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On the Mechanism of the Nitric Oxide Synthase-catalyzed Conversion of N^{ω}-Hydroxy-L-arginine to Citrulline and Nitric Oxide*

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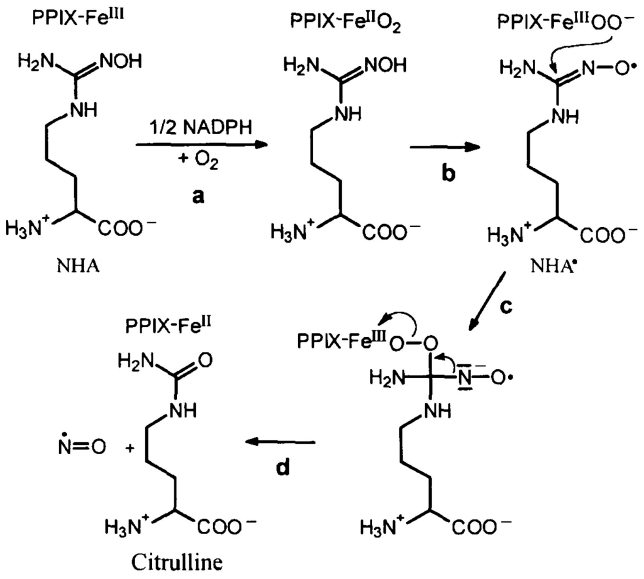
The mechanism of oxidation of N^{ω}-hydroxy-L-arginine (NHA) by the iron-dioxygen complex in nitric oxide synthase (NOS) is still uncertain. The uncertainty has not been helped by a lack of precision in the notation used to describe the oxidation states and electrical charges on the iron and oxygen in some of the suggested mechanisms. These problems of notation are addressed, and, in addition, a cyclic voltammetric measurement of the oxidation potential of NHA, namely +0.10 ± 0.04 V *versus* normal hydrogen electrode, is used to argue that the sometimes postulated oxidation of NHA by the iron-dioxygen complex to form an intermediate radical cation, $NHA^{\cdot+}$, is very unlikely for thermodynamic reasons. Instead, it is suggested that this oxidation occurs by a thermodynamically favored abstraction of the hydrogen atom from the >C=NOH moiety of NHA to form an intermediate iminoxyl radical, >C=NO$^{\cdot}$. A subsequent nucleophilic attack by the iron-hydroperoxide species formed by this H-atom abstraction on the carbon atom of the iminoxyl radical moiety leads to the production of nitric oxide (NO) and citrulline.

In a recent minireview, Marletta (1) has noted that the steps involved in the nitric oxide synthase (NOS)[1]-catalyzed conversion of N^{ω}-hydroxy-L-arginine (NHA) to citrulline and nitric oxide (NO) are far less clear than the earlier steps by which NOS catalyzes the conversion of L-arginine to NHA. Marletta also provides evidence that a P450-type hemoprotein (PPIX-Fe) is implicated in the NHA to citrulline and NO reaction, just as in the L-arginine to NHA reaction (1), and then presents the



SCHEME 1

"mechanistic speculation" shown in Scheme 1. This mechanism is analogous to that proposed for the steps in the demethylation reactions catalyzed by aromatase and related P450 enzymes (2, 3). For example, the 19-methyl group of steroidal androgens (testosterol and androstenedione) is removed with concomitant aromatization of the A ring of the steroid (2–8). This final step involves the attack of an iron peroxide species ($Fe^{II}OO^{\cdot} \leftrightarrow Fe^{III}$-$OO^-$) on the aldehyde produced via two successive hydroxylations of the 19-methyl group (which presumably occur via the "normal" Fe^{IV}=O-induced "oxygen-rebound" mechanism) (9–12). In the Marletta mechanism (see Scheme 1), a similar reaction is invoked between the iron peroxide and the guanidino carbon of the oxime tautomer of NHA (which is thermodynamically more stable than the hydroxylamine) (13). A similar, somewhat more detailed mechanism has recently been proposed by other authors (14).

We believe that the basic features of the Marletta mechanism must be correct. However, we would like to examine in more detail the step that we have labeled **b** in Scheme 1. With regard to this step, Marletta states that "...one distinguishing feature [relative to the P450-catalyzed demethylation reaction] has been introduced, namely [that] NHA provides one electron for the reaction while the other is derived from the reductase via NADPH[.]"

