Characterization of bovine endothelial nitric oxide synthase as a homodimer with down-regulated uncoupled NADPH oxidase activity: tetrahydrobiopterin binding kinetics and role of haem in dimerization

Barbara M. LIST*, Burkhard KLÖSCH*, Christof VÖLKER*, Antonius C. F. GORREN*, William C. SESSA†, Ernst R. WERNER‡, Walter R. KUKOVETZ*, Kurt SCHMIDT* and Bernd MAYER*§

*Institut für Pharmakologie und Toxikologie, Karl-Franzens-Universität Graz, Universitätsplatz 2, A-8010 Graz, Austria, †Department of Pharmacology and Boyer Center for Molecular Medicine, Yale University School of Medicine, New Haven, CT 06536-0812, U.S.A., and ‡Institut für Medizinische Chemie und Biochemie, Universität Innsbruck, Fritz-Pregl-Strasse 3, A-6020 Innsbruck, Austria

The fatty-acylation-deficient bovine endothelial NO synthase (eNOS) mutant (Gly-2 to Ala-2, G2AeNOS) was purified from a baculovirus overexpression system. The purified protein was soluble and highly active (0.2–0.7 μmol of L-citrulline·$mg^{-1}\cdot min^{-1}$), contained 0.77 ± 0.01 equivalent of haem per subunit, showed a Soret maximum at 396 nm, and exhibited only minor uncoupling of NADPH oxidation in the absence of L-arginine or tetrahydrobiopterin. Radioligand binding studies revealed K_D values of 147 ± 24.1 nM and 52 ± 9.2 nM for specific binding of tetrahydrobiopterin in the absence and presence of 0.1 mM L-arginine respectively. The positive co-operative effect of L-arginine was due to a pronounced decrease in the rate of tetrahydrobiopterin dissociation (from 1.6 ± 0.5 to $0.3\pm0.1\ min^{-1}$). Low-temperature SDS gel electrophoresis showed that approx. 80% of the protein migrated as haem-containing dimer after preincubation with L-arginine and tetrahydrobiopterin. Gel-filtration chromatography yielded one peak with a Stokes radius of 6.8 ± 0.04 nm, corresponding to a hydrodynamic volume of 1.32×10^{-24} m^3, whereas haem-deficient preparations (approx. 0.3 equivalent per subunit) contained an additional protein species with a hydrodynamic radius of 5.1 ± 0.2 nm and a corresponding volume of 0.55×10^{-24} m^3, suggesting that haem availability regulates eNOS dimerization.

INTRODUCTION

Nitric oxide, an important effector and signalling molecule in the nervous, immune, and cardiovascular systems, is enzymically generated from the guanidino group of L-arginine by different nitric oxide synthases (NOSs, EC 1.14.13.39) [1–3]. The neuronal (nNOS) and endothelial (eNOS) isoforms are constitutively expressed and require micromolar concentrations of free Ca^{2+} for activity, whereas the isoenzyme first described in murine macrophages (iNOS) is cytokine-inducible and largely Ca^{2+}-independent.

Oxidation of L-arginine occurs via formation of N^G-hydroxy-L-arginine as intermediate and involves reductive activation of molecular oxygen catalysed by a cytochrome *P*-450-like haem iron bound to a cysteine residue in the oxygenase domain of the protein. The five-electron oxidation of L-arginine is accompanied by an eight-electron reduction of molecular oxygen, with three exogenous electrons provided by the nucleotide cofactor NADPH. The electron transfer from NADPH to the haem is catalysed by an FAD- and FMN-containing cytochrome *P*-450 reductase domain, which is located in the C-terminal half of NOS, and requires bound calmodulin for activity. Accordingly, NOS appears to be a self-sufficient cytochrome *P*-450, resembling the soluble cytochrome *P*-$450_{(BM\text{-}3)}$ from *Bacillus megaterium*, which also contains oxygenase and reductase domains within one single polypeptide [4]. In contrast with other *P*-450s, all three NOS isoforms require (6*R*)-5,6,7,8-tetrahydro-L-biopterin (H_4biopterin) as essential cofactor. Although its precise function is not known, current evidence suggests that the pteridine has a dual role and acts as both redox-active cofactor of L-arginine oxidation and allosteric effector of the NOS protein [5].

In the presence of suboptimal concentrations of L-arginine, Ca^{2+}/calmodulin-activated nNOS exhibits NADPH oxidase activity, resulting in formation of superoxide anions and H_2O_2 due to uncoupling of oxygen reduction [6–9]. Unlike NO and L-citrulline formation, uncoupled NADPH oxidation is independent of H_4biopterin. As a consequence, H_4biopterin-deficiency of nNOS leads to generation of oxygen radicals instead of NO even if the enzyme is saturated with L-arginine [7,10]. The finding that iNOS does not exhibit considerable NADPH oxidase activity in the absence of a bound amino acid ligand [11,12] suggested that uncoupled oxygen reduction may be a specific feature of the constitutive isoforms, but so far this issue has not been addressed with the endothelial enzyme.

