Evidence of two distinct oxygen complexes of reduced endothelial nitric oxide synthase*

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Running title: Heme-oxy I and II as oxygenated eNOS_ox complexes
Abstract

Oxygen-binding to the oxygenase domain of reduced endothelial nitric oxide synthase (eNOS) results in two distinct species differing in their Soret and visible absorbance maxima, and in their capacity to exchange oxygen by CO. At 7 °C, heme-oxy I (with maxima at 420 and 560 nm) is formed very rapidly ($k_{on} \approx 2.5 \times 10^6$ M$^{-1}$s$^{-1}$) in the absence of substrate, but in the presence of pterin cofactor. It is capable of exchanging oxygen with CO at -30 °C. Heme-oxy II is formed more slowly ($k_{on} \approx 3 \times 10^5$ M$^{-1}$s$^{-1}$) in the presence of substrate, regardless of the presence of pterin. It is also formed in the absence of both substrate and pterin. In contrast to heme-oxy I, it cannot exchange oxygen with CO at cryogenic temperature. In the presence of arginine, heme-oxy II is characterized by absorbance maxima near 432, 564 and 597 nm. When arginine is replaced by N-hydroxyarginine, and also in the absence of both substrate and pterin, its absorbance maxima are blue-shifted to 428, 560 and 593 nm. Heme-oxy I seems to resemble the ferrous dioxygen complex observed in many hemoproteins, including cytochrome P450. Heme-oxy II, which is the oxygen complex competent for product formation, appears to represent a distinct conformation, in which the electronic configuration is essentially locked in the ferric superoxide complex.
Introduction

Activation of molecular oxygen by nitric oxide synthase precedes NO biosynthesis by a yet incompletely known reaction mechanism. A study of the steps following oxygen binding and NO formation is important, since NO is a mediator of a wide range of physiological and pathophysiological processes in humans and other mammals (1-6). The formation of NO is catalyzed by nitric oxide synthases (NOS1; EC 1.14.13.39) via a two-step mechanism. The substrate, L-Arg, is first converted to \( \text{\(N^G\)} \)-hydroxy-L-Arg (NHA) at the heme active site. In the second step, NHA is further oxidized to NO and citrulline (3). In both steps oxygen binding occurs after a one-electron reduction of the ferric heme. Prior to product formation, an electron stemming from tetrahydrobiopterin (BH4) then further reduces the ferrous oxygen complex.

Despite much effort, the reaction mechanism of these steps is still unclear. Even the spectral properties of the oxyferrous complex are not yet defined: conflicting observations report absorbance maxima differing by up to 15 nm. The origin of these differences is unknown. The strongest differences are those between observations at cryogenic temperatures yielding maxima in the 417-419 nm region (7-9) and observations at higher temperature by rapid-scan techniques yielding considerably red-shifted maxima (430-432 nm) (10-14). However, this distinction is not clear-cut: in some cases low-wavelength maxima are also found by rapid-scan spectroscopy (11) and high-wavelength maxima also by low-temperature UV/vis spectroscopy (9).

To clarify this confusing situation, we re-analyzed the temporal evolution of the spectral changes occurring upon oxygen binding to reduced eNOS oxygenase domain by rapid scanning stopped-flow, assisted by global fit analysis. The present spectral and kinetic data show that – depending on experimental conditions such as temperature and presence of substrate and pteridine cofactor – two different heme-oxy complexes are formed. These complexes are further distinguished by their ability to exchange oxygen by CO at –30 °C.

The implication of two different oxygen complexes in NOS distinguishes this enzyme from other heme-thiolate containing proteins. In addition, the dependence of these oxy-complexes on other reaction partners may be important in the mechanism of uncoupling of NADPH oxidation from product formation. Therefore, formation, stability and decay of the oxygen complexes were followed within both eNOS reaction cycles (using L-Arg and NHA as substrate), and in the presence of different tetrahydrobiopterin (BH4) analogues.
Materials and Methods

Materials
The oxygenase domain of bovine endothelial NOS (eNOSoxy) was expressed in and purified from *Escherichia coli* (15). All chemicals were from Sigma except for pteridines (Schircks Laboratories, Jona, Switzerland), Ar, O₂, and CO (Aga, Toulouse, France).

Sample preparation
Samples were prepared as described (7,9,16). Briefly, NOS samples (2-6 μM eNOSoxy) in KCEM buffer (50 mM KPᵢ at pH 7.5, 1 mM CHAPS, 0.5 mM EDTA, 1 mM 2-mercaptoethanol), in the presence or absence of substrates (1 mM NHA or 0.5 mM L-Arg) and pteridines (100 μM) were deoxygenated under argon atmosphere and then reduced by sodium dithionite (1 mM final concentration) at 15 °C. CHAPS (which does not affect the spectral properties of the enzyme) was used to maintain the structural and functional integrity of NOS (17). For stopped-flow experiments, O₂-saturated stock solutions were prepared by bubbling KCEM buffer with O₂ for 45 min. The concentration of oxygen in these solutions was assumed to be 1 mM at 25°C. Argon-saturated solutions were prepared by the same procedure.

