Kinetics of CO binding to the haem domain of murine inducible nitric oxide synthase: differential effects of haem domain ligands

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The binding of CO to the murine inducible nitric oxide synthase (iNOS) oxygenase domain has been studied by laser flash photolysis. The effect of the (6*R*)-5,6,7,8-tetrahydro-L-biopterin (BH₄) cofactor L-arginine and several Type I L-arginine analogues/ligands on the rates of CO rebinding has been evaluated. The presence of BH₄ in the iNOS active site has little effect on the rebinding of protein-caged haem–CO pairs (geminate recombination), but decreases the bimolecular association rates 2-fold. Addition of L-arginine to the BH₄-bound complex completely abolishes geminate recombination and results in a further 80-fold decrease in the overall rate of bimolecular association. Three of the Type I ligands, *S*-ethylisothiourea, L-canavanine and 2,5-lutidine, displaced the CO from the haem iron upon addition to the iNOS oxygenase domain. The Type I ligands significantly

decreased the rate of bimolecular binding of CO to the haem iron after photolysis. Most of these ligands also completely abolished geminate recombination. These results are consistent with a relatively open distal pocket that allows CO to bind unhindered in the active site of murine iNOS in the absence of L-arginine or BH₄. In the presence of BH₄ and L-arginine, however, the enzyme adopts a more closed structure that can greatly reduce ligand access to the haem iron. These observations are discussed in terms of the known structure of iNOS haem domain and solution studies of ligand binding in iNOS and neuronal NOS isoenzymes.

Key words: CO recombination, fast kinetics, flash photolysis, redox enzyme.

INTRODUCTION

The NO synthases (NOSs) are a family of complex enzymes which catalyse the NADPH-dependent formation of NO and citrulline from L-arginine and molecular oxygen. The reaction involves a two-step haem-based oxygenation, during which *N*^ω-OH-L-arginine is formed as a stable intermediate. The overall reaction is similar to mono-oxygenations catalysed by cytochromes P450 [1,2]. NO is synthesized in a variety of cells and tissues and is an important effector molecule in the nervous, cardiovascular and immune systems. Three distinct NOS isoforms have been purified and characterized, i.e. neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). Although the three NOS isoforms vary in size, amino acid sequence, expression level and regulation, all three are homodimeric enzymes. Each subunit of NOS comprises an N-terminal catalytic oxygenase domain and a C-terminal reductase domain. The oxygenase domain binds haem (iron protoporphyrin IX), (6*R*)-5,6,7,8-tetrahydro-L-biopterin (BH₄) and L-arginine. The reductase domain binds two flavin molecules (FMN and FAD) and NADPH. The two domains are linked covalently by a polypeptide chain that acts as a binding site for calmodulin (CaM) [3].

The FAD and FMN prosthetic groups within the reductase domain of NOS transfer reducing equivalents from NADPH to the haem iron, which then binds oxygen and catalyses the two-step oxidation of L-arginine. CaM functions like a molecular switch, permitting electron transfer from the reductase to the haem domain. Ca²⁺-dependent CaM binding activates both eNOS and nNOS, whereas iNOS is active at all physiological

Ca²⁺ levels as it contains tightly bound CaM [4–6]. In all three isoforms, BH₄-binding and dimerization of the oxygenase domain are essential for catalytic activation [7,8]. BH₄ is located at the dimer interface, where it stabilizes the dimer and facilitates substrate interactions. Structural coupling between BH₄ and the haem propionate indicates that the pterin may have a key catalytic role [9]. A pterin radical (H₃B[•]) has been identified during the reaction of the iNOS oxygenase domain with oxygen [10].

The haem in the active centre of NOS can react in the reduced (ferrous) state with CO to form a characteristic thiolate ferrous haem–CO complex with spectral properties virtually identical with the corresponding complexes of cytochromes P450. CO inhibits both steps of the NO synthesis reaction, thus indicating that the haem is the catalytic centre of the enzyme [11,12]. An understanding of the interactions between cofactors, substrates, inhibitors and the active site of NOS is important in clarifying the mechanism of catalysis. Changes in the haem environment can affect binding of CO to the haem iron. Measuring the kinetics of CO ligation to haem have previously been shown to provide useful information on the structure and dynamics of haem-based enzymes (e.g. myoglobin, cytochromes P450 and, more recently, NOS [13–16]). Measurement of CO rebinding to nNOS by flash photolysis has provided information on the binding of BH₄ and L-arginine to nNOS [16–18].

Each domain of NOS can exist and function independently [19–21], which therefore aids structure–function studies of the enzyme. Crystal structures have been determined for both the monomer and dimer forms of the oxygenase domain of murine

Abbreviations used: NOS, NO synthase; iNOS, inducible NOS; nNOS, neuronal NOS; eNOS, endothelial NOS; BH₄, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; CaM, calmodulin; L-NMMA, *N*^ω-monomethyl-L-arginine; *S*-EITU, *S*-ethylisothiourea.

