Nitroarginine and tetrahydrobiopterin binding to the haem domain of neuronal nitric oxide synthase using a scintillation proximity assay

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Nitric oxide synthases (NOS) have a bidomain structure comprised of an N-terminal oxygenase domain and a C-terminal reductase domain. The oxygenase domain binds haem, (6*R*)-5,6,7,8-tetrahydro-L-biopterin (tetrahydrobiopterin) and arginine, is the site where nitric oxide synthesis takes place and contains determinants for dimeric interactions. A novel scintillation proximity assay has been established for equilibrium and kinetic measurements of substrate, inhibitor and cofactor binding to a recombinant N-terminal haem-binding domain of rat neuronal NOS (nNOS). Apparent K_d values for nNOS haem-domain-binding of arginine and N^{ω}-nitro-L-arginine (nitroarginine) were measured as 1.6 μM and 25 nM respectively. The kinetics of [^{3}H]nitroarginine binding and dissociation yielded an association rate constant of 1.3×10^4 $s^{-1} \cdot M^{-1}$ and a dissociation rate constant of 1.2×10^{-4} s^{-1}. These values are comparable to literature values obtained for full-length nNOS, suggesting that many characteristics of the arginine binding site of NOS are conserved in the haem-binding domain. Additionally, apparent K_d values were compared and were found to be similar for the inhibitors, L-N^G-monomethylarginine, *S*-ethylisothiourea, *N*-iminoethyl-L-ornithine, imidazole, 7-nitroindazole and 1400W (*N*-[3-(aminomethyl) benzyl] acetamidine). [^{3}H]Tetrahydrobiopterin bound to the nNOS haem domain with an apparent K_d of 20 nM. Binding was inhibited by 7-nitroindazole and stimulated by *S*-ethylisothiourea. The kinetics of interaction with tetrahydrobiopterin were complex, showing a triphasic binding process and a single off rate. An alternating catalytic site mechanism for NOS is proposed.

INTRODUCTION

Nitric oxide synthases (NOS; EC 1.14.13.39) are a family of enzymes that catalyse the formation of nitric oxide from L-arginine, O_2 and NADPH with the production of citrulline, nitric oxide and $NADP^+$ [1,2]. NOS are homodimers in their active forms and each subunit has a bidomain structure comprised of an N-terminal oxygenase domain and a C-terminal reductase domain [3]. The oxygenase domain binds haem, (6*R*)-5,6,7,8-tetrahydro-L-biopterin (tetrahydrobiopterin) and arginine and is the site of NO synthesis, while the reductase domain binds FAD, FMN and NADPH and transfers electrons from NADPH to the oxygenase domain during NO synthesis [4–9]. Calmodulin is required to facilitate electron transfer from the flavins within the reductase domain to the haem moiety within the oxygenase domain [10]. Tetrahydrobiopterin may play a dual role in the mechanism of NOS catalysis, acting both as an allosteric activator and a redox active cofactor of L-arginine oxidation [11]. Investigation of tetrahydrobiopterin-free neuronal NOS (nNOS) revealed two identical, highly anti-cooperative pteridine binding sites and cooperativity between arginine and tetrahydrobiopterin binding [12–14].

Limited proteolysis has been used to define the domain boundaries of nNOS [4,5,15]. nNOS domains have been expressed in *Escherichia coli* [6,16–19] and have been used for site-directed mutagenesis studies for the identification of residues involved in haem binding [6,19] and arginine and tetrahydrobiopterin binding [18]. However, little information is available on the kinetics of cofactor binding to the NOS haem domain or its interaction with NOS inhibitors. Scintillation proximity assay (SPA) technology may be used to detect binding under equilibrium conditions, without a requirement for a separation step. Previous methods, such as a filter-binding assay for measuring NOS binding to [^{3}H]nitroarginine [15], or a precipitation assay for binding to [^{3}H]tetrahydrobiopterin [12], involved a separation step. In this paper, we describe the use of SPA technology to measure the binding of [^{3}H]N^{ω}-nitro-L-arginine ([^{3}H]nitroarginine) and [^{3}H]tetrahydrobiopterin to the haem domain of nNOS and thus to measure the kinetics of ligand binding and also competition by inhibitors.

EXPERIMENTAL

Materials

N^G-Nitro-L-[2,3,4,5-^{3}H]arginine hydrochloride, L-[2,3,4,5-^{3}H]-arginine monohydrochloride and Protein A polyvinyltoluene (PVT) SPA beads were obtained from Amersham. (6*R*)-[6-^{3}H]-5,6,7,8-Tetrahydro-L-biopterin was a custom synthesis prepared by Amersham. The antibodies used were: anti-rat IgG, whole molecule (Sigma); anti-glutathione S-transferase (GST) rabbit polyclonal (Molecular Probes); and YL1/2 rat anti-tubulin monoclonal antibody (Serotec). Inhibitors were purchased from Sigma, with the exceptions of: *S*-ethylisothiourea (obtained from Aldrich), *N*-iminoethyl-L-ornithine (L-NIO) (obtained from Alexis Biochemicals) and *N*-[3-(aminomethyl) benzyl] acetamidine (1400W) and 2,4-diamino-5-(3′,4′dichlorophenyl) pyrimidine (11U50) which were synthesised by J. Oplinger and R. Morrison respectively (Glaxo Wellcome, Research Triangle Park, NC, U.S.A.). Glutathione agarose (S-linked) was obtained from Sigma.

