Haem insertion, dimerization and reactivation of haem-free rat neuronal nitric oxide synthase

Benjamin HEMMENS*, Antonius C. F. GORREN*, Kurt SCHMIDT*, Ernst R. WERNER† and Bernd MAYER*[1]

*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

The nitric oxide synthases are dimeric enzymes in which the intersubunit contacts are formed by the *P*-450-haem-containing, tetrahydrobiopterin-dependent oxygenase domain. The dimerization of the neuronal isoenzyme was shown previously to be triggered by Fe-protoporphyrin IX (haemin). We report for the first time the reactivation of the haem-deficient neuronal isoenzyme (from rat, expressed in a baculovirus/insect cell system) after haem reconstitution. We further examined the reconstitution of the enzyme with protoporphyrin IX (PPIX) and its Mn and Co complexes. All of these porphyrins inserted into the haem pocket, as assessed by quenching of intrinsic protein fluorescence. In addition to haemin, MnPPIX stimulated dimerization, as measured by gel filtration and by cross-linking with glutaraldehyde. In contrast, neither CoPPIX nor PPIX stimulated dimerization. The absorbance spectra of the reconstituted enzymes were measured and compared with published results on *P*-450 enzymes reconstituted with the same metals. The results suggest that those metalloporphyrins which caused dimerization were able to acquire a thiolate ligand from the protein, and we propose that this ligation is the trigger for dimerization. Substrate and tetrahydrobiopterin binding sites only emerged with the metalloporphyrins that caused dimerization.

INTRODUCTION

Nitric oxide is a signalling molecule in the nervous and vascular systems and is produced by activated macrophages as a toxic compound to kill pathogens. Its synthesis from molecular oxygen and L-arginine is catalysed by nitric oxide synthase (EC 1.14.13.39; NOS) (reviewed in [1,2]). There are three NOS isoenzymes [neuronal (nNOS), endothelial (eNOS) and inducible (iNOS)], with divergent regulatory properties but a shared domain layout, consisting of a C-terminal flavin-containing reductase region of about 80 kDa and an N-terminal haem-containing oxygenase region of about 45 kDa. All three isoenzymes are homodimeric.

The oxygenase domain has no recognizable sequence homologues and also has a topology very unlike other haemoproteins, based largely on β-sheet [3]. As well as the haem, the oxygenase also contains the intersubunit contacts of the enzyme dimer [4] and the binding site for the (6*R*)-5,6,7,8-tetrahydro-L-biopterin (H_4biopterin) cofactor. There is strong negative cooperativity between the H_4biopterin sites of the two subunits [5]. The enzyme as purified usually contains 1 mole of tightly bound H_4biopterin per mole of dimer.

The haem has a cysteine thiolate proximal ligand and therefore the spectral properties of a *P*-450. Like other *P*-450s there is a high spin/low spin equilibrium which is shifted towards high spin by the binding of the substrate (in this case L-arginine). Unlike other *P*-450s, the NOS haem is already mostly high spin in the absence of L-arginine. This is because H_4biopterin also only binds to the high-spin form [5] .

Underlying these modulatory effects of cofactor and substrate on the conformation of the protein is the initial assembly of the dimer from its subunits. Assembly of mouse iNOS from monomeric subunits was studied by Baek et al. [6]. The monomers contained neither haem nor H_4biopterin and had no NOS activity, but their flavin content and cytochrome *c* reductase activity were normal. The subunits became active when incubated simultaneously with Fe-protoporphyrin IX (haemin), H_4biopterin and L-arginine. The reactivated iNOS was dimeric. In the absence of H_4biopterin the subunits neither bound haem nor dimerized.