All three NOS isoforms are homodimers under native conditions [13–16]. Dimerization appears to be a tightly regulated process showing distinct cofactor requirements. Dimerization of iNOS needs the coincident presence of haem and H_4biopterin [17], and the intracellular availability of these cofactors was shown to limit the expression of active iNOS dimers [18,19]. Dimerization of nNOS appears to be different: although H_4biopterin stabilizes the dimeric structure of the enzyme, dimerization itself is pteridine-independent and solely regulated by haem [15,20,21].

Until recently, the limited availability of eNOS has precluded detailed biochemical and biophysical characterization of this isoform. The enzyme was first purified from membrane fractions of cultured bovine aortic endothelial cells [22]. Molecular cloning

Abbreviations used: NOS, nitric oxide synthase, eNOS, endothelial NOS (type III); iNOS, inducible NOS (type II); nNOS, neuronal NOS (type I); H_4biopterin, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; L-NMA, N^G-methyl-L-arginine; L-NNA, N^G-nitro-L-arginine.

§ To whom correspondence should be addressed.

and site-directed mutagenesis revealed that membrane association of eNOS results from myristoylation at the N-terminal Gly-2 residue [23–29] targeting the protein to the Golgi apparatus [30,31] and/or plasmalemmal caveolae [32,33] of endothelial cells. It was the aim of the present study to establish a baculovirus system for efficient overexpression of eNOS. Infection of Sf9 cells with baculovirus transfected with a cDNA encoding a myristoylation- and palmitoylation-deficient mutant of the enzyme (Gly-2 to Ala-2; [27,34,35]) resulted in high-level expression of a soluble, functionally intact enzyme which was purified and biochemically characterized.

EXPERIMENTAL

Materials

L-[2,3,4,5-^{3}H]Arginine hydrochloride (57 Ci/mmol) was from Amersham, purchased through MedPro (Vienna, Austria). Labelled L-arginine was further purified by cation exchange HPLC with 50 mM sodium acetate (pH 6.5) as eluent. 3′(6*R*)-5,6,7,8-[^{3}H]Tetrahydro-L-biopterin (14 Ci/mmol) was synthesized enzymically from [8,5′-^{3}H]GTP as described previously [36]. Rat nNOS was purified from baculovirus-infected Sf9 cells as described [37,38]. H_4biopterin was obtained from Dr. B. Schircks Laboratories, Jona, Switzerland. Unless indicated otherwise, the material used for molecular biology was from Gibco (Vienna, Austria). All other chemicals were from Sigma.

Expression and purification of eNOS

The Gly-2 to Ala-2 (G2A) mutant of bovine eNOS was made by site-directed mutagenesis as described [27]. The plasmid pcDNA3 containing the G2AeNOS cDNA was digested with *Eco*RI, and the cDNA was subcloned into the *Eco*RI-digested vector pFAST BAC1. The recombinant plasmid, named pFB16, was transformed into the *Escherichia coli* strain DH10 BAC, in which the expression cassette was integrated into the bacmid bMON14272 by a transposition process. Following selection of positive recombinants, recombinant bacmid DNA was isolated from *E. coli* clones and used for transfection of fall armyworm ovary (*Spodoptera frugiperda*; Sf9) cells with CELLFECTIN reagent. After 2 days, recombinant baculovirus was harvested and used for protein expression after amplification. For expression and purification of eNOS, the protocols established previously for preparation of nNOS [37,38] were slightly modified: Sf9 cells (1.5×10^9 cells/l) were infected with the recombinant baculovirus at a ratio of 5 plaque-forming units per cell in the presence of 4 mg/l haemin chloride. Where indicated, haemin was omitted for preparation of haem-deficient eNOS. After 56 h, cells were harvested by centrifugation, washed with 100 ml of serum-free TC-100 medium, resuspended in 15 ml of homogenization buffer (50 mM triethanolamine/HCl, pH 7.4, containing 0.5 mM EDTA and 12 mM 2-mercaptoethanol), and sonicated three times for 10 s followed by preparation of 30000 ***g*** supernatants. The expressed protein was purified from the supernatants by sequential affinity chromatography on 2′,5′-ADP–Sepharose and calmodulin–Sepharose. Final elution was with 10 ml of 20 mM Tris/HCl buffer (pH 7.4) containing 150 mM NaCl and 4 mM EGTA. The enzyme was stored at −70 °C at a concentration of 0.4–1.5 mg/ml. Protein was determined with the Bradford method [39] using BSA as a standard.

Gel filtration chromatography

Aliquots of 0.10–0.20 ml containing 80–100 μg of eNOS were incubated with L-arginine (1 mM) and H_4biopterin (0.2 mM) at ambient temperature for 30 min and then injected into an HPLC system (LiChroGraph L-6200; Merck) equipped with a low pressure gradient controller and a gel filtration column, providing a separation range from approx. 5 to 5000 kDa (Superose 6 HR 10/30; Pharmacia Biotech, Vienna, Austria). The protein was eluted at room temperature with a 50 mM triethanolamine/HCl buffer, pH 7.4, containing 0.50 M NaCl, 0.5 mM EDTA, 0.1 mM L-arginine and 0.2 mM H_4biopterin at a flow rate of 0.3 ml/min and detected by its absorbance at 280 nm (UV/visible detector L-4250, Merck). In some experiments, L-arginine and H_4 biopterin were omitted. The column was calibrated with the gel filtration calibration kit from Pharmacia Biotech (Vienna, Austria), including Blue Dextran 2000 (for the determination of the column void volume), thyroglobulin (669 kDa; Stokes radius = 8.50 nm), ferritin (440 kDa, 6.10 nm), catalase (232 kDa, 5.22 nm), aldolase (158 kDa; 4.81 nm), and BSA (67 kDa, 3.55 nm). Calibration curves were obtained by plotting $(-\log K_{av})^{0.5}$ against the Stokes radii of the above standard proteins [$K_{av} = (V_e - V_o)/(V_t - V_o)$], with V_e, V_o and V_t denoting the elution volume of the protein, the column void volume and the total bed volume of the column respectively [21].

