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DISSECTING STRUCTURAL AND ELECTRONIC EFFECTS IN INDUCIBLE NITRIC OXIDE SYNTHASE

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Abstract

Nitric oxide synthases (NOS) are haem-thiolate enzymes that catalyse the conversion of L-Arginine (LArg) into NO and citrulline. Inducible NOS (iNOS) is responsible for delivery of NO in response to stressors during inflammation. The catalytic performance of iNOS is proposed to rely mainly on the haem midpoint potential and the ability of the substrate L-Arg to provide an H-bond for oxygen activation (O-O scission). We present a comparative study of native iNOS versus iNOS-mesohaem, and investigate the formation of a low-spin ferric haem-aquo or -hydroxo species (P) in iNOS mutant W188H substituted with mesohaem. iNOS-mesohaem and W188H-mesohaem were stable and dimeric, and presented substrate-binding affinities comparable to their native counterparts. Single turnover reactions catalysed by iNOSoxy with LArg (first reaction step) or N-hydroxyarginine (second reaction step) showed that mesohaem substitution triggered faster rates of Fe^{II}O₂ conversion and altered other key kinetic parameters. We elucidated the first crystal structure of a NOS substituted with mesohaem and found essentially identical features compared to the structure of iNOS carrying native haem. This facilitated the dissection of structural and electronic effects. Mesohaem substitution substantially reduced the build-up of species P in W188H iNOS during catalysis, thus increasing its proficiency toward NO synthesis. The marked structural similarities of iNOSoxy containing native haem or mesohaem indicate that

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AUTHOR CONTRIBUTIONS.

L.H. designed the study, performed the experiments and prepared the manuscript; R.C.P and S.M. solved the crystal structure of iNOSoxy-mesohaem and wrote the corresponding experimental and results sections of the manuscript; M.M.H carried out the simulation studies under steady state conditions; K.B. purified proteins and performed experiments; Z.Y. purified proteins and obtained high-quality crystals of iNOSoxy-mesohaem; D.J.S. designed the study and prepared the manuscript. All authors performed critical reading of the manuscript prior to submission and approved the final version of the paper.

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the kinetic behaviour observed in mesohaem-substituted iNOS is most heavily influenced by electronic effects rather than structural alterations.

Keywords

nitric oxide synthase; mesohaem; haem; catalysis; steady state kinetics; reaction mechanism

INTRODUCTION

Nitric oxide synthases are a group of homodimeric enzymes (NOS, EC 1.14.13.39) that catalyse the conversion of L-Arginine and dioxygen into citrulline and nitric oxide. Each monomer is comprised of an N-terminal oxygenase domain (NOSoxy) incorporating binding sites for its substrate L-Arginine (LArg), tetrahydrobiopterin (H₄B), and a Cys-coordinated haem, and a C-terminal reductase domain (NOSred) that hosts sites for NADPH, FAD and FMN. A calmodulin binding sequence (CaM) bridges the NOSoxy and NOSred domains [1, 2].

The sequence of reactions that leads to NO synthesis by NOS involves several steps of reduction and oxidation. Resting NOS is first reduced by electrons coming from the reductase domain. Ferrous haem then reacts with molecular oxygen to form a transient FeO₂ species (Fig. 1). This FeO₂ intermediate is further reduced by the cofactor H₄B to form a ferric-peroxo complex. Scission of the O-O bond follows to form the highly valent compound I species, which ultimately oxidizes L-Arg to form N-hydroxyarginine (NOHA). Conversion of NOHA into citrulline and NO requires a second event of oxygen activation, in which H₄B is proposed to serve as the electron donor (Fig. 1).

All NOS sequenced and crystallized to date possess a conserved tryptophan residue that forms stacking interactions with the porphyrin ring and hydrogen bonds with the haem-thiolate bond. Replacement of this proximal Trp residue by histidine in murine iNOS (W188H) increased the midpoint potential of the haem group by +88 mV and reduced the rate of NO synthesis compared to wild type iNOS. In addition, the W188H mutation stabilized an inert enzyme species, P, that formed downstream of the FeO₂ species, which reacted slowly with L-Arg to form NOHA [3]. Surprisingly, the W188F and W188A mutants of iNOS exhibit defective haem binding, impeding further characterization [4]. Substitution of Trp⁴⁰⁹ by Phe in rat nNOS reduced the haem midpoint potential of the protein, led to a faster formation of the FeO₂ species and to greater rates of Fe(II)NO oxidation compared to the wild type protein [5–7]. A side-by-side characterization of *Bacillus subtilis* NOS and its proximal Trp variants W66H and W66F revealed distinct thermodynamic and kinetic behaviours depending on the nature of the amino acid residue replacing the native Trp (His or Phe)[8–10]. Thus, the proximal Trp residue may play a role in controlling the enzyme's reactivity by tuning the properties and reactivity of its haem. Further, a study performed with *S. aureus* NOS revealed that NOS display a steep dependence between the haem midpoint potential and the rates of oxygen activation under single turnover conditions for the hydroxylation of L-Arg to NOHA [8]. Lang *et al* showed that mutations on the Trp residue proximal to the haem-thiolate bond altered the haem

midpoint potential and the rates of disappearance of the FeO₂ species in trends that correlated with the donating or withdrawing electron density from the haem-thiolate bond that NOS enzymes display [8].

We have employed mesohaem-substituted iNOS to study the effect of the haem midpoint potential in catalysis. Mesohaem incorporates 2,4-ethyl groups instead of the vinyl substituents found in the naturally occurring haem, leading to an increase in electron density. We utilized murine iNOS variant W188H to further examine the thermodynamic component of catalysis and attempted the reversal of the W188H phenotype by substitution with mesohaem. We present a comprehensive examination of the effect of porphyrin replacement on the following features: Structure of wild type iNOSoxy-mesohaem, haem midpoint potential, enzyme oligomeric state, substrate binding, FeO₂ stability, kinetics of haem transitions during catalysis, extent of product formation, reactivity of the Fe-NO complex and NO synthesis. This is the first study to: i) Provide a crystal structure of inducible NOS substituted with mesohaem; ii) Establish the differential influence of structural and electronic components on the kinetic behaviour of iNOS and iii) Demonstrate that the formation of inert enzyme species P formed by iNOS mutant W188H [3, 11] is heavily influenced by an increased midpoint potential of the haem centre.

EXPERIMENTAL

Reagents

H₄B was purchased from Schircks Laboratories (Jona, Switzerland). CO gas was obtained from Praxair, Inc. (Danbury, CT). N-hydroxy-L-Arg (NOHA) and [¹⁴C]-labelled L-Arginine ([¹⁴C]-L-Arg) were purchased from MP Biomedicals (Solon, OH). EPPS was purchased from Fisher Scientific (Pittsburgh, PA). DTT was purchased from RPI Corp. (Mount Prospect, IL). All other reagents were purchased from Sigma (St. Louis, MO).