Stopped-flow spectroscopy.
Rapid-scanning experiments were performed at 7°C using an SFM 300 BioLogic Instrument (Grenoble, France) stopped-flow apparatus equipped with a J&M TIDAS diode array detector (MCS/16–TSPEC/500kHz). Anaerobic solutions of ferrous eNOS oxygenase domain were mixed with equal volumes of oxygen-containing buffer. The final concentration of oxygen was varied by the use of 3 independent mixing syringes: one containing the enzyme, one the oxygen-saturated buffer, and one the argon-saturated buffer. The mixing dead-time was 0.3 ms. After each injection two hundred spectra from 350 to 700 nm were collected with an acquisition time of 2.5 ms. Rapid-scanning data were compiled and fitted to different reaction models using the Specfit global analysis program (provided by the instrument manufacturer), which evaluates the number of different enzyme species, their individual spectra, and the concentration of each species as a function of time, as well as the rate constant for each transition. In some experiments where the kinetics of formation of the oxygenated eNOSoxy complexes were too rapid to be detected with the diode array (lower
limit of integration time: 2.5 ms), the kinetics were followed at a single wavelength (414 nm) with a PMS-250 photomultiplier (BioLogic Science Instruments), which allowed the kinetic resolution of reactions occurring within a couple of milliseconds. All experiments were performed at least twice, with different batches of eNOS$_{oxy}$. The kinetics of formation and decay of oxygen complexes were not affected by the presence of excess dithionite: control experiments using different concentrations of dithionite (0.5 to 5 mM) and of oxygen (0.1 to 0.5 mM) did not reveal any effect of dithionite on the kinetics.

**Low-Temperature Optical Spectroscopy**

Low-temperature UV/visible absorption spectra of the reaction between reduced eNOS$_{oxy}$ and O$_2$ or CO were recorded with a Cary 3E (Varian, Palo Alto, CA) spectrophotometer, adapted for low-temperature studies, according to previously published procedures (7,9,18). To avoid freezing, the experiments were carried out in a 1:1 (v/v) mixture of aqueous buffer/ethylene glycol. The organic cosolvent did not change significantly the spectral properties of NOS, it did not induce a transition to the P420 state, and in its presence the enzyme was still active in a standard assay (19).

**Results**

*Detection of two spectrally distinct oxygen complexes*

Depending on experimental conditions, rapid mixing of reduced eNOS$_{oxy}$ with oxygen containing buffer resulted in two different spectral species. As shown in Figure 1, one species is characterized by a Soret absorbance maximum of 420 nm and a single visible band at 560 nm, the other one by a considerably red-shifted Soret maximum (428 nm), and by two visible bands (560 and 595 nm). For simplicity, we denote the first one heme-oxy I, and the latter one heme-oxy II. Inspection of Table 1 shows that heme-oxy I is formed in the absence of substrate, regardless of the nature of the pterin cofactor. In contrast, heme-oxy II is formed in the presence of substrate (L-Arg or NHA), irrespective of the pterin cofactor. In addition, heme-oxy II is also formed when both substrate and pterin cofactor are absent. A closer analysis of Table 1 reveals small spectral differences of heme-oxy II when formed in the presence of L-Arg or NHA. With L-Arg a Soret maximum is found near 432 nm, and alpha/beta bands at 564 and 597 nm. With NHA, the Soret band culminates at 428 nm and the alpha/beta bands at 560 and 593 nm. Interestingly, when the experiments were carried out at -30 °C, under some conditions different results were obtained. For instance, at low temperature
the presence of NHA and absence of pterin resulted in an oxygen complex of heme-oxy I spectral characteristics.

**Occurrence of heme-oxy II within the second reaction cycle**

Rapid-mixing of reduced eNOSoxy with O₂-saturated buffer in the presence of BH4 and NHA resulted in a gradual red-shift of the Soret band from 414 to ~440 nm, followed by a shift in the opposite direction yielding finally ferric heme (394 nm) (Figure 2). The temporal sequence of spectra was best fitted to a sequential model with three monophasic transitions between four distinct species. The four spectral components revealed by global analysis were ferrous eNOSoxy (λₘₐₓ 414 nm), heme-oxy II (characterized by absorbance peaks at 428, 560, and a shoulder at 600 nm), the Fe(III)-NO complex with λₘₐₓ(Soret) 438 nm and λₘₐₓ(α,β) 547/583 nm, and high-spin ferric heme (λₘₐₓ 394 nm). The Soret and visible absorbance features of the transient species after O₂ addition and of ferric-NO match those of oxygenated (FeᴵᴵΟ₂) and FeᴵᴵΙ-NO complexes of iNOSoxy, nNOSoxy or eNOSoxy characterized previously at 10°C (10,12,20,21). The transient occurrence of heme-oxy II and Fe(III)-NO appears also evident from the time course of the concentration of the four species, which was deduced by global fit analysis (Figure 2C).