Determination of cofactors

Enzyme-bound flavins and H_4biopterin were determined by reversed-phase HPLC and fluorescence detection using authentic FAD, FMN and H_4biopterin as standards [40]. Haem was quantified by reversed-phase HPLC and UV/visible detection using myoglobin as standard [41]. Calculations of molar cofactor/NOS ratios were based on a subunit molecular mass of 134 kDa as calculated from the deduced amino acid sequence of the enzyme [24].

Determination of enzyme activity

NOS activity was determined as formation of L-[2,3,4,5-^{3}H]citrulline from L-[2,3,4,5-^{3}H]arginine [42]. Incubations were for 10 min at 37 °C in 0.1 ml of 50 mM triethanolamine/HCl buffer, pH 7.4, containing 0.1–0.2 μg of eNOS, 0.1 mM L-[2,3,4,5-^{3}H]arginine (approx. 80000 c.p.m.), 0.5 mM $CaCl_2$, 10 μg/ml calmodulin, 0.2 mM NADPH, 10 μM H_4biopterin, 5 μM FAD, 5 μM FMN and 0.2 mM CHAPS. Calmodulin-dependent NADPH:oxygen oxidoreductase activity of nNOS was determined in the absence of L-arginine as described previously [6,43]. Unless otherwise indicated, purified NOS (1–3 μg) was incubated in a final volume of 0.2 ml of 50 mM triethanolamine/HCl buffer, pH 7.4, in the presence of 0.5 mM $CaCl_2$, 10 μg/ml calmodulin, 0.1 mM NADPH and 0.2 mM CHAPS at 37 °C. The changes in absorbance at 340 nm were continuously monitored against calmodulin-deficient blanks, using a molar absorption coefficient of 6.34 $mM^{-1} \cdot cm^{-1}$ for calculating the rates of NADPH oxidation.

H_4biopterin binding

For saturation binding, eNOS (0.5 μg) was incubated for 10 min at 37 °C in the absence and presence of 0.1 mM L-arginine with 10 nM [^{3}H]H_4biopterin (approx. 14 nCi) and increasing concentrations of unlabelled H_4biopterin (10 nM–10 μM) in 0.1 ml of a 50 mM triethanolamine/HCl buffer, pH 7.4, followed by rapid vacuum filtration using the MultiScreen Assay System from Millipore and determination of the radioactivity retained on the filters by liquid scintillation counting. Association kinetics were performed under the same conditions but in the absence of unlabelled H_4biopterin. At the indicated time points, aliquots containing 0.5 μg of protein were removed and immediately

subjected to vacuum filtration. For dissociation experiments, eNOS was equilibrated with 10 nM [^{3}H]H_4biopterin for 10 min, followed by addition of 10 mM unlabelled H_4biopterin and processing of aliquots containing 0.5 μg of eNOS at the indicated time points. Data were corrected for non-specific binding determined in the presence of 1 mM unlabelled ligand. K_D and B_{max} values were calculated using the GIPMAX non-linear least-squares regression curve fitting program [44]. Association and dissociation experiments were fitted according to first-order kinetics.

Gel electrophoresis and haem staining

Purified eNOS was analysed by conventional or low-temperature SDS/PAGE as described [15]. To test for dimerization, eNOS was incubated for 30 min at ambient temperature in 50 μl of 50 mM triethanolamine/HCl buffer (pH 7.4) in the absence or presence of H_4biopterin (0.2 mM) plus L-arginine (1 mM). Incubations were terminated by the addition of 50 μl of chilled Laemmli buffer [45] containing 0.125 M Tris/HCl (pH 6.8), 4 % (w/v) SDS, 20 % (w/v) glycerol and 0.02 % (w/v) Bromphenol Blue. Samples containing 6 μg of eNOS were subjected to SDS/PAGE for 60 min at a constant current of 30 mA on discontinuous 6 % SDS gels (70 mm × 80 mm × 1 mm). Gels and buffers, prepared according to [45], were equilibrated at 4 °C and the buffer tank was cooled during electrophoresis in an ice-bath. Gels were stained either for protein with Coomassie Blue R250 or for haem with 3,3′-dimethoxybenzidine/H_2O_2 following a published method [46] with modifications. Gels were washed for 10 min in methanol/sodium acetate (0.25 M, pH 5.0; 3:7 v/v) and subsequently incubated in the dark for 20 min in a freshly prepared solution, containing 7 parts of 0.25 M sodium acetate, pH 5.0, and 3 parts of 6 mM 3,3′-dimethoxybenzidine dihydrochloride in methanol. Gels were developed for 60 min by adding H_2O_2 to a final concentration of 60 mM, washed for 30 min in water/methanol/acetic acid (8:1:1, by vol.), dried, and photographed. Relative amounts of protein or haem were estimated by densitometric analysis using the vds 800 video system and H1D software of Hirschmann (Analysentechnik Hirschmann, Taufkirchen, Germany).

