Delineation of the arginine- and tetrahydrobiopterin-binding sites of neuronal nitric oxide synthase

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Nitric oxide synthase (EC 1.14.13.39) catalyses the conversion of arginine, NADPH and oxygen to nitric oxide and citrulline, using haem, (6*R*)-5,6,7,8-tetrahydro-L-biopterin (tetrahydrobiopterin), calmodulin, FAD and FMN as cofactors. The enzyme consists of a central calmodulin-binding sequence flanked on the N-terminal side by a haem-binding region that contains the arginine and tetrahydrobiopterin sites and on the C-terminal side by a region homologous with NADPH:cytochrome *P*-450 reductase. By using domain boundaries defined by limited proteolysis of full-length enzyme, recombinant haem-binding regions of rat brain neuronal nitric oxide synthase were expressed and purified. Two proteins were made in high yield: one, corresponding to residues 221–724, contained bound haem and tetrahydrobiopterin and was able to bind N^{ω}-nitro-L-arginine (nitroarginine) or arginine; the other, containing residues 350–724, contained bound haem but was unable to bind tetrahydrobiopterin, nitroarginine or arginine. These results showed that rat brain neuronal nitric oxide synthase contains a critical determinant for arginine/tetrahydrobiopterin binding between residues 221 and 350. Limited proteolysis with chymotrypsin of the former protein resulted in a new species with an N-terminal residue 275 that retained the ability to bind nitroarginine, further defining the critical region for arginine binding as being between 275 and 350. Comparison of the sequences of nitric oxide synthase and the tetrahydrobiopterin-requiring amino acid hydroxylases revealed a similarity in the region between residues 470 and 600, suggesting that this might represent the core region of the pterin-binding site. The stoichiometries of binding of substrate and cofactors to the recombinant domains were not more than 0.5 mol/mol of monomer, suggesting that there might be a single high-affinity site per dimer.

INTRODUCTION

Nitric oxide has been implicated in diverse biological functions in blood vessels, neurons, macrophages and other tissues [1]. Nitric oxide synthases [L-arginine,NADPH:oxygen oxidoreductase (nitric oxide forming), EC 1.14.13.39; NOS] are a family of enzymes that catalyse the conversion of arginine, O_2 and NADPH to citrulline, nitric oxide and $NADP^+$ [1,2]. The reaction is dependent on bound (6*R*)-5,6,7,8-tetrahydro-L-biopterin (tetrahydrobiopterin) and calmodulin, and also requires haem, which is bound to the protein by a cysteine thiolate linkage [1,3–6]. Three distinct isoforms of NO synthases, neuronal (nNOS), endothelial (eNOS) and inducible (iNOS), have been characterized, and the genes encoding them cloned. The amino acid sequences of these isoenzymes shows extensive regions of similarity (overall about 50% amino acid identity between each form), reflecting their common catalytic function and cofactor requirements. There are three main regions within the protein: a C-terminal reductase region containing an FMN-, FAD- and NADPH-binding site, an N-terminal haem region and a calmodulin-binding domain linking the haem and reductase domains [1,6–8].

The FAD and FMN within the reductase domain transfer reducing equivalents from NADPH to the haem iron at the N-terminal portion of the molecule, which is thought to activate oxygen and catalyse the two-step oxidation of L-arginine, via L-hydroxyarginine, to NO and L-citrulline [2]. The FMN to haem interaction and subesequent haem iron reduction is controlled by the binding of calmodulin to the central region of the molecule. Tetrahydrobiopterin is also required, but its precise role remains unclear [9,10].

Limited proteolysis has been used to dissect multidomain enzymes and to aid in the design of constructs for the high-level expression of recombinant domains. Thus recombinant domains of the analogous fatty acid ω-hydroxylase enzyme from *Bacillus megaterium* (cytochrome *P*-$450_{BM\text{-}3}$), which catalyses the NADPH- and oxygen-dependent hydroxylation of fatty acids, using FAD, FMN and haem as cofactors, have been characterized and their structure determined [11]. We and others have previously used limited proteolysis on rat brain NOS to define the domain boundaries of nNOS [7,8,12]. Here we describe how we have used this information to express haem-binding regions in *Escherichia coli*, and also the subsequent characterization of such domains with respect to substrate and cofactor binding, and subunit structure. This information, and that from amino acid sequence comparisons with aromatic amino acid hydroxylases, a family of enzymes that catalyse a similar iron- and tetrahydrobiopterin-dependent hydroxylation [13–15], has enabled us to suggest a more precise delineation of the binding sites for arginine and tetrahydrobiopterin in NOS.

EXPERIMENTAL

Construction of *E. coli* expression vectors for nNOS haem regions

The haem region cDNA species, containing residues 1–715, 221–724 or 350–724, were generated by PCR amplification from

Abbreviations used: nitroarginine, N^{ω}-nitro-L-arginine; NOS, nitric oxide synthase; nNOS, eNOS, and iNOS, neuronal, endothelial and inducible forms of NOS (unless specifically stated otherwise all sequence numbering refers to rat nNOS); tetrahydrobiopterin, (6*R*)-5,6,7,8-tetrahydro-L-biopterin.

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the rat brain nNOS cDNA template, pBSKRBNOS [16]. The following primers were used [the DNA sequence in capitals is the nNOS cDNA, the sequence in lower case is the additional or altered sequence required to generate restriction sites (in bold), stop codons and the EEF purification tag (in italics) and to mutate an internal *Afl*III restriction site]. nNOS 1–715 5′ primer, cgcgt**catATG**GAAGAGAACACGTTTGGGGTTC; nNOS 1–715 3′ primer, acgat**catatg***ctagaattcttc*CACGTGGGTGTTCCATGGATCAGGC; nNOS 221–724 5′ primer, cgcgt**catatg**GGGGGACCAGCCAAAGCAGAGATG; nNOS 221–724 3′ primer, acgat**catatg***ttagaattcttc*CGTGGGGGTCCCGTTGGTGCCCTTC; nNOS 350-724 5′ primer, ctgcg**acatgt**CAAAGGATCAACTCTTCCCTCTA; nNOS 350-724 3′ primer, actag**tctaga***ctagaattcttc*CGTGGGGGTCCCGTTGGTGCCCTTCCAtACaTG.