Protein expression and purification

Wild type and mutant W188H iNOS 65 (deletion of the first 65 amino acids in the N-terminus) proteins containing a His₆ tag were overexpressed in *Escherichia coli* strain BL21(DE3) and purified using Ni-NTA affinity chromatography as previously described [3]. Haem replacement studies required expression in M9 minimal medium supplemented with 2X the amount of glucose and casamino acids suggested by the manufacturer in order to achieve acceptable expression yields. Mesohaem (5 μM final concentration) was added at the time of induction, along with 0.375 mM IPTG. Cells were harvested 24–30 hours post-induction and the cell pellets were frozen at -80 °C until further use. Protein concentration was determined from the absorbance at 444 nm of the ferrous haem-CO complex in proteins carrying native haem (Fe-protoporphyrin IX), using an extinction coefficient of 76 mM⁻¹cm⁻¹ (25). Protein concentration was determined from the absorbance at 432 nm of the ferrous mesohaem-CO complex, using an adjusted extinction coefficient of 55 mM⁻¹cm⁻¹ (obtained by comparison of the respective absorption coefficient for free haem and mesohaem). All proteins were purified to homogeneity (95%) as assessed by SDS-PAGE. Expression yields were low compared to their corresponding proteins containing native haem under the same culture conditions (15–20 mg/L of culture): iNOSoxy-

mesohaem ~ 8 mg/L of culture; W188Hox-mesohaem 2 mg/L of culture; iNOS FL-mesohaem 1 mg/L of culture and W188H FL-mesohaem (0.5 mg/L of culture). Mesohaem to haem ratios were determined by HPLC according to a published procedure [12]. Mesohaem content ranged from 83% to 94% in both wild type and W188H iNOS. Attempts to express these proteins in rich media (Luria Broth or Terrific Broth) resulted in little incorporation of mesohaem (less than 5%). The oligomeric state of wild type and W188H iNOS reconstituted with H₄B and L-Arg was examined by size-exclusion chromatography. Protein samples (~150 µM) in EPPS buffer (40 mM, pH 7.6, 150 mM NaCl) were incubated with 2 mM L-Arg, 400 µM H₄B and 1.2 mM DTT for 15 min. Samples were injected on a Superdex 200 resin pre-equilibrated with EPPS buffer (40 mM, pH 7.6, 150 mM NaCl) supplemented with 100 µM L-Arg, 40 µM H₄B and 120 µM DTT. Under these conditions, all proteins existed predominantly in the dimeric state (80–90%).

Imidazole and L-Arginine binding

Binding affinities of imidazole and L-Arg were studied at 25 °C by perturbation difference spectroscopy according to methods described previously (27;28). NOS samples (around 5 µM) in 40 mM EPPS buffer, pH 7.6, with 10% glycerol, 0.6 mM DTT, 0.2 mM H₄B were titrated by stepwise addition of imidazole, to a final concentration of 10 mM. The K_d of imidazole ($K_{d\text{imid}}$) was calculated by fitting the data to a simple saturation binding equation. The K_d of L-Arg (K_d) was determined under the same conditions, in the presence of 10 mM imidazole. The data were fit to a simple saturation binding equation, and K_d was

calculated according to the following equation: $appK_d = K_d \left(1 + \frac{[imid]}{K_{d\text{imid}}} \right)$, where $appK_d$ is the apparent K_d of the enzyme for L-Arg in the presence of saturating concentrations of imidazole.

Single turnover reactions

L-Arg hydroxylation and NOHA oxidation experiments were carried out in a Hi-Tech SF61-DX2 stopped-flow instrument (Hi-Tech Scientific, Salisbury, UK) coupled to a diode array detector, as previously described [10, 13]. An anaerobic solution of 20 µM ferrous NOS (obtained by titration of ferric NOS with a solution of dithionite), 2 mM L-Arg (or 1 mM NOHA), 0.2 mM H₄B and 1 mM DTT in 40 mM EPPS pH 7.6 containing 10% glycerol and 150 mM NaCl was mixed at 10 °C with a solution containing air-saturated buffer, 2 mM L-Arg, 0.2 mM H₄B and 1 mM DTT. Sequential spectral data were fit to a two-exponential, A→B→C, model using the Specfit/32 global analysis software, version 3.0 (Spectrum Software Associates, Marlborough, MA). Specfit is a multivariate data analysis program for modelling and fitting 3D chemical kinetics data by singular value decomposition (SVD). Rates are the average of at least 5 reactions ± standard deviation.

Determination of Reaction Products by HPLC

End-point conversion of [¹⁴C]-L-Arg to [¹⁴C]-NOHA under single turnover conditions were carried out according to a previously published procedure [10]. [¹⁴C]-L-Arg to [¹⁴C]-NOHA were extracted from the reaction mixtures and the conversion of substrate to product was

monitored by HPLC. The radioactivity of each fraction was counted using a Scintillation counter [10].

Midpoint potential determinations

Spectroelectrochemical titrations were conducted in a glove-box (Belle Technology, Dorset, UK) under N₂ atmosphere, as previously described (16;30). NOS proteins were made air-free by gel filtration in a Sephadex G-25 column (PD 10, GE Healthcare) pre-equilibrated with anaerobic buffer (100 mM phosphate buffer, pH 7.0, 125 mM NaCl). Protein samples were diluted to a 3.5 mL final volume (final concentration $\approx$ 10 μ M) and L-Arg (2 mM) and H₄B (100 μ M) were added. The following electron carrier dyes (0.5–1 μ M) were utilized: phenosafranine (E_m = -252 mV), benzyl viologen (E_m = -358 mV), methyl viologen (-450 mV) and anthraquinone-2-sulfonate (E_m = -225 mV). The titration was performed at 15 °C by step-wise addition of a sodium dithionite solution. Oxidative titrations were carried out under the same conditions using potassium ferricyanide to oxidise the haem centre. Reductive and oxidative titrations yielded midpoint potential values within $\pm$ 2 mV, suggesting the process involves one-electron transfer in either direction. Absorption spectra were collected with a Cary 50 spectrophotometer equipped with a dip-probe detector, and the potentials were measured using a silver/silver chloride microelectrode saturated with 4 M KCl (Fisher Scientific). Midpoint potential values are expressed as midpoint potential $\pm$ error of the fit for a one-electron process.