EXPERIMENTAL PROCEDURES

Cyclic voltammetry was performed with a Bruker 310 instrument on a 7×10^{-3} M solution of N^{ω}-hydroxy-L-arginine (Alexis Corp.) in phosphate buffer (0.1 M; pH 7.5) at 20 °C under argon. Measurements were made using a platinum button as the working electrode and a platinum foil as the counter electrode; the reference electrode was a saturated calomel electrode (SCE). Data acquisition was made with the DigiS software package (GfS, Aachen, Germany) running on a 486 PC equipped with a DAP 1200/4 data acquisition board (Microstar Laboratories). No changes in the general shape of the voltammograms were observed when the scan rates were varied from 50 mV/s to 10 V/s. Plots of the peak potentials *versus* the logarithm of the scan rate and the peak current density *versus* the square root of the scan rate were linear up to 3 V/s, indicating a diffusion-controlled electron transfer process. Plots of

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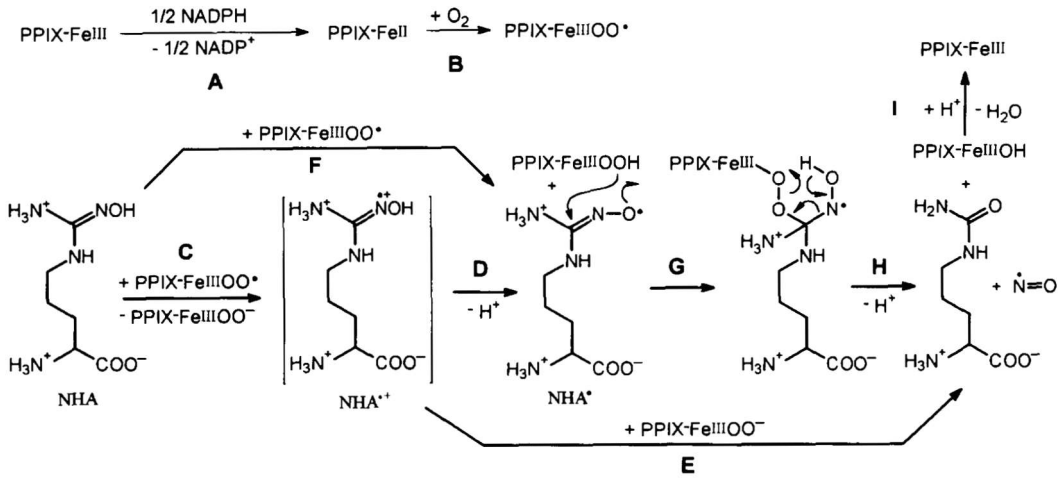
[1] The abbreviations used are: NOS, nitric oxide synthase; NO, nitric oxide; NHA, N^{ω}-hydroxy-L-arginine; PPIX, protoporphyrin IX.



SCHEME 2

the peak potentials E_p *versus* β according to Equation 1 were linear (r^2 = 0.984) up to scan rates of 3 V/s, yielding E^0_{ox1} at β = 0.5.

$$E_p = E^0 + 4(1 - 2\beta)\Delta G^{\ddagger} \quad \text{with} \quad \beta = 1.857\,RT/[F(E_p - E_{p/2})] \qquad \text{(Eq. 1)}$$

RESULTS AND DISCUSSION

Before discussing other possible schemes in detail, we believe it would be helpful to make some general comments concerning the notation of oxidation states and electrical charges used throughout the biochemical literature. There, inconsistencies can often be found with a disregard of the proper electron count and the usual convention when writing valence bond structures. Because the oxidation states of transition metal atoms in complexes (in particular those of biological relevance) very often cannot be determined definitely, their assignment by *formal oxidation numbers* is normally based on a convention relating to the formal heterolysis of bonds (see Refs. 15–17, and references cited therein). In this respect, the mechanistic scheme found in Ref. 14 is particularly confusing/misleading because therein no differentiation has been made between the formal oxidation numbers of the relevant atoms (O, Fe) in the complexes, formal electrical charges, and net ionic charges. In the light of such inconsistencies, the statement "...reducing the *ferrous* oxy complex of the heme..." in Marletta's paper (1) and the formulation as "PPIX-$Fe^{II}O_2$" in his Scheme 1 *might* lead to the impression that in the PPIX-Fe^{II} + O_2 reaction the formal oxidation state of the iron is not altered. In correct valence bond terms, the structures of the PPIX-$Fe^{II}O_2$ complex should be represented as shown in Structures I or II, which formally describe the coordination of triplet and singlet state dioxygen, respectively.