As shown in Figure 3, a similar spectral evolution was observed when BH4 was replaced by ABH4, except that no Fe(III)-NO complex was observed, which is in line with recent observations (22). Therefore, in this case, the reaction contained only two steps: conversion of ferrous heme to heme-oxy II, followed by its decay to ferric heme. Clear isosbestic points characterized both processes.

**Kinetic analysis of heme-oxy I and heme-oxy II formation**

The formation of heme-oxy I was too fast for a precise determination of rate constants from rapid scanning stopped-flow data. Therefore, we used single wavelength stopped-flow. Figure 4A is an example for the kinetic traces observed. Heme-oxy I forms within a few milliseconds and decays within 100 ms. In contrast, the kinetics of formation and decay of heme-oxy II were much slower, and could be analyzed adequately from rapid-scan data. Figure 4B shows the dependence of kₖₒₐₛ of formation of heme-oxy II as a function of oxygen concentration. Clearly, a linear relationship was observed, indicating a pseudo-first order reaction. Accordingly, kₒₐₜ and kₒᵣₑ₉ were determined using Equation 1:

\[ kₒₛₐ = kₒᵣₑ₉ + kₒₐₜ × [O₂]. \] (1)
The kinetic rate constants under the different experimental conditions are listed in Table II. In most cases the formation of heme-oxy II was irreversible as evidenced by a $k_{\text{off}}$ value close to zero\(^2\). Exceptions were the reactions in the presence of substrate and BH4 ($k_{\text{off}} = 23 \pm 1 \text{ s}^{-1}$), and in the presence of NHA and BH2 ($k_{\text{off}} = 9.7 \text{ s}^{-1}$). In order to enable a comparison of the formation rates of heme-oxy I and heme-oxy II, the experimental values of $k_{\text{obs}}$ for heme-oxy I were converted into second order rate constants, using a single concentration of oxygen (150 µM). At higher concentrations oxygen binding occurred within the mixing time, precluding analysis of the [O\(_2\)] dependence.

For both heme-oxy I and heme-oxy II, the decay rate constants (reflecting the transition to ferric high spin or to Fe(III)-NO) were independent of oxygen concentration, i.e., the decay was a first order reaction.

A particular situation was encountered when both substrate and pterin cofactor were absent. In this case, the starting spectrum recorded after 2.5 ms mixing displayed essentially identical spectral features of heme-oxy II. This transient species decayed to form ferric eNOS\(_{\text{oxy}}\), as judged by a shift of the Soret band to a broad spectrum centered at 410 nm and build-up of visible absorbance at 630 nm (data not shown). Using single-wavelength stopped-flow, we showed that the formation kinetics of the oxygenated intermediate were very fast (a couple of milliseconds), similar to the formation of heme-oxy I (Table 2).

Kinetic effects of pterins and substrates

As shown in Table 2, the kinetics of heme-oxy I formation did not depend significantly on the nature of the pterin cofactor. In contrast, its decay rate was about 5 times higher in the presence of BH4 with respect to other pterins. Similarly, the $k_{\text{on}}$ rate of heme-oxy II did not vary strongly under the different conditions. However, its decay rate was tremendously (more than 100 fold) enhanced in the presence of BH4. Similar tendencies were observed for both substrates (L-Arg and NHA).

Oxygen exchange by CO

In order to further characterize heme-oxy I and heme-oxy II, we investigated their reactivity versus CO. To do that, we chose conditions at -30 °C where heme-oxy I or II were predominant and bubbled CO through the sample immediately after formation of the oxygen complex. As shown in Figure 5, the two oxygen complexes behaved differently: heme-oxy I transformed to a spectrum with contributions from two compounds: the Soret region exhibited
maxima at 396 and 444 nm. The 396nm-peak most likely reflects the oxidized form whereas the latter was typical of thiolate-ligated ferrous-CO heme ($\lambda_{\text{max}}$ 444 nm). In contrast, heme-oxy II did not exchange CO: its 427 nm Soret maximum shifted without intermediate to 395 nm, indicating a decay of heme-oxy II to ferric high spin.
Discussion

Are heme-oxy I and heme-oxy II distinct intermediates?