Recently, similar questions were addressed for nNOS [7]. Expression of rat nNOS in a baculovirus system under conditions of haem deprivation allowed the isolation of haem-free nNOS monomers. Like the haem-free iNOS subunits, the haem-free nNOS subunits did not contain bound H_4biopterin, but had a normal flavin content and cytochrome *c* reductase activity. Their UV CD spectrum and hence their secondary structure content was essentially similar to that of native nNOS. In radioligand binding assays, they bound neither H_4biopterin nor the L-arginine analogue N^G-nitro-L-arginine (L-NNA). In contrast to haem-free iNOS monomers, they dimerized on addition of haemin alone, without the need to add H_4biopterin or L-arginine. It appears that the dimerization equilibrium of iNOS is rather more finely balanced than that of nNOS; this is also evident from the fact that nNOS dimers can only be dissociated by conditions which cause general unfolding of the protein, whereas haem-containing monomers of iNOS are stable at certain urea concentrations [1].

In the previous study no enzyme activation was observed after reconstitution of haem-free nNOS with haemin; therefore the first goal of the work described here was to achieve reactivation of the haem-free nNOS. We also wished to characterize the haem recognition by nNOS by presenting haem-free nNOS with

Abbreviations used: NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase (type I); iNOS, inducible nitric oxide synthase (type II); H_4biopterin, (6*R*)-5,6,7,8-tetrahydro-L-biopterin = (6*R*)-5,6,7,8-tetrahydro-6-(L-*erythro*-1,2-dihydroxypropyl)pteridine; haemin, Fe-protoporphyrin IX; PPIX, protoporphyrin IX; DTT, D,L-dithiothreitol; L-NNA, N^G-nitro-L-arginine.

[1] To whom correspondence should be addressed (e-mail mayer@kfunigraz.ac.at).

different porphyrin complexes. Since the dimerization of nNOS can be stimulated by haem without participation of H_4biopterin, we wished to find out whether a particular aspect of haem insertion could be identified as the trigger for the assembly of the nNOS dimer.

MATERIALS AND METHODS

Materials

L-[2,3,4,5-^{3}H]Arginine hydrochloride (57 Ci/mmol) and N^G-nitro-L-[2,3,4,5-^{3}H]arginine hydrochloride (50 Ci/mmol) were from MedPro(Amersham),Vienna, Austria. [^{3}H]Arginine was further purified as described earlier [7]. 3′-(6*R*)-5,6,7,8-[^{3}H]tetrahydro-L-biopterin was synthesized enzymically from [8,5′-^{3}H]-GTP as described in [8]. Unlabelled H_4biopterin was obtained from Dr. B. Schircks Laboratories, Jona, Switzerland. Protoporphyrin IX (PPIX), haemin (ferriprotoporphyrin IX chloride), manganese protoporphyrin IX chloride and cobaltic protoporphyrin IX chloride were from Porphyrin Products Inc., Logan, UT, U.S.A. Glutaraldehyde, Grade II (25% solution) and other chemicals were from Sigma.

Preparation of nNOS

Native haem-containing rat nNOS was prepared by expression in baculovirus-infected Sf9 cells and affinity purification on 2′,5′-ADP–Sepharose and then calmodulin–Sepharose, as previously described [9,10]. Partially haem-deficient rat nNOS (about 0.3 equivalents of haem per subunit) was prepared by expression in the same cells but omitting haemin chloride from the culture medium [7]. The monomeric, haem-free subunits in this preparation were further purified by gel filtration on a Superose 6 HR 10/30 column (Pharmacia, Vienna, Austria) as described [7]. This resulted in a protein preparation with a haem content of less than 0.1 equivalents per subunit; for simplicity we refer to this as 'haem-free' and this was the material used in the experiments. Haem-free nNOS was stored at −70 °C at concentrations of not less than 4 μM.

Determination of enzyme activity

NOS activity was determined by the formation of L-[2,3,4,5-^{3}H]citrulline from L-[2,3,4,5-^{3}H]arginine as described [11].