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iNOS [9,22]. In the present paper, we report the kinetics of CO binding (geminate recombination and bimolecular association) to the structurally defined haem domain of murine iNOS using laser photolysis methods. By exploiting *Escherichia coli* as expression host, we have investigated the kinetics of CO binding in BH_4 -free and BH_4 -reconstituted iNOS haem domain. We have also investigated effects on the CO association kinetics of binding various Type I haem domain ligands, which bind above the haem plane, converting the haem iron into the high-spin state. Type I ligands bind competitively with arginine and many are specific inhibitors of NOS. Hence, the CO association kinetics in the presence of these ligands provide an additional probe for the structure around an important region of the protein.

EXPERIMENTAL

Materials

E. coli bacterial strains were supplied by Novagen (Madison, WI, U.S.A.). Plasmid pSVLiNOS [23], which encodes the full-length murine iNOS cDNA, was obtained from Dr I. Charles (The Wellcome Research Laboratories, Langley Court, Beckenham, Kent, U.K.). The pB13 expression vector is a derivative of plasmid pCWori [24] and was a gift from L. McLaughlin (Biomedical Research Centre, Ninewells Hospital, Dundee, Scotland, U.K.). Restriction enzymes and buffers were from Life Technologies (Gibco BRL), or New England Biolabs. Expand *Pfu* DNA Polymerase was from Boehringer Mannheim and the PCR-Script Amp Cloning Kit was from Stratagene. Primers were synthesized using Applied Biosystems 380B or 394 synthesizers, and automated DNA sequencing was performed using the PRISM ready reaction DyeDeoxy Terminator cycle sequencing kit on an Applied Biosystems 373A DNA Sequencer. BH_4 was from Schircks Laboratories (Jona, Switzerland). The NOS substrate/inhibitors were obtained from Aldrich or Sigma, with the exception of *N*^ω-OH-L-arginine monoacetate, which was obtained from Alexis Biochemicals. CO gas was from Merck. PD-10 columns were purchased from Amersham Pharmacia Biotech. All other chemicals used were of at least analytical grade and were obtained from Sigma or Fisher Scientific.

Cloning, expression and purification of the $\Delta 65$ iNOS haem domain

The DNA sequence encoding the murine iNOS haem domain (residues 66–498) was generated by PCR amplification from the plasmid pSVLiNOS containing the full-length iNOS cDNA. The primer 5'-AAA AAA ACA TAT GGG CCA TCA TCA TCA TCA TCA TCT GGA CAA GCT GCA TGT GAC ATC G-3' was designed to incorporate an *NdeI* restriction site and His-tag sequence at the 5'-end of the PCR product. A second primer, 5'-AAA AAA TCT AGA TTA CTC ATT CTG CCA GAT GTG GGT CTT-3', was synthesized to engineer an *XbaI* site and termination codon at the 3'-end of the PCR product. The PCR product was ligated into a PCR Script holding vector using the PCR Script Amp Cloning Kit. The ligated vector was then transformed into the *E. coli* strain XL-1 Blue and the plasmid DNA purified using a Qiagen mini-prep kit. The PCR Script-iNOS plasmid was digested with *NdeI* and *XbaI*, purified by agarose gel electrophoresis, and ligated into a similarly restricted pB13 expression vector. The sequence of the final recombinant plasmid was confirmed by DNA sequencing.

The pB13 recombinant plasmid encoding the murine iNOS haem domain (residues 66–498) and the additional N-terminal His-tag was expressed and purified as described in [25]. Protein concentrations were determined using the Coomassie Plus

Protein Assay Reagent Kit (Pierce and Warriner, Rockford, IL, U.S.A.). Amino acid analysis, performed as described previously [26], was 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 Coomassie protein assay. Protein concentrations are given as concentration of monomer.

Determination of the spectral binding constant, K_s

The binding constants for the NOS ligands were measured by substrate perturbation difference spectroscopy [27]. Optical spectra were recorded using a PerkinElmer UV/visible dual-beam Lambda 20 spectrophotometer, with a temperature control accessory and 1.0 ml quartz cuvettes. Titration experiments were performed at 25 °C using 2.65 μM protein in 100 mM phosphate buffer (pH 7.5)/100 mM NaCl, containing 26.5 μM BH_4 , 2 mM 2-mercaptoethanol and 380 μM imidazole. Ligands were dissolved in 100 mM sodium phosphate buffer (pH 7.5)/100 mM NaCl. iNOS haem domain (1 ml) was placed in the cuvette, the absorbance difference was adjusted to zero, and a baseline was recorded. Difference spectra were recorded after each addition of ligand at 0.2 nm intervals, from 300 nm to 700 nm, using a 2 nm slit width. The final sample volume changes were less than 3%. The change in absorbance between 393 nm (the wavelength of maximal absorbance change) and 430 nm (the wavelength of minimal absorbance change) was measured and plotted as a function of ligand concentration. The mean data from two titrations were plotted for each ligand. All ligands used were Type I inhibitors/substrates, and therefore optical titrations were performed in the presence of imidazole, a competitive inhibitor. The apparent K_s in the presence of imidazole was determined by fitting the data to eqn (1) using the computer program GraFit [28]:

$$\Delta A = \frac{(\Delta A_{\text{max}})}{2E_o} \cdot \left(F - \sqrt{F^2 - 4E_o S_o} \right) \quad (1)$$

where $F = E_o + S_o + K_s$, ΔA is the measured absorbance change after each titrant addition, ΔA_{max} is the maximal absorbance change at the end of titration, E_o is the total concentration of protein, S_o is the total concentration of titrant and K_s is the apparent spectral binding constant for titrant binding to NOS.