Abbreviations used: DTT, dithiothreitol; nitroarginine, N^{ω}-nitro-L-arginine; NOS, nitric oxide synthase(s); nNOS, neuronal nitric oxide synthase (unless specifically stated all sequence numbering refers to rat nNOS); tetrahydrobiopterin, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; SPA, scintillation proximity assay; PVT, polyvinyltoluene; GST, *Schistosoma japonicum* glutathione S-transferase; L-NIO, *N*-iminoethyl-L-ornithine; 1400W, *N*-[3-(aminomethyl) benzyl] acetamidine; 11U50, 2,4-diamino-5-(3′,4′dichlorophenyl) pyrimidine.

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Expression of nNOS haem regions in *E. coli*

The nNOS haem region, residues 221–724, with a Glu-Glu-Phe C-terminal tag (a tubulin epitope recognized by the rat YL1/2 anti-tubulin antibody) was expressed, purified and characterized as previously described [17]. The expression construct for the nNOS haem region 221–724 as a GST fusion protein with a Glu-Glu-Phe C-terminal tag was generated by inserting the cDNA corresponding to nNOS 221–724 into a pGEX expression plasmid.This nNOS cDNA was amplified from a rat brain nNOS cDNA template (pBSKRBNOS) by PCR using *Pfu* polymerase (Stratagene, Cambridge, U.K.). The following primers were used to introduce an *Eco*RI and an *Not*I (in bold) restriction site at either end of the PCR product. Bases in italics indicate the sequence that introduces the C-terminus Glu-Glu-Phe sequence to the expressed protein.

5′primer: accag**gaattc**GAGGGGGACCAGCCAAAGCAGA-GATG

3′primer: ttttcctttt**gcggccgc***ttagaactcttc*CGTGGGGGTCCCG-TTGGTGCCCTTCCACAC

The resulting PCR product was digested with *Eco*RI and *Not*I, gel purified and inserted into the expression plasmid pGEX-4T-2 (Pharmacia, St. Albans, U.K.), also digested with *Eco*RI and *Not*I. The sequence of the recombinant plasmid over this region, including the junctions, was confirmed by DNA sequencing. The recombinant plasmid was transformed into *E.coli* strain BL21 (Novagen, Madison, WI, U.S.A.) and growth and expression methods were identical with those used for the unfused nNOS haem domain [17].

Purification of nNOS 221–724 Glu-Glu-Phe–GST fusion protein

The cell pellet was thawed and resuspended in an equal volume of 10 mM Tris/HCl/0.1 mM EDTA/50 mM NaCl/0.1 mM L-arginine/1 mM dithiothreitol (DTT)/10 μM tetrahydrobiopterin, pH 7.5 (buffer A). Cells were lysed by sonication and centrifuged at 150000 ***g*** for 60 min. The supernatant was applied to a column (7 × 1.6 cm) of glutathione agarose pre-equilibrated in buffer A. The column was then washed with buffer A supplemented with 200 mM NaCl. The nNOS 221–724 Glu-Glu-Phe–GST fusion protein was then eluted with the same buffer supplemented with 5 mM glutathione and adjusted with Tris base to pH 8. Fractions containing nNOS (14 mg of crude protein) were pooled and concentrated to 2.2 ml using an Amicon ultrafiltration cell containing a PM10 membrane. This sample was applied to a column of Superose 12 (prep grade, 50 × 2 cm) eluted at 0.1 ml/min with 10 mM Tris/HCl/0.1 mM EDTA/250 mM NaCl/0.1 mM L-arginine/1 mM DTT/10 μM tetrahydrobiopterin, pH 7.5. The purest fractions were pooled and concentrated to 8.5 mg/ml protein and stored at −70 °C.

Characterization of recombinant nNOS haem domains

The protein concentration, tetrahydrobiopterin and haem content of recombinant nNOS domains were determined as previously described [17]. The stoichiometry of nitroarginine binding was measured by a filter-binding assay [15].

