Density functional theory (DFT) and combined quantum mechanical/molecular mechanics (QM/MM) studies on the oxygen activation step in nitric oxide synthase enzymes

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Abstract
In this review paper, we will give an overview of recent theoretical studies on the catalytic cycle(s) of NOS (nitric oxide synthase) enzymes and in particular on the later stages of these cycles where experimental work is difficult due to the short lifetime of intermediates. NOS enzymes are vital for human health and are involved in the biosynthesis of toxic nitric oxide. Despite many experimental efforts in the field, the catalytic cycle of this important enzyme is still surrounded by many unknowns and controversies. Our theoretical studies were focused on the grey zones of the catalytic cycle, where intermediates are short-lived and experimental detection is impossible. Thus combined QM/MM (quantum mechanics/molecular mechanics) as well as DFT (density functional theory) studies on NOS enzymes and active site models have established a novel mechanism of oxygen activation and the conversion of L-arginine into NO-ω-hydroxoy-arginine. Although NOS enzymes show many structural similarities to cytochrome P450 enzymes, it has long been anticipated that therefore they should have a similar catalytic cycle where molecular oxygen binds to a haem centre and is converted into an Fe(W)-oxo haem(•+) active species (Compound I). Compound I, however, is elusive in the cytochrome P450s as well as in NOS enzymes, but indirect experimental evidence on cytochrome P450 systems combined with theoretical modelling have shown it to be the oxidant responsible for hydroxylation reactions in cytochrome P450 enzymes. By contrast, in the first catalytic cycle of NOS it has been shown that Compound I is first reduced to Compound II before the hydroxylation of arginine. Furthermore, substrate arginine in NOS enzymes appears to have a dual function, namely first as a proton donor in the catalytic cycle to convert the ferric-superoxo into a ferric-hydroperoxo complex and secondly as the substrate that is hydroxylated in the process leading to NO-ω-hydroxy-arginine.

Introduction
An important messenger molecule in the human brain is nitric oxide, a process that was only discovered in the early 1980s [1–3]. However, due to the high reactivity of nitric oxide it has to be synthesized close to where it is needed. The enzyme NOS (nitric oxide synthase) fulfils this function in the body through the two-step conversion of L-arginine into L-citrulline and nitric oxide via an NO-ω-hydroxoy-arginine intermediate. The biochemical functions of nitric oxide include control of blood pressure and heart rate, and signalling in the brain [4–9]. As a consequence, there are three major NOS isoforms: nNOS (neuronal NOS), eNOS (endothelial NOS) and iNOS (cytokine-inducible NOS) enzymes [5–8,10] that catalyse nitric oxide biosynthesis in these locations. The three isoforms are distinguished by their sequence, catalytic activity, regulation and tissue location.

Structurally, all NOS isoforms contain an oxygenase domain that binds haem, H4B (tetrahydrobiopterin) and arginine [11,12]. Inhibition of NOS enzymes has been correlated with Parkinson’s and Alzheimer’s diseases, epilepsy and stroke [13].

The active site structure of a typical NOS enzyme is shown in Figure 1, which is taken from the 4NSE PDB file [14]. The enzyme binds as a dimer, and the biosynthesis of nitric oxide takes place on one of the haem centres. A covalent bond of the metal (iron) of the haem with a thiolate group of a cysteine side chain (Cys186 in 4NSE) connects the haem to the protein backbone. On the distal site, substrate arginine is bound, i.e. locked in a salt bridge through hydrogen-bonding interactions with Glu363. In addition, the peptide bond in between Trp358 and Tyr359 stabilizes the methylguanidinium unit of L-arginine by keeping it in a constrained orientation. As a consequence, the substrate-binding pocket is tight and only fits the natural substrate (L-arginine) or close analogues. The distal position of the haem is vacant in the crystal structure, but it has been anticipated that molecular oxygen binds here that, via a set of two protonation steps, is converted into...
an Fe(IV)-oxo haem(\(+\cdot\)) active species, called Compound I. This species is the active oxidant in cytochrome P450 enzymes [15,16] and is expected to be involved in the hydroxylation mechanism of arginine to form $N_\omega$-hydroxo-arginine.

