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Linkage Isomerism in Nitrite Reduction by Cytochrome cd_1 Nitrite Reductase

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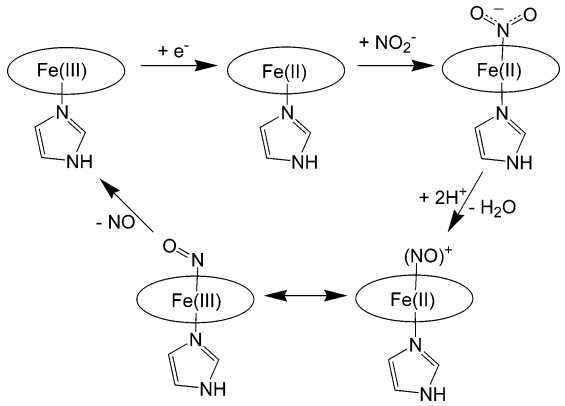
Nitrite reduction by cytochrome cd_1 nitrite reductase (cd_1NIR) is currently accepted to involve coordination of the nitrite nitrogen atom to the ferrous d_1 heme. Here, density functional theory results are reported on the previously unexplored O-binding of nitrite to ferrous and ferric cd_1NIR. Although the N-isomer (nitro) is energetically favored over the O-nitrite (nitrito), even one single strong hydrogen bond may provide the energy required to put the two isomers on level terms. When hydrogen bonding existent at the cd_1NIR active site is accounted for in the computational model, the O-nitrite isomer is found to spontaneously protonate and thus yield a ferric–hydroxo species, liberating nitric oxide. An O-nitrite ferrous cd_1NIR complex appears to be an energetically feasible intermediate for nitrite reduction. O-Coordination would offer an advantage since the end product of nitrite reduction would be a ferric–hydroxo/water complex, rather than the more kinetically inert iron–nitrosyl complex implied by the N-nitrite mechanism.

Introduction

Nitrite reduction by cytochrome cd_1 nitrite reductase (cd_1NIR) has been proposed to occur (cf. Scheme 1) via N-coordination of nitrite to the d_1 heme of cd_1NIR.[1,2] Protonation of a nitrite oxygen atom within the ferrous−nitrite complex would lead to release of a water molecule, forming a weakly bound [FeNO][6] complex, that subsequently decays via NO liberation. Nitrite and nitric oxide adducts of the d_1 heme in cd_1NIR have been characterized experimentally and computationally.[1−6]

A detailed QM/MM examination of the mechanism shown in Scheme 1 has recently been reported.[2] Amino acid residues were identified at the active site that would be important for protonation or hydrogen bond donation events. The N mode of binding of nitrite and the feasibility of N−O bond cleavage were reported upon, thus providing computational support for previous proposals supported by crystallographic data.[1] However, the key issue of the mechanism remained unresolved by the QM/MM study: does the Fe(III)−NO adduct feature an Fe−NO bond labile enough to make it catalytically competent? Experimental data available to date in fact suggests that the answer to the above question may be *no*: the decay of the cd_1NIR Fe(III)−NO adduct appears too slow to be catalytically competent.[6] While the QM/MM study[2] pointed out factors that would influence the structure of the

Scheme 1



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Fe(III)−NO adduct, it failed to confirm the very long Fe−NO bond observed experimentally,[1] and it failed to provide evidence for a low-energy barrier associated with cleavage of the Fe(III)−NO bond. In fact, our own unpublished data also suggest that elongation and bending of the Fe(III)−NO unit from its canonical equilibrium structure to the geometry seen in the NO-bound cd_1NIR crystal structure would require more than 20 kcal/mol, and that such an elongated−bent moiety is not an equilibrium structure even when the oxygen or nitrogen is protonated, or when a hydrogen bond donor such as ammonium is included in the model. These and other (see below) issues may justify revisiting in great detail the Scheme 1 mechanism with QM/MM.

Rather than entertaining a theoretical polemic by again addressing the issue of Fe−NO bond cleavage (for which apparently QM/MM[2] as well as experimental[6] approaches fail to yield the "desired" result) we wish to report here density functional theory results on the previously unexplored *O-binding* of nitrite to ferrous and ferric cd_1NIR. An O-nitrite ferrous cd_1NIR complex appears to be an energetically feasible intermediate for nitrite reduction, offering the extra advantage (compared to an N-nitrite adduct) that it avoids formation of a kinetically undesirable iron−nitrosyl complex. While solid experimental and theoretical evidence exists to support the mechanism shown in Scheme 1, we report here an alternative mechanism that is also consistent with all available experimental data.