SPA binding assay proceedures

Equilibrium binding assays

The standard nitroarginine equilibrium binding SPA was performed in 96-well sample plates (Wallac Part No. 1450-401) as follows: a mixture of Protein A PVT SPA beads (4.2 mg/ml), anti-GST IgG (0.029 mg/ml) and GST–nNOS haem domain fusion protein (6 μg/ml) in 50 mM Tris/HCl, pH 7.5/50 mM NaCl/100 μM tetrahydrobiopterin/1 mM DTT was added to each well. It was ensured that the SPA beads were in homogeneous suspension before pipetting to minimize error between replicates. NOS inhibitors (10 μl) at appropriate concentrations were added to each well to give a volume of 194 μl, and incubated for 10 min before the addition of 6 μl of N^G-nitro-L-[2,3,4,5-^{3}H]arginine hydrochloride (3.33 μM, 7200 Ci/mol) to a final concentration of 0.1 μM. The plates were sealed, shaken for 1 h at 22 °C, centrifuged at 760 ***g*** for 2 min and counted for 10 min in a Wallac 1450 Microbeta scintillation counter set up in SPA mode. The final concentration of GST–nNOS haem domain in the assay was 0.05 μM. Background signal was measured by omitting the GST–nNOS haem domain from the well. We noted that there was a batch-to-batch variation in the anti-GST antibody, causing a variation in the SPA signal obtained. Therefore the assay was optimized with each new batch of antibody.

Arginine binding was measured directly by substituting [^{3}H]-nitroarginine with L-[2,3,4,5-^{3}H]arginine monohydrochloride. In experiments with the Glu-Glu-Phe-tagged nNOS haem domain, anti-rat IgG (0.04 mg/ml) and YL1/2 rat monoclonal antibody (0.01 mg/ml) were substituted for anti-GST IgG in the SPA.

In order to measure radiolabelled tetrahydrobiopterin binding, unlabelled tetrahydrobiopterin in the assay was substituted with 0.075 μM (6*R*)-[6-^{3}H]5,6,7,8-tetrahydro-L-biopterin, and nitroarginine was omitted.The nNOS haem domain was dialysed overnight at 4 °C into 50 mM Tris/HCl, pH 7.5/50 mM NaCl/1 mM DTT to remove tetrahydrobiopterin from the storage buffer. The plate was incubated in the dark at 22 °C for exactly 1 h before centrifugation and counting as above.

The SPA signal obtained from experiments in which the concentration of either [^{3}H]nitroarginine or of [^{3}H]tetrahydrobiopterin was varied at a constant concentration of GST–nNOS haem domain was used to obtain apparent K_d values. Data were fitted to an equation describing formation of the binary complex by non-linear regression using the computer program GraFit [20]. The equation used was

$$\text{SPA signal} = (S_{max}/2A_o)(P - \sqrt{P^2 - 4A_oB_o}) \quad (1)$$

where $P = A_o + B_o + K_d$, S_{max} is the maximum SPA signal, i.e. the signal when nNOS haem is fully complexed with ligand, A_o is the total concentration of nNOS haem domain, B_o is the total concentration of ligand and K_d is the equilibrium dissociation constant for ligand binding to NOS. In these calculations, the concentrations used were the total concentrations of reagents present and did not take into account any changes in local concentration due to binding to SPA beads. The estimates of K_d therefore represent apparent rather than absolute values.

The apparent K_d for binding of compounds to NOS, which competed for binding with either nitroarginine or tetrahydrobiopterin, were calculated from SPA data from experiments in which the inhibitory compound was titrated into SPA assays containing fixed concentrations of both NOS and radioligand. SPA data were fitted to equations defining competitive binding for two ligands, taking the K_d values for nitroarginine and tetrahydrobiopterin with respect to NOS to be 25 nM and 20 nM respectively. The equations used were:

$$\text{SPA signal} = \{(S_{max})[(K_iP + K_dI_o) - \sqrt{(K_iP + K_dI_o)^2 - (4K_i^2A_oB_o)}]\}/[K_i(P - \sqrt{P^2 - 4A_oB_o})] \quad (2)$$

where $P = A_o + B_o + K_d$, S_{max} is the maximum SPA signal, I_o is total concentration of inhibitor and K_i is the equilibrium dissociation constant for equilibrium binding to the nNOS haem

domain. This equation does not take into account effects of removal of inhibitor by binding to protein and so K_i will be an upper estimate for high-affinity inhibitors.

Kinetics of ligand binding

Kinetic experiments were performed essentially as above, but using either 0.1 μM [^{3}H]nitroarginine or 0.1μM [^{3}H]tetrahydrobiopterin. However, after the addition of radiolabel, the plates were shaken for only 10 s and centrifuged at 760 ***g*** for 30 s before placing the plate in the counter. Each sample was counted repetitively at 1–5 min intervals for the duration of the experiment. The delay between adding radioligand and starting counting was timed and was routinely 3 min. In order to measure dissociation of the radiolabel from the nNOS haem domain an excess of unlabelled L-arginine (1 mM) or unlabelled tetrahydrobiopterin (10 μM) was added to the assay, the plates were shaken for 10 s, centrifuged at 760 ***g*** for 30 s and counting was restarted. Nitroarginine association and dissociation data were fitted to a single exponential increase and decrease respectively. A second-order association rate constant was calculated according to $k_{on} = (k_{obs} - k_{off})/[\text{nitroarginine}]$. Tetrahydrobiopterin association and dissociation data were fitted to a double-exponential increase (with offset) and a single-exponential decrease respectively using the curve-fitting program GraFit [20].