PPIX-Fe^{II} ← $\dot{O}$–$\bar{O}$·

STRUCTURE I

PPIX-Fe^{II} ← O=$\bar{O}$

STRUCTURE II

PPIX-Fe^{III} ← $\bar{O}$–$\bar{O}$·

STRUCTURE III

Most spectroscopic and chemical evidence, however, indicates that "end-on" (1:1) heme-dioxygen complexes (*i.e.* those that bind oxygen reversibly) are better formulated as Fe^{III}-superoxide complexes (Structure III) (see Refs. 15–17, and references cited therein) (compare also Scheme 14 of Ref. 2). Structure III may either be regarded simply as a resonance form of I/II or as a distinct chemical species, deriving from an "inner-sphere"

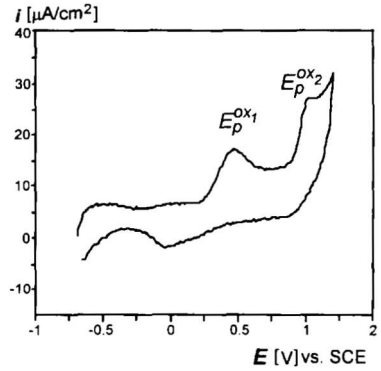


FIG. 1. **Cyclic voltammogram of N^{ω}-hydroxy-L-arginine in phosphate buffer, pH 7.5, at a scan rate of 100 mV/s.** The reduction peak at −0.1 V is due to the solvent.

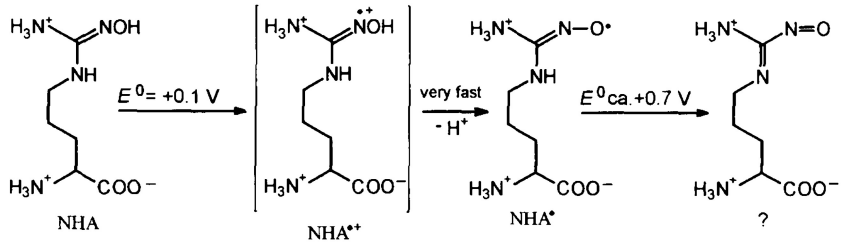
electron transfer in I/II. (The apparent diamagnetism of iron-oxygen complexes of type III can be explained by an antiferromagnetic coupling of the two unpaired electrons (see Refs. 16 and 17, and references cited therein).) Thus, PPIX-$Fe^{II}O_2$ should only be regarded as an abbreviated way of indicating that an end-on complex has been formed from a (high spin) PPIX-Fe^{II} (d^6) species and molecular oxygen; this formula does not and should not be used to define a distinct oxidation state of the iron.

Here we wish to propose small, but significant, modifications to Marletta's original Scheme 1 and the scheme presented by Feldman *et al.* (14). Our complete catalytic cycle is shown in Scheme 2. The electron "count" has been balanced for each elementary step, and each step would appear to be thermodynamically favored. We prefer to represent the PPIX-$Fe^{II}O_2$ adduct in terms of Structure III.

Pufahl and Marletta (18) have provided evidence that it is indeed NADPH which delivers the first electron for the initial reduction of the PPIX-Fe^{III} form of the NOS (step **A**), which is required for the subsequent binding of O_2 (step **B**). Therefore, the second electron, which is needed for the reduction of the PPIX-$Fe^{III}OO^{\cdot}$ species to the (assumed) heme peroxo complex, PPIX-$Fe^{III}OO^{-}$, must be delivered by the substrate (provided that no other, unknown species are involved in this part of the catalytic cycle).

The focal point of the present paper is the way in which the second electron is transferred from the NHA substrate to the PPIX-$Fe^{II}O_2$ complex (step **b** in Scheme 1). Marletta's statements that "...NHA provides one electron..." and "[the] mecha-

SCHEME 3



nism as drawn shows the oxime of NHA reducing the ferrous oxy complex of the heme..." may be interpreted in a sense that a true single electron transfer occurs between NHA and PPIX-$Fe^{II}O_2$ with formation of the radical cation NHA$^{\cdot+}$ of NHA (step **C**). Whereas Marletta did not speculate about any intermediate involved in this step and simply wrote the direct formation of the iminoxyl radical of NHA, NHA$^{\cdot}$ (step **b**, equivalent to steps **C** and **D** in Scheme 2), a radical cation intermediate, NHA$^{\cdot+}$, has been explicitly assumed by Feldman *et al.* (14) in their mechanistic rationalization. In the Feldman scheme, NHA$^{\cdot+}$ is formed and is subsequently attacked by the nucleophilic peroxo complex, PPIX-$Fe^{III}OO^-$ (step **E**). We considered the existence of the NHA$^{\cdot+}$ radical cation as a distinct intermediate to be highly unlikely for two reasons. First, the radical cation of an oxime is expected to lose a proton and form the iminoxyl radical NHA$^{\cdot}$ (step **D**) much more rapidly than it would undergo a nucleophilic attack (step **E**); second, we felt that the redox potential of the PPIX-$Fe^{III}OO^{\cdot}$complex was unlikely to be sufficiently high as to favor such an oxidation (15–17, 19, 20).