For nNOS 1–715 and 221–724, the PCR products were digested with *Nde*I and inserted into the expression plasmid pET11a (Novagen). The recombinant plasmids encoded proteins with sequences MEEN … THVEEF (nNOS 1–715) and MGGP … TPTEEF (nNOS 221–724). For nNOS 350–724, the PCR products were digested with *Afl*III and *Xba*I and inserted into the expression plasmid pTRC99a (Pharmacia) digested with *Nco*I and *Xba*I. It encoded a protein with sequence MSKD … TPTEEF (nNOS 350–724).

Growth of *E. coli* expressing nNOS haem regions

pET-derived recombinant plasmids were transformed into *E. coli* strain BL21(DE3) (Novagen). The pTrc99a-derived plasmid was transformed into strain BL21 (Novagen). Cells were grown at 37 °C to a D_{600} of approx. 0.4 in Terrific Broth [17] with ampicillin at 100 μg/ml, supplemented with 10 μM 5′-δ-aminolaevulinic acid (Sigma), 10 μM tetrahydrobiopterin (Shircks Laboratories; from a stock of 100 mM tetrahydrobiopterin dissolved in 100 mM dithiothreitol) and 5 μg/ml haemin chloride (Sigma; from a stock of 5 mg/ml in 0.1 M NaOH). The temperature was reduced to 20 °C and cells allowed to equilibrate to this temperature for at least 1 h before induction with 100 μM isopropyl β-D-thiogalactoside. After 16 h the cells were harvested by centrifugation; the pellets were stored at −20 °C until required.

Purification of nNOS haem regions

All manipulations were performed at 4 °C. The cell pellet was thawed and resuspended by the addition of 1 vol. of ice-cold 10 mM Tris/HCl (pH 7.5)/50 mM NaCl/0.1 mM EDTA/1 mM dithiothreitol (buffer A), containing additionally 0.1 mM L-arginine, 0.1 mM tetrahydrobiopterin and 1 mM PMSF. The cells were broken by sonication [18] and centrifuged at 150000 ***g*** for 60 min. The supernatant was applied to a column of YL1/2 IgG coupled to Sepharose [18] equilibrated with buffer A, containing 0.1mM L-arginine and 10μM tetrahydrobiopterin. After extensive washing first with that buffer and then with the same buffer supplemented with 150 mM NaCl, protein bearing the C-terminal EEF epitope was eluted with the latter buffer, additionally containing 2 mg/ml Asp-Phe (pH brought back to 7.4 by the addition of Tris base). The haem region protein was concentrated by $(NH_4)_2SO_4$ precipitation (78 % saturation), followed by dialysis against buffer A supplemented with 0.1 mM L-arginine, 10 μM tetrahydrobiopterin and 300 mM NaCl. Protein was quick-frozen in 0.1–0.2 ml aliquots at a protein concentration of 15–30 mg/ml and stored at −70 °C.

Protein chemistry

Electrophoresis and Western blotting were performed as described by Lowe et al. [7]. Amino acid analysis was performed as described by Riveros-Moreno et al. [19] and used to determine absolute protein concentrations on the basis of the known amino acid composition defined by the primary sequences. These were used to standardize the Bio-Rad dye-binding protein assay. BSA was found to give a similar colour yield as the haem regions.

Limited proteolysis

Purified nNOS 221–724 (4 mg/ml protein) in buffer A, supplemented with 0.1 mM L-arginine, 10 μM tetrahydrobiopterin and 300 mM NaCl, was incubated with 50 μg/ml α-chymotrypsin (Sigma Type VII, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one-treated) at 4 °C for 1–4 h. At intervals, samples were removed for analysis by SDS/PAGE and for ability to bind N^{ω}-nitro-L-arginine (nitroarginine). As judged by SDS/PAGE, the major proteolysis product did not change after the first hour.

Determination of tetrahydrobiopterin content

Tetrahydrobiopterin was oxidized under acidic conditions to 6-biopterin and then analysed by reverse-phase HPLC on a Partisil 10 ODS column (25 cm × 0.46 cm) eluted with 0.5 % (v/v) acetonitrile and 0.1 % (v/v) tetrahydrofuran in water [20]. Biopterin was detected by fluorimetry (λ_{ex} 360 nm, λ_{em} 450 nm), and quantified by integrating the peak area relative to that of a pure 6-biopterin (Sigma) standard. For oxidation, samples (100 μl) were mixed with 1 M perchloric acid (100 μl) and 1 % (w/v) I_2 dissolved in 2 % (w/v) KI (200 μl). The mixture was heated at 100 °C for 5 min and then left for 55 min in the dark. Ascorbic acid (2 %, w/v) was then added until the sample was nearly colourless.

Nitroarginine and arginine binding

[^{3}H]Nitroarginine binding was measured as described by Lowe et al. [7], except that 0.1 mM tetrahydrobiopterin was used and 500 mM NaCl was included in all buffers. Arginine binding was measured by isothermal titration calorimetry on a Microcal MCS calorimeter [21]. Samples (approx. 20 mg/ml protein) were dialysed overnight against 50 mM Tris/HCl (pH 7.5)/350 mM NaCl/0.1 mM EDTA/1 mM dithiothreitol. The dialysed protein, diluted with dialysate to a concentration of 40 μM and supplemented with 0.1 mM tetrahydrobiopterin (from a stock of 100 mM tetrahydrobiopterin dissolved in 100 mM dithiothreitol), was placed in the cell, and a solution containing 1.5 mM L-arginine dissolved in dialysate supplemented with 0.1 mM tetrahydrobiopterin was placed in the injection syringe. Blank runs were also performed with the cell filled with dialysate supplemented with tetrahydrobiopterin alone. In each case an injection of 2 μl was made followed by seven to fifteen injections of 5 μl. The heat output from the titration in the absence of arginine was subtracted from that from the titration with L-arginine and the data were fitted to an equation describing binding at a single site, using the Origin software supplied so as to yield estimates of Ka, ΔH and stoichiometry.