Measurement of inhibition constant of L-NIO for rat nNOS activity

Rat nNOS was purified from baculovirus-infected insect cells as described previously [21]. Inhibition of full-length rat nNOS by L-NIO was measured using the oxyhaemoglobin assay [22]. Data from the first 120 s of the time-dependent inhibition were fitted to the integrated rate equation [23] to yield a K_i value.

RESULTS AND DISCUSSION

The 221–724 nNOS haem domain, with a C-terminal Glu-Glu-Phe tag, was expressed and purified in two forms: unfused, which has been characterized previously [17] and shown to contain approximately 0.4 mol of haem per mol, 0.4 mol of tetrahydrobiopterin per mol and 0.3–0.4 mol of nitroarginine per mol, and as a GST fusion protein.

An SPA was set up to measure nitroarginine binding to nNOS haem domains, based on a previously described SPA [24]. An anti-GST antibody is bound to protein A-coated beads containing a fluorophor. The nNOS haem domain–GST fusion protein binds, via the anti-GST antibody, to the beads, and also binds [^{3}H]nitroarginine, causing scintillation to occur. Unbound [^{3}H]nitroarginine does not cause a significant signal. The effect of varying the concentrations of [^{3}H]nitroarginine in the SPA is shown in Figure 1. In the absence of GST–nNOS haem domain, increasing the concentration of [^{3}H]nitroarginine produced a modest linear increase in SPA counts; however, in the presence of GST–nNOS haem a large increase occurred. After subtraction of the blank, without GST–nNOS haem, the SPA signal was saturable with respect to [^{3}H]nitroarginine binding. Curve fitting (shown in Figure 1) to a binding isotherm (eqn. 1) was used to calculate an apparent K_d of 25 nM for [^{3}H]nitroarginine binding to the nNOS haem domain, which is comparable with the K_d of 15 nM reported for full-length nNOS and nitroarginine obtained by kinetic analysis of inhibition of NOS activity [25]. The signal was largely dependent on the presence of both nNOS haem domain (Figure 1) and antibody (results not shown), and signal-to-noise ratios of 10:1 were routinely obtained. The addition of an excess of unlabelled nitroarginine (1 mM) to the assay completely abolished the signal. Measurement of the SPA signal

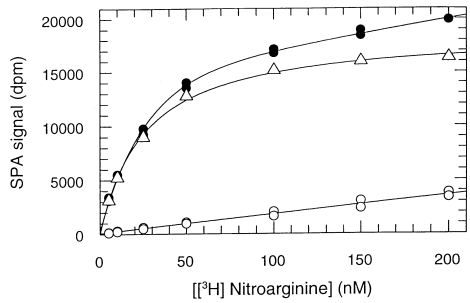

Figure 1 Measurement of [^{3}H]nitroarginine binding using an SPA

SPAs were performed as described in the Experimental section at the indicated concentrations of [^{3}H]nitroarginine. Experiments were performed in the presence (●) and absence (○) of the nNOS haem domain–GST fusion protein. The signal from duplicate experiments was averaged and corrected for background counts and is also plotted (△). The line shown joining the triangles is the best fit to a binding isotherm for nitroarginine binding to GST–nNOS haem domain with an apparent K_d of 25 nM.

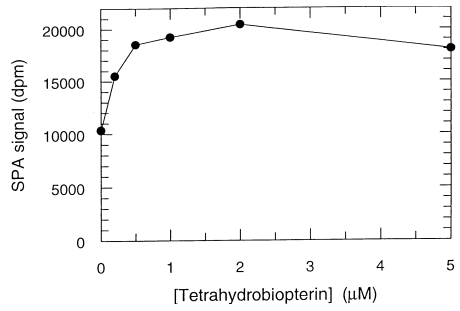

Figure 2 Effect of tetrahydrobiopterin on [^{3}H]nitroarginine binding

SPAs were performed using 0.1 μM [^{3}H]nitroarginine and the indicated concentrations of tetrahydrobiopterin. A background signal obtained in the absence of the nNOS haem domain–GST fusion protein has been subtracted.

at different anti-GST antibody concentrations (up to 0.1 mg/ml) gave a hyperbolic dose–response curve (results not shown). The antibody concentration in the assay was chosen to be close to the maximum signal to minimize variability.

The SPA was also used to detect [^{3}H]arginine binding to nNOS haem domain (results not shown); however, the higher concentration of radioligand needed to saturate the nNOS haem domain (K_d arginine = 1.6 μM) resulted in a lower signal-to-noise ratio. The SPA was also configured using the nNOS haem domain C-terminally tagged with a Glu-Glu-Phe epitope, which was bound to the Protein A PVT beads via a YL1/2 rat antibody and an anti-rat IgG. This assay gave very similar results to the SPA using anti-GST described above, demonstrating that the nNOS haem domain nitroarginine binding site is functional if the domain is either C- or N- terminally tagged.