The K_s values for the titrant in the absence of imidazole were calculated by fitting the data to eqn (2):

$$\Delta A = \left[\frac{(\Delta A_{\text{max}}) \left\{ (K_s Z + K_{\text{Im}} S) - \sqrt{(K_s Z + K_{\text{Im}} S)^2 - 4K_s^2 E_o I_{\text{Im}}} \right\}}{K_s (Z - \sqrt{Z^2 - 4E_o I_{\text{Im}}})} \right] \quad (2)$$

where $Z = (I_{\text{Im}} + E_o + K_{\text{Im}})$, I_{Im} is the imidazole concentration before addition of the titrant, and K_{Im} and K_s are the dissociation constants of imidazole and the titrant respectively.

Laser flash photolysis

Sample preparation was performed within an anaerobic glove box (Belle Technology, Portesham, Dorset, U.K.). Buffer [100 mM sodium phosphate (pH 7.5)/100 mM NaCl] was prepared and deoxygenated in a gas-tight flask by bubbling with Ar gas for > 2 h. Stock protein was transferred into anaerobic buffer by passage down a PD-10 buffer-exchange column pre-equilibrated with anaerobic buffer. Where required, a 10-fold concentration of BH_4 , with respect to iNOS haem domain concentration, was added to the eluted protein sample. Methyl-

viologen was used to measure the amount of $\text{Na}_2\text{S}_2\text{O}_4$ required to reduce the haem iron. Methylviologen ($10\ \mu\text{l}$ of a 2 mM solution) was added to 2 ml of $5.5\ \mu\text{M}$ iNOS haem domain, and the solution was titrated with $\text{Na}_2\text{S}_2\text{O}_4$ (1 mM solution) until the sample remained blue. A 100 mM stock of $\text{Na}_2\text{S}_2\text{O}_4$ was then used to add 2.5 times that concentration to a 4 ml sample of iNOS haem domain. After recording the optical spectrum to ensure complete reduction, CO was bubbled through the sample for 2 min (approx. 1 bubble per second). The optical spectrum was measured to ensure formation of the iNOS–CO complex. NOS ligands were added to the iNOS–CO complex at the desired concentration (see below) and the optical spectrum measured to ensure CO remained bound to the haem iron.

Laser CO photodissociation experiments were carried out using an Applied Photophysics LKS.60 flash-photolysis instrument. The instrument offers tunable wavelength excitation pulses, by using the third harmonic (355 nm) of a Continuum Surelite 1-10 Nd YAG laser and a manually adjusted Surelite Optical Parametric Oscillator system. The apparatus was configured to deliver 545 nm pulses. Kinetic transients were recorded at 445 nm. A pulsed 150 W xenon lamp was used to measure geminate recombination. A conventional tungsten lamp was used to measure bimolecular association of CO with reduced iNOS domain. To maximize the signal/noise ratio, multiple kinetic traces for each sample were measured and averaged prior to data analysis. Transients for geminate recombination are the average of approx. 100 individual measurements; transients for bimolecular association of CO and reduced iNOS domain are the average of 10 measurements. The data were fitted to standard exponential functions using the non-linear least-squares method provided by the instrument manufacturer. Optical spectra were taken before and after flash-photolysis experiments to safeguard against laser-induced degradation of the sample. Transients were recorded at 20 °C with a 1 cm optical pathlength cell. Cell volume and iNOS domain concentration were 4 ml and $5.5\ \mu\text{M}$ respectively.

Stopped-flow spectroscopy

Deoxygenated buffer and the reduced iNOS–CO complex were prepared as described for laser flash photolysis in an anaerobic glove box at 25 °C. Inhibitor samples were prepared in CO-saturated anaerobic buffer. One syringe of the stopped-flow apparatus contained the reduced iNOS–CO complex (in CO-saturated buffer with a 10-fold concentration of BH_4 with respect to iNOS–CO concentration) and the other contained inhibitor in CO-saturated buffer. The sample, when mixed, contained $2.75\ \mu\text{M}$ iNOS–CO complex, $27.5\ \mu\text{M}$ BH_4 and inhibitor at the desired concentration (see below). Stopped-flow reactions were recorded on an Applied Photophysics SX18MV stopped-flow instrument. Transients were fitted to standard exponential functions (see below) using the fitting algorithms supplied by the instrument manufacturer. Absorption changes were monitored at 445 nm. Reaction rate constants were calculated from averaged transients comprising 10 individual kinetic transients.