First catalytic cycle of NOS

The first catalytic cycle of NOS enzymes for conversion of L-arginine into $N_\omega$-hydroxo-arginine is schematically depicted in Figure 2. The catalytic cycle starts from the resting state (1) where a water molecule occupies the sixth binding site of iron (top structure in Figure 2). Substrate binding to the catalytic centre removes the water molecule from the active site (2) and triggers a low-spin to high-spin transition [17,18]. The haem is reduced (3) and binds molecular oxygen (4) followed by a subsequent reduction (5). This ferric-peroxo complex has been characterized by low-temperature EPR/ENDOR (electron nuclear double resonance) and resonance Raman studies [19–21], and is the last stable intermediate in the catalytic cycle. A subsequent protonation gives the ferric-hydroperoxo complex, Compound 0. Experimental as well as DFT (density functional theory) studies have suggested arginine to donate a proton in the catalytic cycle that converts Compound 0 into an Fe(IV)-oxo haem(\(+\cdot\)) species [19,22].

The latter is also called Compound I and is the active species of haem as well as non-haem enzymes and is known to be a very efficient oxidant of hydroxylation reactions [23–26].

QM/MM (quantum mechanics/molecular mechanics) studies by Cho et al. [27] were performed on the formation of Compound I from the ferrous-peroxo species (4 in Figure 2). The two proton transfer steps were both studied from $H_3O^+$ as it was reasoned that a chain of water molecules from the surface of the enzyme to the active site is available. It was found that both protonation steps are exothermic and lead to a $H_3B$ cation radical (\(H_3B^{+\cdot}\)). Moreover, the studies predicted a mechanism of formation of Compound I via a hydrogen peroxide–haem complex via homolytic cleavage of the O–O bond. These studies did not consider the possibility that protonated arginine can donate a proton in the catalytic cycle instead and assumed that arginine remained protonated throughout. Recent thermodynamic studies, however, showed the hydrogen abstraction and proton transfer reactions of protonated arginine by Compound I to be highly endothermic, so it is unlikely that protonated arginine reacts with Compound I in the last step of the catalytic cycle [24]. Thus DFT studies on hydroxylation of protonated L-arginine by Compound I of NOS predicted high reaction barriers due to the strength of the N–H bond of the substrate. However, the N–H bond strength is much weaker for a neutral arginine molecule and as a consequence also lower reaction barriers are observed for hydroxylation of L-arginine by an iron–oxo complex. Therefore, if a protonated arginine donates a proton in the catalytic cycle, e.g. to the ferric-peroxo intermediate, a neutral arginine rest group remains. Neutral arginine has a much weaker N–H bond, so that hydrogen abstraction reactions are easier to perform. DFT studies on the gas-phase proton affinities of the ferric-peroxo (5) and Compound 0 indicate that a proton transfer reaction from protonated arginine to either of these two catalytic cycle intermediates is thermodynamically possible, i.e. exothermic. This mechanism, therefore, creates a Compound
I species with a nearby neutral arginine molecule (Figure 2). Subsequently, instead of a direct hydrogen abstraction reaction from arginine by Compound I, the catalytic cycle proceeds with an electron transfer from the substrate to Compound I to form an arginine cation radical and Compound II. This low-energy mechanism was recently identified with DFT methods [22] and replaces the hydrogen atom abstraction step from arginine by the Fe(IV)-oxo haem(•+) species to form an iron–hydroxo group. The DFT studies further implicated a fast electron transfer from substrate to Compound I thereby forming arginine cation radical and Compound II. The subsequent hydrogen abstraction reaction is more exothermic and proceeds via a low barrier. Finally, the hydroxy group rebinds arginine to form Nω-hydroxo-arginine products to make the catalytic cycle complete.

