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COMPARISON OF OXYGEN-INDUCED RADICAL INTERMEDIATES IN iNOS OXYGENASE DOMAIN WITH THOSE FROM nNOS and eNOS

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Abstract

Inducible nitric-oxide synthase (iNOS) produces the reactive oxygen and nitrogen species (ROS/RNS) involved in bacteria killing and is crucial in the host defense mechanism. However, high level ROS/RNS can also be detrimental to normal cells and thus their production has to be tightly controlled. Availability or deficiency of tetrahydrobiopterin (BH₄) cofactor and L-arginine substrate control coupling or uncoupling of NOS catalysis. Fully coupled reaction, with abundant BH₄ and L-arginine, produces NO whereas the uncoupled NOS (in the absence of BH₄ and/or L-arginine) generates ROS/RNS. In the current work we focus on direct rapid freeze EPR to characterize the structure and kinetics of oxygen-induced radical intermediates produced by ferrous inducible NOS oxygenase domain (iNOS_{ox}) in the presence or absence of BH₄ and/or L-arginine. Fully reconstituted iNOS_{ox} (+BH₄, +L-Arg) forms a dimer and yields a typical BH₄ radical that indicates coupled reaction. iNOS_{ox} (–BH₄) remains mainly monomeric and produces exclusively superoxide, that is only marginally affected by the presence of L-arginine. iNOS_{ox} (+BH₄, –L-Arg) exists as a monomer/dimer mixture and yields both BH₄ radical and superoxide. Present study is a natural extension of our previous work on the ferrous endothelial NOS_{ox} (eNOS_{ox}) [V. Berka, G. Wu, H.C. Yeh, G. Palmer, A.L. Tsai, *J. Biol. Chem.* 279 (2004) 32243–32251] and ferrous neuronal NOS_{ox} (nNOS_{ox}) [V. Berka, L.H. Wang, A.L. Tsai, *Biochemistry* 47 (2008) 405–420]. Overall, our data suggests different regulatory roles of L-arginine and BH₄ in the production of oxygen-induced radical intermediates in NOS isoforms which nicely serve individual functional role.

1. INTRODUCTION

Nitric oxide (NO) is a gaseous signaling molecule and its production is catalyzed by three isoforms of nitric oxide synthase (NOS): neuronal nitric oxide synthase (nNOS/NOSI), inducible NOS (iNOS/NOSII) and endothelial NOS (eNOS/NOSIII). Both nNOS and eNOS are constitutively expressed Ca²⁺-calmodulin dependent enzymes that produce nM levels of

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NO [1–3]. In contrast, expression of Ca^{2+} -independent iNOS, induced by inflammatory mediators, such as lipopolysaccharide (LPS) and cytokines, results in production of μM levels of NO [4, 5] to perform its bacterial killing function [6–11]. The difference in the NO output between eNOS/nNOS and iNOS points to possible differences in their reaction mechanism and deserves further investigation.

All three NOS isozymes catalyze NADPH-dependent conversion of substrate L-arginine and oxygen to NO and L-citrulline through 5-electron oxidation of N-atom of the guanidino group of L-arginine [12] [13]. The bioavailability of substrate L-arginine and cofactor BH_4 is absolutely essential for production of NO from all three NOS isoforms. L-arginine and/or BH_4 deficiency causes “uncoupling”, leading to production of superoxide [14–18] and/or hydrogen peroxide [19] instead of NO. In addition to NOS enzymes, L-arginine is also a substrate for arginase, another key enzyme involved in immunoregulation [20] leading to L-arginine deficiency for NOSs under certain pathological conditions [21–23]. Similarly, BH_4 deficiency, or more precisely the ratio between cellular BH_4 and its oxidized counterpart BH_2 , a competitive inhibitor of NOS, dictates the extent of NOS uncoupling, and is tightly associated with vascular dysfunction [24–26]. Thus, metabolic pathways that control the level of BH_4 and L-arginine play crucial role in regulation of coupling of NOS catalysis and determine the $\text{NO}/\text{O}_2^{\bullet-}$ ratio. While uncoupling could be beneficial for iNOS function in cytoprotection to generate potent product including peroxynitrite formed from NO and $\text{O}_2^{\bullet-}$, it is harmful for eNOS as it leads to vascular dysfunction and is detrimental to nNOS due to its high sensitivity to the ROS or RNS [27–30].

NOS isozymes are composed of two domains; an N-terminal heme-containing oxygenase domain and a C-terminal flavin-containing reductase domain, tethered by a Ca^{2+} /calmodulin-binding loop. Both the oxygenase and reductase domains of NOS can fold and function independently of one another but fully functional wild type (WT) NOSs are active only as homodimers [31, 32]. The BH_4 , and partially L-arginine, are important for dimerization of NOS enzymes, especially for dimerization of iNOS, as BH_4 -deficient iNOS is fully monomeric [33]. The iNOS dimers are less stable than the other two NOS enzymes and thus control of monomer/dimer equilibrium may be, on top of substrate and/or BH_4 availability, an additional factor that controls switching from NO to the ROS/RNS production in iNOS [12, 33].

In addition to the role in NOS dimerization, BH_4 plays a critical role in NO biosynthesis. The proposed mechanism for NOS catalysis, especially the first reaction of L-arginine hydroxylation is relatively well characterized [13, 34, 35] and consists of the following steps: in the NADPH driven reaction, ferric heme is reduced to ferrous form by electron transfer from reductase flavins; oxygen then binds to the ferrous heme to form oxy-ferrous ($\text{Fe}^{\text{II}}\text{O}_2$) intermediate, which then receives an electron from BH_4 to reduce $\text{Fe}^{\text{II}}\text{O}_2$ intermediate to form ferric peroxy intermediate and BH_4 itself is converted to an enzyme-bound cationic BH_4 radical. Several studies from different groups including us characterized spectroscopically the formation and decay kinetics of the $\text{Fe}^{\text{II}}\text{O}_2$ complex [36–43], as well as BH_4 radical formation in all three NOS isoforms [39, 44–46]. Recently published papers provide even deeper insights into understanding of the structure/function aspects of BH_4 radical [47–50]. Timely supply of this reducing equivalent is critical for coupling of the NO

formation since NOS Fe^(II)O₂ intermediates are unstable and may produce superoxide or H₂O₂ if electrons from BH₄ are not delivered to the heme at a sufficient rate or if both substrate and BH₄ are missing [16, 18, 51–55].

With focus on the first step of NOS catalysis, we initiated the single-turnover studies of interaction of ferrous eNOS_{ox} and nNOS_{ox} [46] [56] with oxygen using direct rapid freeze quench EPR (RFQ EPR) rather than indirect spin-trapping method, which provide neither direct structural nor kinetic information for the various radical intermediates. Single-turnover RFQ EPR allowed us to identify, in addition to typical BH₄ radical and superoxide, also novel radical species formed by eNOS_{ox} and nNOS_{ox} [40, 46, 56]. Moreover, the correlational spectral kinetic data enabled quantitative mechanistic interpretation. Our studies demonstrated that cofactor, substrate, and even reductant DTT affect the reactions and structures of radical intermediates of the two constitutive NOS enzymes differently [40, 46, 56].

In light of our previously identified differences between eNOS and nNOS together with the vastly different functional role and biological [NO] from eNOS/nNOS, we extended our study to iNOS_{ox} to reveal similarity and differences in reaction mechanisms of three NOSs. To address and to clarify the roles of BH₄, L-arginine, and DTT in radical formation during reaction of iNOS_{ox} with oxygen, we measured the kinetics of heme transitions and radical formation in the first half of NO synthesis by single-turnover stopped-flow and RFQ EPR.

2. EXPERIMENTAL PROCEDURES

2.1. Materials and General Methods

All reagents and kits for DNA extraction and isolation were products of Qiagen (Valencia, CA). BH₄ and BH₂ were obtained from Schircks Laboratories (Jona, Switzerland). Restriction enzymes were purchased from New England Biolabs (Beverly, MA). The One Shot[®] BL21 Star[™] (DE3) cells were obtained from Invitrogen (Carlsbad, Ca). Ni-NTA Agarose was obtained from Qiagen (Valencia, CA). The other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

2.2. Protein Expression and Purification of human iNOS_{ox}

Human iNOS_{ox} (amino acid 59–504) containing six-histidine (6His) at the C-terminus was expressed in *Escherichia coli* BL21 Star[™] (DE3) using pC_Wori vector. Human iNOS cDNA in pBlueScript (SK(-))TA plasmid, kindly provided by Professor Timothy R. Billiar (University of Pittsburgh), was used as a template for PCR amplification of DNA fragment encoding calmodulin-free oxygenase domain, amino acids 59–504. The iNOS_{ox} domain forward primer 5'-AGGCATATGCTCGTGGAGACGGGAAAG-3' (translation start codon is underlined) also included the *Nde*I (in italics) site. The reverse primer 5'-CCGCAAGCTTTCAGTGATGGTGATGATGGTGCTCGTCCTGCCAGACATGGG-3' has added *Hind*III restriction site (in italics) after the stop codon (underlined) and 6His tag (in bold). The PCR product was first ligated into the TA vector (Invitrogen, Carlsbad, CA) and then introduced into XL10 Gold *Escherichia coli* cells for propagation according to the manufacturer's recommendation. The sequence of iNOS_{ox}-6His DNA was confirmed by a

primer extension sequencing (UT-HSC sequencing Core). The iNOS_{ox}-6His DNA was excised from the plasmid by double-digestion with *Nde*I and *Hind*III restriction enzymes and subsequently ligated into the corresponding sites of the pCW vector. Purified pCW-iNOS_{ox}-6His plasmid was transformed into *E. coli* BL21 StarTM (DE3) cells containing the pT-groE plasmid [57] with a chloramphenicol-resistance gene for protein expression.

The expression and purification of human iNOS_{ox} in the absence of BH₄ was performed similarly as we described previously for nNOS_{ox} [56] using 50 mM HEPES, 0.1 M NaCl, 10 % glycerol, pH 7.8 without DTT as our purification buffer. Our established bacterial expression system for human iNOS_{ox} domain yielded ~15 mg of the purified protein per liter of culture medium, with ~90 % purity, judged by the ratio of A₂₈₀/A₃₉₆, SDS-PAGE analysis and the heme content by pyridine hemochrome assay. For the experiments with BH₄, BH₂ and/or L-arginine, freshly isolated iNOS_{ox} was incubated overnight with 1 mM of biopterin at desired redox state, 1mM β-mercaptoethanol and/or 1mM L-arginine under anaerobic conditions and then stored in liquid nitrogen container. Unless otherwise indicated, excess of biopterin and ligands was removed before the experiments by passing through a 10-DG gel-filtration column.

