Rapid Kinetic Studies Link Tetrahydrobiopterin Radical Formation to Heme-dioxy Reduction and Arginine Hydroxylation in Inducible Nitric-oxide Synthase*

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To understand how heme and (6R)-5,6,7,8-tetrahydro-L-bioppterin (H_{4}B) participate in nitric-oxide synthesis, we followed ferrous-dioxy heme (Fe^{II}O_{2}) formation and disappearance, H_{4}B radical formation, and Arg hydroxylation during a single catalytic turnover by the inducible nitric-oxide synthase (iNOS). In all cases, prereduced (ferrous) enzyme was rapidly mixed with an O_{2}-containing buffer to start the reactions. A ferrous-dioxy intermediate formed quickly (53 s^{-1}) and then decayed with concurrent buildup of ferric iNOSoxy. The buildup of the ferrous-dioxy intermediate preceded both H_{4}B radical formation and Arg hydroxylation. However, the rate of ferrous-dioxy decay (12 s^{-1}) was equivalent to the rate of H_{4}B radical formation (11 s^{-1}) and the rate of Arg hydroxylation (9 s^{-1}). Practically all bound H_{4}B was oxidized to a radical during the reaction and was associated with hydroxylation of 0.6 mol of Arg/mol of heme. In dihydrobiopterin-containing iNOSoxy, ferrous-dioxy decay was much slower and was not associated with Arg hydroxylation. These results establish kinetic and quantitative links among ferrous-dioxy disappearance, H_{4}B oxidation, and Arg hydroxylation and suggest a mechanism whereby H_{4}B transfers an electron to the ferrous-dioxy intermediate to enable the formation of a heme-based oxidant that rapidly hydroxylates Arg.

Nitric oxide (NO) is synthesized from L-arginine by the nitric-oxide synthases (NOS) (EC 1.14.13.39). The reaction consumes 1.5 NADPH and 2 O_{2} for each NO formed from Arg and is catalyzed in two steps (for review see Refs. 1–3). In the first step Arg undergoes mixed function hydroxylation to form water and N^{6}-hydroxy-L-Arg (NOHA) as an enzyme-bound intermediate. NOHA then undergoes mixed function oxidation in the second step to generate water, NO, and citrulline. Both reactions take place within the oxygenase domain of NOS, which contains iron protoporphyrin IX (heme), the cofactor (6R)-5,6,7,8-tetrahydro-L-bioppterin (H_{4}B), and the Arg binding site (1–3). Electrons derived from NADPH are provided to the oxygenase domain by an attached reductase domain that binds FMN, FAD, and NAPDH (4).

How heme and H_{4}B participate in NO synthesis is of wide interest because it involves a novel cooperation between these groups in biologic oxidation. Crystal structures of NOS oxygenase domains (5–7) show that the guanidinium group of Arg is held directly above the heme, consistent with the heme activating O_{2} for substrate oxidation. Accordingly, the mechanisms for Arg hydroxylation in NOS have been modeled after cytochrome P-450 monooxygenase chemistry (Scheme 1). The transfer of an electron to the ferric NOS heme enables O_{2} binding and formation of detectable ferrous-dioxy species (I, Fe^{III}O_{2} (8–12). This species obtains a second electron to form the iron-peroxy species (II), which upon protonation and O–O bond scission generate water and iron-oxo species (III, FeO) that is thought to hydroxylate the guanidino nitrogen of Arg.

In contrast to close proximity between substrate and heme, the H_{4}B cofactor binds away from Arg but next to the heme edge and forms a hydrogen bond between N_{3} of its pterin ring and a heme propionate (5–7). Its position suggests that H_{4}B cannot directly participate in O_{2} binding or substrate oxidation but could function as an electron donor (6, 12). In NOS, the first electron provided to the heme comes directly from the reductase domain, and this transfer does not require that H_{4}B be present (13, 14). However, either the reductase domain or H_{4}B could conceivably provide the second electron to the Fe^{III}O_{2} species (I). Indeed, a recent study with the inducible NOS oxygenase domain (iNOSoxy) showed that bound H_{4}B is oxidized to a radical when ferrous iNOSoxy reacts with O_{2} (15). H_{4}B radical formation was associated with some Arg hydroxylation, implying that these two processes might be related. Separate studies have characterized the spectral properties and formation and decay kinetics of the NOS Fe^{III}O_{2} complex (8–12) or the kinetics of product formation from Arg in an NADPH-driven reaction (16). However, what kinetic and quantitative relationships that may exist between H_{4}B oxidation, Fe^{III}O_{2} formation and disappearance, and product formation remain to be explored.