RESULTS

Infection of Sf9 cells with a G2AeNOS-recombinant baculovirus led to the appearance of a 135 kDa band on Coomassie Blue-stained SDS gels of the supernatants (Figure 1, lane A versus lane B). The eNOS protein was purified to apparent homogeneity by sequential chromatography over 2′,5′-ADP–Sepharose and calmodulin–Sepharose (lanes C and D respectively). As estimated from densitometric analysis of the gels, eNOS accounted for 1–3 % of total soluble Sf9 cell protein.

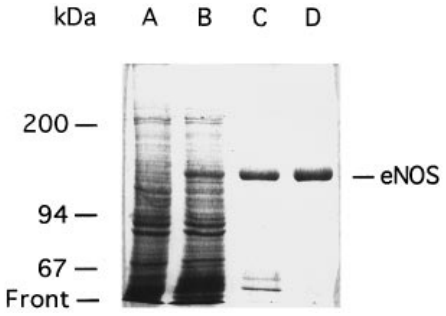

Figure 1 Purification of eNOS monitored by SDS/PAGE analysis

Shown is a representative Coomassie Blue-stained 8% polyacrylamide gel. Lane A, 30 000 ***g*** supernatant from non-infected Sf9 cells (30 μg); lane B, supernatant from G2AeNOS-recombinant baculovirus-infected cells (30 μg); lane C, 2′,5′-ADP–Sepharose eluate (4 μg); lane D, calmodulin–Sepharose eluate (4 μg).

Table 1 Purification of eNOS from baculovirus-infected Sf9 cells

NOS was purified from 1.5×10^9 Sf9 cells, which had been infected with G2AeNOS-recombinant baculovirus for 56 h in 1 litre culture medium supplemented with 4 μg/ml haemin as described in the Experimental section. Enzyme activity was determined as citrulline formation. Data are representative of five similar preparations, but note that specific NOS activity varied within different preparations (0.002–0.005 and 0.2–0.7 μmol of L-citrulline · $mg^{-1} \cdot min^{-1}$ in supernatants and calmodulin–Sepharose eluates respectively).

Fraction	Protein (mg)	Total activity (μmol · min^{-1})	Specific activity (μmol · $mg^{-1} \cdot min^{-1}$)	Yield (%)
30 000 ***g*** supernatant	704	2.52	0.004	100
2′,5′-ADP–Sepharose	7	2.25	0.32	90
Calmodulin–Sepharose	3.0	1.35	0.52	53

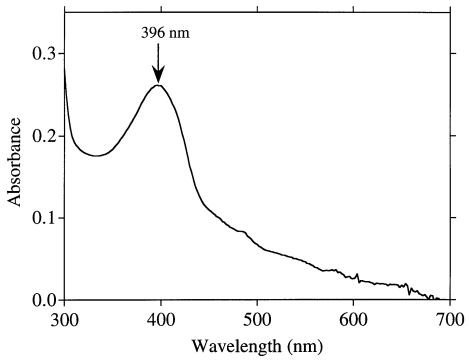

Figure 2 Light absorbance spectrum of recombinant eNOS

The spectrum of purified eNOS (0.4 mg/ml) was recorded at 20 °C and normalized to zero absorbance at 700 nm.

Supernatants of infected Sf9 cells, which exhibited specific NOS activities of 2–5 nmol of L-citrulline · $mg^{-1} \cdot min^{-1}$, were subjected to sequential affinity chromatography on 2′,5′-ADP–Sepharose and calmodulin–Sepharose. The representative purification shown in Table 1 yielded 3 mg of eNOS, which was purified 130-fold from the soluble fractions. The specific activity of this particular preparation was 0.52 μmol of L-citrulline · $mg^{-1} \cdot min^{-1}$, but the activities of different preparations varied considerably (0.2 to 0.7 μmol · $mg^{-1} \cdot min^{-1}$). Also, the purified enzyme showed an unpredictable tendency to precipitate during storage at −70 °C. Precipitation was apparently irreversible, occurred in approximately one out of three preparations, and was not prevented by storage of the protein in the presence of glycerol, detergents or high salt. The enzyme had an apparent K_m for L-arginine of 5.3 ± 0.5 μM and exhibited maximal activity at pH ≈ 7.4. The haem spectrum of eNOS is shown in Figure 2. With a Soret maximum at 396 nm and a molar absorption coefficient of 102 $mM^{-1} \cdot cm^{-1}$, this spectrum is virtually identical with that of rat nNOS obtained from the baculovirus overexpression system [37].

Purified eNOS was not fully saturated with haem. When expressed in the absence and presence of haemin chloride, eNOS

Table 2 Effects of added cofactors on recombinant eNOS activity

Purified eNOS 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 the Experimental section. Data are means $\pm$ S.E.M. from three experiments. n.d., not detectable.