2.3. Stopped-Flow Experiments

Kinetics of the reaction between Fe^(II) iNOS_{ox} and oxygen were conducted at ambient temperature (22 °C) using an Applied Photophysics (Leatherhead, United Kingdom) Bio-Sequential SX-18MV stopped-flow instrument equipped with a rapid-scan diode array detector as previously described [46]. The sample handling and signal detection units were located in the anaerobic chamber filled with 5% H₂ in N₂ pre-filtered with a palladium-based O₂ scrubber. During sample handling and kinetic measurements the gas analyzer (Model 10, Coy Laboratory Products, Inc.) tracked the anaerobic conditions to ensure that oxygen level was 0 ppm. Measurements were carried out using 8–10 μM ferrous enzymes and monitored by diode array detector or at different wavelengths as detailed in the Results section. Anaerobic ferrous samples were prepared in a tonometer with three 5-minute cycles of vacuum and argon replacement followed by stoichiometric titration using anaerobic sodium dithionite solution [58]. Extra care was taken to avoid excess dithionite addition by monitoring the absorbance at 315 nm ($\epsilon_{315\text{ nm}} = 8\text{ mM}^{-1}\text{cm}^{-1}$) [59]. The rates were calculated by fitting the obtained data to a single- or a double- exponential function, based on the profile of the kinetic data.

2.4. Quantitation of Superoxide

The superoxide released from the iNOS_{ox} sample during single-turnover reaction of ferrous iNOS_{ox} (10 μM before reaction) with oxygen (as air saturated buffer, ~240 μM of oxygen at 22 °C) was measured by the cytochrome *c* (at 50 μM) trapping reaction, and was detected by stopped-flow for 5 sec. The total amount of superoxide was calculated using difference extinction coefficient of 21 mM⁻¹ cm⁻¹ at 550 nm [60] at the end of reaction. The optical change at 550 nm during oxidation of ferrous iNOS_{ox} with oxygen in the absence of cytochrome *c* was used as a baseline.

2.5. Hydrogen peroxide detection

The Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine, Molecular Probes), in combination with horseradish peroxidase (HRP), has been used to detect H_2O_2 released from different iNOS_{ox} samples. 3 ml solution of anaerobic ferrous iNOS_{ox} (10 μM) in 50 mM HEPES buffer, 0.1 mM NaCl, 10 % glycerol in tonometer was oxidized by adding 30 μl of aerobic buffer containing 5U of HRP and 20 μM Amplex Red. Resorufin, the red oxidation product of Amplex Red due to H_2O_2 formed by iNOS_{ox} was detected spectrophotometrically by 8453 Hewlett-Packard diode array spectrophotometer 15 sec after mixing. The total amount of hydrogen peroxide was calculated from difference extinction coefficient of 58 $\text{mM}^{-1} \text{cm}^{-1}$ at 571 nm between ferric iNOS_{ox} and peak of resorufin.

2.6. Gel filtration Chromatography

Molecular weight of iNOS_{ox} proteins was estimated by size exclusion chromatography using a Bio-Rad Bio-Sil TSK-250 column. The column was pre-equilibrated with 50 mM HEPES buffer (pH 7.5), containing 10 % glycerol and 0.1 M NaCl at 22 °C. Equal amounts of iNOS_{ox} were injected through a 100 μl loading loop. The isocratic flow rate was 1 ml/min and the column eluate was monitored at 280 nm using Agilent 1100 series UV flow through detector. Molecular weights of protein peaks were estimated relative to protein molecular standards from Sigma Molecular Weight Marker Kit.

2.7. RFQ EPR Kinetic Measurements

Ferrous iNOS_{ox} proteins were prepared as described previously [58]. Briefly, solutions containing 40–50 μM of iNOS_{ox}, bipterin free or BH_4 -reconstituted, in a HEPES buffer (50 mM HEPES, pH 7.5, 0.1 M NaCl, 10% glycerol) was reduced by anaerobic titration with a minimal amount of dithionite, in the presence or absence of 1 mM L-arginine, to shift the heme Soret peak to 413 nm, a marker for ferrous iNOS_{ox} formation. The ferrous iNOS_{ox} was then reacted with air-saturated buffer. Samples were collected for EPR analysis at different reaction time points by the RFQ apparatus (Update Instrument System 1000, Madison, WI) placed inside an anaerobic chamber (Coy Laboratory, MI). All liquid channels including sample syringes, tubings and mixers were kept inside the anaerobic chamber and only the exit end of the nozzle was exposed to air and was unplugged right before the shots. Single push program was used to obtain samples freeze-trapped with reaction time controlled by the length of tubing. At a ram velocity of 1.25 cm/s, the dead time is ~7 ms at room temperature determined by myoglobin azide reaction [61].

2.8. EPR Spectrometry

EPR spectra were recorded at liquid helium or liquid nitrogen temperature on a Bruker EMX EPR spectrometer. For liquid helium system, a GFS600 transfer line and an ITC503 temperature controller were used to maintain the temperature. An Oxford ESR900 cryostat was used to accommodate the sample. For measurements at liquid nitrogen temperature, a silver-coated double-jacketed glass transfer line and a BVT3000 temperature controller were used. Data analysis was conducted using WinEPR program provided by Bruker. Radical spin concentration was routinely quantified by double integration against a copper standard [62].

Progressive power saturation of each radical intermediate was conducted as previously described and data obtained were fitted to:

$$\log(S/P^{1/2}) = -b/2\log(P_{1/2}+P) + b/2\log(P_{1/2}) + \log(K) \quad [1]$$

where $P_{1/2}$ is the power to achieve half-saturation of the signal, b is 1–3 from pure inhomogeneous to pure homogeneous broadening and K is a proportional factor [63].

3. RESULTS

3.1. Stopped-Flow Detection of Transient Species during Single-Turnover Reaction of Ferrous iNOS_{ox} with Oxygen

It is well documented that fully reduced ferrous iNOS_{ox} (with typical Soret peak at 413 nm) binds oxygen rapidly and transiently forms the oxy-ferrous complex (peak at ~ 428 nm), which slowly decays to the original ferric form [36, 39]. This single turnover reaction of ferrous iNOS_{ox} with oxygen follows a simple scheme: $\text{Fe}^{(\text{II})} + \text{O}_2 \leftrightarrow \text{Fe}^{(\text{II})} - \text{O}_2 \rightarrow \text{Fe}^{(\text{III})} + \text{O}_2^-$. We monitored this reaction by two complementary methods: diode array stopped-flow and RFQ EPR technique in the presence or absence of substrate L-arginine and BH₄ cofactor (Fig. 1 – Fig. 4).

In the first experiment (+BH₄, +L-Arg), 10 μM ferrous iNOS_{ox} in the presence of 0.5 mM L-arginine and freshly added 10 μM BH₄ was rapidly mixed with an equal volume of air-saturated buffer. The presence of two sets of isosbestic points indicated two sequential chemical steps. An intermediate with 428 nm peak corresponds to oxy-ferrous complex (Fig 1. inset). iNOS_{ox} Fe^(II)O₂ intermediate was formed within 10 ms after mixing and further converted to the high-spin ferric form with Soret peak at 396 nm within 1s. Global analyses of these kinetic data indicated a best fit to an $A \leftrightarrow B \rightarrow C$ kinetic model, similar to that observed previously for eNOS_{ox} and nNOS_{ox} [46, 56]. The optical spectra of three species as resolved by global analysis are: A - original ferrous form with λ_{max} at 413 nm, B - oxy-ferrous intermediate at λ_{max} 428 nm, and C - final stage ferric form with λ_{max} peak at 396 nm (Fig. 1, left). Kinetics of the Fe^(II), Fe^(II)O₂ and Fe^(III) species also resolved by global analysis (Fig. 1 right) gave estimated rates for oxy-ferrous formation and decay to ferric form at 106 and 18 s⁻¹, respectively (Table 1).

Identical experiments were performed with ferrous iNOS_{ox} (+BH₄, –L-Arg). Diode array spectra showed only a partially formed intermediate with optical absorption maximum at 420 nm (Fig. 2, inset) suggesting that the oxy-ferrous intermediate is too transient to fully develop during rapid scan kinetic measurement and very likely represent a mixture of oxy-ferrous and ferric form of protein. However, de-convolution of the collected data by global analysis using the $A \leftrightarrow B \rightarrow C \rightarrow D$ kinetic model instead of previously used $A \leftrightarrow B \rightarrow C$, with two stages from oxy-ferrous complex (B) to ferric iNOS_{ox} (C and D) yielded the four spectra (Fig. 2, left panel): A - starting ferrous enzyme with λ_{max} at 413 nm, B - oxy-ferrous intermediate with λ_{max} at 428 nm, C - high-spin ferric iNOS_{ox} with λ_{max} at 398 nm and D - ferric iNOS_{ox} with λ_{max} at 403 nm. Kinetics for A, B, C, D species were also resolved by global analysis (Fig. 2, right panel). Estimated rate for oxy-ferrous formation was 108 s⁻¹ and rates for the two-stage decay to ferric form were 54 and 2.6 s⁻¹ (Table 1). The slow

shift, 2.6 s^{-1} , from 398 nm peak to 403 nm indicates that lacking L-arginine, iNOS_{ox} heme pocket is relatively open, allowing water molecule to ligate the heme iron of a portion of the iNOS_{ox} molecules and producing a mixture of high- and low-spin ferric iNOS_{ox}.