The effect of tetrahydrobiopterin on nitroarginine binding in the SPA was assessed using dialysed GST–nNOS haem domain (Figure 2). In the absence of added tetrahydrobiopterin, a signal was obtained which was increased 2-fold by the addition of tetrahydrobiopterin. As the nitroarginine concentration was close to saturating (cf. Figure 2), it is likely that this effect is due to an increase in the available number of binding sites for nitroarginine. A similar observation was made for tetrahydrobiopterin and nitroarginine binding of full-length nNOS [13,15]. The addition of hemin (up to 1 μM) to the SPA gave no increase in signal (results not shown).

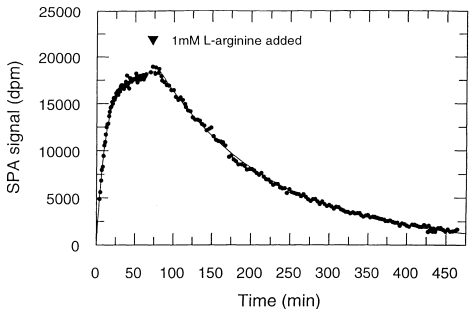

Figure 3 Kinetics of [^{3}H]nitroarginine binding

The kinetics of association to, and dissociation from, the nNOS haem domain were measured in SPAs. The assays were started at time zero by the addition of 0.1 μM [^{3}H]nitroarginine. At 100 min, L-arginine (1 mM) was added to the assay and counting was restarted. For the association, the line shown is the best fit to a single-exponential increase ($k_{obs} = 1.4 \times 10^{-3}$ s^{-1}), from which a second-order association rate constant of 1.3×10^4 s$^{-1} \cdot$ M^{-1} was calculated. For the dissociation, the line shown is the best fit to a single-exponential decrease with a dissociation rate constant of 1.2×10^{-4} s^{-1}.

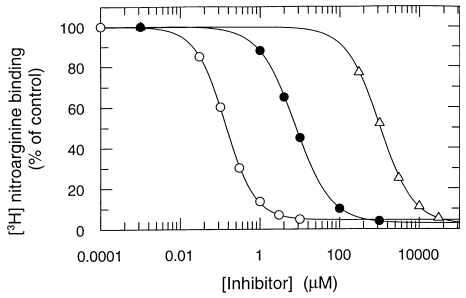

Figure 4 Effect of L-arginine, *S*-ethylisothiourea and imidazole on [^{3}H]-nitroarginine binding

SPAs to measure binding of [^{3}H]nitroarginine by the nNOS haem domain were performed in the presence of the indicated concentrations of L-arginine (●), *S*-ethylisothiourea (○) and imidazole (△). A background signal obtained in the absence of nNOS haem domain–GST fusion protein has been subtracted and the data are expressed as a percentage of the signal in the absence of added inhibitor.

The kinetics of nitroarginine association to nNOS haem domain are shown in Figure 3. The data were fitted to a first-order rate equation to give a rate constant of 1.4×10^{-3} s^{-1}, from which a second-order association constant of 1.3×10^4 s$^{-1} \cdot$ M^{-1} was calculated. The kinetics of dissociation were measured after the addition of excess L-arginine (1 mM) and the data were fitted to a single exponential to give a dissociation rate constant of 1.2×10^{-4} s^{-1}. The K_d for nitroarginine binding calculated from k_{off}/k_{on} was 9 nM. The kinetics of nitroarginine binding to full-length nNOS have been examined previously by inhibition of catalysis, from which $k_{on} = 3.2 \times 10^4$ s$^{-1} \cdot$ M^{-1} and $k_{off} = 6.5 \times 10^{-4}$ s^{-1} were determined at 30 °C [25], and by a precipitation assay, from which $k_{on} = 3 \times 10^4$ s$^{-1} \cdot$ M^{-1} and $k_{off} = 11.1 \times 10^{-4}$ s^{-1} were determined at 37 °C [14]. These are similar to the values determined here for the nNOS domain (at 22 °C), showing that the time-dependent nature of the interaction between NOS and nitroarginine is retained in the isolated haem domain.

The binding of [^{3}H]nitroarginine was competitively inhibited by arginine (Figure 4), and an apparent K_i of 1.6 μM for GST–nNOS haem and arginine binding was calculated from the data, as described in the Experimental section, using eqn. (2). *S*-Ethylisothiourea (apparent K_i 0.03 μM) and imidazole (apparent K_i 197 μM) binding to the nNOS haem domain were also competitive with nitroarginine in equilibrium binding experiments (Figure 4). In the same manner, apparent K_i values were obtained for the known NOS inhibitors, L-N^G-monomethylarginine, L-NIO, 7-nitroindazole and 1400W, and these were compared with inhibition constants for full-length nNOS (Table 1). The affinities for the full-length enzyme and the nNOS haem domain varied maximally only by a factor of 3, suggesting that the active site of the domain has similar modes of binding NOS inhibitors of varied structures as the full-length enzyme.

Table 1 Equilibrium dissociation constants for inhibitors interacting with the haem domain and full-length nNOS

Apparent K_i values, obtained using the SPA assay, are compared with published K_i values for full-length nNOS. The inhibition constant for L-NIO with full-length nNOS was determined as described in the Experimental section.