Radioligand binding

Binding of radiolabelled L-NNA and H_4biopterin to NOS was measured using the Millipore MultiScreen Assay System, which allows vacuum filtration of samples in a microtitre plate format. Samples were preincubated at a concentration of nNOS subunits of 1 μM and a porphyrin concentration of 3 μM in 50 mM sodium phosphate, pH 7.4/0.5 M NaCl, in a volume of 30 μl for 20 min at 20 °C, then diluted 6.4-fold with 50 mM triethanolamine/HCl, pH 7.4, containing 1 mM L-arginine or 1 mM H_4biopterin and incubated for a further 20 min at 20 °C. The samples were then diluted 5-fold into the incubations with radioligand: those preincubated with L-arginine, with [^{3}H]H_4biopterin, and those preincubated with H_4biopterin, with [^{3}H]L-NNA. After incubating for 30 min at 20 °C the samples were pipetted onto the filter membranes and washed immediately with 600 μl of ice-cold 50 mM triethanolamine/HCl, pH 7.4.

Fluorescence spectroscopy

Intrinsic protein fluorescence of nNOS was measured with a Shimadzu RF-5000 spectrophotofluorometer. The buffer used was 10 mM triethanolamine/HCl, pH 7.4, with 0.1 M NaCl. The excitation wavelength was 290 nm; an excitation slit of ±1.5 nm and an emission slit of ±5 nm were used. All spectra were scanned twice and averaged. Fluorescence intensities were standardized using solutions of *N*-acetyl tryptophanamide, diluted to give similar intensities to the samples under investigation. Intensities measured during titrations of haem-free nNOS with porphyrins were corrected for absorption by the porphyrin in the emission range by parallel titration of *N*-acetyl tryptophanamide with each porphyrin, and were also adjusted to take account of volume changes (not more than 3%). The quartz cuvette (sample volume 1 ml) was thermostated at 20.0 °C. In all experiments the concentration of nNOS subunits was 1 μM.

Absorbance spectroscopy

Absorption spectra were recorded with a Hewlett-Packard 8452A diode-array spectrophotometer, using quartz cuvettes with a path length of 1 cm. The cell holder was thermostated at 20 °C. Spectra of Fe(II)–CO complexes were measured using a Thunberg-type quartz cuvette with a sample volume of 2 ml. Samples were evacuated and flushed with CO gas three times before adding a minimal amount of solid sodium dithionite from the side bulb of the cuvette and measuring the spectrum.

Porphyrin solutions

MnPPIX and CoPPIX were dissolved initially to a concentration of 3 g/l in 0.1 M NaOH before diluting in buffer. Haemin and PPIX were dissolved in DMSO.

Reconstitution of nNOS with haemin

Sodium phosphate buffer (50 mM), pH 7.4, was thoroughly degassed and solid D,L-dithiothreitol (DTT) was added to a final concentration of 60 mM. Haem-free nNOS (4 μM) was diluted with an equal volume of this buffer. To 200 μl of this enzyme solution, 2 μl of a 200 μM solution of haemin was added. The solution was incubated at 20 °C for 40 min. Then 2 μl of a 10 mM solution of H_4biopterin in 10 mM HCl, and 2 μl of a 100 mM solution of L-arginine were added. After 4 h of further incubation at 20 °C the samples were assayed for NOS activity. At each stage of this procedure the absorbance spectrum of the sample was recorded. Samples that had been incubated identically, except that no haemin was added, were used as controls.

Cross-linking of nNOS with glutaraldehyde

nNOS (native or haem-free) for cross-linking experiments was prepared in 50 mM Tris/HCl, pH 7.4/150 mM NaCl, with 12 mM 2-mercaptoethanol (native nNOS) or 10 mM DTT (haem-free nNOS) and concentrated to about 10 mg/ml. Before cross-linking, protein so prepared was diluted to the final concentration indicated in 50 mM sodium phosphate, pH 7.4/150 mM NaCl/2 mM DTT. Concentrations of glutaraldehyde and duration of the cross-linking reactions were optimized for these buffer conditions, because higher concentrations of Tris were found to interfere with the cross-linking, whereas the haem-free enzyme sometimes precipitated if stored in phosphate buffer. To start cross-linking reactions, glutaraldehyde was added as a 6% (w/v) solution prepared by diluting the stock solution (25%, w/v) in 50 mM sodium phosphate, pH 7.4/150 mM NaCl. The reactions were stopped by mixing the sample with 0.25 vol. of sample buffer containing glycine [0.32 M Tris/0.5 M glycine, pH 6.8, with 50% (w/v) glycerol, 10% (w/v) SDS, 0.03% (w/v) Bromophenol Blue and 25% (v/v) 2-mercaptoethanol].