Ferrous haem-NO complex oxidation (k_{ox})

Native and mesohaem-substituted iNOSoxy wild type or W188Hoxy ($\sim$ 5 μ M) in 100 mM EPPS (pH 7.6, 150 mM NaCl, 10% glycerol) containing 2 mM L-Arg, 0.2 mM H₄B and 1 mM DTT were carefully titrated with dithionite under anaerobic conditions. The Fe(II)-NO complexes were generated by step-wise addition of anaerobic NO-saturated buffer. Both proteins displayed stable Fe(II)-NO complexes at pH 7.6 (EPPS buffer 100 mM, pH 7.6, 150 mM NaCl, 10% glycerol) and at pH 9.5 (CHES buffer 100 mM, pH 9.5, 150 mM NaCl, 10% glycerol). Fe(II)-NO protein samples were then transferred to an anaerobic stopped-flow instrument using a gastight syringe, and the reactions were initiated by mixing with air-saturated buffer at 10 °C. Spectral data were fit to a single exponential model, A $\rightarrow$ B, using Specfit global analysis software.

NO synthesis

NO synthesis by native and mesohaem-substituted full length proteins was assessed by the oxyhemoglobin NO capture assay using an extinction coefficient of 38 mM⁻¹ cm⁻¹ for methemoglobin minus oxyhemoglobin as described elsewhere [12]. Total nitrite produced by these proteins in the presence of NOHA was determined by the Griess assay [14] according to published protocols [10].

Crystallization and data collection

Mesohaem-substituted iNOS 65 was expressed in minimal medium and purified as described above. Crystals of murine iNOSoxy (residues 66–498) in complex with mesohaem and H₄B were grown by hanging drop vapour diffusion at 293 K in 1 μ l drops (0.5 μ l protein

with 0.5 μ l screen). Hanging drops consisted of a 1:1 ratio mixture of 20 mg/ml iNOSoxy with 10 mM H₄B and a reservoir solution. Crystals grew within 3–6 days in a reservoir solution composed of 700 mM ammonium sulphate, 100 mM MES pH 5.3 and 3.5% octyl-glucoside. Crystals were cryoprotected by brief transfer through reservoir solution supplemented with 30% (w/v) ethylene glycol and flash-frozen in liquid nitrogen. Diffraction data for a crystal in space group *P*6₁22 was collected using a Rigaku MicroMax-007HF generator and a Rigaku Saturn 044+ CCD detector. Data integration, reduction and scaling was performed with d*TREK [15] and processed to a cut-off of 2.78 Å. The resolution cut-off was based on significant drops in non-averaged *I*/ σ (<2.0) and significant increases in *R*_{merge} in shells at higher resolutions.

Structure solution, refinement, validation and deposition

An existing structure of iNOS (PDB ID 3DWJ: chain A) [3] was used as a molecular replacement search model. Phases were calculated by molecular replacement using the *PHASER* [16] component of *PHENIX* [17]. The best molecular replacement solution was subjected to iterative rounds of model building in *COOT* [18] and refinement in *PHENIX*. The refinement protocol used isotropic atomic displacement parameters for all atoms. Stereochemical analysis of the iNOSoxy structure was completed with *MolProbity* [19]. All residues are within the allowed regions of the Ramachandran plot and no poor rotamers or C β deviations were found. *MolProbity* statistics are listed in Table S1. The atomic coordinates for iNOSoxy in complex with mesohaem and H₄B in space groups *P*6₁22 has been deposited in the PDB (accession code 4JS9).

Steady-state kinetics of the reaction of full-length W188H containing native haem

Steady state reactions were carried out according to the conditions described by Abu-Soud et al.[20]. Briefly, full length W188H (~ 2 μ M) was mixed with 1 mM L-Arg and 4 μ M H₄B/DTT in 40 mM EPPS, pH 7.6 containing 125 mM NaCl and 10% glycerol. This solution was mixed with 140 μ M NADPH in the same buffer, and the reaction was monitored at different time intervals by rapid UV-vis scanning.

Simulations of iNOS Distribution during Steady-state NO Synthesis

The NO synthesis kinetics of wild type iNOS were simulated using the global model described earlier [21] and implemented in Gepasi software version 3.30. For mutant W188H, the global model was adjusted to account for the formation and disappearance of inert product, P. The simulations assume constant values for [O₂] = 180 μ M and [NADPH] = 40 μ M. Experimental *k*_{ox} values for wild-type iNOS ferrous haem-NO complexes obtained under half air-saturation conditions were multiplied by a factor of 1.5 (air-saturation) [22]. Likewise, oxygen binding rates *k*₂ and *k*₆ (for wild type enzymes) and *k*₂ and *k*₇ (for W188H mutant enzymes) were multiplied by a factor of 2.0 (air-saturated condition) assuming that this rate is proportional to [O₂]. The selected concentrations of NADPH and dioxygen were kept constant during each simulation, to avoid secondary effects due to their exhaustion.

RESULTS AND DISCUSSION

Expression of mesohaem-containing iNOS proteins

The oxygenase and full-length versions of wild type iNOS and mutant W188H were expressed in *E. coli* grown in minimal medium supplemented with 5 μ M mesohaem. The expression yield was very low, particularly for the full-length proteins (yield ~ 0.5 mg protein/L of culture). Despite this practical hurdle, the purified proteins presented the expected spectral characteristics, suggesting the haem environment was suitable for further characterization. Substitution of iNOS and W188H with Fe(III)-deuteroheemin and Fe(III)-2,4-diacetyl-deuteroheemin proved impossible under our experimental conditions. Gel filtration analysis showed that both iNOSoxy and W188Hoxy containing mesohaem exist predominantly as a dimer (data not shown). Fractions of highly purified proteins were examined for their haem content (Reinheitszahl values, Rz, A_{Soret}/A_{280}). The haem content of both proteins substituted with mesohaem was low compared to their corresponding native counterparts (Rz = 1.5–2.5). Wild type iNOSoxy-mesohaem had a Reinheitszahl value (Rz) of 0.45, whereas W188Hoxy showed ~ 2.5-fold decreased haem content compared to iNOSoxy-mesohaem (Rz = 0.20). This poorer haem load has been observed in W188Hoxy carrying native haem, and the effect was exacerbated upon incorporation of the foreign mesohaem. The lower mesohaem content observed in W188Hoxy seems to be an effect of the Trp to His mutation per se. This could be related to the recently noted misalignment between the imidazole ring of His¹⁸⁸ and the porphyrin ring as opposed to the native arrangement of the indole group of Trp¹⁸⁸ [11]. Conceivably, this structural restriction may affect cellular haem insertion during protein maturation. In fact, iNOSoxy mutants W188A and W188F are isolated as apo-proteins (unpublished data).

