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Binding mode analysis of the NADH cofactor in nitric oxide reductase: A theoretical study

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Abstract

Nitric oxide reductase (Nor), a member of the cytochrome P450 superfamily, takes part in the denitrification process of fungi by reducing NO to N_2O. Evidence indicates that Nor binds NADH, source of the reducing equivalents of the reaction, within its large hydrophilic ligand binding cavity on the distal side of heme and receives electrons directly from the cofactor. Here we present a binding mode analysis of the structure of the Nor–NO–NADH complex, performed in three steps. The NADH cofactor was first docked into the enzyme interior using the Monte Carlo multiple minimum algorithm, refined by low-mode conformational search and the final arrangement was obtained in a 5 ns NPT molecular dynamics simulation. The NADH cofactor, in our results, is positioned – by Arg174, Lys291, Asp393 and several water molecules – within reactive distance of the NO binding spot suggesting a direct hydride shift mechanism between the two. The catalytically required water molecule is captured by NADH and the cofactor not only retains the suggested H-bonded proton transfer pathway between the active site and the solvent, but provides structural restraint for its members. We also found that direct interaction is formed between the cofactor and propionate A of the heme group, which flips from the proximal to the distal side of the heme plane in order to become an H-bonding partner of NADH. The role of Arg64 and Glu71 was suggested to be fixing the residues of the translocated helix B′ to their new position.

Keywords: Heme protein; Nitrix oxide; Nitric oxide reductase; NADH; Molecular dynamics; Monte Carlo multiple minimum search; Low-mode conformational search

1. Introduction

Nitric oxide reductase (Nor) takes part in the denitrification process of certain fungi—it catalyzes the reduction of NO to N_2O [1]. The enzyme has become interesting from several points of view. Nor, although a member of the cytochrome P450 superfamily (P450s) [2], retains important structural differences which result in its inability to carry out a monooxygenation reaction characteristic of the P450s. Instead, it has a unique function of reducing NO, utilizing the same thiolate bound heme center as P450s do. The region corresponding to the substrate access channel of cytochrome P450s is in a different conformation in Nor, forming a spacious distal pocket above the heme [3] lined by an unusually large number of positively charged amino acids and filled with numerous water molecules [4]. Evidence indicates that Nor binds NADH (in case of Nor isozyme of *Fusarium oxysporum* and isozyme 1 of *C. tonkinense*, while isozyme 2 of *C. tonkinense* and isozyme of *T. cutaeum* can utilize either NADH or NADPH [5]), source of the reducing equivalents of the reaction, within this large hydrophilic ligand binding cavity thus receiving the electrons required for catalysis directly—without the aid of a reducing partner such as cytochrome P450 reductase [1]. This mode of NAD(P)H binding – e.g. capturing the cofactor in an ample cavity surrounded by a cluster of positively charged residues – is also unusual, NAD(P)H binding enzymes such as dehydrogenases typically sequester the cofactor within a Rossman fold motif [6].

The heme group of the resting state enzyme (Fe(III)Nor) binds NO in a slightly bent and tilted conformation [7]. In the presence of NAD(P)H, the complex is reduced to an intermediate (*I*) which reacts with a second NO molecule to form the N_2O product [8]:

$$\mathrm{Fe(III)Nor} + \mathrm{NO} \rightarrow \mathrm{(Fe(III)Nor)NO}$$

$$\mathrm{(Fe(III)Nor)NO} + \mathrm{NAD(P)H} \rightarrow I + \mathrm{NAD(P)^+}$$

$$I + \mathrm{NO} + \mathrm{H^+} \rightarrow \mathrm{Fe(III)Nor} + \mathrm{N_2O} + \mathrm{H_2O}$$

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However, it has not been clarified yet whether the NAD(P)H mediated reduction and proton transfer occur simultaneously or sequentially, or if hydride transfer is part of the mechanism. The structure of the reaction intermediate *I* is also still under debate. Averill proposed that following hydride transfer Fe(II)NHO would be formed [9]; the results of Daibner and coworkers also support the hydride transfer mechanism and suggest an intermediate of Fe(III)·NHOH [10]. On the other hand, based on resonance Raman measurements, the Fe(II)$(NO)^{-}H^{+}$ form was suggested by Obayashi et al. [11] who argue against a hydride shift. Theoretical studies reached similar conclusions; both Tsukamoto et al. [12] and Harris [13] concluded in favor of the two electron reduced form of the initial Fe(III)NO complex (see Fig. 1). The proton required for the reaction is thought to be delivered through a hydrogen-bonding network which connects the active site and the bulk solvent, the ultimate source being an ordered water molecule bound in the immediate vicinity of NO [3,7]. Unraveling the details of the mechanism is greatly hampered by the fact that although much is known about the binding of the NAD(P)H cofactor, its exact binding mode is unclear.