Gel electrophoresis

Cross-linked samples were analysed by electrophoresis in a Bio-Rad Mini Protean II apparatus (Bio-Rad, Vienna, Austria). Samples were heated to 95 °C for 5 min before application to the gels. Separating gels of 5.0 % (w/v) and stacking gels of 3 % (w/v) total acrylamide were used. Protein was detected after electrophoresis by Brilliant Blue R staining. Stained gels were photographed with a Hitachi Model KP-M1E/K CCD video camera attached to a Mitsubishi Model CP100E colour video copy processor, and the density of protein bands was measured using the vds 800 Version 1.2 software from Hirschmann Analysentechnik, Taufkirchen, Germany.

RESULTS AND DISCUSSION

Quenching of intrinsic protein fluorescence

The intrinsic fluorescence of the haem-free nNOS subunits was significantly more intense than that of the native enzyme (Figure 1). The emission maximum of the haem-free enzyme was at 334 nm. To investigate the contribution of haem to this difference, haem-free subunits were titrated with haemin, MnPPIX, CoPPIX and PPIX (Figure 2). In every case, the fluorescence intensity decreased linearly with increasing porphyrin concentration, with a definite end-point at molar equivalence of porphyrin to nNOS subunits. The position of the end-point suggests the insertion of one porphyrin molecule into the single haem pocket present in each subunit, and the linearity of the quenching curves (at a protein concentration of 1 μM) implies that the dissociation constants are smaller than micromolar. Even PPIX behaved in the same way, suggesting that the porphyrin–protein contacts contribute significantly to haem recognition. The slopes of the titrations were noticeably steeper for haemin (0.31 ± 0.03) and MnPPIX (0.33 ± 0.01) than for PPIX (0.15 ± 0.04) or CoPPIX (0.22 ± 0.02) (means $\pm$ S.E.M. of three experiments); in other words the binding of a given amount of haemin or MnPPIX caused a stronger quenching than the other porphyrins. The stronger quenching may reflect a smaller average distance of the porphyrin from the tryptophans.

Some additional quenching occurred due to concentrations of porphyrins in excess of the protein concentration, probably reflecting porphyrin binding to sites other than the haem pocket. As the native enzyme contains no more than one mole of haem per mole of subunits, we did not investigate this type of quenching further in the present study.

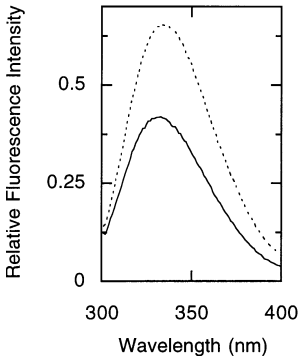

Figure 1 Fluorescence emission spectra of native and haem-free nNOS

Solid line, native haem-containing nNOS (1 μM); dotted line, haem-free nNOS (1 μM). The scale of fluorescence intensity was set so that 1 unit is equivalent to a 21 μM solution of *N*-acetyltryptophanamide (nNOS contains 21 Trp residues). Each spectrum is one of four from separate enzyme preparations.

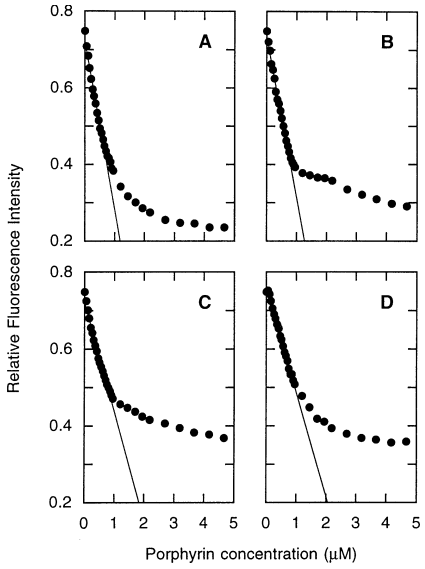

Figure 2 Quenching of nNOS tryptophan fluorescence by haemin and other porphyrins

Haem-free nNOS (1 μM) was titrated with the porphyrins haemin (**A**), MnPPIX (**B**), PPIX (**C**) and CoPPIX (**D**). Fluorescence intensities were measured at 335 nm. The starting point of each titration was adjusted to the average intensity of the haem-free nNOS samples. Results shown are from one of three experiments. Intensities are expressed in the same units as in Figure 1.