Haem determination and spectroscopy

The haem content of the purified proteins was determined by the formation of pyridine haemochrome after alkali denaturation of

the protein. The protein (1 mg/ml), in 100 mM KOH and 30% (v/v) pyridine, was reduced with a few grains of sodium dithionite. The haem concentration was calculated by using an ϵ_{556} of 34700 $M^{-1} \cdot cm^{-1}$ [22].

The reduced CO spectrum was obtained after bubbling CO through a solution of protein (0.1–0.5 mg/ml protein) in degassed 50 mM Tris/HCl, pH 7.5, for 10 min, followed by the addition of a fresh solution of sodium dithionite in degassed buffer to a final concentration of 10–20 mM.

Molecular size analysis

For sedimentation equilibrium analysis, protein was dialysed against 10 mM Tris/HCl, pH 7.5, containing 1 M NaCl, 0.1 mM EDTA, 10 μM tetrahydrobiopterin and 0.1 mM dithiothreitol. Centrifugation was on Beckman Optima XL-A ultracentrifuges at 5 °C. Runs were performed in cells with a 1.5 mm column height at 0.05, 0.1, 0.2, 0.3, 0.5, 0.7, 1.0, 2.0 and 3 mg/ml protein at a rotor velocity of 14000 rev./min (20000 ***g***). Measurements were routinely made at 210, 280 or 300 nm depending on the concentration. For some runs additional measurements were made at 410 nm. The results of absorbance against radius obtained at equilibrium were analysed with the assoc4 software package (Beckman) to give apparent whole-cell weight average molecular masses.

Non-denaturing electrophoresis was performed on 8–25% (w/v) polyacrylamide gels (PhastSystem, Pharmacia). Samples for electrophoresis were prepared in 0.5 M NaCl. The following proteins (M_r) were used as standards: carbonic anhydrase (29000), ovalbumin (43000), BSA (67000 monomer, 134000 dimer), aspartate aminotransferase (100000), pig heart lactate dehydrogenase (140000), yeast alcohol dehydrogenase (150000), β-amylase (200000).

Sequence alignments

The amino acid sequences of all the nitric oxide synthases (seven sequences) and amino acid hydroxylases (16 sequences) were extracted from the SWISS-PROT database Release 33. The two families were compared by using the Pattern-Induced Multi-sequence Alignment (PIMA) algorithm [23,24]. The region of NOS used for the alignments corresponded to residues 276–713 of human nNOS, which we know from the data shown in this paper to contain the haem-, arginine- and tetrahydrobiopterin-binding sites. The region of the eukaryotic aromatic amino acid hydroxylases used in the alignments corresponded to residues 30–453 of the rat phenylalanine hydroxylase. The NOS sequences (residue numbers) used were as follows: NOS2-MOUSE (60–489), NOS2-RAT (62–492), NOS2-HUMAN (65–495), NOS3-HUMAN (46–678), NOS3-BOVIN (46–684), NOS1-RAT (272–711), NOS1-HUMAN (276–713). NOS1, NOS2 and NOS3 refer to nNOS, iNOS and eNOS respectively. The hydroxylase sequences used were: TYR3H-RAT (75 to end), TY3H-MOUSE (75 to end), TY3H-HUMAN (105 to end), TY3H-BOVIN (66 to end), TY3H-PHASP (quail) (67 to end), TR5H-RAT (67 to end), TR5H-MOUSE (10 to end), TR5H-HUMAN (10 to end), TR5H-RABIT (10 to end), PH4H-MOUSE (30 to end), PH4H-RAT (30 to end), PH4H-HUMAN (30 to end), PH4H-DROME (*Drosophilia melanogaster*) (25 to end), TY3H-DROME (69 to end), PH4H-PSEAE (*Pseudomonas aeruginosa*) (1 to end) and PH4H-CHRVO (*Chromobacterium violaceum*) (1 to end). TYR3H, TR5H and PH4H refer to tyrosine, tryptophan and phenylalanine amino acid hydroxylases respectively.

RESULTS

nNOS 1–715

Before having data from limited proteolysis studies, we expressed residues 1–715 of nNOS in *E. coli*. To aid purification, the protein was engineered to contain the sequence EEF at its C-terminus, which is the epitope for the YL1/2 monoclonal antibody [18]. Residues 1–715 comprise the N-terminal half of nNOS ending at the N-terminal side of the calmodulin-binding consensus sequence. This region contains the haem-, arginine- and tetrahydrobiopterin-binding sites, as well as a 220-residue N-terminal extension (relative to the sequences of eNOS and iNOS) within which is the Discs-large Homologous Region [25]. Although a high level of purification of the recombinant protein was obtained by chromatography on the YL1/2 antibody column, the material eluted with Asp-Phe contained many bands when analysed by SDS/PAGE (Figure 1, lane 1). Western blotting with YL1/2 and anti-NOS antibodies AB52–54 (results not shown) showed that the majority of the proteins were degradation products of the main band with an apparent M_r consistent with that for the full-length protein (80141). The two major degradation species were of apparent M_r 58000 and 45000 and both retained the EEF epitope, consistent with degradation within the N-terminal half of the region. The preparation contained bound haem and was capable of binding nitroarginine but was insufficiently homogeneous to establish stoichiometries.

nNOS 221–724

The degradation observed with the expression of nNOS 1–715 (Figure 1) suggested that this region did not represent a single stable domain. This was supported by our data from limited proteolysis of full-length nNOS with trypsin, which showed that a stable haem-containing fragment of M_r 60000 with Gly-221 at its N-terminus was formed [7]. The proteolysis also produced a C-terminally derived protein fragment with residue 728 at its N-terminus. On this basis we chose to express residues 221–724 again tagged with the C-terminal EEF epitope. High levels of expression were seen with little degradation. When expressed at 37 °C the protein was largely insoluble, but at 20 °C in cells grown in the presence of haemin, δ-aminolaevulinic acid and

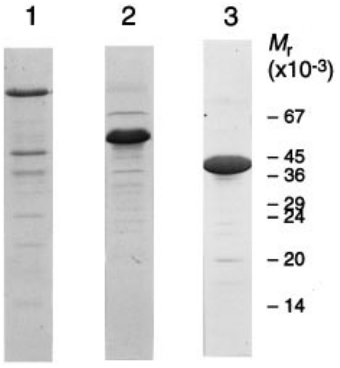

Figure 1 SDS/PAGE of purified nNOS haem-binding regions

Samples of nNOS 1–715 (lane 1), nNOS 221–724 (lane 2) and nNOS 350–724 (lane 3) were purified by immunoaffinity chromatography as described in the Experimental section and analysed by SDS/PAGE followed by Coomassie Blue staining.