To address this issue, we combined stopped-flow, rapid-quench, and rapid-freeze methods to analyze Arg hydroxylation during a single catalytic turnover by ferrous iNOSoxy. Our results reveal and define the temporal and quantitative links that exist between Fe^{III}O_{2} reactivity, H_{4}B radical formation, and Arg hydroxylation and thus clarify how H_{4}B and heme cooperate in the first step of NO synthesis.
the aged reaction samples through a nozzle. A solution containing 50 mM HEPES, pH 7.5, 205 μM ferrous iNOSoxy, 20 mM Arg, 0.5 mM H$_4$B or H$_2$B, and 0.3 mM DTT was mixed with O$_2$-saturated HEPES buffer at 10 °C. The reactions were aged at 10 °C for various times after mixing and then shot into a funnel submerged in an isopentane bath maintained at −135 to −140 °C. The frozen samples were packed into a 707-SQ EPR tube (Wilmad-Labglass, Buena, NJ) and stored in liquid N$_2$ until measurement. The dilution of iNOSoxy after mixing under each aging condition was determined using bromphenol blue standards.

EPR Spectra—EPR spectra were recorded in a Bruker ER 300 electron paramagnetic resonance spectrometer equipped with an ER 035 NMR gauss meter and a Hewlett-Packard 5325B microwave frequency counter. Temperature control was achieved using Oxford Instruments ESR 900 continuous-flow liquid helium cryostat and ITC4 temperature controller. All spectra were obtained at 150 K using a microwave power of 2 milliwatts, a frequency of 9.5 GHz, modulation amplitude of 10 G, and modulation frequency of 100 kHz. 20 scans/sample were accumulated to improve the signal to noise ratio. Spin quantitations were calculated by double integration as compared with a 500 μM Cu-EDTA standard that was analyzed under the same measurement conditions. Radical concentrations versus time were fit to an “A to B to C” kinetic model (where “B” was the radical) using DeltaGraph software to calculate formation and decay rates.

**RESULTS**

We monitored a single catalytic turnover in iNOSoxy. A dithionite-reduced (ferrous) iNOSoxy containing Arg and H$_2$B was rapidly mixed with an O$_2$-containing buffer to initiate the reaction, which was then analyzed by one of three methods: rapid scanning to follow heme transitions, rapid quenching to follow product formation, or rapid freezing to follow buildup of the H$_2$B radical. Fig. 1, A–F, contains representative data from each type of experiment.

Rapid scanning discerned three spectrally distinct species during the reaction: the beginning ferrous iNOSoxy, a transient intermediate, and an ending ferric iNOSoxy (Fig. 1A). The transient species had a Soret peak at 427 nm and visible spectral features that identify it as Fe$^{3+}$O$_2$ iNOSoxy (8–10). Thus, the formation and decay of this intermediate probably represent the buildup of the Fe$^{3+}$O$_2$ complex and its subsequent reaction during the single turnover experiment.

Analysis of the kinetic spectral data showed that it only was fit well to an “A to B to C” kinetic model with monophasic transitions for formation and decay of the Fe$^{3+}$O$_2$ intermediate. This means that no spectrally distinct species were built up during the conversion of the Fe$^{3+}$O$_2$ intermediate to the ferric enzyme. Fig. 1B illustrates how concentrations of the three spectral species changed with time during the single turnover reaction. The Fe$^{3+}$O$_2$ intermediate formed rapidly and reached a maximum after 32 ms. Its conversion to the ferric enzyme was essentially completed by 300 ms after mixing. The calculated rates of formation and/or decay of each of the species are listed in Table I. To understand how H$_2$B influenced this transformation, we used a H$_2$B-saturated iNOSoxy in an otherwise identical stopped-flow experiment. We observed the same three spectral species (data not shown), but in this case conversion of the Fe$^{3+}$O$_2$ intermediate to the ferric enzyme occurred at a rate of 0.3 s$^{-1}$, which is 40 times slower compared with the H$_2$B-containing enzyme.