Cross-linking of native nNOS with glutaraldehyde

Dimerization of NOS has until now been measured mainly by gel filtration. We developed an alternative method involving cross-linking with glutaraldehyde, followed by SDS/PAGE and densitometry. This method has the advantages that less protein is required, more samples can be processed simultaneously, a better separation of the monomer and dimer can be acheived and the aggregation state of the enzyme can be fixed rapidly, allowing kinetic studies of association/dissociation. On treatment of native nNOS with glutaraldehyde, the normal monomeric band of nNOS on SDS/PAGE was converted into the dimer [apparent molecular masses: monomer, 166 ± 10 kDa; dimer, 323 ± 15 kDa (mean $\pm$ S.E.M.of three experiments)]. At an nNOS concentration of 1 μM, a glutaraldehyde concentration of 1 % (w/v) and a reaction time of 1 min was found to be sufficient for the maximal yield of cross-linking observed. The proportion of the cross-linked species formed (68 ± 2 %) agreed with the estimate of the dimer content obtained by gel filtration (73 ± 2 %) (means $\pm$ S.E.M. of three experiments).

Dimerization of nNOS induced by metalloporphyrins

We tested the effects of the different porphyrins on the dimerization of the haem-free nNOS subunits by cross-linking with glutaraldehyde (Table 1). Substantial increases in the dimer content were induced by haemin and MnPPIX, but CoPPIX and PPIX had no significant effects.

Fluorescence and dimerization kinetics

The fluorescence changes were complete within the time of manual mixing of porphyrin with the haem-free nNOS (20 s at

Table 1 Effects of porphyrins on dimerization of haem-free nNOS

Haem-free nNOS (2 μM) was incubated with the porphyrins indicated (3 μM) at 20 °C for 30 min and then analysed by cross-linking with glutaraldehyde. The proportion of dimer found in the control samples was 13 ± 1 %. Figures shown are means ± S.E.M. of four determinations.

Porphyrin	Increase in dimer over control (% of total protein)
FePPIX	61 ± 2
MnPPIX	36 ± 3
PPIX	4.3 ± 1
CoPPIX	2.8 ± 2

most). The rate of dimerization was examined by withdrawing samples at different intervals after adding haemin or MnPPIX to the haem-free enzyme and cross-linking for 30 s. The dimerization required 2–3 min to reach completion, and therefore still continued when the fluorescence changes were already complete. The fluorescence quenching is therefore due predominantly to intrasubunit events, rather than being a consequence of dimerization. This contrasts somewhat with the obervations on iNOS, where a fluorescence decrease observed during reassociation after urea-induced dissociation was interpreted as being due to intersubunit effects and not to haem–protein interactions within individual subunits [12] .

Absorbance spectroscopy

To identify the types of ligand acquired by the central metals of the metalloporphyrins in their complexes with nNOS, we examined the absorbance spectra of the complexes and compared them with previous studies of *P*-450 enzymes.

Haemin

On mixing haem-free nNOS with an equimolar amount of haemin in the presence of 30 mM DTT, new absorption maxima (compared with the sum of the spectra for free enzyme and free haemin, in the same buffer, also containing 30 mM DTT) were observed immediately at 378 nm, 420 nm and 456 nm (Figure 3A). On further incubation, the 420 nm band decreased slowly relative to the others. After saturation of the sample with CO and dithionite reduction, the subunits without added haemin showed only a very weak *P*-450 band (Figure 3B). In contrast, the subunits that had been incubated with haemin had prominent absorption maxima at 419 nm and 446 nm. Based on the molar absorption coefficient of 121 $mM^{-1} \cdot cm^{-1}$ for the CO complex of reduced NOS [13], the *P*-450 peak would correspond to a concentration (in 1 μM nNOS subunits) of 0.6 ± 0.02 μM (mean ± range of duplicate measurements); this could be an overestimate, because the *P*-420 species may also contribute to the absorbance at 446 nm. Control experiments showed that under identical conditions, but in the absence of the protein, no *P*-420 or *P*-450 species were formed.