tetrahydrobiopterin a significant proportion of the protein was soluble. The recombinant protein was highly purified on the YL1/2 column, with a yield of approx. 20 mg per litre of culture. Although complete homogeneity was not obtained (Figure 1, lane 2), the preparation was of much higher purity than that with nNOS 1–715. Under our normal elution conditions (i.e. buffer containing 50 mM NaCl) the recombinant NOS was eluted as a very much broader peak than had been observed with other proteins on the YL1/2 column. However, in the presence of an additional 150 mM NaCl the protein was eluted sharply. It was also noted that at concentrations of over 10 mg/ml the protein often came out of solution at 50 mM NaCl but could be resolubilized by adding NaCl.

The ability of the purified protein to bind cofactors and substrates was examined. The protein contained approx. 0.4 mol of haem per mol, and 0.4 mol of tetrahydrobiopterin per mol. Neither was significantly decreased when the protein was dialysed overnight against buffer in the absence of haem or tetrahydrobiopterin. As measured by the filter-binding assay, 0.3–0.4 mol of nitroarginine bound per mol of protein. We also performed a purification in which haemin was added to the broken cells before purification. The purified protein contained more haem (0.7 mol/mol) but the nitroarginine, arginine and tetrahydrobiopterin binding stoichiometries were not increased. As haemin can bind non-specifically to many proteins, the extra binding observed might also not be specific.

The visible spectra of both native protein and reduced protein after reaction with CO are shown in Figure 2 (upper panel). The native protein showed a broad peak at 400 nm, whereas the reduced CO-spectrum showed a sharp peak at 442 nm, which is characteristic of a haem thiolate. There was also a shoulder at 420 nm, which slowly increased in intensity over a period of 1 h.

The ability to bind arginine was measured directly by isothermal titration calorimetry (Figure 3). To minimize artifacts due to differences in composition between the solution titrated and the solution in the cell, the protein was dialysed overnight against buffer in the absence of tetrahydrobiopterin, and then just before the experiment fresh tetrahydrobiopterin was added to both protein solution and arginine solution in the cell (see the Experimental section). The results could be fitted to a single-binding-site model with a K_d of approx. 2.2 μM and a stoichiometry of 0.2–0.3 mol of arginine bound per mol of monomer. This stoichiometry might be a slight underestimate of the maximum possible, as the overnight dialysis might have resulted in some irreversible loss of binding activity.

In some initial experiments bacteria expressing nNOS 221–724 were grown at 25 °C in the absence of haemin and tetrahydrobiopterin and also purified in the absence of tetrahydrobiopterin. Under these conditions, we noted that the Asp-Phe dipeptide alone was unable to elute the protein from the antibody column, but elution could be achieved at high ionic strength. Binding of nitroarginine was very low (approx. 0.03 mol/mol of NOS). Furthermore the protein produced contained less than 0.01 mol of tetrahydrobiopterin per mol of NOS (as measured by 6-biopterin in the oxidized samples) However, it did contain an unidentified fluorescent compound (after oxidation) that did not co-elute with authentic 6-biopterin, but was very probably a related pterin. This compound was not seen in other protein samples or when tetrahydrobiopterin itself was oxidized. If the fluorescence yield was the same as 6-biopterin, the amount of this compound present was 0.05 mol/mol.

As full-length NOS has been reported to be a dimer [19,26,27], it was of interest to examine the oligomeric structure of nNOS 221–724. Sedimentation equilibrium runs at low ionic strength showed significant aggregation, particularly at low concentrations of protein. Inclusion of 1 M NaCl reduced the amount of aggregation but did not eliminate it. Analysis of single data files showed an acceptable fit to a monomer–dimer–tetramer equilibrium, but it was not possible to obtain a global fit of all data obtained at different protein concentrations and in different centrifugations. However, a molecular mass analysis in the presence of 1 M NaCl showed that at low concentrations (0.1–0.2 mg/ml) the apparent whole-cell weight average M_r was between 60000 and 80000, whereas at 1 mg/ml it was approx. 100000. This value is lower than that predicted for a dimer (116000), possibly because it has an elongated shape causing deviation from ideal behaviour. On electrophoresis on a non-denaturing gradient gel, the protein ran as a single band with apparent M_r of approx. 150000, consistent with a dimer of elongated shape. Although not conclusive, these results suggest that at low concentrations the protein is predominantly mono-

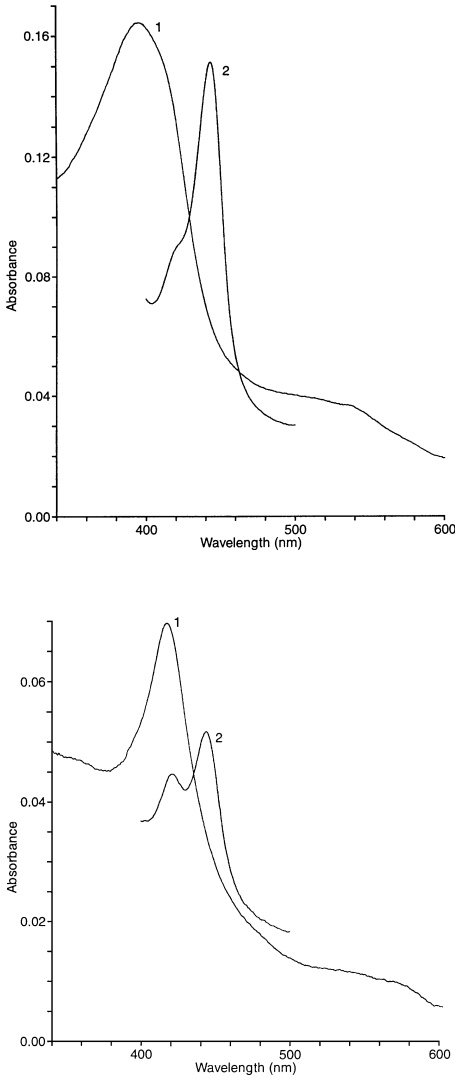

Figure 2 Visible spectra of nNOS haem-binding regions

Upper panel, spectra of native nNOS 221–350 (trace 1) and of the same protein after reduction with dithionite and reaction with CO (trace 2); lower panel, as in the upper panel but with nNOS 350–724.