X-ray Crystal Structure of iNOSoxy-mesohaem

Crystals of iNOSoxy 65 substituted with mesohaem were analysed and compared to the reported crystal structures of iNOSoxy 65 carrying native haem (PDB 1NOD [2]), as well as with the structure of iNOS mutant W188Hoxy (PDB 3DWJ, [3]). X-ray crystallographic analysis revealed a highly conserved iNOSoxy structure upon replacement with mesohaem. The structures of iNOSoxy-mesohaem and iNOSoxy superimpose within 0.3 Å rmsd (Table S1), suggesting an almost identical structure (Fig. 2, panel A). The haem centre displays a similar H-bonding network compared to native iNOSoxy, which is suitable for catalysis. Tetrahydrobiopterin is also oriented within its pocket in the right configuration for electron transfer (Fig. 2B and 2C). Weak electron density was observed for the guanidinium moiety and backbone atoms of the natural substrate L-Arg, although the density was deemed too weak for inclusion in the final coordinates. The electron density for L-Arg is consistent with the position observed in wild type iNOS, with the guanidinium moiety sitting in close proximity to the iron centre (Fig. 2C).

Redox Potentiometry

An anticipated consequence of replacing native Fe-protoporphyrin IX with Fe-mesoporphyrin IX is a lowering of the haem midpoint potential. Redox titrations in the presence of L-Arg and H₄B were performed for mesohaem-containing iNOSoxy and W188Hoxy by stepwise addition of dithionite (reduction) or potassium ferricyanide

(oxidation). Nernst plots for the reductive curves are shown in Fig. 3 and the raw data is provided in Fig. S1. The calculated midpoint potentials were -295 ± 3 mV for iNOSoxy and -239 ± 6 mV for W188Hoxy. Thus, substitution of native haem by mesohaem led to a significant decrease in the haem midpoint potential compared to the corresponding proteins harbouring native Fe-protoporphyrin IX (iNOSoxy = -261 ± 2 mV; W188Hoxy = -173 ± 2 mV [3]). As expected, substitution of W188Hoxy with mesohaem did shift its midpoint potential to a value that is closer to that of wild type iNOSoxy, reversing the effect of the mutation, thus enabling the dissection of electronic versus structural effects. This provided with a unique opportunity to interrogate the role of the midpoint potential on the formation of inert enzyme species P that forms downstream of the FeO₂ species.

Spectroscopic Properties and Substrate Binding of mesohaem-containing iNOS proteins

UV-visible data for wild type iNOSoxy and W188Hoxy-containing mesohaem are given in Table 1. The UV-vis spectral features of iNOS proteins substituted with mesohaem are essentially identical to those reported for nNOSoxy-mesohaem [12, 23]. Substitution with mesohaem led to a 12 nm blue-shift in the UV-visible spectra of the Fe(III), Fe(III)-imidazole, Fe(II), Fe(II)-CO and Fe(II)-NO complexes of iNOS proteins, consistent with spectral changes observed in another variant of iNOS [24] and in other proteins substituted with this haem analogue [25–30]. In the presence of L-Arg and H₄B both wild type iNOSoxy and W188Hoxy substituted with mesohaem exist in the typical Fe(III) high-spin configuration, suggesting the integrity of the haem electronic environment remained comparable to that of their native counterparts (Fig. 4). Reduction with dithionite and exposure to CO resulted in the formation of the characteristic Fe(II)-CO complex with a Soret band appearing at 428–431 nm. UV-visible spectra of W188H proteins displayed the previously observed 2–5 nm blue shift compared to wild type iNOS [3], regardless of the haem centre used. Binding dissociation constants, K_d , for imidazole and L-Arg in the presence of H₄B were measured spectrophotometrically. Both wild type iNOSoxy and mutant W188Hoxy substituted with mesohaem displayed competent binding for imidazole ($K_{d,iNOSoxy} = 56 \pm 9$ μ M; $K_{d,W188Hoxy} = 39 \pm 2$ μ M) and for L-Arg ($K_{d,iNOSoxy} = 2.6 \pm 0.4$ μ M; $K_{d,W188Hoxy} = 5.6 \pm 0.3$ μ M). The lower affinity of W188Hoxy-mesohaem for LArg compared to its wild type counterpart is not unprecedented [3]. However, substitution with mesohaem produced a stronger association with the natural substrate compared to W188Hoxy possessing native haem ($K_d \sim 29 \pm 3$ μ M) [3], thus partially reversing the effects of the structural replacement of Trp¹⁸⁸ by a His residue.

Stopped-flow Analysis of a Single Turnover L-Arg Hydroxylation Reaction in the Presence of H₄B

We next investigated the enzyme haem species that occur in iNOSoxy and W188Hoxy substituted with mesohaem. Of particular interest was to examine the formation (or absence) of the inert enzyme species P whose exact nature remains elusive [3, 11]. L-Arg hydroxylation reactions of mesohaem-containing iNOSoxy could be best fit to a two-exponential model $A \rightarrow B \rightarrow C$, with Fe(II), Fe(II)-O₂ and Fe(III) as the only detectable species (Fig. 5). We investigated whether reversal of the midpoint potential in W188Hoxy-mesohaem impacts the formation and stability of species P, which forms downstream of the Fe-O₂ species [3, 11]. L-Arg hydroxylation reactions of mesohaem-containing W188Hoxy

were fit to a three-exponential model $A \rightarrow B \rightarrow C \rightarrow D$, with Fe(II), Fe(II)-O₂, a presumptive species P with absorption maximum at 411 nm and Fe(III) as the only detectable species (Fig. 6). A summary of the measured rate constants is given in Table 2. Fit of the spectral data to a two-exponential model for reaction times 2 s and 0.6 s confirmed the formation of an enzyme species with a marked shoulder at 411 nm (data not shown). For both mesohaem-containing proteins, disappearance of the Fe(II)-O₂ species was faster compared to their native counterparts. The effect of the midpoint potential on the stability of the Fe^{II}O₂ species has been studied in-depth in several enzyme systems [10, 31–35]. The most widely used approach involves the introduction of mutations to modify the strength of the haem-thiolate bond in both NOS and cytochrome P450 enzymes, which leads to a selective, tuneable increase or decrease in the haem midpoint potential. The Fe^{II}O₂ species is stabilized in proteins with higher haem midpoint potentials, due to a decrease in the driving force for its reduction to Fe^{III}O₂⁻. Likewise, a lower midpoint potential favours the ferric form of the protein, with the concomitant faster disappearance of the Fe-O₂ species with respect to native haem-containing proteins. This general relationship between the stability of the FeO₂ species and the haem midpoint potential has been well documented both in mammalian and bacterial NOSs and in related enzymes [5–7, 10]. A good illustration of this phenomenon is given by the haem transition rates for the conversion of FeO₂ to its downstream species: k_2 W188Hoxy-mesohaem = 10.5 s^{-1} ~ k_2 iNOSoxy native haem = 12.5 s^{-1} ; i.e. restoring the midpoint potential of mutant W188H to resemble that of wild type iNOSoxy regenerated the wild type-like behaviour of the FeO₂ species in W188Hoxy. This suggests that changes in haem electronics posed by the Trp¹⁸⁸ → His mutation can be at least partly reversed by substitution with mesohaem, making electron density the primary factor for the longer-lived FeO₂ species seen in W188Hoxy harbouring native haem (k_2 = 2.03 s^{-1} [3]). Reversal of the midpoint potential in W188Hoxy did not result in the abolition of inert species P that forms downstream of the FeO₂ species (Fig. 6), arguing in favour of a structural effect of the mutation *per se* [11]. However, a decrease in the midpoint potential in W188Hoxy significantly diminished the stability of species P compared to native W188Hoxy (k_3 W188Hoxy mesohaem = 0.198 s^{-1} ; k_3 W188Hoxy = 0.104 s^{-1}), highlighting the contribution of electronic effects.