RESULTS

Type I ligand titrations

The iNOS haem domain was isolated from the recombinant *E. coli* strain as described and consequently was purified in the BH_4 -free form. The purified domain exhibited a Soret absorbance peak around 421 nm, representing a fully low-spin ferric haem iron. Addition of BH_4 (10-fold molar excess) to the iNOS haem domain sample produced a broad Soret band centred on

Figure 1 Chemical structures of the Type I ligands used

Figure 2 Spectral analysis of the titration of iNOS haem domain pre-equilibrated with imidazole with the Type I ligand L-arginine

L-Arginine (0–1 mM) was titrated against a sample of iNOS haem domain pre-equilibrated with imidazole, as described in the Experimental section. (A) Difference spectra calculated by subtracting the spectrum obtained in the absence of L-arginine from that after each addition of substrate. Traces shown are representative of 0, 40, 120, 300 and 1000 μM L-arginine. (B) Plot of the absorbance change observed at 393 and 430 nm versus L-arginine concentration. The data plotted are the mean of two titrations. The line is a least squares fit to eqn (2). Calculated K_s value for L-arginine is $9.19 \pm 0.14\ \mu\text{M}$.

400 nm, indicating a mixture of high- and low-spin haem iron. Addition of Type I ligands (e.g. arginine) to iNOS haem domain in the presence of BH_4 converted the remaining low-spin haem into its high-spin form. The predominantly high-spin haem was represented in the absorption spectrum by a narrow band centered at 396 nm [29].

Imidazole and Type I ligands such as L-arginine [and its analogues (Figure 1)] bind to mutually exclusive sites. Imidazole binds to the haem iron at the sixth axial ligand site causing a Type II optical difference spectrum. Titration of the imidazole-induced low-spin state NOS domain with Type I ligands resulted in the restoration of the enzyme to the high-spin state. Spectral binding constants (K_s) of the Type I ligands were consequently measured by equilibrium titration of the low-spin iNOS haem domain–imidazole complex with increasing concentrations of ligand. An example of the optical titration assay is shown in

Table 1 Equilibrium spectral binding constants for ligand binding to the iNOS haem domain

The spectral binding constants (K_s) for several NOS ligands were measured by perturbation difference spectroscopy, as described in the Experimental section. Values are given for the apparent K_s in the presence of a saturating concentration of imidazole and for the K_s calculated for the ligand in the absence of imidazole.

Substrate/inhibitor	K_s (μ M)	
	K_s calculated	K_s apparent
L-Arginine	9.19 ± 0.14	165 ± 3
<i>N</i> ^ω -OH-L-arginine	2.18 ± 0.03	38.2 ± 0.6
L-NMMA	1.12 ± 0.01	19.2 ± 0.4
L-Canavanine	499 ± 6	9050 ± 110
Aminoguanidine	564 ± 16	10200 ± 300
4-Methyl-pyrimidine	447 ± 7	8140 ± 150
2,5-Lutidine	46.2 ± 0.5	836 ± 10

Figure 3 Geminate recombination of CO to the iNOS haem domain following flash photolysis

Averaged flash-photolysis kinetic transients recorded in the presence and absence of Type I haem domain ligands. Ligand concentrations are given in Table 2. (A) Averaged transient obtained for reactions performed in the presence of BH₄. (B) Averaged transient obtained for reactions performed in the presence of *N*^ω-OH-L-arginine and BH₄. (C) Averaged transient obtained for reactions performed in the presence of L-NMMA and BH₄. (D) Averaged transient obtained for reactions performed in the presence of 4-methylpyrimidine and BH₄. The transients shown are the averages of 100 measurements. The experimental conditions are described in the Experimental section. Geminate recombination rate constants are presented in Table 2. In (A, B and D) fits to a single exponential function are illustrated.

Figure 2. The apparent K_s values for each ligand and the values calculated in the absence of imidazole are presented in Table 1.

Geminate recombination of CO

Laser flash photolysis was used to measure the rate constant for geminate recombination of CO to the iNOS haem domain. Figure 3 shows the transients for the iNOS–CO complex with BH₄ alone, and in the presence of other Type I NOS ligands. Where CO was seen to rebind within the geminate timescale, the data fitted well to a single exponential expression. The geminate

Table 2 Rate constants for geminate recombination of CO to the iNOS haem domain and fraction of recombined product obtained

Rate constants for the geminate recombination of CO to the iNOS haem iron were determined from laser flash-photolysis experiments as described in the Experimental section. k_{gem} is the geminate recombination rate constant for protein-caged haem–CO pairs and $a_{gem}/\Delta A_{(0)}$ is the fraction of geminate recombination with respect to total CO dissociation. BH₄ (0.055 mM) was present in all the samples except the BH₄-free sample.

