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Hartree–Fock and density functional theory calculations for the reaction mechanism of nitric oxide reductase cytochrome P450nor from *Fusarium oxysporum*

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Abstract

A reaction mechanism of a nitric oxide reductase, cytochrome P450nor (P450nor) from *Fusarium oxysporum*, was clarified by using Density functional theory and Hartree–Fock calculations. In this reaction mechanism, molecular orbital (MO) analysis revealed that the NO ligand dissociates from the heme iron immediately after one-electron reduction by NADH, and MO energy analysis revealed that NADH acts as a one-electron reducer, not as a two-electron reducer, and that NADH has a pivotal role different from other one-electron reducers. The role of NADH is to act as a double one-electron donor (i.e. one-electron transfer occurring twice) and to combine with the NO^- molecule by charge recombination reaction. Our quantum chemical calculations indicated that all reactions occurring in the heme pocket are too fast to become rate-limiting. Therefore, the rate-limiting steps in the proposed reaction mechanism are the process of capturing NO and NADH into the heme pocket and the process of expelling a product generated in the heme pocket. Kinetics of these processes was discussed based on large-amplitude vibration, which helps capturing and expelling processes in a widely opened heme pocket of P450nor. The reaction mechanism proposed here well explains published experimental data.
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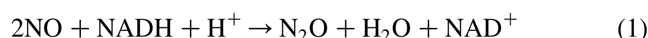
Keywords: Nitric oxide reductase; Cytochrome P450; Quantum chemical calculation; Reaction mechanism

1. Introduction

An enzyme is an elaborate ‘machine’ whose reaction cycles are highly effective, as evidenced by its nearly 100% quantum-efficiency in the photosynthesis of purple bacteria and by its highly efficient metabolism of toxic NO. Intravital reactions such as photosynthesis have been widely used as models for highly functional materials. To control the emission of nitrogen oxides (NO_x), artificial materials that have clearly defined and well-documented highly effective reaction cycles need to be developed.

In the highly efficient metabolism of toxic NO, a heme protein named cytochrome P450 nitric oxide reductase (or P450nor) plays a central role by catalyzing an NO reduction reaction in which two NO molecules are converted into a N_2O molecule as follows:



$$2NO + NADH + H^+ \rightarrow N_2O + H_2O + NAD^+ \quad (1)$$

The high turnover number makes this enzymatic cycle unique. In typical monooxygenase P450s, the maximum turnover number is $\sim 10\ s^{-1}$, whereas in P450nor for NO reduction reaction, the maximum turnover number is $\sim 1000\ s^{-1}$. If the mechanism responsible for this high turnover number in the NO reduction reaction system of P450nor can be imitated, then high performance *nano-machines* can be developed. Although several reaction mechanisms of P450nor have been proposed, none can explain the observed high performance detoxification of NO.

P450nor has been isolated and purified from the denitrifying fungus *Fusarium oxysporum* by Shoun et al.

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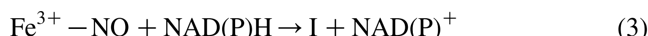
[1]. As identified by its name [2], this P450 enzyme contains protoheme in its active site with the heme iron linked on its proximal side to a sulfur atom of a nearby cysteine residue. Based on cDNA analysis, this enzyme is in the superfamily of cytochrome P450s [2] (cytochrome P450 55A1 is its systematic name based on its cDNA sequence), and its amino acid sequence shows about 25% identity with that of *Pseudomonas putida* P450cam and about 40% with that of *Streptomyces* P450SU2.

Despite these similarities in its structure and properties with other P450s, P450nor has unique biological functions. One example is its NO reductase activity; typical P450s activate O_2 to catalyze a monooxygenation reaction. P450nor can reduce NO to N_2O but cannot catalyze the monooxygenation reaction. Another example is its high turnover number in the NO reduction reaction and its direct electron transfer (ET) reaction with NADH in the NO activity, in sharp contrast with the indirect ET in the monooxygenation reaction by other P450s through a flavoprotein or an iron–sulfur protein. No redox co-factor has been detected in P450nor except for protoheme, in contrast with flavo-hemoproteins detected in other P450s, such as in P450BM3 [3] and NO synthase [4]. The most unique structural characteristic in P450nor is the widely opened heme pocket, thus allowing P450nor to receive electrons from NADH at the heme-distal side, while still being able to receive electrons from the proximal side, namely, able to undergo monooxygenation reaction. This widely opened heme pocket is presumed to act as an access channel to NAD(P)H. Based on its crystal structure, the heme-distal pocket of P450nor is in a highly hydrophilic environment compared with that of other P450s, and has a positively charged cluster of several charged amino acid residues [5].