Stopped-flow Analysis of a Single Turnover NOHA Oxidation Reaction in the Presence of H₄B

The fact that mutant W188H displays altered behaviour in terms of NO synthesis compared to wild type iNOS [3] prompted us to examine NOHA oxidation reactions under single turnover conditions in proteins substituted with mesohaem. Reaction of wild type iNOSoxy-mesohaem with NOHA and H₄B could be best fit to a sequential model with three well-defined haem transitions (Table 2), similarly to the reaction pattern observed for this protein carrying native haem. The observed reaction rates (k_1 , k_2 and k_3) suggested the reaction is slower in iNOSoxy-mesohaem compared to the native haem-containing protein. A stable Fe^{III}-NO complex was identified downstream of the FeO₂ species, with a broad absorption maximum at 431 nm (Fig. 7). Single turnover reactions of W188Hoxy carrying native haem in the presence of NOHA and H₄B do also occur via the formation of three distinct species as observed with wild type iNOSoxy, though formation and disappearance of the FeO₂ species is substantially slower in the mutant (Table 5). A remarkable effect of mesohaem

substitution in mutant W188Hoxy was the change to an extremely short-lived FeO_2 species and the stabilization, for the first time, of a $\text{Fe}^{\text{III}}\text{-NO}$ complex (Fig. 8). The observed rates of formation and disappearance of $\text{Fe}^{\text{III}}\text{-NO}$ in iNOSoxy-mesohaem were proportionally lower (~1.5 fold) than in the native enzyme. The $\text{Fe}^{\text{III}}\text{-NO}$ complexes identified during single turnover reactions of iNOSoxy and W188Hoxy containing mesohaem are practically identical to the same species generated by addition of NO to an anaerobic solution of the corresponding $\text{Fe}^{\text{III}}\text{-NOS}$ enzyme (Insets to Fig. 7 panel A, and Fig. 8 panel B). Global analysis of the single turnover reactions of mutant W188Hoxy-mesohaem was compatible with a sequential 2-exponential mechanism, with the reaction being best described as: $\text{Fe}^{\text{II}} \rightarrow \text{P} + \text{Fe}^{\text{III}}\text{-NO} \rightarrow \text{Fe}^{\text{III}}$, where P defines an enzyme species with a Soret absorption maximum at ~420 nm that forms in the reactions of W188H carrying native haem [3, 11]. We originally referred to this species as a “reaction intermediate”, but recent work [11] and the experimental findings presented herein strongly suggest that species P is a non-native low-spin enzyme species that forms right after NO is made, and thus masks or antagonizes the formation of the enzyme $\text{Fe}^{\text{III}}\text{NO}$ product complex that otherwise transiently builds up in the wild-type reaction. Previous studies provided strong evidence that species P is a hydroxide-bound haem whose formation is accompanied by $\text{H}_4\text{B}^{\bullet+}$ radical buildup, rather than it being a ferryl or a peroxo-heme species [11]. It was proposed that the heme-hydroxide species was stabilized by the Trp¹⁸⁸ to His mutation, and that the hydroxide ligand prevents NO binding to the heme before it escapes the active site channel [11]. Our findings argue against a pure structural effect of the Trp¹⁸⁸ to His mutation, by showing that the stability of this presumptive hydroxide-bound haem species is challenged by sole reversal of the midpoint potential in W188H. This in turns rescues the enzyme’s ability to synthesize NO by shifting oxygen activation toward the productive cycle of NOS. Nonetheless, our data supports the proposal that species P could be indeed an hydroxide-bound haem derived from NOHA (product complex). Binding of substrate and pterin-free W188Hoxy to the NOHA analogues phenylguanidine and methoxyphenylguanidine (4 mM) led to perturbation of the UV-visible spectra, as expected. Binding of W188Hoxy to phenylguanidine produced a complex with absorption maximum at 424 nm, suggestive of Fe-N coordination. Binding to methoxyphenylguanidine, which possesses an available oxygen atom for axial ligation, resulted in a complex with absorption maximum at 419 nm reminiscent of species P (Fig. S2). This finding is in line with binding studies carried out with NOHA [11] and points to the hydroxo group as the axial ligand of species P.

Extent of L-Arg Hydroxylation

An examination of [¹⁴C]-NOHA formation from [¹⁴C]-L-Arg under single turnover conditions at infinite time (10 min) showed that the yield of product formation for both mesohaem-substituted proteins (iNOSoxy = 0.40 NOHA per haem; W188Hoxy = 0.48 NOHA per haem) is in accord with previously reported data for iNOSoxy (NOHA/haem 0.4–0.6 [36–39]) and W188Hoxy (0.37 NOHA/haem [3]) carrying native haem. Substitution of mesohaem in W188H led to a slightly greater yield of L-Arg hydroxylation. This correlates well with the formation of a stable $\text{Fe}^{\text{III}}\text{-NO}$ complex during single turnover reactions with NOHA and the improved NO synthesis rate by this mutant (see next section).

NO synthesis

NO synthesis by full-length iNOS and W188H substituted with mesohaem was examined by the oxy-hemoglobin capture assay. The rate of NO synthesis by wild type iNOS-mesohaem ($34.9 \pm 3 \text{ min}^{-1}$) was lower than that reported for the native enzyme ($51\text{--}66 \text{ min}^{-1}$ [3, 40]). In contrast, the NO synthesis rate of W188H-mesohaem was $22 \pm 5.4 \text{ min}^{-1}$, which is approximately 4-fold higher than the documented value for native W188H [3]. While wild type iNOS has a higher NO synthesis capacity than mutant W188H with mesohaem, the difference is less than in their counterparts with native haem. Thus, replacement of native haem by mesohaem improved the NO synthesis yield of iNOS mutant W188H.