Ligand	[Ligand] (mM)	k_{gem} (s ⁻¹)	$a_{gem}/\Delta A_{(0)}$
BH ₄ -free	None	$2.3 \pm 0.1 \times 10^7$	0.11 ± 0.01
BH ₄	0.055	$2.7 \pm 0.1 \times 10^7$	0.21 ± 0.01
L-Arginine	1	0	0
<i>N</i> ^ω -OH-L-arginine	1	$0.8 \pm 0.1 \times 10^7$	0.08 ± 0.00
L-NMMA	0.5	0	0
L-Canavanine	50	0	0
Aminoguanidine	100	0	0
S-EITU	0.1	0	0
4-Methylpyrimidine	50	$1.3 \pm 0.1 \times 10^7$	0.23 ± 0.01
2,5-Lutidine	10	$1.6 \pm 0.1 \times 10^7$	0.13 ± 0.01

Figure 4 Flash-photolysis transients for the bimolecular association of CO to the iNOS haem domain

(A) Data for iNOS–CO in the absence of BH₄ and other ligands. (B) Data for iNOS–CO in the presence of BH₄ and *N*^ω-OH-L-arginine. I, average of 10 biomolecular association transients recorded following the photolysis of the iNOS–CO complex; II, plot of residuals from fitting to a single exponential function; III, plot of residuals from fitting to a double exponential function; IV, semi-logarithmic plots of the kinetic data illustrating the fit to a double exponential function. Rate constants are given in Table 3. Conditions are described in the Experimental section.

recombination rate constant (k_{gem}) and fraction of rebound CO [$a_{gem}/\Delta A_{(0)}$] for these samples are summarized in Table 2. Geminate recombination rate constants were not affected by the presence/absence of BH₄, but the fraction of geminate re-

Table 3 Bimolecular association of CO to the iNOS haem domain

Rate constants for the bimolecular binding of CO to the iNOS haem iron were determined from laser-flash photolysis experiments, as described in the Experimental section. The bimolecular association rate constants k_1 and k_2 are shown alongside the fractions of the total absorbance change relating to each phase, $\Delta A_1/\Delta A_{\text{tot}}$ and $\Delta A_2/\Delta A_{\text{tot}}$ respectively. BH_4 (0.055 mM) was present in all the samples, except the BH_4 -free sample.

Ligand	k_1 (s^{-1})	$\Delta A_1/\Delta A_{\text{tot}}$	k_2 (s^{-1})	$\Delta A_2/\Delta A_{\text{tot}}$
BH_4 -free	1055 ± 12	0.82	324 ± 8	0.18
BH_4	558 ± 5	0.93	171 ± 11	0.07
L-Arginine	7.35 ± 0.08	0.59	4.64 ± 0.08	0.41
N^G -OH-L-arginine	7.21 ± 0.08	0.73	4.92 ± 0.15	0.27
L-NMMA	11.8 ± 0.1	0.84	6.32 ± 0.14	0.16
L-Canavanine	40.5 ± 0.7	0.78	9.20 ± 0.43	0.22
Aminoguanidine	22.6 ± 0.2	0.48	14.4 ± 0.1	0.52
S-EITU	247 ± 58	0.30	33.1 ± 3.8	0.70
4-Methyl-pyrimidine	123 ± 5	0.20	8.15 ± 0.03	0.80
2,5-Lutidine	246 ± 16	0.55	55.3 ± 3.2	0.45

combination in the presence of BH_4 was almost twice that of the BH_4 -free domain. Geminate recombination was not seen for reactions performed in the presence of L-arginine and its analogues N^G -monomethyl-L-arginine (L-NMMA), L-canavanine, and the Type I ligands aminoguanidine and S-ethylisothiourea (S-EITU). Geminate recombination was observed in the presence of the catalytic intermediate N^G -OH-L-arginine, but the rate constant was less relative to the domain in the presence of BH_4 alone, and a smaller proportion of the dissociated CO was able to rebind. Geminate recombination was also observed with the aromatic heterocyclic ligands, 4-methylpyrimidine and 2,5-lutidine. Again, recombination rate constants were less than with BH_4 alone, but in the case of 4-methylpyrimidine the fraction of rebound CO was similar.

Bimolecular association of CO

The effect of several NOS ligands on the rate constant for bimolecular association of CO to the iNOS haem domain was also measured by the laser flash-photolysis method. Figure 4 shows the transients for the BH_4 -free iNOS-CO complex and that complexed with N^G -OH-L-arginine and BH_4 . Linear displays of the residuals between single and double exponential fits to the data are shown below each averaged transient. Small deviations are seen on fitting the data to a single exponential expression, and semi-logarithmic plots of the kinetic transients clearly demonstrate that a double exponential is more appropriate. The bimolecular association rate constant for each of the iNOS complexes is summarized in Table 3.

The presence of BH_4 in the iNOS-CO complex reduces the bimolecular association rate constant by approx. 50% compared with the BH_4 -free enzyme. The presence of another ligand, additional to the BH_4 cofactor, in the iNOS active site further decreases the CO association rate constant. The rate constants for L-arginine and the catalytic intermediate N^G -OH-L-arginine are essentially identical, with the predominant CO association rate constant for iNOS-CO complexes containing either of these Type I compounds being approx. 80 times less than that for the iNOS-CO complex with only BH_4 bound. The rates observed for complexes with L-NMMA and aminoguanidine are also significantly slower than for the BH_4 -bound enzyme. CO association in the presence of L-arginine is, however, slightly slower than with these two ligands, being 1.5 and 3.0 times slower than L-NMMA and aminoguanidine respectively. The kinetics of CO association

Figure 5 Spectral changes accompanying addition of Type I ligands to the iNOS haem domain-CO complex

Heavy line, spectrum of the iNOS haem domain-CO complex prior to addition of the ligand; light line, spectrum of the iNOS haem domain-CO complex after addition of the ligand. (A) Spectral changes accompanying addition of L-canavanine. (B) Spectral changes accompanying addition of 2,5-lutidine.