One of the most salient observations in the present paper is the detection of intermediates in the oxygenation of reduced eNOSoxy of different spectral and kinetic properties (heme-oxy I and heme-oxy II). The question now is whether these two spectral forms represent distinct reaction intermediates. There are prior indications for the existence of different NOS oxygen complexes. Intermediates with absorbance maxima at 417-419 nm (of heme-oxy type I) have been observed in several low-temperature optical studies under a variety of conditions (7-9). A maximum at 417 nm was also found by rapid-scan spectroscopy for full-length nNOS in the absence of substrate and BH4 (11). However, most stopped-flow/rapid-scan studies yielded intermediates with Soret maxima at 427-431 nm, i.e. of heme-oxy type II (10-12,14,20,21,23,24). These spectral differences cannot be exclusively explained by different experimental temperature or the presence of cryogenic cosolvent (8,9,11,12,25), since red-shifted oxygen complexes with absorbance maxima up to 432 nm have been reported in low-temperature studies as well (9). Some of the reported spectral discrepancies may have been artificial in those cases, when incomplete formation of a 428/432 nm intermediate resulted in an apparent maximum at lower wavelength, as discussed previously (9). However, the extreme stability of some of the observed intermediates appears to preclude such an explanation in many instances. The presence and identity of substrate and pteridines may also have affected the spectral properties of the oxyferrous complexes (9,24), in line with prior observations for cytochrome P450 (26).

Several observations in the present study argue in favor of two distinct heme-oxy complexes: i) the observed spectra can be clearly distinguished in two groups with well-defined properties in the Soret and α,β regions; ii) the effects of substrate and pterines on the spectra follow regular patterns; (iii) although in most cases the red-shifted spectra exhibited much greater stability than the blue-shifted intermediates, this property was not universal, since a short-lived 428 nm intermediate was formed in the absence of substrate and pterin, which rules out that the blue species are experimental artifacts; iv) at low temperature, formation of heme-oxy I is reversible, and oxygen can be replaced by CO. This is not the case for heme-oxy II. Particularly, the different reactivity versus CO constitutes a strong argument for the existence of two different oxygen complexes.
**Nature of heme-oxy I and II**

All spectral species that were formed in the absence of pteridines and in the presence of BH2 must have been in the same \( [\text{Fe}\cdot\text{O}_2]^{2+} \) redox state, since under those conditions further reduction of the oxy-complex was impossible. In those instances, where a second electron transfer was possible (L-Arg/NHA+BH4/ABH4) a different interpretation (for heme-oxy II) would be theoretically possible but highly improbable, in view of the near identity with the spectra obtained under non-productive (BH2 or absence of pterin) conditions (see Table 1). Indeed, electron paramagnetic resonance (EPR) spectra corresponding to the oxy-complexes observed at low temperature showed no trace of a ferrous-superoxy or ferri-peroxy complex (27). We conclude that all spectral species that were designated heme-oxy I and II by us in the present study were isoelectronic, formally presented by the \( [\text{Fe}\cdot\text{O}_2]^{2+} \) redox state.

The spectral properties of heme-oxy I are reminiscent of the oxy-complexes of other hemoproteins, such as cytochrome P450 and globins (28). In those cases, there is spectroscopic evidence (29) that the predominant electronic state is the ferric superoxide \( (\text{Fe}^{3+}\cdot\text{O}_2^-) \), suggesting a similar electronic structure for heme-oxy I. However, this species must still have a considerable contribution of the ferrous-dioxygen \( (\text{Fe}^{2+}\cdot\text{O}_2) \) mesomer, since the complex readily exchanged \( \text{O}_2 \) for \( \text{CO} \), as was also observed for cytochromes P450 and globins (30).

The marked red-shift of heme-oxy II with respect to the “classical” ferrous dioxygen complex of cytochrome P450 has been noted before for neuronal NOS (13) and also for chloroperoxidase (30), and was attributed to a more polar heme pocket on the basis of Resonance Raman spectral analysis. Increased polarity of the heme pocket could lead to a charge redistribution in favor of ferric superoxide, \( \text{Fe}^{3+}\cdot\text{O}_2^- \). This would be consistent with the \( \text{O}_2 / \text{CO} \) exchange experiment: in contrast to heme-oxy I, heme-oxy II was unable to exchange \( \text{O}_2 \) by \( \text{CO} \) and decayed directly to ferric heme. This suggests that in heme oxy II the complex is locked in the ferric superoxide \( (\text{Fe}^{3+}\cdot\text{O}_2^-) \) state and that the ferrous dioxygen mesomer is hardly populated.

We would like to stress at this point that the \( \text{Fe}^{2+}\cdot\text{O}_2 \) and \( \text{Fe}^{3+}\cdot\text{O}_2^- \) electronic states, which we suppose to (partly) determine the spectral signatures of heme oxy I and II, are in fact mesomers that are not by themselves expected to be isolable species. It is the structure of the heme pocket and the way in which it is affected by substrates and pteridines (discussed below), that is reflected in a changed electron distribution of the heme oxy complex, resulting in the observation of two distinct spectral species. It should also be borne in mind that the ability of the oxycomplex to yield the ferrous CO complex will depend not only on the
electron distribution between the heme and the oxy ligand, but also in the relative ease with which O$_2$ and O$_2^•$ will dissociate from Fe(II) and Fe(III). In this respect it may be relevant that dissociation of O$_2^•$ is generally regarded as insignificant for cytochrome P450 (31), whereas it appears to be the main pathway of uncoupling for NOS (32).