Inhibitor/substrate	Apparent K_i (μM) measured in SPA assay	K_i (μM)	Reference
Arginine	1.6	1.2 (K_m value)	[25]
Nitroarginine	0.025	0.015	[25]
L-N^G-Monomethylarginine	0.11	0.17	[26]
S-Ethylisothiourea	0.03	0.03	[27]
L-NIO	1.3	0.5	This study
Imidazole	197	95	[28]
7-Nitroindazole	2.5	2.8	[29]
1400W	4.1	2	[30]

In SPAs similar to those with [^{3}H]nitroarginine, an SPA signal was obtained for the binding of [^{3}H]tetrahydrobiopterin to the nNOS haem domain (results not shown). After subtraction of background, with GST–nNOS haem domain omitted from the SPA signal, a saturable increase in counts was produced with increasing [^{3}H]tetrahydrobiopterin, and an apparent K_d of 20 nM was calculated for [^{3}H]tetrahydrobiopterin binding to nNOS haem domain. The addition of 1 mM unlabelled tetrahydrobiopterin to the assay completely abolished the signal (results not shown). However, the background signal was higher in the tetrahydrobiopterin SPA than in the nitroarginine SPA, as there was some non-specific binding of tetrahydrobiopterin to the SPA beads. 7-Nitroindazole, which was shown to be competitive for nitroarginine binding to nNOS haem domain (see above), was also competitive with [^{3}H]tetrahydrobiopterin for binding to nNOS haem domain (Figure 5), with an apparent K_i of 0.5 μM. This compound was previously shown to be an inhibitor of nNOS competitive with both arginine and tetrahydrobiopterin [29,31], with a K_i of 0.12 μM for full-length nNOS [29]. In our experiments, 7-nitroindazole has a lower apparent K_i as an inhibitor of tetrahydrobiopterin than of nitroarginine binding. Mayer et al. [29] observed a similar effect and proposed that this was due to the presence of tetrahydrobiopterin in the nitroarginine assay acting as a competitive inhibitor of 7-nitroindazole binding. The same explanation is possible in our own experiments, as the concentration of tetrahydrobiopterin is 100 μM in nitroarginine binding but only 0.075 μM in tetrahydrobiopterin binding experiments. Additionally, 11U50, originally synthesized as one of a series of inhibitors of dihydrofolate reductase [32], and which was found to be a pterin-site inhibitor of NOS (L. J. Russell, personal communication) was competitive with [^{3}H]-tetrahydrobiopterin for binding to the nNOS haem domain (Figure 5) and an apparent K_i of 0.12 μM for 11U50 binding was

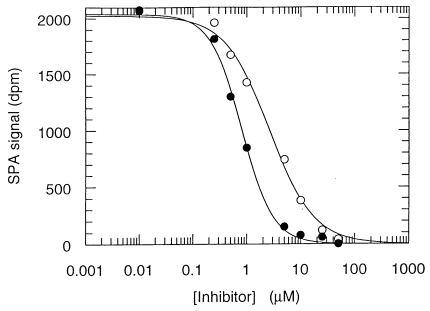

Figure 5 Effect of 7-nitroindazole and 11U50 on [^{3}H]tetrahydrobiopterin binding

SPAs to measure binding of [^{3}H]tetrahydrobiopterin by the nNOS haem domain were performed in the presence of the indicated concentrations of 7-nitroindazole (○) and 11U50 (●). A background signal obtained in the absence of nNOS haem domain–GST fusion protein has been subtracted. Apparent K_i values were calculated as 0.5 μM (7-nitroindazole) and 0.12 μM (11U50).

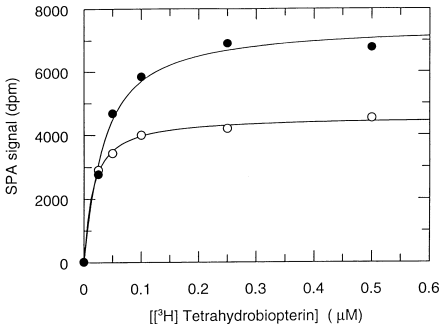

Figure 6 Effect of *S*-ethylisothiourea on [^{3}H]tetrahydrobiopterin binding

SPAs were performed at the indicated concentrations of [^{3}H]tetrahydrobiopterin in the absence (○) and presence (●) of 5 μM *S*-ethylisothiourea. A background signal obtained in the absence of nNOS haem domain–GST fusion protein has been subtracted. The lines shown are the best fits to binding isotherms for tetrahydrobiopterin binding to nNOS haem domain with apparent K_d values of 20 nM (in the absence of *S*-ethylisothiourea) and 28 nM (in the presence of 5 μM *S*-ethylisothiourea).

calculated. The titration of up to 500 μM 11U50 into the SPA measuring [^{3}H]nitroarginine binding to nNOS haem had no effect on the signal, indicating that 11U50 is competitive only with tetrahydrobiopterin (results not shown).