Oxidation Rate of the Ferrous-NO complex (k_{ox})

During NO synthesis, a $\text{Fe}^{\text{II}}\text{-NO}$ complex forms and can be reduced by NOS reductase at rates comparable to that of $\text{Fe}^{\text{II}}\text{-NO}$ dissociation [41, 42]. Reaction of the ferrous-NO complex with O_2 resumes the catalytic cycle, ultimately leading to NO release. We investigated the oxidation of anaerobic $\text{Fe}^{\text{II}}\text{-NO}$ complexes by O_2 ($[\text{O}_2] \sim 120 \mu\text{M}$) by stopped-flow spectroscopy at pH 7.6 and pH 9.5 (Fig. S3). Oxidation of $\text{Fe}^{\text{II}}\text{-NO}$ by O_2 occurred as a single step. Observed rate constants were derived by fitting the data to a first-order exponential model. $\text{Fe}^{\text{II}}\text{-NO}$ complexes were stable at both pHs, with rates in the following rank order: iNOS-mesohaem pH 7.6 = $8.89 \pm 0.43 \text{ s}^{-1}$ > iNOS-mesohaem pH 9.5 = $5.08 \pm 0.09 \text{ s}^{-1}$ > W188H-mesohaem pH 7.6 = $4.68 \pm 0.05 \text{ s}^{-1}$ > W188H-mesohaem pH 9.5 = $2.89 \pm 0.16 \text{ s}^{-1}$. At both pHs, the $\text{Fe}^{\text{II}}\text{-NO}$ complex of W188Hoxymesohaem reacted more slowly with O_2 compared to wild type iNOSoxymesohaem, and more generally, the mesohaem-containing proteins displayed a faster conversion of $\text{Fe}^{\text{II}}\text{-NO}$ into Fe^{III} compared to the native-haem containing versions (iNOSoxy $k_{ox} = 3.11 \text{ s}^{-1}$ at pH 7.6)[43]. Overall, the $\text{Fe}^{\text{II}}\text{-NO}$ oxidation rates by dioxygen in mutant W188H-mesohaem are ~ 2-fold slower compared to wild type iNOS-mesohaem at both pH 7.6 and pH 9.5. This decreased k_{ox} rate is nonetheless relatively fast compared to the predicted haem reduction rate for W188H-mesohaem (0.3 s^{-1}), thus of predictably little effect on the enzyme's steady-state behaviour and NO synthesis activity.

Haem reduction rates of iNOS and W188H containing native haem (k_r)

The measured haem reduction rates for iNOS and W188H under our experimental conditions were $k_r = 0.5 \pm 0.03 \text{ s}^{-1}$ and $1.79 \pm 0.03 \text{ s}^{-1}$, respectively (Fig. S4). The k_r values reported for iNOS under similar conditions are 0.6 to 1.2 s^{-1} [20, 42, 43]. Poor expression yields of the corresponding full length mesohaem-containing proteins prevented our measuring their haem k_r , therefore, estimated values were calculated by extrapolation of k_r values from a previous study performed with nNOS, where k_r for nNOS containing native or mesohaem were: nNOS native = 6.6 s^{-1} , nNOS mesohaem = 1.1 s^{-1} [12]. Assuming a similar trend would apply to iNOS, we estimated values of k_r would be 0.1 s^{-1} and 0.3 s^{-1} for iNOS-mesohaem and W188H-mesohaem, respectively. While assumptions carry an intrinsic limitation, it is unlikely that heme reduction rates in iNOS will deviate substantially from the trend observed in nNOS for two fundamental reasons: a) the heme electronic environment and the surface amino acid residues involved in reductase-oxygenase interactions necessary for electron transfer are highly conserved in both enzymes [44] and b)

x-ray structural analysis presented herein indicates that substitution of native haem with mesohaem in iNOSoxy did not alter the bonding interactions around the porphyrin moiety where reduction by the reductase domain would occur. We utilised these estimates of k_r to model steady-state reactions that otherwise could not be studied experimentally.

Steady State Analysis of W188H Carrying Native Haem

The enzyme species formed under steady state conditions were examined in full length W188H in the presence of L-Arg, H₄B and NADPH. Spectra collected during 2 minutes (300 scans) (Fig. S5, panel A) were subjected to global analysis using Specfit software as described earlier. The reaction could be best fit to a 3-exponential sequential model, with Fe^{III} as the initial and final species, and Fe^{II} and inert product P as the other two detectable haem species (Fig. S5, panel B). This is the first study to demonstrate the formation of species P in a NOS enzyme under steady-state conditions. The long-life (Fig. S5, panel C) and spectral features of species P (Fig. S5, panels B and D) are consistent with those observed during single turnover reactions with L-Arg and NOHA, thus confirming that product P forms downstream of the Fe^{II}O₂ intermediate in both phases of oxygen activation in NOS catalysis.

Steady State Simulations of Wild Type and W188H iNOS

To examine the predicted partition of enzyme species under our experimental conditions, the experimental kinetic parameters obtained for native and mesohaem-containing wild type and W188H iNOS (Tables S2 and S3) were submitted to Gepasi software version 3.30 pre-loaded with our global model for NOS catalysis as described in Experimental Procedures. The results of these simulations are presented in Fig. 9. Substitution of mesohaem significantly increased the partition of the respective enzymes toward the ferric state, Fe^{III}, and reduced the build-up of the Fe^{II}O₂ intermediate. Replacement of native haem with mesohaem in W188H restored the relative partition of enzymes species to a pattern that resembles that of wild type iNOS, i.e., it led to a substantial decrease in build-up of species P, with the concomitant stabilization of ferric enzyme. While we observe Fe^{III}-NO complex formation during single turnover reactions with NOHA in W188H, this species is unlikely to build up under steady-state conditions where product P is the predominant species when L-Arg is used as the substrate and NADPH as the source of electrons (Fig. S5). Our global model for W188H was built to depict this experimental finding (Fig. S7). We next ran simulations of the steady-state reactions for all four proteins utilized in this study (Figs. S6 and S7). We found that the relative distribution of each enzyme species is consistent with their different kinetic parameters and is sensitive to mesohaem replacement both in wild type and in W188H iNOS (Fig. 9).