Ferrous iNOS_{ox} (–BH₄, –L-Arg) deficient of both BH₄ and L-arginine, when reacted with O₂, again only partially converted to oxy-ferrous complex (422 nm) (Fig. 3). However, the rapid scan stopped-flow was not able to record any kinetics of oxy-ferrous complex formation, which was essentially completed in the dead time of our stopped-flow instrument. Nevertheless, diode array rapid scan was able to reliably capture the decay from oxy ferrous intermediate to ferric iNOS_{ox} manifested as a spectral shift from 422 nm to 419 nm (Fig. 3 inset). This spectral shift to 419 nm, instead of typical five coordinate high-spin ferric heme iNOS_{ox} (396 nm), indicates that the distal side of the heme iron in the BH₄- and L-arginine-free enzyme was likely occupied by a water to form a six coordinate low-spin ferric heme iNOS_{ox}. Global analysis of obtained spectra, using the model $A \leftrightarrow B \rightarrow C$, was able to predict oxy ferrous intermediate (B) at 426 nm and initial ferrous iNOS_{ox} (A) at 413 nm (Fig. 3, left panel). The kinetics of A, B and C were also resolved by global analysis (Fig. 3, right panel). During our experiments, estimated rate constant for formation of putative oxy-ferrous complex (B, observed at 422 nm) was 400 s^{-1} based on the $\sim 1.5 \text{ ms}$ dead time of our stopped-flow, and rate of decay to ferric form C (419 nm) was monophasic with estimated rate constant at 7.3 s^{-1} (Table 1).

The rapid scan stopped-flow data of iNOS_{ox} (–BH₄, +L-Arg) showed that build-up of oxy-ferrous complex was very fast, again almost finished in the $\sim 1.5 \text{ ms}$ dead time of the stopped-flow. However, the presence of L-arginine stabilized oxy-ferrous intermediate exhibiting λ_{max} at 428 nm, which decayed to final ferric form with λ_{max} at 420 nm (Fig. 4 inset). The $A \leftrightarrow B \rightarrow C$ analysis, with fixed $k_1 = 400 \text{ s}^{-1}$, revealed a beginning ferrous iNOS_{ox} with Soret peak at 413 nm, an oxy-ferrous intermediate at 428 nm and the final water-coordinated low-spin ferric form at 420 nm (Fig. 4, left panel). Thus, the presence of L-arginine in iNOS_{ox}(–BH₄) caused a bathochromic shift in the Soret peak of oxy-ferrous species relative to those observed in the iNOS_{ox} (–BH₄, –L-Arg) (422 nm vs 428 nm) and also slowed down the rate of oxy-ferrous decay to ferric iNOS_{ox} from 7.3 to 3.3 s^{-1} (Fig. 4, right panel and Table 1). The analysis of rate constants indicates that even in the presence of L-arginine, iNOS_{ox} reacts extremely fast with oxygen. However, monophasic decay rate of intermediate B (at 428 nm) to final ferric form C points to homogenous population of iNOS_{ox} (–BH₄, +L-Arg). Comparison of iNOS_{ox} (+BH₄, +L-Arg) vs. iNOS_{ox} (–BH₄, +L-Arg) revealed not only differences in rates of oxy-ferrous heme formation (106 s^{-1} vs. $>400 \text{ s}^{-1}$) but also differences in decay rates (18 s^{-1} vs. 3.3 s^{-1}). The ~ 5 times faster decay observed with BH₄-reconstituted vs. BH₄-deficient iNOS_{ox} is in agreement with the previous reports about the ability of BH₄ to speed up the decay of oxy-ferrous complex in NOSs [36, 39].

The cofactor BH₄ not only provides one electron for L-arginine hydroxylation, but is also essential for iNOS_{ox} dimerization [33, 64–66], that is required for full coupling of iNOS catalysis. HPLC analyses of iNOS_{ox} samples confirmed that BH₄ is required for dimerization of iNOS_{ox} (Fig. 5). However, while iNOS_{ox} fully dimerized in the presence of BH₄ and L-arginine (Fig. 5, solid line), omission of L-arginine led to a monomer/dimer

mixture (Fig. 5, dash line). In the absence of BH_4 , iNOS_{ox} was present exclusively in monomeric form, independent of L-arginine (Fig. 5, dotted line).

3.2. Superoxide Production during Single Turnover Reaction of iNOS_{ox} with Oxygen

We next examined how the formation of superoxide, a primary product of uncoupling, is modulated by different conditions during a single turnover reaction. Superoxide can be oxidized by cytochrome c (cyt c), and thus production of superoxide by iNOS_{ox} was monitored at 550 nm as reduction of cyt c (Fig. 6). Experimental conditions were similar to those described in Fig. 1 – 4. The final concentration of iNOS_{ox} after reaction with air saturated buffer containing 50 μM cyt c was 5 μM . In the control experiments, we added also 5 U/mL of superoxide dismutase (SOD) that has a very high turnover rate for superoxide and prevents it from reacting with cyt c. A lack of absorbance increase at 550 nm (Fig. 6, dotted line) confirms that cyt c reduction observed in the experiments without SOD was indeed due to the superoxide release from iNOS_{ox} during reaction with oxygen. Small decrease in the absorbance at 550 nm in the experiment with SOD observed within the first 0.5 s (Fig. 6, dotted line) reflects optical changes corresponding to transition from ferrous to ferric iNOS_{ox} at 550 nm.

Percentages of superoxide production of iNOS_{ox} under different experimental conditions were calculated from the ratio of cyt c reduction to the amount of NOS heme and are listed in Table 1. The highest amounts of superoxide, ~ 80% and 91%, were detected in the single turnover reactions of iNOS_{ox} ($-\text{BH}_4$, $-\text{L-Arg}$) and iNOS_{ox} ($-\text{BH}_4$, $+\text{L-Arg}$), respectively. These data clearly indicate that in the absence of BH_4 , the main product of iNOS_{ox} is superoxide and substrate L-arginine has only a marginal effect. Small increase from 80% to 91% indicate that L-arginine-mediated stabilization of the oxy-ferrous intermediate (Fig. 3 vs. Fig. 4), which slows down the release of superoxide leading to more efficient superoxide interaction by cyt c before superoxide self-dismutation to H_2O_2 and O_2 . In the assumed coupled reaction, i.e., iNOS_{ox} ($+\text{BH}_4$, $+\text{L-Arg}$), no superoxide production was expected and indeed, less than 8% of superoxide was detected in our system (Table 1).

3.3. Production of Hydrogen peroxide during Single Turnover Reaction of Fe^{II} iNOS_{ox} with Oxygen

Several studies had suggested, that under the experimental conditions, $+\text{BH}_4$, $-\text{L-Arg}$, BH_4 provide electron to $\text{Fe}^{\text{II}}\text{O}_2$ complex yielding H_2O_2 as an alternative product of iNOS_{ox} instead of superoxide [51, 55, 67]. To test this uncoupling mechanism, we checked production of H_2O_2 by iNOS_{ox} (10 μM) using Amplex Red assay. Produced amount of H_2O_2 was detected spectrophotometrically by 8453 Hewlett-Packard diode array spectrophotometer. As expected, there was no detectable amount of H_2O_2 produced by the fully reconstituted iNOS_{ox} ($+\text{BH}_4$, $+\text{L-Arg}$) (Fig. 7A) and approximately 33% of H_2O_2 were produced by iNOS_{ox} ($+\text{BH}_4$, $-\text{L-Arg}$) (Fig. 7B, solid line). Surprisingly, a similar amount of H_2O_2 was produced by ferrous iNOS_{ox} ($-\text{BH}_4$, $\pm\text{L-Arg}$) (data not shown). This seemed at odds with the results of cyt c assay (Fig. 6) that showed mainly (80–91%) superoxide production. However, self-dismutation of superoxide is a rapid spontaneous process with the second order rate constant $3 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$ at pH 7.4 [68], thus we were not able to

distinguish by a rather slow Amplex red assay between H_2O_2 originated from either iNOS_{ox} (+ BH_4 , -L-Arg) uncoupling from $\text{Fe}^{\text{II}}\text{O}_2^-$ or via self-dismutation of superoxide.

3.4. EPR Characterization of Oxygen-Induced Radical Species of Ferrous iNOS_{ox}

iNOS_{ox} catalysis involves either BH_4 radical formation, heme oxidation, or superoxide production, detectable directly by RFQ EPR, depending on different coupling/uncoupling conditions (Fig. 8). The EPR spectra of samples freeze trapped at ~ 80 ms reaction time between 50 μM iNOS_{ox} and O_2 in the presence or absence of L-arginine or BH_4 recorded at 10 K showed clear differences in both the heme and radical signals. At fully-coupled condition (+ BH_4 , +L-Arg) (top spectrum), the evolving rhombic high-spin heme signals at $g = 7.88$ and 3.80 and a symmetric radical signal $g \sim 2$ are the dominant EPR features. Little low-spin ferric heme was observed, matching the dominant 396 nm Soret peak observed for the ferric heme species (Fig. 1). EPR for the sample containing BH_4 only (+ BH_4 , -L-Arg) (middle spectrum), ferric heme present at both rhombic high- and low-spin ($g = 7.88/3.80$ and $2.40/2.27$) and a symmetric radical signal were conspicuous, indicating a mixture of high- and low-spin heme formed together, corroborating with the 403 nm optical data of the evolving ferric heme in Fig. 2. For the reactions of iNOS_{ox} (- BH_4 , $\pm$ L-Arg) the EPR data showed dominant low-spin ($g = 2.41$ and 2.27) heme and a very big asymmetric radical signal ($g \sim 2$), typical for axial superoxide radical (bottom spectrum). The dominant low-spin heme matches the observed 419 nm peak for the ferric heme formation (Figs. 3 and 4).

To characterize in detail the EPR data of the various radical species observed in Fig. 8 at $g = 2$ region, EPR spectra of rapid freeze samples were recorded at various microwave power and 400 G range at 115 K (Fig. 9). iNOS_{ox} (+ BH_4 , +L-Arg) produced a typical BH_4 radical [39, 40, 44–46, 48] with 39 G wide symmetric EPR centered at $g = 2.0023$ (Fig. 9A) and a homogeneous microwave power dependence with a $P_{1/2} \sim 26$ mW (Table 2). Similar to eNOS_{ox} and nNOS_{ox} , iNOS_{ox} (- BH_4 , $\pm$ L-Arg) produced only superoxide radical (Fig. 9C) with a broad $g_{\parallel}$ (or g_z) = 2.080 and $g_{\perp}$ (or g_x/g_y) = 2.006 and the very high $P_{1/2} \sim 155$ mW (-L-Arg) and ~ 126 mW (+L-Arg) (Table 2), all typical features for an isolated superoxide anion radical (35). Thus, L-arginine had only a minimal effect on the chemical property of the radical intermediate. Ferrous iNOS_{ox} (+ BH_4 , -L-Arg) generated a radical intermediate with a 39 G wide spectrum centered at $g_{x/y} = 2.005$ and an additional broad peak at $g_z = 2.076$ and a broad trough at $g = 1.99$ (Fig. 9B). The microwave power dependence ($P_{1/2}$), calculated from the center peak of the derivative signal, was 37 mW (Table 2). After normalizing against their spin concentrations, subtracting of 40 % of the superoxide radical formed in the absence of L-arginine and BH_4 (spectra at Fig. 9C) from the EPR spectra obtained in the presence of BH_4 (Fig. 9B) yielded a radical spectra showing a 39 G wide symmetric spectrum centered at 2.002 (Fig. 9D). The EPR line shape and power saturation of the serial difference spectra ($P_{1/2} = 24$ mW) showed strong similarity with typical BH_4 radical of iNOS_{ox} (Fig. 9D vs. Fig. 9A). Thus the arithmetic treatment of the EPR spectra revealed that iNOS_{ox} (+ BH_4 , -L-Arg) probably produced a mixture of superoxide and BH_4 radical.