Rapid quench experiments revealed that H$_2$B-containing iNOSoxy oxidized [14C]Arg to NOHA but not to citrulline during the single turnover reaction (Fig. 1C). This conversion was substantially inhibited with respect to heme (0.55 ± 0.06 NOHA generated/heme, n = 4) even when Arg and H$_2$B achieved saturation binding (see “Experimental Procedures”). NO NOHA was generated in reactions where H$_2$B was substituted for H$_2$B or when iNOSoxy was not reduced or was absent (data not shown). The time course of [14C]Arg oxidation and NOHA formation were essentially...
kinetics of H₄B radical formation and H₂B was rapidly mixed with an O₂-containing solution at 10 °C to start the reaction. Subsequent transformations were followed either by stopped-flow rapid-scanning spectroscopy, rapid-quenching and high pressure liquid chromatography analysis, or rapid-freezing and EPR spectroscopy. Our results also show how coupled these processes are linked to Arg hydroxylation, which occurs at essentially the same rate.

The buildup of the oxygenated intermediate (Soret at 427 nm) clearly preceded both H₂B radical formation and Arg hydroxylation. Indeed, at the point of its maximum buildup (32 ms) only 10–15% bound H₂B had formed a radical. This means that the oxygenated intermediate cannot already contain an electron from H₂B and is in fact Fe⁵O₄⁺ iNOSoxy. The disappearance of the Fe⁵O₄ intermediate followed the same kinetics as the H₂B radical buildup and was much slower in H₂B iNOSoxy. Together, these results indicate that Fe⁵O₄ formation is a prerequisite for H₂B oxidation, and H₂B oxidation represents electron transfer from H₂B to the Fe⁵O₄ intermediate.

Because H₂B oxidation also occurred at the same rate as Arg hydroxylation and without buildup of additional heme-oxy intermediates, all steps required to form the ultimate oxidant Arg must be as fast as (or faster than) the electron transfer from H₂B. Thus, Fe⁵O₄ reduction is rate-limiting when one starts the reaction with ferrous enzyme. A similar situation holds true in many cytochrome P-450 enzymes where steps beyond FeIIO₂ reduction occur faster than the electron transfer to the heme (18, 19).

Our results also show how coupled these processes are within the single turnover. For example, practically all bound H₂B had remained in the enzyme or if the transfer between H₄B and FeIIO₂ was uncoupled, we should have observed less radical buildup and/or biphasic decay of the Fe²O₂ intermediate. A complete well coupled electron transfer between H₂B and Fe²O₂ is consistent with their close proximity and the irreversible nature of the single turnover reaction.

Given the above results, one would expect that Arg hydroxy-
lation should match H₄B radical formation and generate 1 NOHA/heme. However, we and others (8, 12, 15) typically observe substoichiometric NOHA formation in single turnover reactions ranging from 0.2 to 0.8 NOHA formed per heme. Here the estimated stoichiometry was about 0.6 NOHA formed per heme. Our current work shows that events leading up to and including the reduction of the FeᴵᴵᴵO₂ intermediate by H₄B were tightly coupled and complete. This rules out incomplete electron transfer from H₂B as a possible explanation and instead implies that subsequent steps (i.e. conversion of the iron-peroxo intermediate to iron-oxo or its reaction with Arg) become uncoupled in the iNOSoxy reaction. Further studies should resolve this issue.

The ability of H₄B to speed the “decay” of the FeᴵᴵᴵO₂ species was first observed while conducting O₂ binding studies with tetrahydro reduction state (13). Our results suggest that H₄B radical to the ferric heme. Oxygen then binds to form the FeᴵᴵIO₂ species, which receives an electron from H₂B to form a heme-peroxo radical. Subsequent steps that lead to and include Arg hydroxylation are rapid and generate ferric iNOSoxy containing NOHA and the H₂B radical.

In the NADPH-driven reaction, the slow step is the transfer of an electron from the reductase flavins (FMNH₂) to the ferric heme. Oxygen then binds to form the FeᴵᴵIO₂ species, which receives an electron from H₂B to form a heme-peroxo intermediate. Subsequent steps that lead to and include Arg hydroxylation are rapid and generate ferric iNOSoxy containing NOHA and the H₂B radical.

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In conclusion, our work demonstrates the kinetic and quantitative relationships among H₂B oxidation, FeᴵᴵIO₂ disappearance, and Arg hydroxylation in NOS. H₂B appears to be a kinetically competent and complete source of the second electron to reduce the FeᴵᴵIO₂ intermediate. It will now be important to identify the structural features of the enzyme that control these processes. The electron transfer between H₂B and the FeᴵᴵIO₂ intermediate should depend on their relative redox potentials and structural proximity. Crystallography has identified residues that surround H₂B or stack with the NOS heme (5–7), and mutagenesis studies suggest that some of these residues help modulate H₂B and heme function (24–26). The kinetic approach described here should help define how these and other structural features enable the cooperation between heme and H₂B during oxygen activation in NOS.

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