In the presence of DTT, a complex with maxima at 376 nm and 456 nm has been observed previously for nNOS [14] and was attributed to bisthiolate ligation of the haem Fe(III) by the proximal cysteine of the enzyme and a distal DTT thiolate, by analogy with model dimercaptide–haem complexes [15], and cytochrome *P*-450*cam* [16,17]. The band at 420 nm, which disappeared slowly on incubation with DTT, is a low-spin complex, possibly with a water molecule as a distal ligand [18]. The acquisition of the cysteine thiolate ligand by the haem was

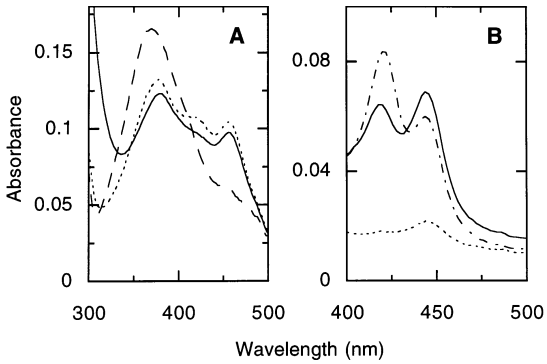

Figure 3 Absorption spectra of complexes formed between haem-free nNOS and haemin

(**A**) Broken line, sum of the spectra of haem-free nNOS (2 μM) and free haemin (2 μM); dotted line, spectrum immediately after mixing; solid line, spectrum of mixture after 2 h incubation (each spectrum is from one of three experiments). (**B**) CO complexes of the dithionite-reduced enzyme: dotted line, haem-free subunits (1 μM); solid line, haem-free subunits (1 μM) reconstituted with haemin in the presence of 30 mM DTT; broken line, haem-free subunits reconstituted with haemin in the absence of DTT (experiments were done in duplicate).

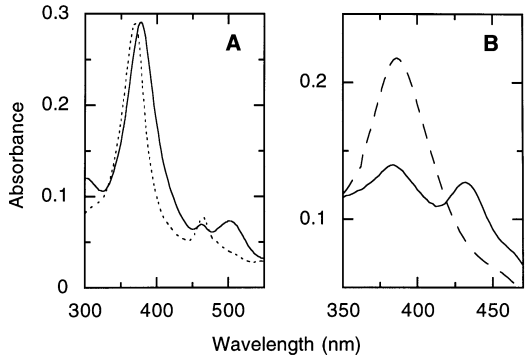

Figure 4 Difference spectra of non-iron metalloporphyrins complexed to haem-free nNOS subunits

For each of the spectra of the complexes with nNOS subunits, the spectrum of the free enzyme has been subtracted to show the changes in the porphyrin spectrum due to its complex formation with the enzyme. Each spectrum is from one of three experiments. (**A**) MnPPIX: dotted line, free MnPPIX (3.2 μM); solid line, complex of MnPPIX (3.2 μM) with nNOS subunits (4 μM). (**B**) CoPPIX: broken line, free CoPPIX (3.2 μM); solid line, complex of CoPPIX (3.2 μM) with nNOS subunits (4 μM)

confirmed by the formation of a *P*-450 band with CO after dithionite reduction. However, the observation of a strong *P*-420 band indicated that a substantial amount of an N-ligated species was also formed. If the subunits were prepared by gel filtration in the absence of DTT, less *P*-450 compound and more *P*-420 was formed upon addition of haemin (Figure 3B), suggesting that the availability of the thiolate proximal ligand was improved by mildly reducing conditions.