Methods

All models consisted of a truncated heme d_1 (porphyrin substituents, excepting the oxo atoms, were replaced by hydrogen atoms) coordinated axially by nitrite and protonated imidazole, respectively. Starting atomic coordinates were from the crystal structure of nitrite-bound cytochrome cd_1 nitrite reductase (pdb entry 1AOM). Low-spin states were assumed throughout (cf. refs 2 and 10). The UBP86 functional (the gradient-corrected exchange functional of Becke (1988), the correlation functional of Perdew (1986)),[7−9] and the DN** numerical basis set (comparable in size to 6-31G**) were used. In SCF calculations, a fine grid was used; the convergence criteria were 10^{-6} (root-mean square of electron density) and 10^{-8} (energy), respectively. For geometry optimization, convergence criteria were 0.001 au (maximum gradient criterion) and 0.0003 (maximum displacement criterion).[10]

To reflect potential second-sphere (steric) effects that the protein may impose on the heme and on its histidine ligand, all heavy atoms except for the Fe−nitrite unit were frozen in the models reported in text. This approach conserves potentially important features at the active site, features that are lost upon full geometry optimization: a relatively short Fe−imidazole bond with the imidazole plane skewed relative to the heme normal, and a distorted porphyrin ring.

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(10) We found that use of UB3LYP/LACVP** or UB3LYP/6-31G** (as implemented in the Jaguar and Gaussian98 packages), either for geometry optimization or simply for energy calculation at the UBP86/DN** geometries, did not (1) affect the geometries of the iron−nitrite moieties (N−O bond cleavage included) nor (2) bias the energies toward the N-nitrite isomers. In fact, the B3LYP functional (regardless of basis set) reduced the gap between the N-nitrite and O-nitrite to 2−3 kcal/mol, further arguing for the viability of the O-nitrite isomer.

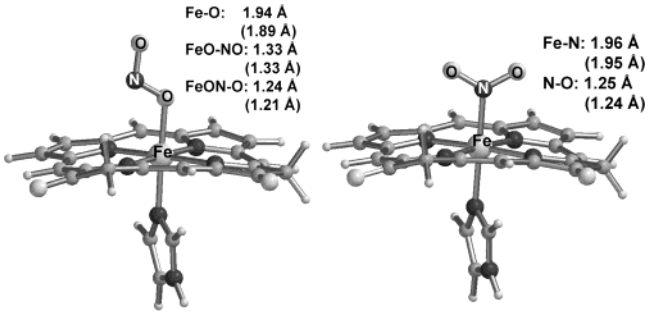

Figure 1. Optimized geometries for O- and N-ferrous−nitrite models of the cd_1NIR active site. Distances for the corresponding ferric−nitrite models are given in parentheses.

Geometry optimization in the absence of the above-mentioned geometrical constraints, or by reducing the number of frozen atoms to five (the four meso carbons of the heme plus the non-iron-bound nitrogen of the imidazole) led to iron-nitrite moieties essentially identical to those reported in text. On the other hand, free geometry optimization resulted in an essentially planar heme and an imidazole ligand aligned on the heme normal. These latter features were however not lost in the model where only the four meso carbons and the NH nitrogen were frozen.

The deviations from heme planarity perhaps deserve an extra comment here. In a recent report on *Paracoccus pantotrophus* cd_1NIR, an essentially planar heme d_1 moiety appears to have emerged from QM/MM calculations.[2] However, all the >20 cd_1NIR crystal structures deposited in the Protein Data Bank feature heme d_1 active sites distorted essentially the same way as in the models we employ here. The origin of these distortions and the reason why cd_1NIR would use an atypical heme (i.e., d_1) at its active site are unknown at this time and perhaps deserve further investigation. While the equilibrium structures obtained at the QM/MM level fail to reproduce the experimentally observed porphyrin distortions, our approach of constraining the heme to its experimentally observed geometry is likely to provide a more sound account of the perturbations induced by the protein on the porphyrinic ring.

Results and Discussion

Figure 1 shows optimized geometries for ferrous and ferric N- and O-nitrite heme d_1 models. N-Nitrite complexes of hemes (including cd_1NIR) have been characterized elsewhere[2,11] and are discussed here only to the extent to which a comparison with the O-nitrite isomers is required.

Compared to the N-nitrite isomer, the ferrous O-nitrite complex featured one longer (Fe)O−NO bond (1.33 Å) and one shorter N−O bond (1.23 Å), suggestive of nitrite activation toward O−N bond cleavage and nitric oxide release. The N-isomer was favored over the O-isomer by 6 kcal/mol in the ferrous and 4.5 kcal/mol in the ferric model.