**Mechanism of formation and possible physiological significance**

The mechanism by which the two oxy compounds are formed is not quite clear yet. Considering the Fe$^{2+}$-O$_2$ and Fe$^{3+}$-O$_2^•$ states as separate species, a sequential mechanism from ferrous heme to ferric superoxide via ferrous dioxygen is, of course, inevitable. However, these being resonance states, their interconversion will not be reflected in the kinetics, and any sequential mechanism must rather involve the (slow) transformation of the heme oxy I conformational species into the heme oxy II species, or vice versa. Although, in most cases, formation of heme-oxy II was much slower than that of heme oxy I, there was no clear spectral or kinetic evidence for a transient formation of heme-oxy I prior to heme-oxy II and formation of heme-oxy II was strictly [O$_2$]-dependent. This strongly suggests that heme oxy II (like heme oxy I) is formed directly from the reaction between ferrous heme and O$_2$.

A sequential mechanism cannot be excluded altogether, since heme-oxy I forms in a rapid equilibrium reaction, which would allow subsequent oxy II formation to depend on O$_2$, provided the preceding rapid heme oxy I formation is non-saturating. Indeed, although satisfactory fits of the spectral transitions were obtained with just one oxy complex, we found that in most cases the introduction of an oxy I complex prior to oxy II formation improved the fits somewhat (results not shown). However, the improvements were rather marginal, and in most cases the spectrum of the putative intermediate could not be discerned from a mixture of the initial deoxy-reduced and the final heme-oxy II spectra. The slight deviations from pure first-order behavior underlying the improvements that were attained by introduction of an additional transition, may probably be explained equally well by assuming two populations of reduced heme that both react with O$_2$ to heme-oxy II with different rates. Therefore, the simplest interpretation of the kinetics is that both oxy I and oxy II complexes are formed directly by a reaction between reduced NOS and O$_2$.

Heme-oxy II is the catalytically relevant species, since it is formed in the presence of both substrate (L-Arg or NHA) and BH$_4$. One may speculate why species II needs to be formed during NOS catalysis, and why it does not appear to be formed in the reaction of reduced globins or even cytochrome P450 with oxygen. A possible explanation is the specific
requirement for BH4 as a second electron donor in the NOS catalytic cycle. Indeed, one of the reasons why a role for BH4 as a 1-electron donor to the heme was for a long time discounted is the expected high redox potential of the BH4/BH3* couple (33), and it has been suggested that electron transfer from BH4 to Fe(II)•O2 may be thermodynamically uphill (34,35). Shifting electron density away from the heme to the oxy-ligand might ameliorate that situation by increasing the redox potential of the heme. In this way, formation of heme-oxy II could prime the enzyme for the second electron transfer step. Knowledge of the redox potential of NOS-bound BH4, as well as of heme-oxy I and II, will be required to investigate this intriguing possibility.

Effects of substrate and pterin cofactor
In heme-thiolate proteins such as NOS, chloroperoxidase and cytochrome P450, the visible absorbance spectrum of oxygen complexes depends on the negative charge of the thiolate ligand and on the electronic environment of the heme pocket. Certainly, the thiolate ligand is preserved in both heme-oxy I and II. Indeed, Resonance Raman spectroscopy has shown that an intact thiolate ligand is essential for enzyme function (36,37). In addition, substrate binding within the heme pocket may well modulate the absorbance characteristics. For instance, the oxygen complex of cytochrome P450scc depends on the nature of substrate (cholesterol, 22-hydroxycholesterol, or 20,22-dihydroxycholesterol) (38). For NOS, bound substrate appears to impose the heme-oxy II state. L-Arg and, to a lesser degree, NHA contribute a positive charge at neutral pH. This charge may interact with the partial negative charge of the oxygen ligand, and freeze an eventual ferrous dioxygen – ferric superoxide equilibrium in the ferric superoxide state. This hypothesis is supported by the 2-4 nm smaller blue-shift of heme-oxy II spectra in the presence of NHA with respect to those in the presence of L-Arg, which has a more pronounced positive charge (39).