Compound omitted	NO synthase activity (μmol of citrulline · $mg^{-1} \cdot min^{-1}$)
None	0.36 ± 0.091
Calmodulin	n.d.
NADPH	n.d.
FAD + FMN	0.15 ± 0.034
H_4biopterin	0.10 ± 0.024
FAD + FMN + H_4biopterin	0.051 ± 0.012

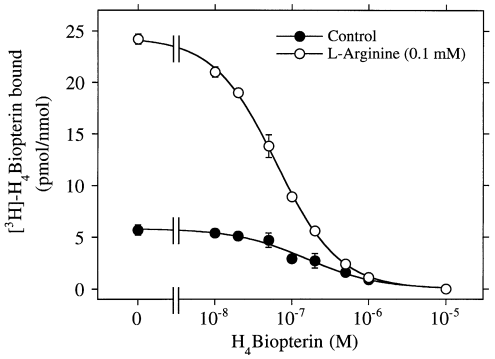

Figure 3 Saturation binding of [^{3}H]H_4biopterin

For saturation binding of [^{3}H]H_4biopterin, 0.5 μg of eNOS was incubated for 10 min at 37 °C with 10 nM [^{3}H]H_4biopterin (approx. 14 nCi) and increasing concentrations of the unlabelled ligand (10 nM–10 μM) in 0.1 ml of a 50 mM triethanolamine/HCl buffer, pH 7.4, in the absence and presence of 0.1 mM L-arginine. The amount of specifically bound H_4biopterin was determined as described in the Experimental section. Data are means $\pm$ S.E.M. of three experiments.

contained 0.32 ± 0.07 and 0.77 ± 0.01 equivalent of haem per subunit respectively ($n = 3$ each). The H_4biopterin and flavin content of haem-containing eNOS was as follows (equivalent per monomer; $n = 3$ each): 0.37 ± 0.05 H_4biopterin, 0.61 ± 0.02 FAD and 0.49 ± 0.04 FMN. The effect of exogenously added cofactors on enzyme activity is shown in Table 2. Under regular assay conditions (see Experimental section) this particular preparation had a specific activity of 0.36 μmol of L-citrulline · $mg^{-1} \cdot min^{-1}$. L-Citrulline formation was reduced to approx. 50 % in the absence of added flavins, to 30 % in the absence of added H_4biopterin and to 15 % if both flavins and H_4biopterin had been omitted.

To see whether the uncoupling of NADPH-dependent oxygen reduction is specific for nNOS or a feature common to the constitutive isoforms, we studied Ca^{2+}/calmodulin-dependent NADPH oxidation catalysed by both isoforms in the absence and presence of L-arginine, H_4biopterin and the two L-arginine analogues, N^G-methyl-L-arginine (L-NMA) and N^G-nitro-L-arginine (L-NNA). Table 3 shows that eNOS exhibited very little NADPH oxidase activity in the absence of L-arginine (0.07 μmol · $mg^{-1} \cdot min^{-1}$). The presence of 10 μM H_4biopterin had no significant effect. Addition of L-arginine (0.1 mM) led to a pronounced increase in NADPH oxidation to 0.18 μmol · $mg^{-1} \cdot min^{-1}$, which was further stimulated to 0.52 μmol · $mg^{-1} \cdot min^{-1}$ by H_4biopterin. NADPH oxidation was not inhibited by 0.1 mM L-NMA, although formation of L-citrulline was reduced by the inhibitor by approx. 75 %. L-NNA (0.05 mM) inhibited the oxidation of NADPH to approx. 35 % of control and almost completely blocked L-citrulline formation (7.5 % residual activity). For comparison, the NADPH oxidase activity of recombinant rat nNOS was determined under the same conditions (Table 3). When activated with Ca^{2+}/calmodulin, nNOS exhibited considerable NADPH oxidase activity even in the absence of L-arginine. The presence of either the amino acid substrate or H_4biopterin had no significant effect; the presence of both induced a moderate stimulation of NADPH oxidation. L-NNA (50 μM) completely inhibited formation of both L-citrulline and NADPH, whereas L-NMA (0.1 mM) had no appreciable effect on NADPH oxidation in spite of a pronounced inhibition of L-citrulline formation, down to 25 % of controls.

Radioligand binding studies performed with [^{3}H]H_4biopterin showed that pteridine binding was monophasic and increased by L-arginine (Figure 3). The apparent K_D values in the absence and presence of L-arginine were 147 ± 24.1 and 52 ± 9.2 nM and the corresponding B_{max} values were 96 ± 19.6 and 161 ± 8.2 pmol/nmol of protein respectively ($n = 3$ each). The kinetic experiments shown in Figure 4 demonstrate that the presence of L-arginine had no great effect on the rate of association (0.25–0.5 min^{-1}) (Figure 4A) but decreased the rates

Table 3 NADPH oxidase activities of eNOS and nNOS

The purified enzymes (1–3 μg) were assayed for NADPH oxidation and L-citrulline as described in the Experimental section in a 50 mM triethanolamine/HCl buffer at pH 7.0 (nNOS) or pH 7.5 (eNOS) in the presence of 0.5 mM $CaCl_2$, 10 μg/ml calmodulin, 0.1 mM NADPH and 0.2 mM CHAPS at 37 °C. The NADPH oxidation data were corrected for calmodulin-deficient blanks. L-Arginine, H_4biopterin, L-NMA and L-NNA were present as indicated. Data are means $\pm$ S.E.M. from three experiments. H_4B: H_4biopterin; L-Arg/H_4B: L-arginine (0.1 mM) plus H_4biopterin (10 μM); n.d.: not determined.