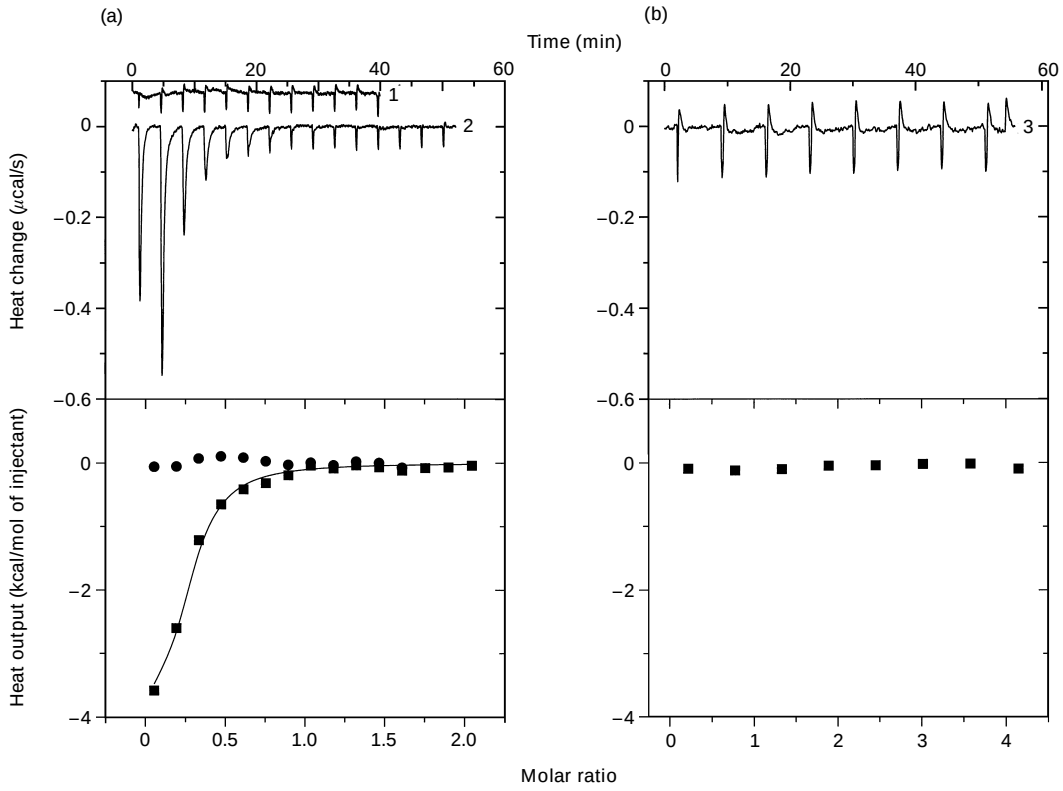

Figure 3 Measurement of arginine binding by isothermal titration calorimetry

Arginine was titrated into buffer [(a), trace 1], nNOS 221–724 [(a), trace 2] or nNOS 350–724 [(b), trace 3] and heat changes were recorded continuously with time on a Microcal isothermal titration calorimeter as described in the Experimental section. In each case, an injection of 2 μl of arginine solution was followed by subsequent injections of 10 μl. The measured heats from each peak were integrated and plotted in the lower panels as heat output per mol of injected arginine against the molar ratio of arginine to nNOS monomer (■). To allow plotting of the data for the buffer control (●), the points have been positioned directly in line with data from the parallel experiment in which nNOS was present. The solid line through the points from the titration with nNOS 221–724 is a least-square fit to the data, assuming a single-site binding model.

meric but at higher concentrations in high salt it is predominantly dimeric.

Limited proteolysis of nNOS 221–724 by chymotrypsin produced a single major protein with residue 275 at its N-terminus. This cleavage occurred at the same position as with full-length nNOS [7]. During proteolysis there was no loss of ability to bind nitroarginine, demonstrating that residues 221–274 are not required for arginine binding.

nNOS 350–724

Our limited trypsin proteolysis experiments on full-length nNOS also indicated stable fragments with N-termini of 344 and 350 [7]. To investigate the properties of such fragments we expressed in *E. coli* residues 350–724 of rat brain nNOS tagged with the EEF epitope. The recombinant protein was almost fully soluble and was purified to near homogeneity by chromatography on YL1/2 Sepharose (Figure 1, lane 3) with a yield of 50 mg/l protein.

The haem content was similar to that of nNOS 221–724. The reduced CO spectrum (Figure 2, lower panel) was very similar to that of nNOS 221–724 except that the ratio of peaks at 442 and 420 nm was lower. With time, the proportion of the 420 nm peak increased. However, the native spectrum differed in that that of nNOS 350–724 showed a sharper peak and it was at 420 nm rather than 400 nm (Figure 2). For comparison, the native and CO-reduced spectra of the haem domain of cytochrome *P*-$450_{BM\text{-}3}$ (a gift from Dr. Sandeep Modi) were recorded under the same conditions. This showed a peak at 418 nm in the native spectrum that shifted to 446 nm in the CO-reduced spectrum.

We were unable to detect any significant tetrahydrobiopterin bound either to the nNOS 350–724 protein as purified or when measured after equilibrium dialysis against 10 μM tetrahydrobiopterin. Furthermore the protein did not bind nitroarginine sufficiently tightly to be measurable by the filter-binding procedure, and no significant amount of arginine binding could be detected by isothermal titration calorimetry (Figure 3).