CONCLUSIONS

We employed two different strategies to investigate the kinetic behaviour of iNOS in depth: i) Mutation of the proximal tryptophan residue Trp188 to histidine and b) Replacement of native protohaem with the electron- rich mesohaem. The kinetic behaviour of W188Hoxymesohaem with NOHA and H₄B differed from its native counterpart in two ways: a) A Fe^{II}O₂ species could not be detected during single turnover reactions, and b) A stable Fe^{III}-

NO was observed, for the first time. The reduced stability of the $\text{Fe}^{\text{II}}\text{O}_2$ species, also observed in the half-reaction with L-Arg has been noted in the case of nNOS substituted with mesohaem [12]. Build-up of the $\text{Fe}^{\text{III}}\text{NO}$ complex was faster in W188Hoxy-mesohaem compared to wild type iNOSoxy-mesohaem, and the same was true for its transition to Fe^{III} . This is favourable for NO synthesis in W188H, by diminishing diversion of the available NO into the unproductive NO-bound form of the enzyme [7, 41], but not sufficient to account for its greater NO synthesis performance compared to its native counterpart. A similar relationship has been identified in nNOS mutant W409F [6, 7], *B. subtilis* mutant W66F [10] and in nNOS harbouring Fe-mesoporphyrin IX [12], all of which display reduced haem midpoint potentials compared to their respective native or wild type counterparts. While our results support the recent proposal that structural changes are partly responsible for the build-up of species P in iNOS W188H [11], the remarkable effect of mesohaem insertion in W188H leading to partial restoration of catalytic activity points to haem electronics as a key factor in controlling the formation and lifetime of species P. This study advanced our understanding of the nature of species P by demonstrating that: 1) the heme midpoint potential is a fundamental contributor to the formation of species P, 2) species P forms during steady-state conditions, and 3) decreasing the buildup of species P leads to an increase in NO synthesis by W188H. From a mechanistic standpoint, we conclude that species P is a hydroxo-heme ligated state that halts the reaction at a new point, competing with both the productive and futile cycles of NOS.

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Abbreviations

DTT	dithiothreitol
EPPS	4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid
CHES	N-Cyclohexyl-2-aminoethanesulfonic acid
H₄B	(6R)-5,6,7,8-tetrahydro-L-biopterin
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NOS	nitric oxide synthase
eNOS	endothelial nitric-oxide synthase
iNOS	inducible nitric-oxide synthase
nNOS	neuronal nitric-oxide synthase

wt wild-type

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Summary statement

Wild type inducible NOS oxygenase and mutant W188H were substituted with the electron rich analogue mesohaem. The absence of structural changes upon mesohaem replacement and the decrease in the enzyme's midpoint potential permitted the dissection of electronic and structural effects.

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Fig. 1. Model for catalysis by mammalian NOS

Ferric haem is first reduced by NADPH via intramolecular electron transfer from the reductase domain. Ferrous haem binds O₂ to form Fe^{II}O₂. This haem-oxy complex rearranges to form a short-lived Fe^{III}-O-O⁻ species. A second electron derived from H₄B generates a ferric peroxo species and the corresponding pterin radical, H₄B⁺. The second semi-reaction that converts NOHA into citrulline and NO is less well understood; however, some of the reaction intermediates are common to the L-Arg hydroxylation pathway.

Fig. 2. X-ray crystal structure of iNOSoxy 65 with mesohaem (PDB 4JS9)

Panel A: The structures of native iNOSoxy 65 (PDB 1NOD, grey) and iNOSoxy 65-mesohaem (magenta) are superimposed and with backbones shown as ribbons. Native haem (orange), mesohaem (cyan) and H₄B groups from native iNOSoxy 65 (blue) and iNOSoxy 65-mesohaem (yellow) are shown as sticks. Panel B: A close-up of the iNOSoxy 65-mesohaem active site highlights the spatial arrangement of haem and H₄B groups (coloured as in panel A). Panel C: Electron density for ligands in the X-ray crystal structure of iNOSoxy 65 with mesohaem (PDB 4JS9). The structure of iNOSoxy 65-mesohaem (magenta) is shown as ribbons while mesohaem (cyan) and H₄B groups (yellow) are shown as sticks. Electron density (2Fo-Fc, drawn at 1 σ) is drawn as a mesh (grey). A proposed location for L-Arg (not included in the deposited coordinates) places the backbone and

guanidinium groups into two areas of weak electron density situated above the haem iron and is consistent with the position of L-Arg in native iNOSoxy structure (PDB 1NSI).

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Fig. 3. Determination of the midpoint potentials of wild type iNOSoxy and mutant W188Hoxo substituted with mesohaem

The fraction of oxidized protein at each redox potential upon titration with dithionite was fitted to the Nernst equation. The calculated values for the midpoint potentials are (Panels A and B, respectively): iNOSoxy-mesohaem: $-295 \pm 3 \text{ mV}$ and W188Hoxo-mesohaem: $-239 \pm 6 \text{ mV}$.

Fig. 4. UV-vis spectral properties of wild type iNOSoxy and mutant W188Hoxy substituted with mesohaem, in the presence of H_4B and L-Arg

Substitution with mesohaem led to a blue shift of ~ 12 nm in the UV-vis spectra of both wild type and W188Hoxy proteins. Panel A: Wild type iNOSoxy-mesohaem. Panel B:

W188Hoxy-mesohaem. Absorption maxima are provided in Table 2.

Fig. 5. Single turnover reactions in iNOSoxy-mesohaem with L-Arg and H₄B

Global analysis with SpecFit 3.0 software identified three distinct species: Fe(II) (Soret maximum ~402 nm), Fe-O₂ (Soret maximum ~ 415 nm) and Fe(III) (Soret maximum ~ 378 nm). Haem transitions occurred faster than in native iNOSoxy (Table 2), with a return to the resting state within 0.4 s. Panel A: Spectral changes observed during single turnover reactions. Panel B: Haem enzyme species identified by global analysis. Panel C: Time courses for each haem enzyme species calculated by global analysis.

Fig. 6. Single turnover reactions in W188Hoxo-mesohaem with L-Arg and H₄B

Panel A: Spectral changes observed for a 2 s reaction time. Panel B: Global analysis of the reaction kinetics identified an additional species (P + Fe^{III}) in addition to those observed in the single turnover reactions of wild type iNOSoxy-mesohaem. Species P previously identified during hydroxylation of L-Arg to NOHA appeared downstream of the Fe-O₂ complex, with an absorption maximum at 411 nm (shoulder). This inert enzyme species P has a shorter half-life (panels C and D) compared to the same reaction performed with native haem [3] and as a result, its net build-up is diminished. The enzyme returns to the resting, ferric state with an observed rate of 0.198 s⁻¹.