Addition	NADPH oxidation (μmol · $mg^{-1} \cdot min^{-1}$)		L-Citrulline formation (μmol · $mg^{-1} \cdot min^{-1}$)	
	eNOS	nNOS	eNOS	nNOS
None	0.07 ± 0.009	0.48 ± 0.032	n.d.	n.d.
H_4B (10 μM)	0.06 ± 0.006	0.58 ± 0.060	n.d.	n.d.
L-Arg (0.1 mM)	0.18 ± 0.003	0.56 ± 0.019	0.09 ± 0.005	0.38 ± 0.032
L-Arg/H_4B	0.52 ± 0.011	0.67 ± 0.080	0.30 ± 0.032	0.81 ± 0.087
L-Arg/H_4B + L-NMA (0.1 mM)	0.69 ± 0.053	0.44 ± 0.029	0.07 ± 0.003	0.21 ± 0.020
L-Arg/H_4B + L-NNA (0.05 mM)	0.18 ± 0.030	0.02 ± 0.004	0.02 ± 0.003	0.02 ± 0.004

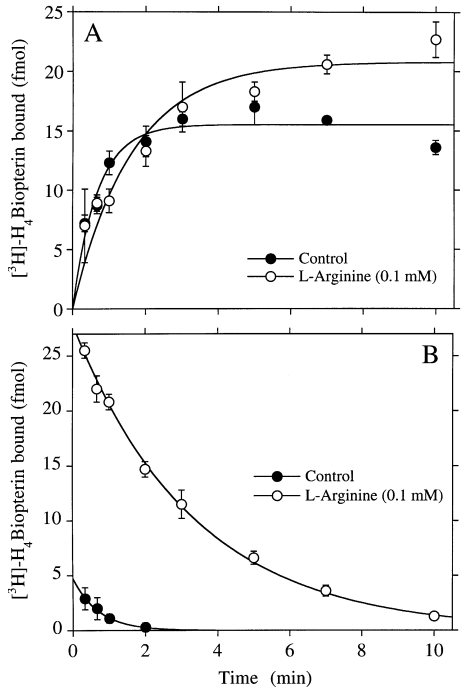

Figure 4 Kinetics of [^{3}H]H_4biopterin binding

(**A**) For association kinetics, eNOS (5 μg) was incubated with 10 nM [^{3}H]H_4biopterin (approx. 14 nCi) in 1 ml of a 50 mM triethanolamine/HCl buffer, pH 7.4, in the absence and presence of 0.1 mM L-arginine at 37 °C. At the indicated time points, 0.1 ml aliquots were removed and assayed for enzyme-bound [^{3}H]H_4biopterin as described in the Experimental section. (**B**) For dissociation kinetics, eNOS (5 μg) was equilibrated with 10 nM [^{3}H]H_4biopterin (approx. 14 nCi) in 1 ml of a 50 mM triethanolamine/HCl buffer, pH 7.4, in the absence and presence of 0.1 mM L-arginine at 37 °C. After 10 min, 10 mM unlabelled H_4biopterin was added, followed by determination of enzyme-bound radioligand in 0.1 ml aliquots. Data are means $\pm$ S.E.M. of three experiments.

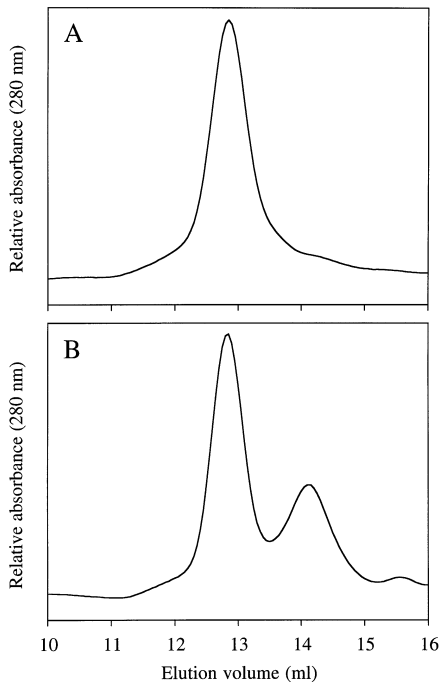

Figure 5 Separation of dimeric and monomeric eNOS by gel-filtration chromatography

Control (**A**) and haem-deficient (**B**) preparations of purified eNOS (0.10–0.20 ml, containing 80–100 μg of protein) were incubated at ambient temperature for 30 min with a combination of L-arginine (1 mM) and H_4biopterin (0.2 mM) before HPLC analysis and determination of Stokes radii as described in the Experimental section. The chromatogram shown is representative of six.

of dissociation approx. 5-fold (from 1.6 ± 0.05 to 0.3 ± 0.1 min^{-1}; $n = 3$ each; Figure 4B).