DISCUSSION

The aim of this work was to express truncated forms of the N-terminal half of nNOS in *E. coli* to define more precisely the binding sites for haem, arginine and tetrahydrobiopterin. As might have been expected on the basis of our reported limited proteolysis experiments [7], the region from residues 1–715 was not expressed as a stable protein. However, the recombinant 221–724 region was much more stable than the longer fragment and retained the binding sites for tetrahydrobiopterin, arginine (and nitroarginine) and haem. As with the full-length nNOS [7], chymotrypsin trimmed off a portion from the N-terminus of this protein, resulting in a fragment starting with residue 275. This protein retained binding of nitroarginine, demonstrating that the arginine site was N-terminal of residue 275. This conclusion is supported by the ability of a recombinant iNOS fragment

(containing residues 59–504), corresponding to nNOS 275–724, to bind both nitroarginine and tetrahydrobiopterin [28].

Amino acid residue 350 was chosen as the N-terminus of a shorter recombinant nNOS haem domain fragment as this residue was the N-terminus of a very stable limited proteolysis product of apparent M_r 35000 derived from full-length NOS [7]. The C-terminus of the limited proteolysis fragment was not defined but as estimated from its apparent M_r was N-terminal of residue 724. Recombinant nNOS 350–724 was produced as a very stable pure protein (Figure 1). It contained very similar amounts of bound haem as the 221–724 fragment (Figure 2), but in contrast with the latter did not possess the ability to bind tetrahydrobiopterin or arginine (Figure 3), whether added separately or together. The reduced CO haem spectra of nNOS 221–724 and nNOS 350–724 (Figure 2) were quite similar both to each other and to that of the haem domain of cytochrome P-$450_{BM\text{-}3}$, suggesting that in all three proteins the haem is bound as expected via a haem thiolate linkage. It has been reported that tetrahydrobiopterin-deficient nNOS is unstable; the Soret absorption maximum in its reduced CO spectrum shows a shift from 443 to 421 nm over time [29]. It is noteworthy that nNOS 350–724, which contains no bound tetrahydrobiopterin, had a significant peak at 420 nm in its reduced CO spectrum, which also increased with time (Figure 3). Interestingly, the native spectra differed in that both cytochrome P-$450_{BM\text{-}3}$ and nNOS 350–724 showed sharp peaks at approx. 420 nm, whereas nNOS 221–724 had a broader peak at approx. 400 nm. We presume that this difference is related to the ability of only nNOS 221–724 to bind arginine and tetrahydrobiopterin.

These results suggested that all the requirements for haem binding are contained within residues 350–724, whereas residues 275–350 contain a critical determinant for arginine and tetrahydrobiopterin binding. The binding of these two cofactors seems to be synergistic [9]. In view of this, there are three possible ways in which residues 275–350 could be essential. This region could constitute a critical part of just one of these binding sites, but loss of binding of that particular component prevents the binding of the other. Alternatively, part of both binding sites could be located in it. Finally, both binding sites might lie in the region between residues 350 and 724, but the sequence 275–350 could confer an altered conformation on it, thereby affecting both binding sites. The importance of the sequence 275–350 is consistent with the data of Chen et al. [4,30], which showed that mutation of Cys-99 of eNOS (equivalent to Cys-331 in rat nNOS) did not grossly affect the ability to bind haem but severely impaired both arginine and tetrahydrobiopterin binding. Our results are in apparent conflict with the data on nitroarginine binding obtained by Nishimura et al. [31], who showed nitroarginine binding to their glutathione S-transferase (GST) fusion of nNOS 558–721 but not to a region between residues 220 and 557. However, we note that the nitroarginine binding observed with GST–nNOS 558–721 was not tetrahydrobiopterin-dependent, whereas that seen by others with full-length NOS and by us with recombinant nNOS 221–724 is tetrahydrobiopterin related. Furthermore neither the affinity nor the stoichiometry of nitroarginine binding was assessed with the 558–721 fragment. The results could be reconciled if the arginine-binding site were at least partly within residues 558–721 but binding were made tetrahydrobiopterin-dependent by the addition of the region comprising residues 221–557, and the region 221–350 were to contain a critical determinant for the binding of either tetrahydrobiopterin or arginine, or both.

Spectral analysis of purified full-length nNOS has indicated that this enzyme contains a haem iron bound to the protein via a cysteine thiolate linkage similar to that in the cytochromes P-450 [3]. Although a low similarity between the haem regions of cytochrome P-$450_{BM\text{-}3}$ and NOS has been proposed [32], this alignment seems to be unlikely as it predicts the thiolate ligand of NOS to be a conserved Cys residue near the C-terminus of the domain (residue 672) and not Cys-415 of rat nNOS, which we now know from mutagenesis studies to be the residue involved [5,6]. This suggests that if there is any similarity between the haem regions of NOS and cytochrome P-$450_{BM\text{-}3}$, it is lower than can be detected by overall alignments. If the NOSs and cytochromes P-450 are related, one might have expected that the sequences immediately adjacent to the haem-binding cysteine would show the high similarity exhibited within the cytochromes P-450. However, although there is some similarity in the sequence immediately adjacent to Cys-415 [33] the similarity is quite low [5]. This suggests that although NOS does possess a typical cytochrome P-450 spectrum, it might not be so closely related to cytochromes P-450 structurally. A further key difference between NOS and all cytochromes P-450 is that the latter are monomeric, whereas all three NOS isoforms are dimeric. Moreover the results obtained in the present study strongly suggest that the nNOS haem-binding region itself is predominantly dimeric. This is supported by other reports that the haem-binding region of iNOS is dimeric [8,28]. The recombinant domains produced in this study showed a haem-binding stoichiometry of 0.4 mol/mol. This stoichiometry could indicate a mixture of protein with and without haem. However, electrophoresis on non-denaturing gels showed only one band and ultracentrifugation analysis showed no difference when monitored at 280 nm and at 410 nm, arguing against the protein's being heterogeneous. Alternatively, only one haem molecule might bind per dimer (see below). If this is so, the binding site might be across the subunit interface and therefore one would not expect to see conservation in linear sequence alignments with cytochromes P-450. It is pertinent that haem binding, but not tetrahydrobiopterin binding, seems to be essential for dimerization in all NOS isoforms [28,34–36]. If this is a direct role, it would be consistent with haem's binding across the subunit interface.