L-Arginine had previously been found to increase the amount of tetrahydrobiopterin able to be bound by full-length nNOS [14]. Therefore, the effect of L-arginine on [^{3}H]tetrahydrobiopterin binding to the nNOS haem domain was investigated. Addition of up to 1 mM L-arginine had no effect on the SPA signal. Interestingly, the addition of *S*-ethylisothiourea (5 μM) to the SPA increased the [^{3}H]tetrahydrobiopterin binding by nNOS haem domain almost 2-fold (Figure 6). From this experiment the apparent K_d for tetrahydrobiopterin in the presence of *S*-ethylisothiourea was calculated as 28 nM, which is similar to the apparent K_d for tetrahydrobiopterin in the absence of *S*-ethylisothiourea (20 nM). The addition of excess unlabelled tetrahydrobiopterin (100 μM) completely abolished the signal. Concentrations of up to 5 mM *S*-ethylisothiourea had no effect on the assay blank (without nNOS haem domain), nor did it have any effect in an unrelated SPA measuring the binding of Ras to Raf [24], confirming that a specific effect was observed. The *S*-

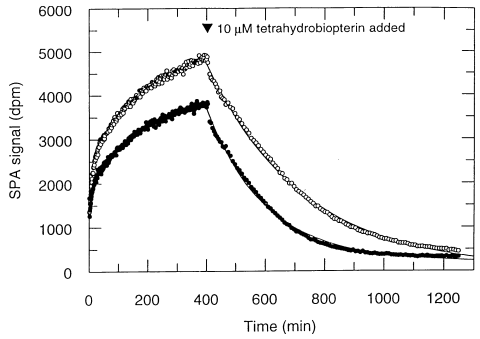

Figure 7 Effect of *S*-ethylisothiourea on the kinetics of [^{3}H]tetrahydrobiopterin binding

The kinetics of association to, and dissociation from, nNOS haem domain were measured in SPAs. The assays were performed in the absence (●) and presence (○) of *S*-ethylisothiourea (5 μM). The assays were started at time zero by the addition of [^{3}H]tetrahydrobiopterin (0.1 μM). At 400 min, 10 μM unlabelled tetrahydrobiopterin was added to the assay and counting was restarted. The visible lines represent the best fit to a single-exponential decrease for the dissociation of [^{3}H]tetrahydrobiopterin from the nNOS haem domain. The lines for the best fit to the association phase are masked by the data points. All the kinetic parameters calculated are shown in Table 2.

ethylisothiourea-dependent stimulation of tetrahydrobiopterin binding to nNOS haem was not reversed by the addition of up to 100 mM L-arginine, suggesting that S-ethylisothiourea has effects on nNOS at a site other than the arginine-binding site. Previous investigations of *S*-ethylisothiourea inhibition have shown that it is a rapidly reversible inhibitor of NOS activity competitive with arginine [27]. Interactions with tetrahydrobiopterin were not previously examined. Our data show that there is an interaction between *S*-ethylisothiourea and NOS, additional to that which is competed by arginine.

The kinetics of [^{3}H]tetrahydrobiopterin binding to the nNOS haem domain and dissociation after the addition of 10 μM unlabelled tetrahydrobiopterin are shown in Figure 7. The binding of tetrahydrobiopterin shows an initial very rapid phase (< 5 min), followed by a slower phase that could be fitted to a double-exponential increase (Figure 7); the kinetic parameters are shown in Table 2. Thus, there is triphasic binding of [^{3}H]tetrahydrobiopterin to the nNOS haem domain. The dissociation of [^{3}H]tetrahydrobiopterin could be fitted to a single exponential curve (Figure 7 and Table 2). The experiment was repeated in the presence of 5 μM *S*-ethylisothiourea (Figure 7). The on-rates of phases 2 and 3 in the absence and presence of 5 μM *S*-ethylisothiourea were similar, but the amplitude of the phases was greater in the presence of *S*-ethylisothiourea (Table 2). The off-rate, however, was slower in the presence of *S*-ethylisothiourea.

The kinetics of tetrahydrobiopterin binding to nNOS (porcine) were first investigated by Klatt and co-workers [12,13]. Although they reported simple monophasic second-order association kinetics for radiolabelled tetrahydrobiopterin binding, they also postulated that NOS contained a tightly bound, non-exchangeable tetrahydrobiopterin site, as well as a binding site at which the radiolabelled ligand was binding. However, more recently, the same laboratory re-examined the kinetics of binding [14] and reported that the association kinetics are more complex, showing, particularly in the presence of arginine, at least two phases of binding, i.e. a very rapid burst followed by a zero-order increase. Gorren et al. [14] have proposed a complex model which includes a spin transition of the haem and two sites for both arginine and

Table 2 Kinetic parameters for [^{3}H]tetrahydrobiopterin binding to the nNOS haem domain in the presence and absence of *S*-ethylisothiourea

Rate constants and amplitudes were calculated from the data shown in Figure 7 using curve fitting as described in the Experimental section.