The haem content of purified eNOS was reduced to 0.32 ± 0.07 equivalent per subunit when the enzyme was expressed in the absence of haemin chloride. We analysed both eNOS containing approx. 0.8 equivalent of haem and the partially haem-deficient enzyme by gel permeation chromatography in the presence of L-arginine and H_4biopterin to study the possible role of haem in subunit dimerization. As shown in Figure 5, the protein containing approx. 0.8 equivalent of haem per subunit showed only one major peak with a Stokes radius of 6.8 ± 0.04 nm ($n = 3$; Figure 5A), whereas a second peak with a radius of 5.1 ± 0.2 nm ($n = 3$) appeared in the partially haem-deficient preparation (Figure 5B). The hydrodynamic volumes of the two protein species (1.32×10^{-24} and 0.55×10^{-24} m^3) differed by a factor of 2.4, indicating the presence of dimers and monomers respectively. Similar results were obtained by preincubation and chromatography of the preparations in the absence of L-arginine and H_4biopterin (not shown). Quantification of the relative amount of monomers present in haem-deficient preparations was hampered by insufficient separation, but was estimated to range from 30 % to maximally 50 % of total protein.

The SDS gel shown in Figure 6 (upper panel) demonstrates that eNOS migrated as a 135 kDa band if it had been boiled before electrophoresis (lane B). The unboiled enzyme exhibited an additional high-molecular-mass band that migrated slightly below catalase (232 kDa) and accounted for approx. 55 % of total protein (lane C). The relative amount of this putative dimer was increased to 80 % of total protein after preincubation of the enzyme with a combination of L-arginine (1 mM) and H_4biopterin (0.2 mM) (lane D). When haem-deficient eNOS containing approx. 0.3 equivalent of haem was preincubated

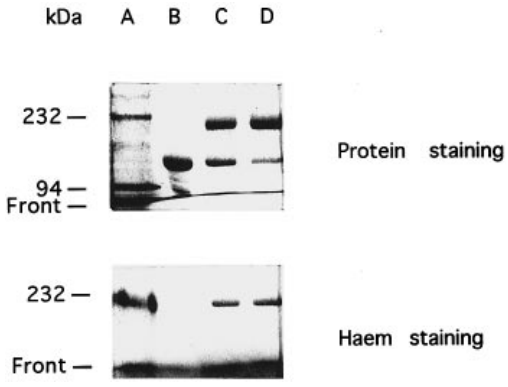

Figure 6 Low-temperature SDS/PAGE of eNOS

Purified eNOS (6 μg) was analysed by low-temperature SDS/PAGE (6% gels) as described [15]. Upper panel, protein staining with Coomassie Blue; lower panel, haem staining with 3,3′-dimethoxybenzidine/H_2O_2. Lane A, marker proteins (catalase; 232 kDa and phosphorylase B; 94 kDa); lane B, boiled eNOS; lane C, unboiled eNOS; lane D, eNOS preincubated for 30 min at ambient temperature with L-arginine (1 mM) and H_4biopterin (0.2 mM). The gels shown are representative of three.

with L-arginine/H_4biopterin, dimers accounted for 58 % of total protein (results not shown). Haem staining of the gels with dimethoxybenzidine/H_2O_2 showed that the haem co-migrated exclusively with the dimeric protein (Figure 6, lower panel).

DISCUSSION

Expression of a fatty-acylation-deficient mutant of bovine eNOS in baculovirus-infected Sf9 cells yielded a soluble, functionally intact enzyme, but expression levels were approx. 10-fold lower than those achieved with nNOS. The specific activities reported for eNOS purified from different sources are in the range 10–200 nmol of L-citrulline $\cdot$ mg^{-1} $\cdot$ min^{-1} [22,47–52]. The enzyme we obtained from baculovirus-infected Sf9 cells exhibited an activity of up to 0.7 μmol L-citrulline $\cdot$ mg^{-1} $\cdot$ min^{-1}, a value that is not much lower than that reported for other NOS isoforms, including one paper describing exceptionally high activity of eNOS isolated from bovine aortic endothelial cells [53]. Considering the variability of eNOS activity observed in the present study, the discrepancies in the literature may be due to differences in the experimental conditions rather than intrinsic differences in the properties of the isolated proteins.

Binding studies with [^{3}H]H_4biopterin used as a radioligand showed that the presence of L-arginine increased the apparent affinity of eNOS for H_4biopterin, indicating that pteridine and substrate binding to eNOS is positive co-operative as previously reported for nNOS [54]. The effect of L-arginine was mainly due to a pronounced, approx. 5-fold decrease in the rates of H_4biopterin dissociation ($t_{\frac{1}{2}}$ approx. 20 s versus 2.3 min), whereas association rates were not significantly affected. The very fast dissociation observed in the absence of L-arginine explains why saturation experiments yielded significantly lower B_{max} values under these conditions.