MnPPIX

The spectrum of free MnPPIX had maxima at 369 nm and 465 nm. When MnPPIX was added to the haem-free enzyme in the presence of 30 mM DTT, the main Soret band shifted to 378 nm, the intensity of the 465 nm band decreased markedly, and a new rather broad band appeared, centred at 502 nm (Figure 4A).

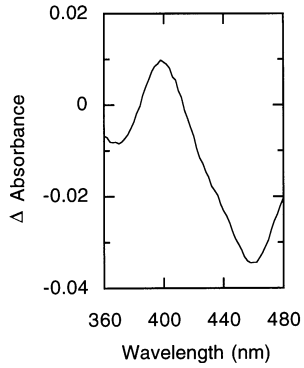

Figure 5 Spectral changes induced by H_4biopterin and L-arginine in reconstituted nNOS

Difference spectrum of nNOS reconstituted with haemin (as shown in Figure 4A) after incubation with H_4biopterin and L-arginine for 4 h minus the spectrum before the incubation (spectrum is from one of three experiments).

Table 2 Effects of porphyrins on binding of [^{3}H]L-NNA and [^{3}H]H_4biopterin to nNOS subunits

Values given are means ± S.E.M. of six determinations.

	Bound ligand (pmol/nmol nNOS)	
	L-NNA	H_4biopterin
Control (no porphyrin added)	0.3 ± 0.02	3.7 ± 0.5
PPIX	0.3 ± 0.03	1.4 ± 0.06
FePPIX	1.1 ± 0.04	10.4 ± 0.4
MnPPIX	12.4 ± 0.9	25 ± 1
CoPPIX	0.3 ± 0.01	2.2 ± 0.2

These spectral changes are very similar to those observed previously for Mn-*P*-450*cam* [19], in which the Soret band of MnPPIX was shifted from 370 nm to 378 nm, the band observed at 470 nm disappeared and a new band appeared at 490 nm. This strongly suggests that the proximal ligand in the Mn–nNOS was a thiolate.

CoPPIX

Free CoPPIX had a Soret maximum at 382 nm. When CoPPIX was added to the haem-free nNOS, a new band appeared at 428 nm (Figure 4B). This contrasts sharply with Co-*P*-450*cam*, which formed a bisthiolate complex with a hyperporphyrin spectrum very like that seen for native Fe(III)-containing *P*-450s and NOS, with maxima at 467 nm and 573 nm [20]. In fact, the Soret wavelength of the Co–nNOS is the same as that of oxyCo-haemoglobin [21], in which the proximal ligand is a histidine. These results suggest that the CoPPIX was not ligated by a thiolate in its complex with nNOS.

Reactivation

When haem-free nNOS reconstituted with haemin was treated with 100 μM H_4biopterin and 1 mM L-arginine in the presence of 30 mM DTT over a period of 4 h, a decrease in the absorbance at 456 nm and an increase at 400 nm were seen (Figure 5). The enzyme was then found to have a specific activity in the citrulline assay of 0.344 ± 0.012 μmol·min^{-1}·mg^{-1} compared with 0.017 ± 0.007 μmol·min^{-1}·mg^{-1} (mean ± S.E.M. of three experiments) for control samples incubated under the same conditions but without haemin. For comparison, the activity of the native enzyme is usually between about 0.8 and 1.2 μmol·min^{-1}·mg^{-1}. Thus the final activity attained is still only about one-third of that expected for a preparation of the native enzyme; the main limitation may be the relative amounts of *P*-420 and *P*-450 formed.