Fig. 7. Single turnover reactions in iNOSoxy-mesohaem with NOHA and H₄B

Global analysis of the spectral changes identified four species during the course of this reaction: Fe(II) (Soret maximum ~402 nm), Fe-O₂ (Soret maximum ~415 nm), Fe(III)-NO (Soret maximum ~431 nm) and Fe(III) (Soret maximum ~378 nm). Panel A: Species identified by global analysis. The inset to panel A shows authentic Fe^{III}-NO complex generated by addition of NO to anaerobic Fe^{III} iNOSoxy-mesohaem. Panel B: Time courses for the haem enzyme species calculated by global analysis. Panel C: Overlay of the experimental and global analysis curves for the time course of Fe(III)-NO species during single turnover reactions with NOHA and H₄B.

Fig. 8. Single turnover reactions in W188Hoxy-mesohaem with NOHA and H₄B

Panel A: Spectral changes observed during a 0.6 s reaction time. Panel B: Global analysis of the spectral changes showed an extremely short-lived Fe^{II}O₂ species, with three major observable haem transitions: Fe(II) (Soret maximum ~402 nm), Fe(III)-NO (Soret maximum ~418–422 nm) and Fe(III) (Soret maximum ~376–380 nm). Build-up of a Fe^{III}-NO complex was observed for the first time in the reactions of W188Hoxy, and it is thought to be a result of changes in the electronic environment of the haem. The inset to panel B shows authentic Fe^{III}-NO complex generated by addition of NO to anaerobic Fe^{III} W188Hoxy-mesohaem. Panel C: Time courses for each species calculated by global analysis. Panel D: Overlay of the experimental trace for the formation and disappearance of Fe(III)-NO complex and its calculated fit obtained by global analysis.

	WT iNOS	WT iNOS-meso
Fe ^{III}	87 %	94 %
Fe ^{II}	0.3 %	0.2 %
Fe ^{II} O ₂	1.7 %	0.9 %
Fe ^{III} NO	10 %	4.6 %
Fe ^I NO	1 %	0.3 %

	W188H iNOS	W188H iNOS-meso
Fe ^{III}	15.4 %	95.3 %
Fe ^{II}	0.2 %	0.2 %
Fe ^{II} O ₂	7.7 %	1.2 %
P	76.7 %	3.3 %

Fig. 9. Partition of haem- enzyme species during the reactions of wild type and W188H iNOS simulated with Gepasi software version 3.3

The reactions of wild type iNOS are characterized by Fe^{III} as the predominant species, with a shorter-lived FeO₂ species in iNOS containing mesohaem compared to its native counterpart. In contrast, mutant W188H features the formation and build-up of an inert species, P, which competes with both the productive, NO –synthesis pathway and the futile cycle of iNOS. Incorporation of mesohaem into W188H reduces the formation of and accelerates the decay of enzyme species P enabling a larger fraction of the oxygen-activated substrate to be channelled into the productive cycle of NO synthesis.

Table 1
Spectral properties of iNOS wild type and mutants W188H substituted with mesohaem

UV-vis absorption maxima in the presence of H4B and L-Arg or imidazole (Imid) of wild type iNOSoxy and W188Hoxo substituted with mesohaem in EPPS buffer (40 mM, pH 7.60) containing 150 mM NaCl and 10% glycerol.

	Soret (nm)	Visible (nm)
<i>iNOS-mesohaem^a</i>		
Fe(III)	381	506, 536, 642
Fe(III)-Imid	417	540
Fe(II)	404	549
Fe(II)-CO	431	545
Fe(II)-NO	422 (broad)	559
<i>W188H-mesohaem</i>		
Fe(III)	377	507, 536, 638
Fe(III)-Imid	414	540
Fe(II)	400	547
Fe(II)-CO	428 ^b , 413	546
Fe(II)-NO	420 (broad)	556

^aThe values for iNOS-mesohaem are in agreement with a previous study [24].

^bThe species at 428 nm shifts slowly to an uncharacterized species with absorption maximum at 413 nm.

Table 2

Single turnover reactions

Observed rate constants (s^{-1}) for haem transitions during single turnover reactions in wild type iNOSoxy and W188Hoxy substituted with mesohaem. Values for native and mesohaem-containing nNOS are provided for comparative purposes.

iNOS				
	$Fe^{(II)} \xrightarrow{k_1} FeO_2 \xrightarrow{k_2} Fe^{(III)}$	$Fe^{(II)} \xrightarrow{k_1} FeO_2 \xrightarrow{k_2} Fe^{(III)}$	$Fe^{(II)} \xrightarrow{k_1} FeO_2 \xrightarrow{k_2} Fe^{(III)}$	Reference
	L-Arg/H4B		NOHA/H4B	
iNOSoxy	52.7 ± 2.2 ; 12.5 ± 0.2^a		47.3 ± 3.1 ; 36.7 ± 1.8 ; 2.30 ± 0.09	This work
iNOSoxy-mesohaem	37.8 ± 2.3 ; 22.9 ± 0.8		35.6 ± 0.6 ; 18.1 ± 0.2 ; 1.70 ± 0.12	This work
	$Fe^{(II)} \xrightarrow{k_1} FeO_2 \xrightarrow{k_2} Int \xrightarrow{k_3} Fe^{(III)}$	$Fe^{(II)} \xrightarrow{k_1} FeO_2 \xrightarrow{k_2} Fe^{(III)}$	$Fe^{(II)} \xrightarrow{k_1} FeO_2 \xrightarrow{k_2} Fe^{(III)}$ Or $Fe^{(II)} \xrightarrow{k_1} FeNO \xrightarrow{k_2} Fe^{(III)}$	
W188Hoxy	46.0 ± 1.1 ; 2.03 ± 0.04 ; 0.104 ± 0.003^b		43.1 ± 2.1 ; 2.97 ± 0.27 ; 0.239 ± 0.010	This work
W188Hoxy-mesohaem	45.3 ± 8.6 ; 10.5 ± 0.9 ; 0.198 ± 0.06		37.7 ± 1.2 ; 5.75 ± 0.08^c	This work
nNOS				
nNOSoxy	$183 \pm 11 s^{-1}$ $17.5 \pm 0.2 s^{-1}$		$133 \pm 6 s^{-1}$ $23.8 \pm 0.4 s^{-1}$ $5.1 \pm 0.1 s^{-1}$	[12]
nNOSoxy-meso	$168 \pm 7 s^{-1}$ $52.1 \pm 0.7 s^{-1}$		$153 \pm 13 s^{-1}$ $117 \pm 6 s^{-1}$ $8.2 \pm 0.1 s^{-1}$	[12]

^{a,b}Values taken from reference [3].

^cThe FeO2 species could not be observed during single turnover reactions of W188Hoxy with NOHA and H4B.