Because we had shown previously that radical formation by nNOS_{ox} (+ BH_4 , -L-Arg) is affected by DTT [56], we repeated the experiment with iNOS_{ox} in the presence of 1 mM

DTT. The oxygen-induced radical intermediate species produced by iNOS_{ox} (+BH₄, -L-Arg) were not affected by the presence of DTT and the obtained spectra were identical to those shown in Fig. 9B. The EPR parameters, the P_{1/2} and g values, for all iNOS_{ox} radical intermediates are summarized in Table 2 together with our previous data for eNOS_{ox} and nNOS_{ox} for comparison.

3.5. Correlation of Kinetics of Heme Redox Changes and Oxygen-Induced BH₄/superoxide Radical Intermediates in iNOS_{ox}

As mentioned earlier, a single turnover catalytic reaction of ferrous iNOS_{ox} with oxygen follows a simple reaction scheme: $\text{Fe}^{(\text{II})} + \text{O}_2 \leftrightarrow \text{Fe}^{(\text{II})} - \text{O}_2 \rightarrow \text{Fe}^{(\text{III})} + \text{O}_2^{\bullet-}$. The kinetics of heme oxidation measured by stopped-flow were compared with kinetics of 1) heme oxidation state detected by RFQ EPR as high spin $g = 7.88$ or low spin $g = 2.27$ signal at 10 K; and 2) BH₄ and/or superoxide radicals obtained by rapid freeze experiments and detected by EPR as $g = 2$ signal at 115 K. Table 1 summarizes the rate constants of iNOS_{ox} obtained in the presence or absence of BH₄ and/or L-arginine.

A representative single wavelength stopped-flow and EPR kinetics of ferrous iNOS_{ox} (+BH₄, +L-Arg) during reaction with oxygen are shown in Figure 10. The rate constant of oxy-ferrous decay to the ferric form of iNOS_{ox} (396 nm) obtained by stopped-flow was 18 s⁻¹ (Fig. 10; dashed line). The BH₄ radical formation, as EPR signal change at $g = 2$, had a rate constant of 20 s⁻¹ and was followed by a slow decay of 0.8 s⁻¹ (Fig. 10, dotted line and Table 1). A rate constant of the ferric high-spin heme formation at $g = 7.88$ was 15 s⁻¹ (Fig. 10, solid line). These data confirms that decay of oxy-ferrous heme complex correlates nicely with the BH₄ radical formation and the formation of ferric heme in iNOS_{ox} (+BH₄, +L-Arg).

Stopped-flow data showed only putative formation of oxy-ferrous complex produced by ferrous iNOS_{ox} (-BH₄, -L-Arg) and iNOS_{ox} (-BH₄, +L-Arg) during reaction with oxygen (Figs. 3 and 4). Increases of absorbance at 419 nm represent decay from these complexes to low-spin ferric iNOS_{ox} (-BH₄, -L-Arg) (Fig. 11, dash line); data for iNOS_{ox} (-BH₄, +L-Arg) are very similar (not shown). The oxy-ferrous complex decay rates obtained by stopped-flow were 7.3 s⁻¹ and 3.3 s⁻¹, for these two iNOS_{ox} samples, respectively (Table 1). Kinetics of superoxide radical production by iNOS_{ox} (-BH₄, -L-Arg) and iNOS_{ox} (-BH₄, +L-Arg) obtained by RFQ EPR are also very similar for both samples; majority of the formation kinetics of superoxide radical was lost in the dead time of our rapid freeze apparatus, ~ 7 ms, and rates for radical decays were of 4.5 and 2.5 s⁻¹, respectively (Table 1 and Fig. 11 square symbols for representative kinetics). The total amount of detected superoxide at 8 ms, was less than ~30% compared to the amount of iNOS_{ox} heme. Significant amount of superoxide signal missing in the dead time of RFQ EPR probably due to self dismutation of superoxide to hydrogen peroxide and oxygen. The superoxide scavenger cyt c further decreased detectable superoxide by RFQ EPR to ~15% which decayed at 6.5 s⁻¹ (Fig. 11, circles). Loss of kinetic data of oxy-ferrous complex formation, heme oxidation (Fig. 11, stars symbols) as well as superoxide radical formation in the dead time of the instruments point to a very fast Fe^(II)O₂ bond cleavage in oxy-ferrous iNOS_{ox} (-BH₄, ±L-Arg) complexes. These data indicate that in monomeric, BH₄-deficient

iNOS_{ox} the heme pocket is wide open for immediate water ligation to the iron. The result of this process is a transient formation of high-spin (396 nm) ferric iNOS_{ox} undetectable by stopped-flow. Appearance of Soret peak at 419–420 nm represents final stage of conversion of high-spin to the terminal water-ligated low-spin ferric iNOS_{ox} (–BH₄, ±L-Arg) (Figs. 3, 4 and 8).

In iNOS_{ox} (+BH₄, –L-Arg), that produced unusual EPR spectra representing a mixture of BH₄ and superoxide radical (Fig. 9B), the RFQ kinetic measurements revealed a radical formation rate of 66 s^{–1} (Fig. 12, squares) and a heme oxidation rate of 70 s^{–1} (Fig. 12, stars). The 66 s^{–1} rate of formation of BH₄/superoxide radical mixture is faster than the 20 s^{–1} rate of BH₄ radical formation by iNOS_{ox} (+BH₄, +L-Arg) but slower than that of iNOS_{ox} (–BH₄, –L-Arg), which is not detectable by RFQ-EPR. The oxy-ferrous decay rate obtained by stopped-flow was biphasic with rates 54 s^{–1} (~ 85 %) and 2.6 s^{–1} (~15%) (Fig. 12, dash line and Table 1). Thus again, stopped-flow and RFQ data showed comparable rates of oxy-ferrous complex decay (54 s^{–1}) and radical formation (66 s^{–1}). As we described earlier (Figs. 2 and 8), the second slow face of oxy-ferrous decay (2.6 s^{–1}) represents conversion from five coordinate high-spin to partially water ligated low-spin ferric iNOS_{ox} (+BH₄, –L-Arg).

4. DISCUSSION

4.1. New information derived from our single turnover stopped-flow and RFQ EPR kinetic measurements of iNOS_{ox}

In our previous studies, we simulated physiological as well as pathophysiological conditions by supplying or depleting of BH₄ and L-arginine during the reactions of reduced eNOS_{ox} or nNOS_{ox} with oxygen [46, 56]. It was clear that the substrate and/or cofactor control the formation and decay of oxy-ferrous intermediate and various EPR detectable radical species from eNOS and nNOS oxygenase domains (Tables 2 and 3) [46, 56]. Thiol reductant also showed discrete effect on the oxygen-induced radical intermediates in the nNOS_{ox}.

In the current study, we focused on the study of iNOS_{ox}. To obtain direct structural and correlated kinetic information of the heme center, BH₄ cofactor and the various radical intermediates for individual steps of the catalysis in the presence or absence of BH₄ and/or L-arginine, single turnover rather than the steady-state conditions were used. Our stopped-flow data from iNOS_{ox} catalysis measured at 22 °C indicate that formation of fully developed oxy-ferrous intermediate, judged by absorbance change at 428 nm, is detectable only in the presence of L-arginine and BH₄ (Fig. 1). In the absence of BH₄, the rate of formation of Fe(II)O₂ was very fast (> 400 s^{–1}), and resulted in a Soret maximum between 422–428 nm (Figs. 3 and 4). This outcome expands our conclusion derived from the previous study of nNOS_{ox} and eNOS_{ox} further to iNOS_{ox}, that heme, L-arginine and BH₄ “triad” reinforces each other in rigidifying the heme pocket scaffold via H-bonding network and thus the Fe-O bond strength in the Fe(II)O₂ intermediate. The significance of BH₄ in NOS is further underscored by the faster decay rate of oxy-ferrous to ferric form in the presence of BH₄. The oxy-ferrous intermediate of all NOS isoforms is kinetically unstable and spontaneously decays to superoxide and ferric enzyme if it is not reduced in a timely manner [13]. Thus, faster reduction of the iNOS_{ox} oxy-ferrous intermediate in the presence

of BH₄ (18 s⁻¹) compared to BH₄-deficient iNOS_{ox} (3.3 s⁻¹) prevents the uncoupling of catalysis and emphasizes the role of BH₄ as an effective electron source to provide the second reducing equivalent in the monooxygenation reaction in NOS proteins.