Here we present a model for the ternary complex of Nor, NO and NADH. Results demonstrate that the inner landscape of Nor is architectured to hold the cofactor adjacent to the substrate leaving enough room for specific coordination of water molecules and that the heme prosthetic group also participates in the communication between the protein matrix and NADH. Our results provide a framework for further theoretical studies of the enzyme mechanism, where the effect of the cofactor could also be taken into consideration.

2. Methods

The structure of the Nor–NO–NADH complex was calculated in three steps. The NADH cofactor was first docked into the enzyme interior using the Monte Carlo multiple minimum algorithm and was further refined by low-mode conformational search. Solvent effects were modeled by using the GB/SA continuum solvation model. The structure thus derived was solvated by explicit water molecules, and the final arrangement was obtained in a 5 ns NPT molecular dynamics simulation.

2.1. Calculation of the charge set of the heme, NO and the NADH cofactor

ESP charges [14] were calculated by the B3LYP density functional method (LACVP** basis set [15]) – as implemented in the program Jaguar [16] – for the crystallographically determined geometry of the Nor(Fe(III))–NO complex (Nor of *F. oxysporum*, PDB code:1cl6 [7]). Two models were formed differing in the representation of the proximal Cys352 ligand, the residue anchoring the heme group to the protein matrix. In Model 1, an SH^{-} group, in Model 2, $CH_3CH_2S^{-}$ group was used. Both systems included five water molecules, three of these coordinate the heme propionates and two waters were found in the vicinity of the NO ligand. The carbonyl oxygen of Ala239 is 2.92 Å from the oxygen of NO, therefore an additional water molecule was built into the model to represent its effect; as in the case of the OG atom of Ser286. Since the active site of Nor is exposed to the solvent and is lined by a number of charged residues, calculations were carried out in water (using the SCRF method of Jaguar [17,18]). Charges of the NADH cofactor were obtained using the same method.

In all further calculations (carried out using the program MacroModel [19]) the heme, NO and NADH carried the charges calculated by the described protocol.

2.2. Monte Carlo multiple minimum (MCMM) search

The input structure of the MCMM search [20,21] was that of the crystal structure of the Nor–NO complex [7] with the NADH cofactor positioned manually, the internal torsions of residues 60–90, 170–180, 230–250, 282–294 (the residues building up the ligand binding pocket of Nor) and all internal torsions of NADH were considered. Monte Carlo search involved the random variation (within the range of 0°–180°) of a randomly selected subset of all torsional angles (a minimum

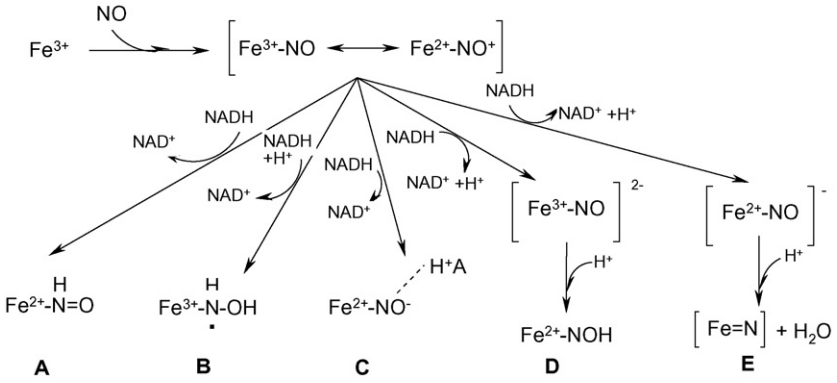

Fig. 1. Schematic representation of proposed reaction paths (A–E) leading to the formation of the reactive species (*I*) ready to attack the second NO molecule according to Averill [9], Daibner et al. [10], Obayashi et al. [11], Tsukamoto et al. [12] and by Harris [13], respectively. Schemes A and B proceed by a direct hydride shift mechanism, while in the others, donation of the hydrogen of the NADH cofactor (to a protein residue or water) will initiate a cascade leading to protonation of the active site.