The effect of the pterin cofactors is somewhat harder to explain. Crystallographic studies have repeatedly demonstrated that BH4 has no major effects on the structure of NOS in general, or on the active site structure in particular, despite its close proximity (39,40). Perhaps the clue to understanding the effects of pterin on the nature of the oxyferrous complex may be found in its interaction with one of the propionates of the heme, which it shares with bound substrate (39,41,42). Conceivably, a shift in the position of this propionate in the presence of pterin and/or substrate may decrease the polarity in the vicinity of the heme-bound ligand. As discussed above, the presence of the substrate guanidinium group in close proximity to heme-bound oxygen will nevertheless favor the ferric superoxide state of
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heme-oxy II. In contrast, in the presence of pterin alone, the ferrous oxygen state of heme-oxy I may be favored. Changes in the position of the propionate accompanied by elimination of BH4 binding have been reported for the complex of eNOSoxy with 3-bromo-7-nitroindazole (39).

Kinetically, the most striking effects were observed in the combined presence of substrate and pteridine. In the presence of substrate (L-Arg or NHA), BH4 caused a marked destabilization of heme-oxy II, as a result of the unique capability of these productive combinations to allow the reaction cycle to continue. A similar requirement for both BH4 and substrate has been reported for phenylalanine hydroxylase, which also utilizes BH4 as a cofactor. In the absence of one of both, reductive oxygen activation is inhibited (43). Moreover, the presence of substrate in unproductive combinations resulted in a strong (2 to 3 orders of magnitude) stabilization of the oxygen complex. Interestingly, the complex was more stable in the presence than in the absence of the inhibitory pterin analogs BH2 and ABH4.

Comparison with other NOS isoforms

Our results differ markedly from results reported for nNOSoxy (10) and for full-length eNOS (23). Although a similar stabilizing effect of L-Arg on the oxyferrous complex of BH4-free nNOSoxy has been reported, BH4 (in the absence of substrate) was reported to strongly destabilize the complex (10) in contrast to the moderate effect observed by us. Moreover, for both nNOSoxy and full-length eNOS, substrate was reported not to affect the decay rate of the complex in the presence of BH4, and the red-shifted heme-oxy II complex was formed under all conditions. The origin of these differences with our results is unclear. They seem to involve mainly the substrate- and pterin-deficient states. In contrast, the reported stabilities in the presence of L-Arg and BH4 are comparable with our results.

Coupled versus uncoupled electron transfer

The lifetime of the oxyferrous complex and its modulation by substrate and pterin cofactor are major factors in the susceptibility to uncoupling. The ability of the enzyme to suppress uncoupling in the presence of substrate and BH4 will depend on the relative rates of the productive reaction and the production of O2−. The productive reaction occurs with rates of 6.2 and 14.5 s⁻¹ for L-Arg and NHA, respectively, whereas the uncoupled reaction exhibits a rate of 60 s⁻¹ in the absence of substrate and pterin (see Table 2). Consequently, uncoupling
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would still occur unless substrate and/or BH4 decrease the rate of the uncoupled reaction. The increased stability of the oxyferrous complex in the presence of BH4 or Arg demonstrates that this is indeed the case. In the presence of both, uncoupling will be inhibited even further, as judged form the stability of the complex in the presence of substrate and BH2 or 4-aminobH4. Assuming a similar effect of BH4 in combination with substrate, the inhibition of uncoupling can be estimated to amount to $\geq 98\%$. The ability of the enzyme to sustain NADPH oxidation in the absence of BH4 and/or pterin will depend on the stability of the oxyferrous complex and the rate of electron transfer through the enzyme. For eNOS, the latter rate is very slow (23), suggesting that even fairly low decay rates of the oxyferrous complex should be sufficient to allow uncoupling to occur without significant effects on the rate of NADPH oxidation. We recently observed strong inhibition of NADPH oxidation by recombinant human nNOS in the combined presence of 4-amino-BH4 and substrate (L-Arg or NHA) (27), which we tentatively explained by a mechanism-based component of 4-aminobH4-induced inhibition. The present results suggest that the extraordinary stability of the oxyferrous complex under those conditions may have reduced the rate of catalysis.

**Conclusion**

The observations presented in this study are consistent with two structurally and functionally distinct oxygen complexes: heme-oxy I and heme-oxy II. Heme-oxy I seems to closely resemble the oxy complex of cytochrome P450. Heme-oxy II, which is formed in the presence of substrate, appears to be the obligatory intermediate in both NOS reaction cycles. It seems to differ from heme-oxy I in having a more polar active site pocket and a higher electron density on the dioxygen ligand. These electronic changes may increase the redox potential of the heme, which may be essential for facile reduction of the oxy-complex by BH4. They are also expected to result in stronger uncoupling, a side effect that appears to be counteracted by the presence of BH4 (or any pteridine) at its binding site.