EPR study of radical formation revealed that ferrous iNOS_{ox} is also capable of forming multiple radical intermediates as the other two NOS isoforms [46, 56] upon reaction with oxygen depending on the availability of L-arginine and BH₄. In addition to typical BH₄ radical formed by iNOS_{ox} (+BH₄, +L-Arg) or superoxide radical formed by iNOS_{ox} (-BH₄, ±L-Arg), iNOS_{ox} (+BH₄, -L-Arg) formed a mixture of BH₄ and superoxide radical. This is clearly a specific property of iNOS_{ox}, because eNOS_{ox} and nNOS_{ox} produced new radical intermediates under similar conditions (Table 2). The new wide 75 G radical formed by the nNOS_{ox} (+BH₄, -L-Arg) in the presence of thiol, detected by RFQ-EPR [56], was interpreted by pulsed EPR/ENDOR studies as the BH₄ radical signal broadened by an adjacent low-spin ferric heme via dipolar or/and exchange coupling (Krzyaniak, M.D., Bowman, M.K., et al, manuscript in preparation). This is in contrast to the typical 39 G BH₄ radical observed for fully coupled NOS (46, 56), which is magnetically coupled to a fast relaxing high-spin ferric heme (Krzyaniak, M.D., Bowman, M.K., et al, manuscript in preparation). Furthermore, in iNOS_{ox}(+BH₄, -L-Arg) w/o thiol there is no such magnetic interaction with a nearby low-spin ferric heme, since RFQ-EPR of iNOS_{ox}(+BH₄, -L-Arg) clearly showed a mixture of BH₄ and superoxide radical rather than the new wide 75 G radical formed by the nNOS_{ox} (+BH₄, -L-Arg) in the presence of thiol. This characteristic might be a consequence of the critical role of BH₄ in stabilization of NOS dimers, which is more prominent in iNOS_{ox} than in the constitutive NOS isozymes [33]. Interaction of BH₄ with the heme propionate and the helix α7a initiates dimer formation and the dimer is further reinforced through the interaction of dihydroxypropyl side chain interaction with the enzyme's N-terminal hook to create a tight active iNOS_{ox} dimer [69]. The role of BH₄ in iNOS_{ox} dimer reinforcement is significantly stronger than that of L-arginine [33]. However, the relative strength of all three NOS homodimers follows eNOS > nNOS > iNOS [33, 70] indicating that iNOS forms the least stable dimers and even the smallest decrease of BH₄ and/or L-arginine levels can lead to dissociation of the dimeric iNOS_{ox} and consequently to an uncoupling reaction (Table 1 and Table 2). This weaker interaction between two monomers in iNOS than nNOS also corroborates the EPR/ENDOR observation for nNOS_{ox}(+BH₄, -L-Arg) plus thiol, implying that the BH₄ radical formed interacting magnetically with the nearby low-spin heme center of the other subunit, whereas the distance is too long between BH₄ radical and the heme iron in iNOS_{ox}(+BH₄, -L-Arg) w/o thiol due to much looser dimer formation.

Data from all our experiments show that a fully reconstituted iNOS_{ox} (+BH₄, +L-Arg) forms dimer and only BH₄ radical in Fe^(II) iNOS_{ox} is induced by oxygen. In the absence of BH₄ ± L-arginine, iNOS_{ox} remains monomeric and produces superoxide up to 80% the amount of heme. In the presence of BH₄ only, iNOS_{ox} (+BH₄, -L-Arg) exists as a monomer/dimer mixture and thus, not unexpectedly, produces a mixture of BH₄ and superoxide radical (Fig. 7 and Table 2). Our data clearly show that in addition to the reductase domain of iNOS [15], iNOS oxygenase domain is also able to produce a large amount of superoxide when uncoupled by a lack of BH₄ or L-arginine. Earlier reports demonstrate that exogenous addition of superoxide to iNOS greatly decreases NO generation through BH₄ oxidation

associated with shift of the enzyme from the active dimer to the inactive monomer [71]. Thus, in correlation with our data, superoxide internally produced from iNOS_{ox} in the absence of BH₄ or L-arginine may damage protein itself and consequently increases the production of superoxide by iNOS even further. On the other hand, it is well known that the reaction of NO with superoxide generates the potent oxidizing peroxide, peroxynitrite (ONOO⁻) [11, 72]. Interestingly, ONOO⁻ induces the irreversible inactivation of iNOS and decreases both NO and O₂⁻ production [71].

We have shown here that Fe^(II)iNOS_{ox} forms only BH₄ or superoxide radical or a mixture of these two radicals upon reaction with oxygen (Scheme 1). The heme-BH₄-L-arginine triad interplay seems to dictate the monomer/dimer ratio as well as the identity and amount of the oxygen-induced radical species, an interesting and intricate structure/function relationship of NOS.

It is worth mentioning that WT NOS is active only as a homodimer and the electron transfer is conceivable between NOS reductase and oxygenase domain only in a “inter-molecular” fashion where electron transfer occurs between flavin and heme groups located on adjacent subunits in the dimer [73]. Nevertheless, engineered NIH 3T3 cells incapable of *de novo* BH₄ biosynthesis are able to express human iNOS, in a predominantly monomeric form inactive in NO synthesis, or an active dimers form in cytosols when supplemented with BH₄ alone [66]. In the absence of L-arginine and BH₄ the heme iron in an iNOS is solvent-exposed [64] and has a low midpoint potential (–347 mV), difficult to accept electrons from the reductase domain flavins FADH/FMNH₂ (–290 mV) [74]. On the other hand, binding of BH₄ or L-arginine alone increases the heme midpoint potential to more favorable –295 mV or –235 mV, respectively. In the presence of both BH₄ and L-arginine, these values in iNOS_{ox} domain and in WT iNOS are –263 mV and –270 mV, respectively [74, 75]. Thus, thermodynamically heme reduction should be achieved only in the presence of L-arginine and BH₄. However, a midpoint potential of –340 mV for NADPH can provide sufficient push, and is able to reduce NOS heme even in L-arginine and/or BH₄ deficient iNOS. Indeed, NADPH oxidation was observed for macrophage NOS in the absence of L-arginine, even though only ~16% relative to L-arginine bound NOS [76].

Based on iNOS_{ox} data presented here, it is possible to assume that part of heme containing monomeric WT iNOS can be reduced by NADPH and in the absence of L-arginine, iNOS produces superoxide instead of NO, thus justifies the biological significance in using isolated iNOS_{ox} to study the dynamics of oxygen-induced radical species. Moreover, inactive iNOS is not more preferentially degraded than active iNOS. Protein turnover rates of iNOS monomers and dimers are similar, with cellular half-life of 1.6 ± 0.3 h [77].

4.2. Comparison between eNOS_{ox}, nNOS_{ox}, and iNOS_{ox}

Although stopped-flow conducted at decreased temperature or protein concentration helped to slow down the reaction to acquire more kinetic data for oxy-ferrous heme formation [38, 42, 43, 78, 79], we decided to use identical temperature, 22 °C, and similar final protein concentration after $2 \times$ dilution in stopped-flow or $\sim 4 \times$ dilutions in rapid freeze EPR experiments to enable direct comparison of oxy-ferrous decay with heme oxidation and

radical formation, as well as comparison with data from other two NOS_{ox} isoforms. These comparative studies led to important conclusions at two different aspects.

4.2.1. Kinetics of oxygen-induced heme and radical changes—Our results, summarized in Table 3, indicate that the decay rates of oxy-ferrous intermediates are different among the three NOS_{ox} (+BH₄, +L-Arg) enzymes and display a rank of nNOS_{ox} (34.9 s⁻¹) > iNOS_{ox} (18.0 s⁻¹) > eNOS_{ox} (15.0 s⁻¹). The rate of BH₄ radical formation follow the identical trend, nNOS_{ox} (67.0 ± 10.5 s⁻¹) > iNOS_{ox} (20.0 ± 2.5 s⁻¹) > eNOS_{ox} (13.1 ± 2.7 s⁻¹). Our data obtained at 22 °C are in agreement with the previous results acquired at 10 °C [43]. Regardless of the intrinsic differences between NOS isozymes and huge differences in NO production, these data support the existence of a common kinetic mechanism for L-arginine hydroxylation in the three NOSs: the BH₄ radical forms only after O₂ binds to the ferrous heme and the rate of BH₄ radical formation matches the rate of oxy-ferrous disappearance in all three NOSs.

4.2.2. Structures of radical intermediates—The radical species from all three NOS isoforms obtained by rapid-freeze EPR are summarized in Table 2. Only nNOS_{ox}, not iNOS_{ox} or eNOS_{ox}, showed thiol-dependent radical EPR structural changes [56]. Under fully coupled conditions, i.e. +BH₄, +L-Arg, eNOS_{ox}, nNOS_{ox}, and iNOS_{ox} produce solely BH₄ radical with a 39 G wide symmetric spectrum and crossover g_x/g_y of 2.002 (Table 2, first column). There are only subtle differences among NOS_{ox} isozymes in the reaction between oxygen and ferrous NOS_{ox} (-BH₄, ±L-Arg). All three isozymes produce superoxide EPR with g_z of 2.077–2.08 and crossover g_x/g_y of 2.005–2.006. The EPR line shape and the very high P_{1/2} in the range of 54–155 mW are typical features of a superoxide radical (Table 2, second column). Only exception is the data from nNOS_{ox}(-BH₄, +L-Arg) where in addition to a typical superoxide radical our analysis of rapid-freeze EPR spectra revealed formation of a novel (38 gauss) radical with 3-line hyperfine structure, probably involving the guanidine nitrogen nucleus of L-arginine (Table 2, second column and [56]). Thus, L-arginine decreases the amount of superoxide produced by nNOS_{ox} by converting it to a new radical. The most interesting data were obtained in the presence of BH₄ and absence of L-arginine, NOS_{ox}(+BH₄, -L-Arg) (Table 2, last column and [46, 56]). Reconstitution with BH₄ only (-L-Arg) yields a novel BH₄-based radical in eNOS_{ox} and also a new 75G radical in DTT-containing nNOS_{ox}, as an ensemble of magnetically coupled BH₄ radical and a low-spin ferric heme. On the other hand, iNOS_{ox} and also nNOS_{ox} in the absence of DTT produce a mixture of superoxide and typical BH₄ radical that couples to a high-spin ferric heme. This gives the implication that DTT also enhances a tighter dimer formation in nNOS_{ox}.

4.2.3. Conclusions

In summary, our spectroscopic analyses showed that during normal catalytic cycle, iNOS_{ox} produces a typical BH₄ radical and consequently NO, whereas under uncoupling conditions it produces either superoxide radical alone or a mixture with BH₄ radical. No additional new radical was observed under any of the tested conditions. iNOS is unique for its potential in forming large (μM) quantities of both superoxide and NO and further the potent oxidizing peroxynitrite, ONOO⁻, efficient for killing pathogens. However, production of large

quantities of these radicals also induces disease conditions such as sepsis, oxidative stress, tissue damage, or chronic inflammatory diseases. One way to avoid these unwanted side-effects is to prevent the expression or to enhance degradation of iNOS itself due to its inductive nature and fast protein turnover, facilitated by its less rigid protein scaffold than nNOS and eNOS.