In order to establish that the gas-phase calculations are indeed transferable to enzymatic systems, we recently performed a further set of calculations on the environmental effects of the stability of Compound II and its hydroxylation mechanism of arginine using QM/MM methods (C.S. Porro, T.P. Murray, J. Pang and S.P. de Visser, unpublished work). Optimized geometries of Compound II in the quartet and doublet spin states are shown in Figure 3. Calculations were performed using the initial co-ordinates from the 1NSI PDB file [28] that was set up for the Fe(IV)-oxo haem species with a 5 Å (1 Å = 0.1 nm) layer of solvent. The total system contained 12949 atoms of which the inner core was calculated with DFT methods and the outer range with a universal force field. The inner region contained protoporphyrin IX (without side chains), iron–oxo, thiolate for Cys186, acetate for Glu363 and methylguanidinium for substrate arginine. These procedures were similar to earlier QM/MM calculations on non-haem enzyme models in our group [29]. QM/MM optimized geometries are close to those obtained with small model complexes. In particular, the Fe–O distance was calculated to be 1.694 Å for the DFT model complexes, whereas here similar values of 1.700 and 1.637 Å are reported. The high-spin structure gives an Fe–S distance of 2.594 Å, which is close to the DFT models where 2.460 Å was found and also is in good agreement with studies on cytochrome P450 Compound I [16,22]. Interestingly, the Fe–S bond is considerably weakened in the low-spin state...
to 3.551 Å and thereby significantly longer than typical Fe–S bonds.

The group spin densities shown in Figure 3 confirm our structures as a Compound II system with a nearby arginine cation radical and two unpaired electrons on the FeO group. In the quartet spin state, the amount of radical on the arginine group is large (\(\rho_{\text{Arg}} = 0.72\)), whereas in the doublet spin state it is only \(\rho_{\text{Arg}} = -0.56\). In addition, the low-spin structures give significant spin density on the cysteinate group, whereas it is negligible in the quartet spin state. Therefore the quartet spin state is a pure Compound II configuration with arginine cation radical, whereas in the doublet spin state this state mixes somewhat with a Compound I and neutral arginine electronic configuration. Clearly, the formation of Compound II from Compound I and neutral arginine is an exothermic process that is even retained in an enzyme-mimicking environment.

In conclusion, QM/MM and DFT studies on the Fe(IV)-oxo species of NOS enzymes reveal that the most stable structure has a closed-shell haem and an arginine cation radical, i.e. is a Compound II system. The protein environment in NOS enzymes stabilizes the electron transfer mechanism from arginine substrate to Compound I and forms Compound II with an arginine cation radical couple. This is energetically favourable in the gas phase as well as with the protein surroundings included in the model. These studies confirm the conclusions derived from gas-phase DFT calculations on model complexes and establish the final steps in the catalytic cycle of NOS enzymes to include an electron abstraction by Compound I, followed by hydrogen abstraction by Compound II and radical rebound to form \(N^\omega\)-hydroxo-arginine products.

**Second catalytic cycle of NOS**

In a second catalytic cycle, \(N^\omega\)-hydroxo-arginine is converted into \(L\)-citrulline and nitric oxide by using another molecule of molecular oxygen and one electron [5,8]. Gauld and co-workers have published several DFT studies on the mechanism for the second catalytic cycle leading to nitric oxide products [30–32]. Thus their density functional studies started from the protonated \(N^\omega\)-hydroxo-arginine system with another molecule of oxygen bound to the metal-haem. Proton transfer leads to a haem-bound hydrogen peroxide system that via a Ping Pong peroxidase-type mechanism leads to an Fe(IV)-oxo species. A subsequent tetrahedral transition state leads to \(L\)-citrulline and nitric oxide products.

**Summary**

Recent advances in theoretical chemistry have made it possible to study large biochemical systems such as NOS enzymes. These studies are at the forefront of chemistry and use computational challenging methods such as QM/MM. The first catalytic cycle of NOS enzymes, where \(L\)-arginine is hydroxylated to \(N^\omega\)-hydroxo-arginine shows many similarities to cytochrome P450s beyond the formation of Compound I. However, due to the fact that NOS enzymes have a unique substrate that is highly polar and involved in electron and proton transfer processes, the catalytic cycle diverges from the cytochrome P450s beyond Compound I. Thus arginine substrate in NOS enzymes appears to have a dual function in the catalytic cycle, where it donates a proton (presumably the second one for the formation of Compound I) and in the next step it donates an electron to Compound I. As a consequence, in NOS enzymes the active oxidant is Compound II, whereas in cytochrome P450 enzymes the catalysis is performed solely by Compound I.

**References**


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