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Footnotes:

1 The abbreviations used are: NOS, nitric-oxide synthase; eNOS\textsubscript{oxy}, the oxygenase domain of recombinant bovine endothelial NOS; nNOS, neuronal NOS; iNOS inducible NOS; NHA, \textit{N}\textsuperscript{G}-hydroxy-L-arginine; BH4, tetrahydrobiopterin [(6\textit{R})-5,6,7,8-tetrahydro-6-(\textit{l}-erythro-1’,2’-dihydroxypropyl)pterin]; 4-ABH4, 4-amino-tetrahydrobiopterin [(6\textit{R})-2,4-diamino-5,6,7,8-tetrahydro-6-(\textit{l}-erythro-1’,2’-dihydroxypropyl)pteridine]; BH2, 7,8-dihydro-L-biopterin; 4-amino-BH2, 4-amino-7,8-dihydro-L-biopterin; UV/vis, ultraviolet/visible; KCEM-buffer, a mixed reaction buffer consisting of 50 mM phosphate, 1 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.5 mM EDTA, 1 mM \(\beta\)-mercaptoethanol.

2 With BH4 analogues or in the absence of pterins, a negative intercept value was estimated from plots of \(k_{\text{obs}}\) versus \(O_2\) concentration for eNOS\textsubscript{oxy} in the presence of substrates. In order to indicate the irreversible process of \(O_2\) binding under these conditions, we tabulated the \(O_2\) dissociation constant value as zero.
Figure legends

**Figure 1:** Absorbance spectra for the reaction of ferrous eNOS oxygenase domain in the presence of BH4 alone and NHA alone with oxygen. An anaerobic solution containing 6 µM enzyme was rapidly mixed at 7 °C with an equal volume of oxygen-saturated buffer in the presence of 50 µM BH4 (Panel A) or 500 µM NHA (Panel B). For clarity only the spectra of the reduced forms (ferrous), of the oxygenated complexes (intermediate) after 2.5-ms mixing, and of the reoxidized forms (ferric) are shown. The intermediates in panels A and B are representative of heme-oxy I and II respectively.

**Figure 2:** Rapid-scan stopped-flow analysis of the reaction between BH4-bound eNOS oxygenase domain and O₂ in the presence of NHA. Panel A, left: expanded spectral traces for the Soret band and the visible region (absorption magnified by a factor 3) within the first 0.1 s; right: overlay of absorbance spectra recorded within the time period of 0.1 to 5 s. The arrows indicate the direction of the spectral shifts with time. Panel B: Spectra associated with the kinetic components from Specfit analysis. Species A to D correspond to the ferrous, heme-oxy II, Fe³⁺-NO and ferric eNOSoxy, respectively. Panel C: calculated variation of concentration for the above species over the course of the reaction.

**Figure 3:** Rapid scan stopped-flow analysis of the reaction of ferrous eNOS oxygenase domain in the presence of saturating NHA and 4-amino-BH4 with oxygen. Panel A, left: expanded spectral traces for the Soret band and the visible region (absorption magnified by a factor 3) within the first 0.1 s; right: overlay of absorbance spectra recorded within the time period of 0.1 to 5 s. The arrows indicate the direction the spectral shifts with time. Panel B: calculated spectra associated with the kinetic components from Specfit analysis: the ferrous (A), heme-oxy II (B) and ferric eNOSoxy (C), respectively. Panel C: calculated variation of concentration for the above species over the course of the reaction.

**Figure 4:** Kinetic analysis of the formation and decay of the heme-oxy I and II complexes as a function of oxygen concentration for ferrous eNOS oxygenase domain. Reactions were initiated by mixing a solution of 6 µM ferrous eNOSoxy with a buffer of desired O₂ concentration. Panel A: Stopped-flow trace for the formation and decay of the heme-oxy I at 0.15 mM O₂. The reaction was monitored at 414 nm in the presence of 50 µM
BH4. The temporal sequence of each transition is indicated by the maximum absorbance value of individual eNOSoxy species detected by rapid scanning methods (see figure 2). Panel B plots the observed rate constants of the heme-oxy II formation (filled symbols) and disappearance (open symbols) versus O₂ concentration. The above rates were given by Specift analysis of the time courses of spectral changes recorded for each O₂ concentration. Experiments contained BH4-bound eNOSoxy in the presence of 250 µM L-Arg (●,○) or 500 µM NHA (▲,Δ), respectively. The lines are a least squares fit for each data set.