In order to check on these two points, we measured the oxidation potential of NHA by cyclic voltammetry. A typical cyclic voltammogram obtained from a 7×10^{-3} M solution of NHA in buffer (pH 7.5) is shown in Fig. 1. At 100 mV/s two irreversible oxidation peaks are observed at E_p^{ox1} = +0.47 and E_p^{ox2} = +1.05 V (*versus* SCE).[2] There was absolutely no sign of even a partial reversibility of either step at scan rates up to 10 V/s. We interpret this result as being in agreement with our first point (Scheme 3), that is, the removal of an electron from NHA at E_p^{ox1} is followed by a rapid, irreversible deprotonation. The iminoxyl radical, NHA$^{\cdot}$, thus formed[3] is further oxidized at the higher potential and the resulting cation is again capable of loosing a proton irreversibly (a possible product is indicated in Scheme 3).

The standard potential E^0_{ox1} connected with the first irreversible oxidation step was estimated from the dependence of the peak potential on the scan rate by the procedure of Tanaka *et al.* (21–23), giving a value of E^0_{ox1} = +0.10 ± 0.04 V *versus* normal hydrogen electrode (+0.34 V *versus* SCE). Standard reduction potentials of P450-dioxygen (PPIX-$Fe^{II}O_2$) complexes are generally found to be around -0.4 V *versus* normal hydrogen electrode (see Refs. 16, 17, 19, and 20, and references cited therein), significantly (~0.5 V) lower than the potential we measured. Therefore, the PPIX-$Fe^{III}OO^{\cdot}$ species is very unlikely to be capable of oxidizing NHA to its radical cation; indeed, this electron transfer reaction would be energetically about 12 kcal/mol uphill. Therefore, we conclude that the radical cation of N^{ω}-hydroxy-L-arginine is very unlikely to be an intermediate in the NOS-catalyzed formation of nitric oxide from N^{ω}-hydroxy-L-arginine.

Since the intermediate formation of the iminoxyl radical, NHA$^{\cdot}$, is an attractive assumption for the NOS cycle, we propose that its formation occurs by a direct reaction between NHA and PPIX-$Fe^{III}OO^{\cdot}$, that is, via a free radical hydrogen atom abstraction by the peroxyl radical-type species PPIX-$Fe^{III}OO^{\cdot}$ (path **F**). The O–H bond strengths of sterically unhindered oximes are 85 ± 1 kcal/mol (24), whereas the O–H bond strengths of hydrogen peroxide and various organic hydroperoxides are 88 ± 1 kcal/mol (see Refs. 25–29, and references cited therein). A hydrogen atom abstraction from NHA by the PPIX-$Fe^{III}OO^{\cdot}$ peroxyl radical is therefore likely to be ~3 kcal/mol exothermic and hence should be a kinetically competent step in the overall process of NO synthesis. Thus, the "second electron" that is required to make NO is transferred as a hydrogen atom. This means that it will be the resulting hydroperoxy species, PPIX-$Fe^{III}OOH$, which acts as a nucleophile towards NHA$^{\cdot}$ (path **G**) rather than PPIX-$Fe^{III}OO^-$ as predicted in Marletta's scheme (step **c**). The release of $^{\cdot}$NO and citrulline then could occur from an intermediate, neutral aminyl radical (step **H**) rather than from a nitroso radical anion (step **d**). Finally, protonation of the such formed PPIX-Fe^{II}-$^{\cdot}$OH species regenerates the initial PPIX-Fe^{III} stage of the catalytic NOS cycle (step **I**).

In conclusion, we wish to emphasize that Marletta's mechanism for the NOS-catalyzed conversion of NHA to citrulline and NO (Scheme 1) as modified in the present work (Scheme 2) is elegant, simple, and involves only the thermodynamically acceptable and chemically known (or perfectly reasonable) elementary reaction steps: **A**, **B**, **F**, **G**, **H**, and **I**, in sequence. This cannot be said about any alternative mechanistic proposal of which we are aware.

[2] Virtually identical cyclic voltammograms were obtained from pivaloylketone oxime in acetonitrile solution, although the peak potentials are higher by ~0.7 V.

[3] The generation of iminoxyl radicals by oxidation of oximes is a common procedure for ESR purposes.

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