Table 4 Rate constants from stopped-flow spectroscopy for CO dissociation from the iNOS haem domain

The dissociation of CO from the iNOS haem domain after rapid mixing with a NOS inhibitor was measured by stopped-flow spectroscopy as described in the Experimental section.

Ligand	[Ligand] (mM)	k_1 (s^{-1})	k_2 (s^{-1})
L-Canavanine	25	39 ± 0	
S-EITU	0.05	203 ± 3	
2,5-Lutidine	5	1.1 ± 0.0	0.11 ± 0.00

in complexes with 4-methylpyrimidine is also similar to that with L-arginine.

Relative stabilities of the iNOS-CO complex in the presence of Type I ligands

An absorbance peak centered at 444 nm accompanies formation of the iNOS-CO complex. Optical spectra of the iNOS samples were measured before and after flash photolysis to monitor the stability of the CO complex and to check for degradation of the iNOS protein. Loss of CO from the iNOS complex generates a new absorbance peak centered at 412 nm, which is representative of the reduced (ferrous) haem iron. If the protein is destabilized, the spectrum of the protein is perturbed and a new absorbance peak at 420 nm accumulates, indicating that the proximal thiolate linkage to the haem iron has weakened or become broken [30,31]. Although the BH_4 -free sample appeared to be stable over the length of the flash-photolysis experiments, on leaving the sample for extended periods of time (> 2 h), it gradually became unstable, as monitored by perturbation of the optical spectrum (i.e. loss of the absorbance peak at 444 nm and formation of the 420 nm absorbance peak). The presence of a Type I ligand and/or BH_4 in the iNOS active site stabilized the enzyme, since conversion of the 444 nm absorbance peak into the 420 nm species was not observed even following prolonged incubation (> 4 h).

Optical spectra were also taken before and after addition of each Type I ligand to the iNOS-CO complex. The spectra showed that addition of some of the Type I ligands resulted in the displacement of the majority of CO from the ferrous haem iron (Figure 5). Only a small proportion of the iNOS haem present in samples containing L-canavanine, S-EITU, or 2,5-lutidine would, therefore, have been complexed with CO prior to

Figure 6 Stopped-flow transients for ligand induced dissociation of CO from the iNOS haem domain-CO complex

Kinetic traces are the average of 10 measurements. (A) Averaged transient obtained on mixing with L-canavanine (25 mM). Data are fitted to a single exponential expression. (B) Averaged transient obtained on mixing with 2,5-lutidine (5 mM). Data are fitted to a double exponential expression and plots of the residuals are illustrated.

flash photolysis. The CO association rate constants measured for iNOS complexed with any of these three compounds were larger than those containing the other Type I ligands. The rate constants were, however, still substantially less than the BH_4 -bound complex without the additional ligand. Although the inhibitors used in this study are Type I ligands that do not bind directly to the haem iron, addition of some of the inhibitors to the iNOS-CO complex clearly results in the displacement of CO from the haem. Stopped-flow spectroscopy was used to demonstrate and measure the rate constants for CO dissociation from the iNOS-CO complex after the addition of these inhibitors (Table 4 and Figure 6). Stopped-flow transients were either monophasic or biphasic, depending on the identity of the ligand. The stopped-flow data indicate that *S*-EITU is the most effective Type I ligand at displacing CO from the iNOS haem-CO complex (Table 4), with relatively large rate constants being obtained at low concentrations of inhibitor (50 μM).

DISCUSSION

The haem domain of murine iNOS is isolated from *E. coli* in the low-spin state, suggesting water or hydroxide forms the sixth ligand to the haem iron [32]. Addition of BH_4 converts the haem iron predominantly into the high-spin state, a structural change that is probably facilitated by the formation of hydrogen bonds between BH_4 and one of the haem propionate groups [9,33]. Addition of Type I ligands to BH_4 -bound iNOS converts the remaining low-spin haem into the high-spin form. Optical titrations with Type I ligands were performed with imidazole-bound (low-spin) iNOS haem domain to establish equilibrium spectral binding constants prior to flash-photolysis experiments. The measured value for L-arginine is in good agreement with the published K_m or K_s values published previously for both full-length murine iNOS (12.5 μM ; [34]) and murine iNOS haem domain (11 μM ; [25]). Formation of the iron-CO bond in murine iNOS haem domain produced a typical absorption spectrum with a maximal peak centred at 444–445 nm. In the absence of ligands the enzyme gradually converted into the 420 nm species, indicating that the interaction of the thiolate of cysteine-194 with the haem iron observed in the crystallographic structure of the haem domain is destabilized [9,22,30]. Conversion into the 420 nm species was prevented by addition of BH_4 and/or L-arginine, a finding that is consistent with previous observations with both iNOS [31] and nNOS [35]. It is clear, therefore, that

BH_4 and L-arginine are key ligands in stabilizing the haem-thiolate bond in the NOS enzymes. This is also evident in studies of nNOS, which was shown to form a stable six-co-ordinate NO complex in the presence of L-arginine or BH_4 , but to convert into a five-co-ordinate NO complex in the absence of these ligands [36].