The cd_1NIR active site features a network of hydrogen bonds consisting of two histidine, one tyrosine, and water molecules, all within hydrogen bonding distance from the heme-coordinated nitrite.[1] Such hydrogen bonding may be expected to provide enough energy to render the O-binding of nitrite entirely physiologically relevant, for both the ferrous and ferric states. To further examine the importance of hydrogen bonding for the ferrous−nitrite adducts, we optimized

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the geometry of a ferrous−O-nitrite model similar to that in Figure 1, but with the iron-bound oxygen atom protonated (i.e., a neutral HONO ligand; this is a limiting structure, describing a state where hydrogen bonding has resulted in complete protonation of nitrite). Upon geometry optimization, this system converged toward a ferric−hydroxo state, with a neutrally charged NO entity placed 1.7 Å away from the oxygen atom of the iron-bound hydroxide. By contrast, single protonation of one of the oxygen atoms in the ferrous N-nitrite complex resulted in significantly shorter (presumably stronger) N−O bonds: 1.45 Å for N−OH and 1.20 Å for N−O (in agreement with previous QM/MM investigations).[2] Further, we optimized the geometry of an O-nitrite ferrous model similar to that of Figure 1 but including a water molecule hydrogen-bonded to the Fe-bound oxygen atom of the nitrite, plus an imidazole ring hydrogen bonded to the water, as seen at the cd_1NIR active site.[12] Upon geometry optimization, the water molecule donated one proton to the iron-bound nitrite oxygen atom (while the imidazolium in turn donated its proton to the water). Thus, our results give a dramatic account of the difference in reactivity between nitrito and nitro isomers. While QM/MM results[2] have suggested that N−O bond cleavage in ferrous−nitro cd_1NIR would be a facile process, we now find that such cleavage is *even more facile in the nitrito isomer*.

Taken together the above data suggest that O-nitrite complexes would be energetically feasible, and at least as catalytically competent as N-nitrite complexes, in cd_1NIR. In fact, unlike the previously characterized N-nitrite isomers, ferrous O-nitrite complexes of cd_1NIR would feature such a weak O−NO bond that they would be unlikely to be isolated with anything but the most rapid kinetic techniques; instead, one would expect to either isolate a ferric−hydroxo species (if a proton is readily available) or the N-isomer (via isomerization, if proton delivery is delayed). Experimental precedent exists for our proposed reaction mechanism: a Co(III) porphyrin model compound (isoelectronic with our ferrous heme) reacts with nitrite via transient formation of an O-adduct, followed by formation of the thermodynamically stable N-nitrite isomer.[13]

The currently accepted cd_1NIR catalytic cycle (Scheme 1) implies formation of a $[FeNO]^6$ complex. This $[FeNO]^6$ complex at the cd_1NIR active site was reported to feature an unusually long (and presumably weakened) Fe−NO bond, thus being primed for NO release from the active site.[1,2] As mentioned before, computational efforts so far, including QM/MM approaches,[2] have failed to confirm this hypothesis. There is in fact no direct experimental evidence to show that a $[FeNO]^6$ species would indeed form *under turnover conditions* with cd_1NIR, or that the Fe−NO bond in this species would cleave at rates compatible with catalytic turnover.[4,6] One exception in this respect may however be the findings of Hollocher and co-workers,[14−16] who demonstrated oxygen atom exchange between nitrite and water, as well as nitrosation reactions, for which cd_1NIR appeared to be responsible. Hollocher and co-workers proposed two explanations for their findings. One[14] involved formation of a nitrito−iron adduct, identical to the one that we now propose to be involved in the cd_1NIR mechanism. While acknowledging that this nitrito explanation cannot be ruled out,[14] Hollocher and co-workers in fact elaborated more on a second explanation, which involved the "canonical" mechanism shown in Scheme 1.[14−16] These isotope substitution and nitrosation experiments were carried out either on whole cells in the presence of acetylene as an exogenous inhibitor of nitrous oxide reductase or on purified cd_1NIR. The electron donor system used in the turnover assays with purified cd_1NIR consisted of ascorbate + *p*-phenylenediamine, chemicals which were shown not to interfere in their reduced "resting" state with the isotope exchange and/or nitrosation processes.[14] However, under turnover conditions a steady-state level of aromatic amine radicals is likely to be generated in these experiments. The reactivity of such radicals is complex (see, e.g., ref 17) and may have affected the results of Hollocher and co-workers. Furthermore, since a nonphysiological electron source was used for turnover, electron delivery to the cd_1NIR active site may have been either slower or not sufficiently concerted with substrate binding and/or proton delivery, thereby biasing the active site toward formation of thermodynamically favored species such as N-nitrite over O-nitrite. Also, the extent to which cd_1NIR activity was affected in vivo by a nonphysiological stress such as acetylene (which blocks nitrite metabolism at a step *immediately after* cd_1NIR) may have been underestimated.