Nishimura et al. [31] identified residues 558–721 of nNOS as a putative pterin-binding region, on the basis of a weak sequence alignment between dihydrofolate reductase and NOS. However, they could not demonstrate that this region was able to bind tetrahydrobiopterin. Cho et al. [26] suggested that residues 448–480 of iNOS (corresponding to residues 669–701 of rat brain nNOS) show similarity with a sequence present in all three aromatic amino acid hydroxylases and also to a sequence present in dihydrofolate reductase. However, the sequence alignment described between NOS and dihydrofolate reductase [26] is quite different from that shown by Nishimura et al. [31]. The sequence alignment reported by Cho et al. [26] between the hydroxylases and iNOS was not stringent, as several gaps were allowed in both sets of short sequences being compared. Although two residues in this region were subsequently mutated, resulting in loss of NO synthase activity and tetrahydrobiopterin and arginine binding, the primary impact of these mutations remained unresolved. It was unknown which of the interrelated functions of tetrahydrobiopterin binding, arginine binding and intermonomer interaction was affected [26].

We have compared the amino acid sequences of all NOS isoforms, within the region from 221 to 724, with the sequences of all the phenylalanine, tyrosine and tryptophan aromatic amino acid hydroxylases, a family of enzymes that utilize tetrahydrobiopterin, in order to investigate possible conserved regions between the two protein types. These might indicate a 'tetrahydrobiopterin-binding consensus sequence' or amino acid residues that might be important in tetrahydrobiopterin binding (Figure 4). We found a low similarity over an extended region of

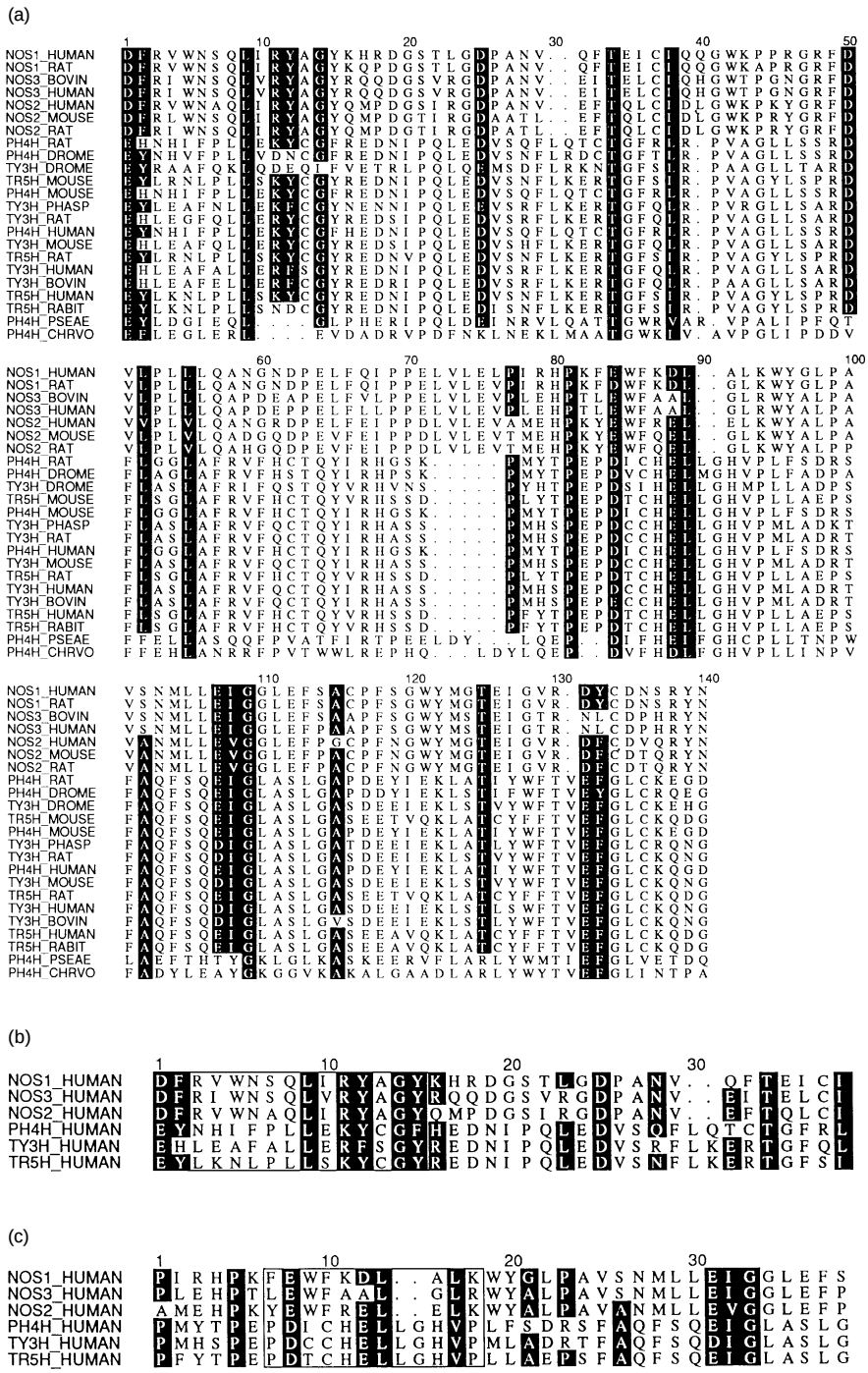

Figure 4 Sequence alignments of NOS isoenzymes and aromatic amino acid hydroxylases