Figure 5: Optical absorbance spectra of the eNOS oxygenase domain after bubbling with O₂ and then CO at –30°C. Reduced eNOSoxy (3 µM) was cooled to –30°C, and precooled oxygen was bubbled through the solution. Oxygen binding and subsequent spectral evolution was recorded within 1 min after oxygen addition, followed by flushing precooled CO gas. (A) Reaction with 500 µM NHA. Shown are the spectrum immediately after O₂ addition (······), spectra recorded after CO exchange (-----) and after intervals as specified. (B) Same experiments with 500 µM NHA and saturating BH2 (50 µM). The inserts expand the spectral evolution in the range of Soret band during the reaction.
Table 1: Spectral properties of heme-dioxy complexes detected in single turnover reactions of eNOS oxygenase domain with oxygen*.  

| Substrate | Pterin         | Soret | α,β | Intermediate   |
|-----------|----------------|-------|-----|----------------|
| -         | BH4            | 421   | 560 | heme-oxy I     |
| -         | 4-amino-BH4    | 420   | 561 | heme-oxy I     |
| -         | BH2            | 421   | 560 | heme-oxy I     |
| Arg       | -              | 433   | 565/595 | heme-oxy II  |
| Arg       | BH4            | 432   | 563/597 | heme-oxy II  |
| Arg       | 4-amino-BH4    | 432   | 565/600 | heme-oxy II  |
| Arg       | BH2            | 431   | 563/598 | heme-oxy II  |
| NHA       | -              | 428 (419) | 560/595 (560) | heme-oxy II (I) |
| NHA       | BH4            | 428   | 560/590 | heme-oxy II  |
| NHA       | 4-amino-BH4    | 429 (425) | 560/593 (557/595) | heme-oxy II  |
| NHA       | BH2            | 429 (426) | 560/594 (557/595) | heme-oxy II  |
| -         | -              | 428   | 560/595 | heme-oxy II  |

*The λ_max values of the Soret and visible (α,β) bands are those of the intermediates discerned by Specfit global analysis of the kinetic data. In parentheses are the corresponding values for the oxy complexes detected under cryogenic conditions (-30°C).
Table 2: Kinetic parameters of the intermediates after oxygen addition to reduced eNOS oxygenase domain in the presence and absence of substrates and pterins. Ferrous eNOS\textsubscript{oxy} in the presence or absence of BH4 and/or biopterin analogs was rapid-mixed with an equal volume of O\textsubscript{2}-containing buffer at 7°C. The association and dissociation rate constants were extracted from the least squares fit of the formation rates of the heme-oxycomplex versus O\textsubscript{2} concentration. The rate constants of the disappearance of each component were determined by global analysis of rapid scanning spectra (Specfit program) using a sequential model. Standard deviations fell within 5% of the reported mean value in all cases.

| Conditions          | heme-oxy | Fe\textsuperscript{III}-NO |
|---------------------|----------|-----------------------------|
|                     | $k_{on}$ | $k_{off}$ | $k_{decay}$ | $k_{decay}$ |
|                     | mM$^{-1}$.s$^{-1}$ | s$^{-1}$ | s$^{-1}$ | s$^{-1}$ |
| NHA                 |          |            |            |            |
| + BH4               | 342      | 22         | 14.5       | 2.5        |
| + 4-amino-BH4       | 272      | 0          | 0.086      |            |
| + BH2               | 227      | 9.7        | 0.134      |            |
| no pterin           | 145      | 0          | 0.358      |            |
| Arginine            |          |            |            |            |
| + BH4               | 265      | 24         | 6.2        |            |
| + 4-amino-BH4       | 410      | 0          | 0.0536     |            |
| + BH2               | 452      | 0          | 0.181      |            |
| no pterin           | 338      | 0          | 1.49       |            |
| No substrate/pterin | 2480$^b$ | N.D$^a$    | 60/2.1     |            |
| BH4                 | 2333$^b$ | N.D$^a$    | 144/18     |            |
| 4-amino-BH4         | 2758$^b$ | N.D$^a$    | 27/0.3     |            |
| BH2                 | 2600$^b$ | N.D$^a$    | 34/0.3     |            |

$^a$Not determined; $^b$pseudo first order rates estimated in the presence of 0.15 mM O\textsubscript{2}
Figure 1

A

B

Heme-oxy I and II as oxygenated eNOS_oxy complexes
Figure 2

(A) Absorbance spectra of Heme-oxy I and II as oxygenated eNOS\textsubscript{oxy} complexes.

(B) Extinction coefficient (10\textsuperscript{-4} M\textsuperscript{-1} cm\textsuperscript{-1}) for different conditions.

(C) Concentration over time for different conditions (µM)
Figure 3

A

Absorbance

wavelength (nm)

B

Extinction coefficient (10^{-4} \cdot M^{-1} \cdot cm^{-1})

wavelength (nm)

C

Concentration (\mu M)

Time (s)

A

B

C

X3
Figure 4

A

Absorbance at 414 nm

Fe$^{2+}$

Fe$^{2+}$O$_2$

Fe$^{3+}$

time (s)

0.00 0.05 0.10 0.15 0.20 0.25 0.30 0.50 1.00

B

$\kappa_{obs}$ (s$^{-1}$)

0 50 100 150 200

[O$_2$], mM

0.0 0.1 0.2 0.3 0.4 0.5

$\Delta$ $\bullet$ $\bigtriangleup$ $\bigtriangleup$ $\bigtriangleup$ $\bigtriangleup$
Figure 5

A

B
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