N-Nitrite as well as nitric oxide adducts of cd_1NIR have been observed with X-ray crystallography[1,5] and spectroscopic techniques, including stopped-flow, under essentially single turnover conditions and with *excess nitrite*.[3,6] Indeed, our results suggest that N-binding of nitrite is *thermodynamically* preferred over O-binding. Furthermore, since these iron−NO adducts are relatively stable,[6] and since NO is the product of catalysis at the active site, the reported observation of nitric oxide adducts rapidly forming at the d_1 heme is not surprising. By contrast, our results suggest that O-binding of nitrite to the d_1 heme would be extremely transient, with Fe−ONO bond formation triggering a concerted proton-assisted N−O bond cleavage. Alternatively, under physiologically irrelevant conditions, alteration of the proton delivery machinery at the active site may allow time for a nitrito-to-nitro rearrangement. Such rearrangement may be followed by N−O bond cleavage to yield the observed relatively stable Fe(III)−NO adduct.

(12) Heavy-atom coordinates for this extended model were taken from pdb entry 1AOM.; besides the heme, truncated as in Figure 1, and the imidazole ligand, the imidazolium ring of H345 and a water molecule placed instead of the Y25 phenolic oxygen were included in this extended model. Ranghini et al. have already demonstrated that at least one of the two histidine residues at the cd_1NIR active site is diprotonated (i.e., imidazolium, rather than imidazole).

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The physiological function of cd_1NIR is currently accepted to be nitrite reduction to nitric oxide.[18] Yet, under certain in vitro conditions, nitric oxide may also be further reduced by cd_1NIR, to produce N_2O.[19] Using computational arguments put forth elsewhere[20] (correlated with the presence of a neutral axial ligand at the d_1 heme, and the presence of a nearby electron source, the cytochrome *c* domain), cd_1NIR may indeed be expected to competently reduce nitric oxide to nitrous oxide. Our alternative mechanism, involving O-coordination of nitrite to the heme, would explain well why nitric oxide reduction to nitrous oxide does not normally occur subsequent to nitrite reduction in cd_1NIR, *under physiological conditions*: an iron−NO bond is according to our mechanism never formed upon nitrite reduction. Others[21] have pointed out however that the redox potential of the d_1 heme may also play a role in this respect.

The crystal structures of cd_1NIR[1] reveal various conformational changes at the heme d_1 active site (mainly involving movement of Y25) upon changes in redox state, with at least one conformation where N-binding, but not O-binding, of nitrite would be sterically obstructed by the Y25 side chain; the latter would, nevertheless, be ideally placed to participate in protonation of an O-coordinated nitrite.

Conclusions

Nitrite reduction by cytochrome cd_1 nitrite reductase (cd_1NIR) is currently accepted to involve coordination of the nitrite nitrogen atom to the ferrous d_1 heme. We do not provide here *new* evidence to suggest that such a mechanism is not feasible. However, we report DFT results suggesting that an alternative mechanism may exist, which is more efficient than the canonical one while also correlating well with the available experimental data. This alternative mechanism involves binding of nitrite to the iron via an oxygen atom (nitrito). Although the N-isomer (nitro) is energetically favored over the nitrito, even one single strong hydrogen bond may provide the energy required to put the two isomers on level terms. When hydrogen bonding existent at the cd_1-NIR active site is accounted for in the computational model, the O-nitrite isomer is found to spontaneously protonate and thus yield a ferric−hydroxo species, liberating nitric oxide. A nitrito ferrous cd_1NIR complex thus appears to be an energetically feasible intermediate for nitrite reduction. Moreover, O-coordination would offer an advantage since the end product of nitrite reduction would be a ferric−hydroxo/water complex, rather than the more kinetically inert iron−nitrosyl complex implied by the N-nitrite mechanism. It now remains to be demonstrated experimentally whether any of the two mechanisms is preferred by cd_1NIR, or if the enzyme in fact offers an example of catalytic promiscuity (reminiscent of the TSR described by Shaik and co-workers for other hemoproteins[22]), where the same reaction is catalyzed at the same site with different mechanisms.

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Supporting Information Available: Atomic coordinates of models discussed in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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