To investigate the NO reduction reaction mechanism in P450nor, various experiments and quantum chemical calculations have been conducted. Based on UV/Vis spectroscopic and kinetics experiments, Shiro et al. reported three characteristic spectra, namely, a Soret peak at 414, 431, and 444 nm, and proposed an overall reaction mechanism, which can be separated into the following three partial reactions [6]:

$$Fe^{3+} + NO \rightarrow Fe^{3+} - NO \quad (2)$$



$$Fe^{3+} - NO + NAD(P)H \rightarrow I + NAD(P)^{+} \quad (3)$$

$$I + NO + H^{+} \rightarrow Fe^{3+} + N_2O + H_2O \quad (4)$$

In Eq. (2), the ferric resting form (Fe^{3+}; ferric resting species) of P450nor binds the substrate NO to form a ferric iron–nitric oxide complex (Fe^{3+}–NO; ferric NO species). Ferric NO species is therefore reduced by NADH to form a specific intermediate (*I*) in Eq. (3), and finally reacts with the second NO to form the product, N_2O, in Eq. (4). Shiro et al., respectively, attributed the three spectra to ferric resting species (414 nm), ferric NO species (431 nm), and an unknown intermediate *I* (444 nm). This identity of ferric resting species and ferric NO species was confirmed by experimental results [6–8], whereas the identity of intermediate *I* is still controversial [6,9–11].

Fig. 1 schematically shows the reaction pathways for several reaction mechanisms of P450nor that have been proposed based on experimental (solid lines) and calculation results (dashed lines) summarized by Silaghi-Dumitrescu [12]. The complex $[FeNO]^6$ denotes ferric NO species, $[FeNO]^7$ denotes reduced ferric NO or ferrous NO species, and $[FeNO]^8$ species denotes further reduced ferric NO species.

The reaction mechanism proposed by Harris (dashed lines in the top half of Fig. 1) based on density functional theory (DFT) calculations assumes that the $[FeNO]^8$ species is formed by ET from NADH into the initial $[FeNO]^6$ complex [10]. In this mechanism, the $[FeNO]^8$ species is the intermediate *I* and reacts with NO, eventually liberating the unstable $ONNO^{2-}$, which in turn decomposes to N_2O and H_2O. Obayashi et al. [9] proposed a reaction pathway (solid line) based on Raman spectroscopic studies. Harris [10] proposed a formation of $[FeNO]^8$ species based on Obayashi's reaction pathway. However, unlike in Harris's mechanism, in Obayashi's mechanism, the $[FeNO]^8$ reacts with two protons and the second NO.

The reaction mechanism proposed by Daiber et al. (solid line in the bottom half of Fig. 1) based on kinetics study suggests that $[Fe–(H)NOH]^8$ is the intermediate *I* [11]. Initially, NO binds to ferric resting species, followed by reduction of the $[FeNO]^6$ complex by NADH. Subsequent

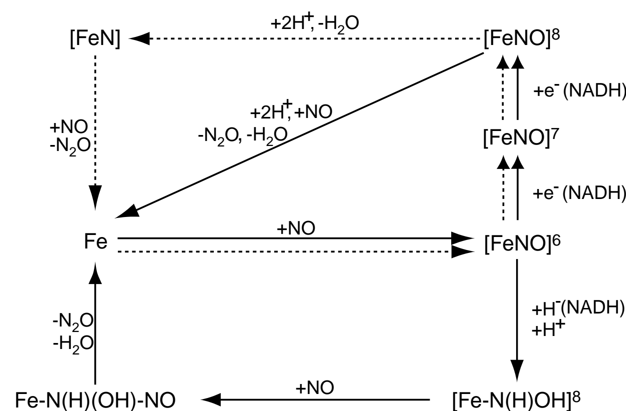


Fig. 1. Previously proposed reaction pathways of P450nor based on experimental and calculation results. Dashed line in upper half of figure indicates NO reduction reaction mechanism proposed by Harris based on DFT calculations [10]. Species unambiguously known to be involved in the catalytic cycle are highlighted. $[FeNO]^{8,}$ [FeN], $[Fe–N(H)OH]^8$ and Fe–N(H)(OH)–NO have been proposed to constitute the experimentally observed intermediate *I*. Solid line in top of half indicate reaction pathway proposed by Obayashi et al. [9] based on Raman spectroscopic studies. Enemark–Feltham notation is used for iron-nitrosyl species $[FeNO]^n$, and to distinguish the oxidation number of ferric species. Superscript denotes the total number of Fe d and NO π^*. Solid line in the bottom half indicates reaction mechanism proposed by Daiber et al. [11].

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