner, F. and Kukovetz, W. R. (1991) Br. J. Pharmacol. **102**, 373–380
42 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
43 Werner, E. R., Werner-Felmayer, G. and Wachter, H. (1993) Proc. Soc. Exp. Biol. Med. **203**, 1–12
44 Mayer, B., List, B. M., Klatt, P., Harteneck, C. and Schmidt, K. (1996) Methods Enzymol., in the press
45 Pinnix, I. B., Guzman, G. S., Bonkovsky, H. L., Zaki, S. R. and Kinkade, J. M. (1994) Arch. Biochem. Biophys. **312**, 447–458
46 Hart, R. A., Kallio, P. T. and Bailey, J. E. (1994) Appl. Environ. Microbiol. **60**, 2431–2437
47 Smith, A. T., Santama, N., Dacey, S., Edwards, M., Bray, R. C., Thorneley, R. N. and Burke, J. F. (1990) J. Biol. Chem. **265**, 13335–13343
48 Abu-Soud, H. M., Yoho, L. L. and Stuehr, D. J. (1994) J. Biol. Chem. **269**, 32047–32050
49 Hevel, J. M. and Marletta, M. A. (1992) Biochemistry **31**, 7160–7165


Received 21 August 1995/15 November 1995; accepted 21 November 1995