In contrast, nM quantities of NO produced by constitutively expressed eNOS and nNOS enzymes are sufficient to fulfill their physiological functions. Thus the need for a limited, tightly controlled production of NO from these two NOS isoforms is crucial and is particularly profound in the case of nNOS since even small changes in quantities of NO or superoxide is toxic to neuronal cells with decreased production of glutathione [80]. Thiols are also important to avoid NOS uncoupling and preserving NOS activity in three ways; 1) to prevent BH₄ oxidation [40] and 2) to enhance the monomer interaction in the dimer and 3) to prevent irreversible oxidation of surface cysteines [56]. The latter two findings are specific only for nNOS and further underscore the neuroprotective role of glutathione. The differences between iNOS and the constitutive NOS isoforms can be expanded also to explain how these enzymes handle unavoidable uncoupling due to L-arginine deficiency. A self-controlling mechanism evolved in eNOS and nNOS to reduce superoxide production by chemical conversion to a novel surrogate radical species involving BH₄ radical via magnetic interaction with an adjacent low-spin ferric heme, and such mechanisms are not employed by iNOS. The differences among all three isoforms are even more apparent under BH₄ deficiency, i.e., -BH₄, +L-Arg. While L-arginine has marginal effect on the rate or the amount of superoxide production by iNOS_{ox}, it decreases only the rate and not the total amount of superoxide radical produced in eNOS_{ox} [46], whereas L-arginine decreased superoxide production by forming a new radical in nNOS_{ox} [56].

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Abbreviations

NOS	nitric oxide synthase
iNOS_{ox}, eNOS_{ox}, nNOS_{ox}	the oxygenase domain of inducible, endothelial and neuronal NOSs, respectively
L-Arg	L-arginine
BH₄	(6 <i>R</i>)-5,6,7,8-tetrahydro-L-biopterin
BH₂	7,8-dihydro-L-biopterin
iNOS_{ox} (+BH₄,+L-Arg)	BH ₄ reconstituted and L-Arg containing iNOS _{ox}
iNOS_{ox} (+BH₄, -L-Arg)	BH ₄ reconstituted and L-Arg deficient iNOS _{ox}
iNOS_{ox} (-BH₄, -L-Arg)	BH ₄ and L-Arg deficient iNOS _{ox}
iNOS_{ox} (-BH₄,+L-Arg)	BH ₄ deficient and L-Arg containing iNOS _{ox}

cyt c	cytochrome c
SOD	superoxide dismutase
HRP	horseradish peroxidase
ROS	reactive oxygen species
RNS	reactive nitrogen species
RFQ EPR	rapid freeze quench EPR
DTT	dithiothreitol
BME	β -mercaptoethanol
HEPES	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid
WT	wild type
Fe^(II)O₂	oxy-ferrous complex

References

1. Forstermann U, Gath I, Schwarz P, Closs EI, Kleinert H. *Biochem. Pharmacol.* 1995; 50:1321–1332. [PubMed: 7503779]
2. Bredt DS, Snyder SH. *Proc. Natl. Acad. Sci. U. S. A.* 1990; 87:682–685. [PubMed: 1689048]
3. Pollock JS, Forstermann U, Mitchell JA, Warner TD, Schmidt HH, Nakane M, Murad F. *Proc. Natl. Acad. Sci. U. S. A.* 1991; 88:10480–10484. [PubMed: 1720542]
4. Moncada S, Palmer RM, Higgs EA. *Pharmacol. Rev.* 1991; 43:109–142. [PubMed: 1852778]
5. Nathan C, Xie QW. *Cell.* 1994; 78:915–918. [PubMed: 7522969]
6. MacMicking J, Xie QW, Nathan C. *Annu. Rev. Immunol.* 1997; 15:323–350. [PubMed: 9143691]
7. Pautz A, Art J, Hahn S, Nowag S, Voss C, Kleinert H. *Nitric Oxide.* 2010; 23:75–93. [PubMed: 20438856]
8. Schulz R, Rassaf T, Massion PB, Kelm M, Balligand JL. *Pharmacol. Ther.* 2005; 108:225–256. [PubMed: 15949847]
9. Robinson MA, Baumgardner JE, Otto CM. *Free Radic. Biol. Med.* 2011; 51:1952–1965. [PubMed: 21958548]
10. Wink DA, Hines HB, Cheng RY, Switzer CH, Flores-Santana W, Vitek MP, Ridnour LA, Colton CA. *J. Leukoc. Biol.* 2011; 89:873–891. [PubMed: 21233414]
11. Pacher P, Beckman JS, Liaudet L. *Physiol. Rev.* 2007; 87:315–424. [PubMed: 17237348]
12. Alderton WK, Cooper CE, Knowles RG. *Biochem. J.* 2001; 357:593–615. [PubMed: 11463332]
13. Daff S. *Nitric Oxide.* 2010; 23:1–11. [PubMed: 20303412]
14. Pou S, Pou WS, Bredt DS, Snyder SH, Rosen GM. *J. Biol. Chem.* 1992; 267:24173–24176. [PubMed: 1280257]
15. Xia Y, Roman LJ, Masters BS, Zweier JL. *J. Biol. Chem.* 1998; 273:22635–22639. [PubMed: 9712892]
16. Xia Y, Tsai AL, Berka V, Zweier JL. *J. Biol. Chem.* 1998; 273:25804–25808. [PubMed: 9748253]
17. Xia Y, Dawson VL, Dawson TM, Snyder SH, Zweier JL. *Proc. Natl. Acad. Sci. U. S. A.* 1996; 93:6770–6774. [PubMed: 8692893]
18. Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BS, Karoui H, Tordo P, Pritchard KA Jr. *Proc. Natl. Acad. Sci. U. S. A.* 1998; 95:9220–9225. [PubMed: 9689061]
19. Heinzel B, John M, Klatt P, Bohme E, Mayer B. *Biochem. J.* 1992; 281(Pt 3):627–630. [PubMed: 1371384]

20. Morris SM Jr. *Sci Signal*. 2010; 3:27.
21. Bronte V, Zanovello P. *Nat Rev Immunol*. 2005; 5:641–654. [PubMed: 16056256]
22. Gobert AP, McGee DJ, Akhtar M, Mendz GL, Newton JC, Cheng Y, Mobley HL, Wilson KT. *Proc. Natl. Acad. Sci. U. S. A.* 2001; 98:13844–13849. [PubMed: 11717441]
23. Chaturvedi R, Asim M, Lewis ND, Algood HM, Cover TL, Kim PY, Wilson KT. *Infect. Immun.* 2007; 75:4305–4315. [PubMed: 17562760]
24. McNeill E, Channon KM. *Thromb. Haemost.* 2012; 108:832–839. [PubMed: 23052970]
25. Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, Mitch WE, Harrison DG. *J. Clin. Invest.* 2003; 111:1201–1209. [PubMed: 12697739]
26. Ionova IA, Vasquez-Vivar J, Whitsett J, Herrnreiter A, Medhora M, Cooley BC, Pieper GM. *Am J Physiol Heart Circ Physiol*. 2008; 295:2178–2187.
27. Manoury B, Montiel V, Balligand JL. *Cardiovasc. Res.* 2012; 94:304–315. [PubMed: 22227153]
28. Bian K, Murad F. *Front. Biosci.* 2003; 8:264–278.
29. Thippeswamy T, McKay JS, Quinn JP, Morris R. *Histol. Histopathol.* 2006; 21:445–458. [PubMed: 16437390]
30. Olson N, van der Vliet A. *Nitric Oxide*. 2011; 25:125–137. [PubMed: 21199675]
31. Baek KJ, Thiel BA, Lucas S, Stuehr DJ. *J. Biol. Chem.* 1993; 268:21120–21129. [PubMed: 7691806]
32. Ghosh DK, Stuehr DJ. *Biochemistry*. 1995; 34:801–807. [PubMed: 7530045]
33. Panda K, Rosenfeld RJ, Ghosh S, Meade AL, Getzoff ED, Stuehr DJ. *J. Biol. Chem.* 2002; 277:31020–31030. [PubMed: 12048205]
34. Stuehr DJ, Santolini J, Wang ZQ, Wei CC, Adak S. *J. Biol. Chem.* 2004; 279:36167–36170. [PubMed: 15133020]
35. Feng C, Chen L, Li W, Elmore BO, Fan W, Sun X. *J. Inorg. Biochem.* 2014; 130:130–140. [PubMed: 24084585]
36. Abu-Soud HM, Gachhui R, Raushel FM, Stuehr DJ. *J. Biol. Chem.* 1997; 272:17349–17353. [PubMed: 9211873]
37. Sato H, Sagami I, Daff S, Shimizu T. *Biochem. Biophys. Res. Commun.* 1998; 253:845–849. [PubMed: 9918817]
38. Ledbetter AP, McMillan K, Roman LJ, Masters BS, Dawson JH, Sono M. *Biochemistry*. 1999; 38:8014–8021. [PubMed: 10387045]
39. Wei CC, Wang ZQ, Wang Q, Meade AL, Hemann C, Hille R, Stuehr DJ. *J. Biol. Chem.* 2001; 276:315–319. [PubMed: 11020389]
40. Berka V, Yeh HC, Gao D, Kiran F, Tsai AL. *Biochemistry*. 2004; 43:13137–13148. [PubMed: 15476407]
41. Moreau M, Boucher JL, Mattioli TA, Stuehr DJ, Mansuy D, Santolini J. *Biochemistry*. 2006; 45:3988–3999. [PubMed: 16548526]
42. Bec N, Gorren AC, Voelker C, Mayer B, Lange R. *J. Biol. Chem.* 1998; 273:13502–13508. [PubMed: 9593685]
43. Wei CC, Wang ZQ, Durra D, Hemann C, Hille R, Garcin ED, Getzoff ED, Stuehr DJ. *J. Biol. Chem.* 2005; 280:8929–8935. [PubMed: 15632185]
44. Hurshman AR, Krebs C, Edmondson DE, Huynh BH, Marletta MA. *Biochemistry*. 1999; 38:15689–15696. [PubMed: 10625434]
45. Schmidt PP, Lange R, Gorren AC, Werner ER, Mayer B, Andersson KK. *J Biol Inorg Chem*. 2001; 6:151–158. [PubMed: 11293408]
46. Berka V, Wu G, Yeh HC, Palmer G, Tsai AL. *J. Biol. Chem.* 2004; 279:32243–32251. [PubMed: 15166218]
47. Wei CC, Wang ZQ, Tejero J, Yang YP, Hemann C, Hille R, Stuehr DJ. *J. Biol. Chem.* 2008; 283:11734–11742. [PubMed: 18283102]
48. Stoll S, NejatyJahromy Y, Woodward JJ, Ozarowski A, Marletta MA, Britt RD. *J. Am. Chem. Soc.* 2010; 132:11812–11823. [PubMed: 20669954]
49. Brunel A, Santolini J, Dorlet P. *Biophys. J.* 2012; 103:109–117. [PubMed: 22828337]