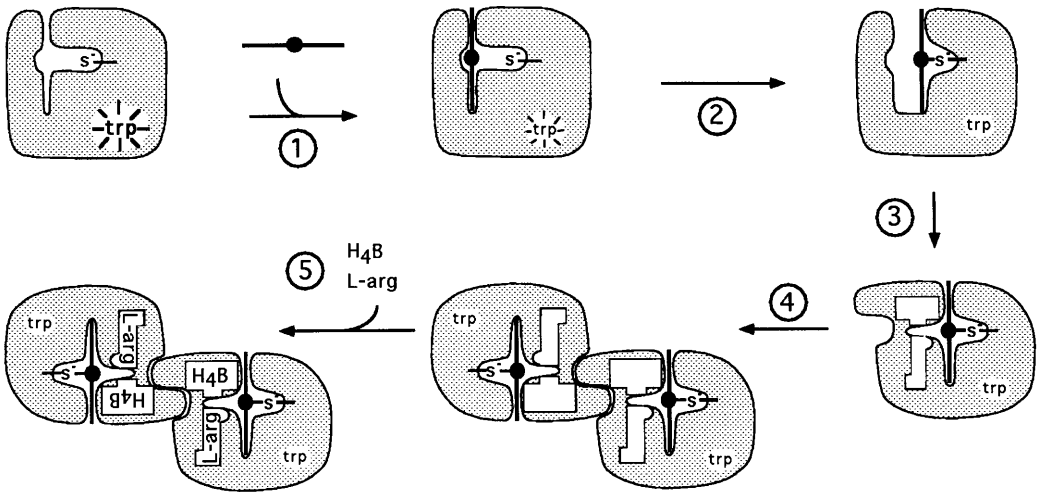

Figure 6 Assembly of nNOS

Porphyrin insertion can occur without the involvement of the metal (step 1). Ligation of the haem iron (step 2) is accompanied by further quenching of the intrinsic protein fluorescence (which under our conditions occurred within 10–20 s) and also triggers the switch to the correct conformation of the dimeric contact points (step 3); the binding sites for L-arginine and H_4biopterin might already exist at this point, although binding of these ligands (step 5) has not been observed in the absence of dimerization (step 4).

Metal ligation and dimerization

Because the structure of the NOS oxygenase is so different from other haemoproteins, it could not be anticipated which factors would be important for haem insertion and assembly of NOS. Our results suggest that the recognition of the haem by the enzyme involves a similar combination of porphyrin–protein and metal–protein interactions to other haemoproteins: the globins, for example, also exhibit metal-independent porphyrin binding [22]. It contrasts with the reported behaviour of iNOS, for which no haem binding could be detected in the absence of H_4biopterin and L-arginine.

However, the insertion of a porphyrin into the haem pocket is insufficient to trigger further assembly of the enzyme. Dimerization was only observed in the cases in which the central metal of the porphyrin acquired a thiolate ligand from the protein; we propose that this metal ligation is necessary and sufficient to tip the enzyme in the direction of assembly. Linkage of haem ligation to subunit association is not without precedent: haemoglobin [23] and the cytochrome *c′* of *Chromatium vinosum* [24] both use haem ligation to modulate the strength of intersubunit bonds.

Substrate and pteridine binding sites

The ability of the different porphyrins to induce the formation of binding sites for H_4biopterin and the L-arginine analogue L-NNA was tested by radioligand binding assays. Haemin and MnPPIX, but not CoPPIX or PPIX, stimulated binding of both H_4biopterin and L-NNA significantly above control levels (Table 2). The catalytic activities of the two oxygenase active sites of the nNOS homodimer are independent of each other [14], so that the emergence of anticooperative H_4biopterin binding is the only undisputed functional consequence of dimerization. Thus it is important to understand how the high-affinity H_4biopterin site is created. The insertion of PPIX or CoPPIX into the haem pocket is insufficient to create the binding site, perhaps because the thiolate proximal ligand is needed to position the porphyrin correctly within the haem pocket. On the other hand, it is clear that the formation of binding sites is not absolutely specific for iron; in fact with manganese the increase in binding capacity was much larger than with iron. This probably reflects the weaker affinity of Mn(III) for a sixth ligand [25], so that in the MnPPIX–nNOS complex the distal side of the metal remains unobstructed.

An overview of nNOS assembly is given in Figure 6. There are still many questions to be addressed in NOS assembly. A rigorous study of the stoichiometry of haem binding and dimer formation is still outstanding: apparently-dimeric NOS with less than a full complement of haem has sometimes been observed, but may be the result of non-specific aggregation of the haem-free enzyme. Also, if NOS, substituted with manganese or other metals, could catalyse any part-reactions related to the normal NOS mechanism, some insights might be gained into the division of roles between haem and H_4biopterin.

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