The low-temperature SDS/PAGE experiments suggest that eNOS is a homodimer, confirming previous results on co-precipitation of truncated mutants with full-length eNOS subunits transiently expressed in COS cells [16]. In the absence of added L-arginine/H_4biopterin, the enzyme migrated as two bands of virtually the same staining intensity. The upper band, most likely representing the dimeric form, migrated slightly below catalase (232 kDa), while the lower band co-migrated with 135 kDa monomers. Preincubation of the enzyme with a combination of L-arginine and H_4biopterin resulted in a shift towards dimers, which accounted for approx. 80 % of total protein under these conditions. Ortiz de Montellano and colleagues reported on an apparent failure of H_4biopterin to induce formation of SDS-resistant eNOS dimers [52], but their findings may be explained by the absence of L-arginine in preincubation buffers leading to rapid dissociation and hence lack of effect of added H_4biopterin. Haem staining of the low-temperature SDS gels showed that the haem co-migrated with dimers, whereas monomers were not stained at all.

Gel-filtration chromatography of the native protein also suggested that the haem is important for dimerization, but the data were less clear. Preparations containing approx. 0.8 equivalent of haem per subunit showed only one protein peak, apparently representing the dimer. Haem deficiency (0.3 equivalent) resulted in the appearance of an additional protein species that was identified as the monomer, based on an approx. 2.4-fold smaller hydrodynamic volume. Of note, the monomeric fraction accounted for 30–50 % of total protein but certainly not for 70 % as would have been expected from the haem content. Low-temperature SDS/PAGE analysis showed that 40 % and 60 % of haem-deficient eNOS migrated as monomers and dimers respectively, a ratio that would be expected if one single prosthetic haem group was sufficient to stabilize the dimeric form. Unfortunately, the protein concentrations in the column eluates were too low for a reliable haem determination. In any case, based on the present results as well as previous observations made with iNOS [17,19] and nNOS [15,21], we propose that (1) haem is the crucial cofactor for NOS dimerization, (2) H_4biopterin stabilizes the dimers by preventing dissociation of the haem, and (3) L-arginine acts by increasing the affinity for H_4biopterin. According to this model, the distinct cofactor requirements for dimerization of the different isoforms are explained by different kinetics of haem and H_4biopterin binding.

Ca^{2+}/calmodulin activation of neuronal NOS in the presence of suboptimal concentrations of L-arginine or H_4biopterin leads to generation of superoxide and hydrogen peroxide, in addition to NO [6–10,55]. Superoxide and NO rapidly combine to form peroxynitrite, suggesting that nNOS functions as peroxynitrite synthase under conditions of limited substrate or pteridine availability [56]. Our present findings demonstrate that eNOS does not catalyse appreciable uncoupling of oxygen reduction in the absence of L-arginine or H_4biopterin, unless the substrate analogues L-NMA and L-NNA were present. Both compounds completely inhibited L-citrulline formation but showed no (L-NMA) or only partial (L-NNA) inhibition of NADPH oxidation. These results indicate that eNOS resembles iNOS [11,12] rather than nNOS with respect to uncoupled oxygen reduction. Martasek et al. [51] recently reported on a similarly low NADPH oxidase activity of eNOS in the absence of L-arginine and H_4biopterin. However, in apparent conflict with our results, they observed no appreciable stimulation of NADPH oxidation by L-arginine, although the presence of 1–10 μM H_4biopterin led to an approx. 2-fold increase in activity. The reason for this discrepancy is unclear.

Our findings that uncoupled oxygen reduction is a unique feature of the neuronal isoform raise a number of questions. Based on our results obtained with nNOS, others suggested that uncoupling of the NOS reaction also occurs in endothelial cells. Cosentino and Katusic found that L-NNA-inhibitable relaxation of coronary arteries became sensitive to catalase upon pteridine depletion of the tissue, indicating that activation of H_4biopterin-deficient eNOS led to relaxation mediated by H_2O_2 instead of NO [57]. Pritchard et al. reported that native low-density lipoprotein induced a pronounced increase in endothelial superoxide release that was blocked by inhibition of NOS [58]. Although these studies provide only indirect evidence for uncoupling of eNOS, it is possible that the enzyme in endothelial cells is different from our expressed protein. Considering that eNOS lacks the 30 kDa N-terminal extension present in nNOS, it is unlikely that substitution of one amino acid residue (Gly-2) should have a dramatic effect on catalytic function. However, the possibility cannot be excluded that post-translational modifications leading to membrane targeting of the wild-type protein affect its biochemical properties.

The present results may have important implications for the controversy over the role of NO in neurotoxicity and/or neuroprotection. Recent studies performed with knockout mice suggested that nNOS contributes to excitotoxicity while eNOS is protective [59,60]. Protection was attributed to an increase in cerebral blood flow by endothelial-derived NO, but it is conceivable that the different biochemical properties of the two isoforms contribute to their distinct physiology, because nNOS might generate oxygen radicals and peroxynitrite under conditions of reduced L-arginine or pteridine availability, whereas eNOS may only slow down its rates of NO production under these conditions without switching to peroxynitrite formation. The finding that nNOS is the sole NOS isoform, which does not

down-regulate NADPH oxidation at low concentrations of L-arginine or H_4biopterin, suggests that this particular feature of the enzyme may be physiologically important for brain function or development.

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