Stabilization of the thiol-iron bond in the Fe^{2+} -CO complex by L-arginine and BH_4 appears to be linked to their capacity to constrain CO binding [31]. In the present paper, we have used laser flash-photolysis methods to analyse the effect of binding L-arginine and a number of L-arginine analogues and NOS inhibitors on the stability and rebinding of CO to the iNOS haem domain. We have shown that BH_4 alone has little effect on the rates of CO association, whereas reformation of the iron-CO bond is much slower when both L-arginine and BH_4 are bound to the enzyme. In general, the effect of various L-arginine analogues is similar to that of L-arginine itself and can be rationalized in terms of a molecular crowding of the haem pocket that decreases the overall rate of CO association, as seen also with the nNOS haem domain [16,17,31]. Geminate recombination rates measured for iNOS haem domain in our studies are similar (approx. 10^7 s^{-1}) to those reported for the nNOS isoform [16]. However, with the iNOS haem domain, L-arginine binding prevents geminate recombination, whereas rebinding of a fraction of the protein-caged iron-CO pairs is observed in L-arginine-bound nNOS [16]. The fraction of geminate recombination with respect to total CO dissociation is generally greater for various ligand-bound and free states of nNOS compared with the equivalent iNOS forms. (Although probably not a source of the kinetic differences observed between iNOS and nNOS haem domains we cannot rule out the effect of different laboratory conditions for protein preparation in our own work and that of others.)

Babu et al. [37] have compared the binding of a number of L-arginine analogues with the three NOS isoenzymes and have suggested that the binding site for L-arginine is more 'open' in nNOS than the corresponding sites in iNOS or eNOS. In the present study, the lack of an effect of BH_4 -binding on the rate of geminate recombination (compared with the BH_4 -free enzyme) suggests that the BH_4 cofactor may lower the overall bimolecular association rate by modifying the access channel (thereby affecting ligand entry). The essentially equivalent geminate recombination rate constants in the presence and absence of BH_4 argue against structural change in the immediate vicinity of the haem iron, but the smaller fraction of geminate recombination with respect to total CO dissociation in BH_4 -free iNOS haem domain indicates that CO is more readily lost from the active site (through a more open access channel) to solvent.

The binding of L-arginine and its analogues to iNOS haem domain has major effects on CO recombination. The binding of L-arginine, L-NMMA, L-canavanine, aminoguanidine and *S*-EITU clearly prevent geminate recombination (Table 2), but also compromise substantially the rate of binding by bimolecular association (Table 3). In these cases, the observed effects on CO rebinding may not only be due to changes in ligand entry, but are likely also to be affected by conformational changes that perturb ligand binding at the haem iron. Resonance Raman spectra of the nNOS haem domain have revealed two distinct conformations of the iron-CO complex that are indistinguishable in optical spectra [32]. It has been suggested that in the absence of L-arginine and BH_4 , nNOS adopts an 'open' structure, whereas the presence of these two ligands affects the immediate environment of CO, producing a 'closed' conformation. The relatively fast rates of geminate CO recombination for substrate-free iNOS and nNOS are consistent with an open distal pocket.

The affect of Type I ligands in the NOS active site is to shift the distribution between the open (fast-reacting) and closed (slow-reacting) conformations. In the open structure, the lack of constraint allows the iron-bound CO to adopt an optimal, linear conformation perpendicular to the haem plane. In the closed structure the geometry of the Fe^{2+} -CO interaction is likely to be less optimal, owing to substrates/inhibitors exerting either strong polar and/or steric effects on the haem-bound CO. Evidence in support of open and closed states of the iNOS haem domain is available from FTIR spectroscopy studies, which have revealed the affect of both BH_4 and L-arginine binding on the CO stretch vibrational mode of the iNOS-CO complex [33].

In addition to those discussed above, one needs to be mindful of alternative explanations for the effects of ligands on CO binding. Apart from exerting a direct steric effect, the ligands may also influence the electronic properties of the haem porphyrin ring, thereby modifying the redox potential of the haem iron. Tetreau et al. [18] have argued that the rate of CO-binding is sensitive to the charge on the proximal ligand. The cysteine residue that forms the proximal thiolate linkage to the haem iron is hydrogen bonded to the peptide backbone. Binding of ligands such as L-arginine on the distal side of the haem may affect local structure on the proximal side of the haem and, in turn, alter the charge density on the thiolate ligand.