50. Tejero J, Stuehr D. IUBMB Life. 2013; 65:358–365. [PubMed: 23441062]
51. Stuehr D, Pou S, Rosen GM. J. Biol. Chem. 2001; 276:14533–14536. [PubMed: 11279231]
52. Vasquez-Vivar J, Hogg N, Martasek P, Karoui H, Pritchard KA Jr, Kalyanaraman B. J. Biol. Chem. 1999; 274:26736–26742. [PubMed: 10480877]
53. Vasquez-Vivar J, Martasek P, Whitsett J, Joseph J, Kalyanaraman B. Biochem. J. 2002; 362:733–739. [PubMed: 11879202]
54. Rosen GM, Tsai P, Weaver J, Porasuphatana S, Roman LJ, Starkov AA, Fiskum G, Pou S. J. Biol. Chem. 2002; 277:40275–40280. [PubMed: 12183447]
55. Weaver J, Porasuphatana S, Tsai P, Pou S, Roman LJ, Rosen GM. Biochim. Biophys. Acta. 2005; 1726:302–308. [PubMed: 16216417]
56. Berka V, Wang LH, Tsai AL. Biochemistry. 2008; 47:405–420. [PubMed: 18052254]
57. Yasukawa T, Kanei-Ishii C, Maekawa T, Fujimoto J, Yamamoto T, Ishii S. J Biol Chem. 1995; 270:25328–25331. [PubMed: 7592692]
58. Du M, Yeh HC, Berka V, Wang LH, Tsai AL. J. Biol. Chem. 2003; 278:6002–6011. [PubMed: 12480940]
59. Di Iorio EE. Methods Enzymol. 1981; 76:57–72. [PubMed: 7329277]
60. Kuthan H, Ullrich V, Estabrook RW. Biochem J. 1982; 203:551–558. [PubMed: 6288006]
61. Tsai AL, Berka V, Kulmacz RJ, Wu G, Palmer G. Anal. Biochem. 1998; 264:165–171. [PubMed: 9866678]
62. Kulmacz RJ, Tsai AL, Palmer G. J Biol Chem. 1987; 262:10524–10531. [PubMed: 3038886]
63. Tsai AL, Berka V, Chen PF, Palmer G. J. Biol. Chem. 1996; 271:32563–32571. [PubMed: 8955082]
64. Ghosh DK, Wu C, Pitters E, Moloney M, Werner ER, Mayer B, Stuehr DJ. Biochemistry. 1997; 36:10609–10619. [PubMed: 9271491]
65. Presta A, Siddhanta U, Wu C, Sennequier N, Huang L, Abu-Soud HM, Erzurum S, Stuehr DJ. Biochemistry. 1998; 37:298–310. [PubMed: 9425051]
66. Tzeng E, Billiar TR, Robbins PD, Loftus M, Stuehr DJ. Proc. Natl. Acad. Sci. U. S. A. 1995; 92:11771–11775. [PubMed: 8524846]
67. Pignitter M, Gorren AC, Nedeianu S, Schmidt K, Mayer B. Free Radic. Biol. Med. 2006; 41:455–463. [PubMed: 16843826]
68. Bielski BHJ, Allen AO. The Journal of Physical Chemistry. 1977; 81:1048–1050.
69. Crane BR, Arvai AS, Ghosh DK, Wu C, Getzoff ED, Stuehr DJ, Tainer JA. Science. 1998; 279:2121–2126. [PubMed: 9516116]
70. Venema RC, Ju H, Zou R, Ryan JW, Venema VJ. J. Biol. Chem. 1997; 272:1276–1282. [PubMed: 8995432]
71. Sun J, Druhan LJ, Zweier JL. Arch. Biochem. Biophys. 2010; 494:130–137. [PubMed: 19932078]
72. Beckman JS. Ann. N. Y. Acad. Sci. 1994; 738:69–75. [PubMed: 7832457]
73. Siddhanta U, Presta A, Fan B, Wolan D, Rousseau DL, Stuehr DJ. J. Biol. Chem. 1998; 273:18950–18958. [PubMed: 9668073]
74. Presta A, Weber-Main AM, Stankovich MT, Stuehr DJ. J. Am. Chem. Soc. 1998; 120:9460–9465.
75. Gao YT, Smith SM, Weinberg JB, Montgomery HJ, Newman E, Guillemette JG, Ghosh DK, Roman LJ, Martasek P, Salerno JC. J. Biol. Chem. 2004; 279:18759–18766. [PubMed: 14715665]
76. Abu-Soud HM, Stuehr DJ. Proc. Natl. Acad. Sci. U. S. A. 1993; 90:10769–10772. [PubMed: 7504282]
77. Kolodziejwski PJ, Koo JS, Eissa NT. Proc. Natl. Acad. Sci. U. S. A. 2004; 101:18141–18146. [PubMed: 15601772]
78. Marchal S, Gorren AC, Sorlie M, Andersson KK, Mayer B, Lange R. J. Biol. Chem. 2004; 279:19824–19831. [PubMed: 15004019]
79. Sorlie M, Gorren AC, Marchal S, Shimizu T, Lange R, Andersson KK, Mayer B. J. Biol. Chem. 2003; 278:48602–48610. [PubMed: 14514694]
80. Aoyama K, Watabe M, Nakaki T. J Pharmacol Sci. 2008; 108:227–238. [PubMed: 19008644]

Highlights

1. We studied the effect of BH₄ and L-arg on radical formation during the reaction of O₂ with iNOS_{ox}.
2. Parallel single turnover stopped-flow and rapid freeze trap EPR measurements were performed.
3. We report differences between O₂ intermediates formed by 3 NOSs isoforms.

Fig. 1. Optical absorbance spectra and kinetics of the heme species observed during single turnover reaction of $\text{iNOS}_{\text{ox}}(+\text{BH}_4, +\text{L-Arg})$ with oxygen

Anaerobic ferrous iNOS_{ox} (8–10 μM) containing 500 μM of L-Arg and 10 μM of BH_4 was rapidly mixed with an air-saturated buffer at 22 $^\circ\text{C}$. The stopped-flow spectra were collected at 2.5 ms intervals using a rapid scan diode-array detector. Data are representative of three experiments.

A) UV-VIS spectra of starting ferrous Fe^{II} (solid line), oxy-ferrous intermediate $\text{Fe}^{\text{II}}\text{O}_2$ (dashed line) and final ferric Fe^{III} (dotted line) species resolved by global analysis.

Inset: Original rapid-scan diode-array data. Arrows indicate directions of the spectral shift with time.

B) Kinetics of $\text{Fe}^{(\text{II})}$ (solid line), $\text{Fe}^{(\text{II})}\text{O}_2$ (dashed line) and $\text{Fe}^{(\text{III})}$ (dotted line) resolved from global analysis. Data are expressed as percentage of the total heme concentration.

Fig. 2. Optical absorbance spectra and kinetics of the heme species observed during single turnover reaction of iNOS_{ox} (+BH₄, -L-Arg) with oxygen

The reaction was identical to that described in Fig. 1 except that L-Arg was not added.

A) UV-VIS spectra of starting ferrous Fe^(II) (solid line), oxy-ferrous intermediate Fe^(II)O₂ (dashed line) and final ferric Fe^(III) (dotted line) species resolved by global analysis.

Inset: Original rapid-scan diode-array data.

B) Kinetics of Fe^(II) (solid line), Fe^(II)O₂ (dashed line) and Fe^(III) (dotted line) obtained from the global analysis. Data are expressed as percentage of the total heme concentration.

Fig. 3. Optical absorbance spectra and kinetics of the heme species observed during single turnover reaction of iNOS_{ox} (-BH₄, -L-Arg) with oxygen

The reaction was identical to that described in Fig. 1 except that both BH₄ and L-Arg were absent.

A) UV-VIS spectra of starting ferrous Fe^(II) (solid line), oxy-ferrous intermediate Fe^(II)O₂ (dashed line) and final ferric Fe^(III) (dotted line) species resolved by global analysis.

Inset: Original rapid-scan data. Arrows indicate directions of the spectral shift with time.

B) Kinetics of Fe^(II) (solid line), Fe^(II)O₂ (dashed line) and Fe^(III) (dotted line) obtained from the global analysis. Data are expressed as percentage of the total heme concentration.

Fig. 4. Optical absorbance spectra and kinetics of the heme species observed during single turnover reaction of iNOS_{ox}(-BH₄, +L-Arg) with oxygen

The reaction was identical to that described in Fig. 1 except that no BH₄ was added.

A) UV-VIS spectra of starting ferrous Fe^(II) (solid line), oxy-ferrous intermediate Fe^(II)O₂⁻ (dashed line) and final ferric Fe^(III) (dotted line) species resolved by global analysis.

Inset: Original rapid scan traces.

B) Kinetics of Fe^(II) (solid line), Fe^(II) O₂ (dashed line) and Fe^(III) (dotted line) obtained from the global analysis. Data are expressed as percentage of the total heme concentration.

Fig. 5. Gel filtration analysis of 59 iNOS_{ox}

iNOS_{ox} (100 µg) were loaded on a Bio-Sil TSK-250 column as described under “Material and Methods”. Dotted line, the elution profile which shows that iNOS_{ox}(-BH₄, -L-arg), as isolated, is monomer. Dash line, the elution profile of protein following an anaerobic overnight incubation in 50 mM HEPES buffer (pH 7.5), containing 10 % glycerol, 0.1M NaCl and 100 µM of BH₄ at 4 °C, iNOS_{ox}(+BH₄, -L-arg), representing dimer-monomer mixture. Solid line, the elution profile of protein following an anaerobic overnight incubation with 1 mM L-arginine and 100 µM of BH₄, iNOS_{ox}(+BH₄, +L-arg), representing

dimer. Two vertical lines are visual guides to indicate the retention time of the iNOS_{ox} dimer and monomer.