(**a**) NOS and aromatic amino acid hydroxylases sequences from the SWISS-PROT database were aligned, over the regions described in the Experimental section, by using the Pattern-Induced Multisequence Alignment (PIMA) algorithm [23,24]. The region of highest similarity is shown. The sequences are as follows: NOS1_HUMAN (residues 475–609), NOS1_RAT (471–605), NOS3_BOVIN (241–375), NOS3_HUMAN (239–373), NOS2_HUMAN (256–390), NOS2_MOUSE (250–384), NOS2_RAT (253–387), PH4H_RAT (205–338), PH4H_DROME (204–337), TY3H_DROME (258–391), TR5H_MOUSE (195–328), PH4H_MOUSE (205–338), TY3H_PHASP (244–377), TYR3H_RAT (251–384), PH4H_HUMAN (205–338), TY3H_MOUSE (251–384), TR5H_RAT (192–325), TY3H_HUMAN (281–414), TY3H_BOVIN (243–376), TR5H_HUMAN (192–325), TR5H_RABIT (192–325), PH4H_PSEAE (45–174) and PH4H_CHRVO (62–191). The shaded columns in the multiple alignment show where at least 75% of the residues are identical. (**b**) A portion of the alignment shown in (**a**) for the three human NOS isoforms and the three human amino acid hydroxylases. The sequences are NOS1_HUMAN (residues 475–510), NOS3_HUMAN (239–274), NOS2_HUMAN (256–291), PH4H_HUMAN (205–242), TY3H_HUMAN (281–318) and TR5H_HUMAN (192–229). The residues DE, KRH, NQ, ST, ILV, FWY and AG were grouped together and those positions at which over half (four or more) residues are of the same type are shaded. The region containing the motif [DE][FYH]X(6)LX[RK][FY]XG[FY] is boxed. (**c**) As in (**b**), except that the regions are NOS1_HUMAN (residues 549–584), NOS3_HUMAN (313–348), NOS2_HUMAN (330–365), PH4H_HUMAN (275–312), TY3H_HUMAN (351–388) and TR5H_HUMAN (262–299). The region containing the PROSITE biopterin-dependent aromatic amino acid hydroxylase signature is boxed.

sequence between residues 470 and 600 of nNOS. Within this there were two regions of particularly high similarity corresponding to human nNOS residues 475–510 (rat nNOS 471–506) (Figure 4b) and to human nNOS residues 589–624 (rat nNOS 584–620) (Figure 4c).

The sequence alignment shown in Figure 4(a) did suggest several possible motifs, and a few of these have been investigated as diagnostic motifs for the NOS and aromatic amino acid hydroxylase sequence families. The motif [DE][FYH]X(6)-LX[RK][FY]XG[FY] (shown in Figure 4b), when scanned against SWISS-PROT, recovered 18 hits. The hits comprised all seven NOS sequences and 11 out of the 16 aromatic amino acid hydroxylases in the database. No false positives were obtained. Of the five remaining aromatic amino acid hydroxylases, two were from *Drosophila* and two were from bacterial species. The other sequence that was not selected by the motif was that of rabbit tryptophan hydroxylase, which stands out in the alignment as being unique among the mammalian hydroxylases in not having the sequence LX[RK][FY]XG. However, on examining the original literature [37], it seemed that there was an error in SWISS-PROT sequence TR5H-RABIT in this region. In SWISS-PROT the sequence of residues 200–205 is given as LSNDCG, whereas in the original paper [37] it is given as LSKYCG. With the corrected sequence, the motif above now selects all mammalian aromatic amino acid hydroxylases. Examination of the multiple sequence alignment (Figure 4a) to see why the four non-mammalian sequences did not fit the motif showed that they diverged within the same region, and indeed the sequence X[RK][FY]X is deleted from the two bacterial enzymes. It is also noteworthy that these four non-mammalian enzymes seem to be evolutionarily far removed from the mammalian equivalents [38,39]. For example, the bacterial enzymes are monomeric and copper-dependent [38], whereas the mammalian counterparts are oligomeric and iron-dependent.

There is indirect evidence that residues 263–289 of rat phenylalanine hydroxylase might contain a sequence required for tetrahydrobiopterin binding [14,40] and it has been suggested that this region shows some similarity to a sequence from the pterin-binding enzyme dihydropteridine reductase [14]. Furthermore a PROSITE biopterin-dependent aromatic amino acid hydroxylase signature has been described (shown in Figure 4c) that will detect all aromatic amino acid hydroxylases with the exception of one of the bacterial enzymes. This motif is within the sequence 263–289 of rat phenylalanine hydroxylase and moreover is within one of the regions picked out as showing the highest similarity between NOS and hydroxylase sequences (Figure 4c). These two classes of enzyme share two features: they both bind tetrahydrobiopterin and catalyse a mono-oxygenation reaction. Taking all the results together, despite the weak overall similarity, we suggest that the region outlined in Figure 4(a) is closely involved in the binding of tetrahydrobiopterin.

The recombinant nNOS 221–724 fragment bound approximately equimolar amounts of haem, tetrahydrobiopterin and nitroarginine. The stoichiometry was approx. 0.4 mol/mol of monomer. As we measured the protein concentration by amino acid analysis, we are confident that this represents a value significantly less than 2 mol/mol of dimer. It is noteworthy that several other groups who have carefully evaluated binding stoichiometries of cofactors on full-length NOS have also found values significantly less than 1 mol/monomer. For example, stoichiometries of 0.3–0.6 mol of nitroarginine bound/mol [41,42], 0.06–0.48 mol of tetrahydrobiopterin/mol [10,43,44] and 0.4–0.6 mol of haem/mol [3,5,34] have been reported. These results suggest the possibility that all three cofactors bind tightly with a stoichiometry of one mol per dimer but do not exclude weaker binding of a second molecule. A similar proposal has recently been made on the basis of a study of recombinant full-length nNOS [45]. This is further supported by the evidence for the presence of two types of binding site for tetrahydrobiopterin, both from direct binding studies and also from the observation that tetrahydrobiopterin can increase NO synthase activity on enzyme fully complemented with tetrahydrobiopterin [9,42]. At one site, ligand (approx. 0.2–0.5 mol/mol) binds with such high affinity that exchange is difficult, and there is a a second weak-affinity site with a more rapid exchange. It might also be relevant that amino acid hydroxylases have two sites with differing affinities for tetrahydrobiopterin [13,15]. The observation of substrate inhibition of NOS activity by arginine [19] would also be consistent with the presence on NOS of two different arginine-binding sites.

In summary, we can define a minimal nNOS haem/arginine/tetrahydrobiopterin-binding region encompassing residues 275–724, and conclude that residues 275–350 contain a critical determinant of substrate/cofactor binding, whereas residues 350–724 contain all information required for haem binding. We suggest that there is a similarity between this region of nNOS and the catalytic domain of aromatic acid hydroxylases and that this is related to the ability of both proteins to bind tetrahydrobiopterin. We further speculate that nNOS is an asymmetric dimer in which there is either only one active site per dimer, possibly with the haem binding across the dimer interface, or that there are two sites with differing affinities for cofactors.

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