	Association						
	Phase 1		Phase 2		Phase 3		Dissociation
	k_1 (s^{-1})	Amplitude (d.p.m.)	$10^3 \times k_2$ (s^{-1})	Amplitude (d.p.m.)	$10^3 \times k_3$ (s^{-1})	Amplitude (d.p.m.)	$10^3 \times k_{off}$ (s^{-1})
− *S*-Ethylisothiourea	> 10	1566	0.96	503	0.07	2775	0.08
+ *S*-Ethylisothiourea	> 10	1545	0.97	1077	0.08	2514	0.05

tetrahydrobiopterin, each differing in affinity. However, the interpretation of the data by Gorren et al. [14] on the kinetics of tetrahydrobiopterin binding is open to some criticism for two main reasons: firstly, the experiments involved a separation step, such that rapidly dissociating ligand might not be detected, and secondly, the association kinetics were performed at a ratio of < 0.1 mol of ligand per mol of NOS. This is likely to result in the preferential detection of high-affinity sites, as weaker sites will have a low occupancy. For the above reasons it is possible that Gorren et al. [14] only saw two of the three phases of binding that we observed under conditions with no separation step and an excess of ligand over NOS. Further experiments would be required to ascertain the precise molecular mechanism for the complex binding kinetics. However, as the dissociation rate constant of tetrahydrobiopterin is similar to the pseudo-first-order association rate constant for the slowest phase, it is possible that the latter represents exchange of already bound (unlabelled) tetrahydrobiopterin with [^{3}H]tetrahydrobiopterin. The first two phases might measure binding to unoccupied sites.

The observation that *S*-ethylisothiourea causes an increase in the amount of tetrahydrobiopterin able to bind to the nNOS haem domain (Figures 6 and 7) is novel. Kinetic analysis (Figure 7) shows that the effect of *S*-ethylisothiourea on the amount of tetrahydrobiopterin bound is neither on the rapid phase nor on the slowest phase (k_3), but on the intermediate phase defined by k_2. In the presence of *S*-ethylisothiourea this phase is doubled in amplitude. *S*-Ethylisothiourea has little effect on the overall equilibrium binding affinity of tetrahydrobiopterin, showing that binding of *S*-ethylisothiourea results in an increase in the total binding sites for tetrahydrobiopterin. Gorren et al. [14] reported a similar effect of arginine on tetrahydrobiopterin binding to full-length nNOS. Curiously, with the recombinant haem domain we found no effect of arginine on tetrahydrobiopterin binding. This is the only significant difference we have noted between properties of the domain and those reported for full-length NOS. It is possible, though we have no evidence for it, that the haem domain already contains tightly bound arginine. Interestingly, in the mechanism proposed by Gorren et al. [14], they postulate a slow-binding step for arginine, which would be consistent with the existence of a very-high-affinity binding site for arginine.

Full-length NOS and isolated haem domains of NOS are both dimeric ([8] and refs. therein). Evidence has recently been summarized, which suggests that once cofactors are bound, NOS acts as an asymmetric dimer, such that tetrahydrobiopterin and arginine might each bind at one high-affinity site and one weak-affinity site [17]. We would highlight the data reported here which are consistent with such a hypothesis. Firstly, the isolated haem domain contains approximately 0.4 mol of tightly bound (i.e. not lost on dialysis) tetrahydrobiopterin per mol of NOS. However, the capacity for binding of nitroarginine to this protein is doubled by the addition of tetrahydrobiopterin, which we postulate is binding at the second 'weak' site. A similar conclusion was reached by Gorren et al. [14]. Secondly, the kinetics of tetrahydrobiopterin binding are complex, showing three phases, which suggests several sites of differing affinity. Interestingly, the dissociation phase appeared to consist of a simple single-exponential decrease in bound tetrahydrobiopterin, suggesting that once all sites are occupied by tetrahydrobiopterin they become equivalent. Thirdly, *S*-ethylisothiourea causes an increase in the amount of tetrahydrobiopterin able to bind to the nNOS haem domain (Figures 6 and 7). The complex interaction between substrates and cofactors, the 'half-site reactivity' (i.e. one mol of tetrahydrobiopterin or nitroarginine bound at high affinity per dimer), and the existence of substrate analogues, such as nitroarginine and *S*-ethylisothiourea, that bind with a very high affinity, are remininiscent of observations made with mitochondrial ATPase [33,34]. Hence, we would like to speculate that NOS possesses a single active site in each dimeric unit and that the enzyme has a form of alternating catalytic-site mechanism.

In summary, a novel SPA assay has been described which can be used to measure equilibrium binding of NOS inhibitors to the nNOS haem domain and also the kinetics of nitroarginine and tetrahydrobiopterin association to and dissociation from the nNOS haem domain. This assay has been used to characterize the cofactor or inhibitor binding of nNOS domains with a degree of detail that has not been possible to date, and allows comparison with full-length NOS. The data contained in this paper provide evidence for the similarity between the arginine-binding site of the nNOS haem domain and the full-length enzyme in arginine and inhibitor binding and in the effect of tetrahydrobiopterin on nitroarginine binding. This SPA could also be extended to provide comparisons of nNOS with the inducible and endothelial isoforms and to analyse the effects of single-point mutations of amino acids.

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