Fig. 6. Cyt c reduction by oxygen-induced superoxide radical during single turnover reaction of iNOS_{ox} in the presence/absence of BH₄ and L-arg

Single wavelength stopped-flow at 550 nm obtained during reaction of 10 μ M ferrous iNOS_{ox} mixed with an air-saturated buffer containing 50 μ M of cyt c at 22 °C. Data in the presence of 1U of SOD are shown as a negative control for that of iNOS_{ox} (-BH₄, -L-Arg). Detected amounts of superoxide from these reactions expressed as percentage of the total heme concentration are summarized at Table 1. Data are similar from three separate experiments.

Fig. 7. Detection of Hydrogen Peroxide after reaction of ferrous iNOS_{ox} with oxygen

A) Optical spectra of 10 μM ferric iNOS_{ox} (+ BH_4 , +L-arg) (dotted line) after anaerobic stoichiometric reduction with dithionite (dash line); oxidized by adding 30 μl of aerobic buffer containing 5U of HRP, 20 μM Amplex Red and opened to air (solid line). B) Optical spectra of 10 μM iNOS_{ox} (+ BH_4 , -L-arg) in similar experiment. For details see “Materials and Methods.”

Fig. 8. EPR spectra of $i\text{NOS}_{\text{ox}}(\pm\text{BH}_4, \pm\text{L-arg})$ formed at 80 ms in the reaction between ferrous $i\text{NOS}_{\text{ox}}$ and oxygen

Fully reduced ferrous 50 μM $i\text{NOS}_{\text{ox}}$ containing equimolar BH_4 and 500 μM L-Arg (top spectrum), with BH_4 alone (middle spectrum), or in the absence of BH_4 (bottom spectrum) was reacted with air saturated 50 mM HEPES buffer (pH 7.5), containing 10 % glycerol, 0.1M NaCl, pH 7.4 at a 1:1 ratio at 22 $^{\circ}\text{C}$, and the reaction mixture was freeze-trapped at 80 ms reaction time in the prechilled isopentane. EPR conditions were: 4 mW microwave

power, 10 G modulation and 11K. The g values of both the high-spin (7.88 and 3.80) and low-spin (2.41 and 2.27) heme species are labeled by *solid lines*.

Fig. 9. Progressive power-dependent EPR spectra of the radical intermediates formed in the reaction between ferrous iNOS_{ox} and oxygen

The reaction was identical to that described in Fig. 8. EPR spectra were recorded at microwave powers ranging from 0.025–100 mW with 3 dB increment for 40–50 μM iNOS_{ox} with equimolar concentration of BH_4 and 500 μM L-Arg present (A) or with BH_4 alone (B) or with L-Arg alone (C) at 115 K. Almost identical EPR spectra shown in (C) were obtained for iNOS_{ox} in the absence of both BH_4 and L-Arg (data not shown). Difference spectra obtained by subtracting of 40% spectra (C) from spectra (B) to maximally remove the $g=2.005$ sharp dip signal are shown at panel (D). Spectra D are very similar to typical EPR spectra of BH_4 radical of iNOS_{ox} (A). Vertical dash lines are visual guides. Insets in (B) and (C) are plots to obtain the half-saturation power via non-linear regression to Equation 1. Symbols are the data and lines are the fit.

Fig. 10. Kinetic correlation between heme redox changes and BH₄ radical during reaction of reduced iNOS_{ox}(+BH₄, +L-arg) with oxygen

Single wavelength stopped-flow at 396 nm obtained during reaction of 10 μ M iNOS_{ox}(+BH₄, +L-arg) with an air-saturated buffer (dash line) at 22 °C and parallel RFQ EPR kinetics of 50 μ M protein to obtain amplitudes of BH₄ radical, $g=2$ signal (square), and high spin ferric heme, $g=7.8$ signal (star) recorded at 10K. The lines and corresponding rate constants were obtained by a one- exponential fit for the $g=7.8$ signal and an irreversible $A \rightarrow B \rightarrow C$ sequential mechanism for $g=2$ signal and $A \leftrightarrow B \rightarrow C$ for A₃₉₆ stopped-flow data.

Fig. 11. Kinetic correlation between the heme redox changes and superoxide radical production during reaction of reduced iNOS_{ox}(-BH₄, -L-arg) with oxygen

Single wavelength stopped-flow at 419 nm obtained during reaction of 10 μ M iNOS_{ox}(-BH₄, -L-arg) with an air-saturated buffer (dash line) at 22 °C and parallel RFQ EPR kinetics of 50 μ M protein to obtain amplitudes of superoxide radical $g=2$ signal (square) recorded at 115K and low-spin ferric heme $g=2.27$ signal (star) recorded at 10 K. The lines and corresponding rate constants were obtained by a one-exponential fit to the $g=2$ signal and an $A \leftrightarrow B \rightarrow C$ sequential mechanism for A₄₁₉ stopped-flow data.

Fig. 12. Kinetic correlation between heme redox changes and BH_4 /Superoxide radical during reaction between reduced $\text{iNOS}_{\text{ox}}(+\text{BH}_4, -\text{L-arg})$ and oxygen

Single wavelength stopped-flow at 403 nm obtained during reaction of 10 μM $\text{iNOS}_{\text{ox}}(+\text{BH}_4, -\text{L-arg})$ with an air-saturated buffer (dash line) at 22 $^\circ\text{C}$ and parallel RFQ EPR kinetics of 50 μM protein to obtain amplitudes of radical (mixture of two radicals, BH_4 and superoxide), $g=2$ signal (square), and ferric heme, $g=7.8$ signal (star), recorded at 10 K. The lines and corresponding rate constants were obtained by an $\text{A} \leftrightarrow \text{B} \rightarrow \text{C} \rightarrow \text{D}$ sequential mechanism for stopped-flow data, one-exponential fit for the $g=7$ signal, and an irreversible $\text{A} \rightarrow \text{B} \rightarrow \text{C}$ sequential mechanism for $g=2$ signal.

Scheme 1.

Radical formation mechanism of iNOS_{ox} in the presence or absence of BH₄ and/or L-arginine.

Table 1

Observed rate constants for different oxygen-induced radical intermediates of iNOS_{ox} obtained during single turnover reaction by Stopped-flow and RFQ EPR.

iNOS _{ox} ¹	+BH ₄ , +L-arg	+BH ₄ , -L-arg	-BH ₄ , +L-arg	-BH ₄ , -L-arg
Fe ^(II) -O ₂ formation (s ⁻¹)	106 ± 7	108 ± 1	ND ³	ND
Fe ^(II) -O ₂ decay (s ⁻¹)	18.0 ± 3.7	54 ± 8 and 2.6 ²	3.3 ± 2.5	7.3 ± 1.2
Reduction of cyt c	8 ± 4 %	36 ± 12 %	91 ± 10 %	80 ± 17 %
<i>Type of radical</i>	<i>BH₄⁺</i>	<i>BH₄⁺ and O₂⁻</i>	<i>O₂⁻</i>	<i>O₂⁻</i>
Radical formation (s ⁻¹)	20.0 ± 2.5	66 ± 16	ND	ND
Radical decay (s ⁻¹)	0.8 ± 0.2	1.2 ± 0.3	2.5 ± 0.5	4.5 ± 1
Heme oxidation (s ⁻¹)	15 ± 4	70 ± 19	ND	ND

¹ Anaerobic ferrous iNOS_{ox} was rapidly mixed with air-saturated buffer at 22 °C. Observed changes were followed either by Stopped-flow or RFQ EPR. For more details see Material and Methods.

² Biphasic decay, 2nd slow face ~15%.

³ ND - Not Detectable

* Standard error, n = 3

Table 2

EPR parameters for different oxygen-induced radical intermediates in eNOS_{ox} [46], nNOS_{ox} [56] and iNOS_{ox}.

	+BH ₄ , +L-arg	-BH ₄ , -L-arg	-BH ₄ , +L-arg	+BH ₄ , -L-arg
radical species	BH ₄ ⁺	O ₂ ⁻	O ₂ ⁻	new radical
<i>e</i> NOS _{ox} [*]	2.002	2.078 ^a , 2.006 ^b	2.078 ^a , 2.006 ^b	2.005 ^b , 1.99 ^c
P _{1/2} (mW)	13.8 ± 1.0	54 ± 3	51 ± 2	4.0 ± 1.5
radical species	BH ₄ ⁺	O ₂ ⁻	O ₂ ⁻ and new 38G	BH ₄ ⁺ and O ₂ ⁻
<i>n</i> NOS _{ox}	2.002	2.077 ^a , 2.005 ^b	2.077 ^a , 1.99	2.076 ^a and 2.005
-DTT	27.0 ± 2.7	100.0 ± 4.7	100 and 10 ± 1	26 ± 7 and 12 ± 2
radical species	BH ₄ ⁺	O ₂ ⁻	O ₂ ⁻ and new 38G	wide 75 G and 20% O ₂ ⁻
<i>n</i> NOS _{ox}	2.002	2.077 ^a , 2.005 ^b	2.077 ^a , 1.99	2.003 ^b , 1.97 ^c
+DTT	27.0 ± 2.7	100.0 ± 4.7	100 and 10 ± 1	37 ± 5 and 14.7
radical species	BH ₄ ⁺	O ₂ ⁻	O ₂ ⁻	BH ₄ ⁺ and O ₂ ⁻
<i>i</i> NOS _{ox} [*]	2.002	2.080 ^a , 2.006 ^b	2.080 ^a , 2.006 ^b	2.076 ^a and 2.005
P _{1/2} (mW)	26 ± 1	155 ± 7	126 ± 14	37 ± 4

^a Broad peak.

^b Crossover.

^c Satellite signal.

* No Difference between samples with or without DTT. Standard error, n = 3

Table. 3

Comparison of rate constants from single-turnover reactions catalyzed by three NOS_{ox} isoforms obtained by stopped-flow and RFQ EPR.

	nNOS _{ox} ^(a)	iNOS _{ox} ^(b)	eNOS _{ox} ^(c)
Fe ^(II) -O ₂ formation	>200	106 ± 7	207
Fe ^(II) -O ₂ disappearance	34.9	18.0 ± 3.7	15
BH ₄ radical formation	67.0 ± 10.5	20.0 ± 2.5	13.1 ± 2.7
BH ₄ radical decay	0.4	0.8 ± 0.2	2.0

Anaerobic ferrous NOS_{ox} solutions that contained both L-arginine and BH₄ were rapid mixed with air saturated buffers at 22 °C.

^(a) Data from [56] in the presence of DTT,

^(b) data from Table 1,

^(c) data from [46].