It is expected that most of the L-arginine analogues and Type I ligands described in the present paper will bind in the same site as L-arginine itself, thereby inducing the formation of a more closed conformation. Indeed, crystal structures of the iNOS dimer have been solved complexed with N^{ω} -OH-L-arginine and L-arginine [38]. Both ligands bind similarly to iNOS, in an extended conformation, with the amino acid side chain fitting tightly into a narrow part of the active-site cavity. The presence of the hydroxy group in N^{ω} -OH-L-arginine, however, is responsible for small differences in the interaction geometries, which are evident in our flash-photolysis studies with the CO complexes (Table 2) inasmuch as geminate recombination is only seen with N^{ω} -OH-L-arginine. The hydroxyl oxygen of N^{ω} -OH-L-arginine is likely to form a hydrogen bond with glycine-365, and steric constraint in the N^{ω} -OH-L-arginine di-oxygen complex suggests that small structural movements are required to simultaneously accommodate N^{ω} -OH-L-arginine and di-oxygen. Iron-bound O_2 is within 2.1 Å of the reactive nitrogen (N^{ω}) of N^{ω} -OH-L-arginine, which is pointed towards the centre of the porphyrin ring. However, the terminal oxygen of bound O_2 is greater than 4 Å from the hydroxyl proton of N^{ω} -OH-L-arginine (1 Å = 0.1 nm). It has been suggested that a covalent conjugate is formed during haem ligation, involving di-oxygen, the reduced haem iron and the N^{ω} nitrogen of N^{ω} -OH-L-arginine. Another important factor has been highlighted in ENDOR studies which suggest, unlike L-arginine, N^{ω} -OH-L-arginine does not exist as a resonance-stabilized cation when bound to NOS [39]. Crystal structures of the iNOS monomer show that aminoguanidine fits into the active site of iNOS, in close proximity to the haem, at the position occupied by the guanidino portion of L-arginine [22]. Sennequier and Stuehr [40] have studied the binding of L-arginine analogues to the murine iNOS isoform, and suggested that the amino, C α and carboxyl regions of L-arginine may position the guanidino moiety in a suitable position relative to the haem iron to effect catalysis. The lack of these key interactions with the peptide backbone may partially explain the weak binding affinity of iNOS for aminoguanidine (Table 1).

Three of the inhibitors (*S*-EITU, L-canavanine or 2,5-lutidine) used in the present study were found to displace CO from the haem iron when added to the iNOS-CO complex. This may occur either through a direct steric interaction with the haem-

bound CO, or by effecting a conformational change within the active site that destabilizes the iron-CO linkage. Little is known about the binding of 2,5-lutidine to the NOS haem domain. 2,5-Lutidine is structurally very similar to 4-methylpyrimidine, and differs only through its possession of an additional methyl group. Both these Type I ligands bind sufficiently close to the haem iron to displace haem-bound imidazole. However, addition of 2,5-lutidine to the iNOS-CO complex slowly displaces iron-bound CO, but the Fe^{2+} -CO linkage remains intact after addition of 4-methylpyrimidine. We suggest that the additional methyl substituent of 2,5-lutidine may be oriented towards the haem where it sterically disrupts the iron-CO linkage.

S-EITU is a potent inhibitor of murine iNOS ($K_i = 5.2$ nM [41]). The iNOS isoform has a high affinity for L-arginine analogues containing uncharged (*S*-EITU) or weakly basic (N^{ω} -OH-L-arginine) functional groups. In comparison with L-canavanine and 2,5-lutidine, the very tight binding of *S*-EITU may explain the fast dissociation of CO after the addition of this ligand to the iNOS complex. Crystal structures of human iNOS complexed with *S*-EITU have been determined [42]. These show that, like L-arginine, *S*-EITU forms a bi-dentate interaction with a glutamate residue. However, unlike the substrate side chain, which is oriented toward an opening in the main cavity, the ethyl group of *S*-EITU is packed near the haem and a phenylalanine residue in the active site. Consistent with this observation, Babu et al. [37] showed that occupancy of the region near the haem by $=\text{S}$ compared with $-\text{NH}_2$, $-\text{CH}_3$, or $=\text{O}$ is energetically favourable. Proposals for key interactions made by several other analogues of L-arginine in the active site of NOS have also been made [37]. L-Arginine was shown to bind to NOS as a protonated species, which can donate a proton to haem-bound di-oxygen through the reactive nitrogen (N^{ω}). L-Canavanine (an analogue of L-arginine in which the δ -methylene group is replaced by oxygen) adopts the imino structure with the guanidinium double bond orientated toward the N-5 nitrogen (Figure 1). The hydrogen bond between the N-5 nitrogen and peptide glutamate is lost with L-canavanine owing to the positioning of the double bond and the loss of the N-5 hydrogen. This probably explains the decrease in binding affinity of iNOS for L-canavanine compared with its interaction with L-arginine (Table 1). Loss of CO on binding L-canavanine is unlikely to be triggered by steric interaction, since its mode of binding is similar to that of L-arginine.

Concluding remarks

Our studies of Type I ligand binding to the iNOS-CO complex support the view that the haem domain exists in different conformational states, corresponding to 'open' and 'closed' forms of the ligand access channel. Moreover, our binding equilibrium data support the existence of common binding modes for the Type I ligands, inasmuch as each elicits formation of the high-spin form from the iNOS haem domain-Im complex. Our flash photolysis and stopped-flow studies highlight subtle differences in binding that affect the stability of the iNOS-CO complex and the exchange of CO from the active site to solvent, and the role of BH_4 in contributing to domain stability and the conformation of the ligand access channel. These detailed kinetic studies of the ligand interactions will facilitate further studies of the dynamics of the iNOS haem domain in the context of the crystallographic structure.

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