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Dedicated to professor Gh. Marcu at his 80th anniversary

HEMES REVISITED BY DENSITY FUNCTIONAL APPROACHES. 2. A PARADIGM FOR AXIAL LIGATION IN HEMOPROTEINS

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ABSTRACT. Reported here are DFT descriptions of dioxygen reduction at the active site of cytochr]ome cd_1 nitrite reductase, an enzyme with a histidine-ligated heme active site, known to act as cytochrome oxidase. Also explored is the possibility of nitric oxide reduction by histidine-ligated hemes. The energetics of these two processes correlate well with previous findings on other hemoproteins, and allow further elaboration on the newly proposed "thiolate obstruction" theory *[Silaghi-Dumitrescu, Eur. J. Inorg. Chem. 2003, 1048]*. This theory, orthogonal if not opposed to the classical "thiolate push" dogma *[Dawson et al., J. Am. Chem. Soc. 1976, **98**, 3707]* , argues that efficient reduction of diatomics such as dioxygen and nitric oxide is more readily accomplished by histidine-ligated hemes than by thiolate– ligated hemes. Generalizing, for reductive processes involving a small diatomic as the sole substrate, neutral ligands (e.g., histidine in cytochrome oxidases and heme oxygenase, lysine in cytochrome *c* nitrite reductase) are found to always be preferable over anionic ligands. By contrast, in enzymes designed to deal with more than one substrate, anionic ligands are preferable (e.g., cysteine or tyrosine in monooxygenases), since they allow the safety switches needed to avoid uncoupling in their race against entropy.

INTRODUCTION

Proteins such as cytochrome P450 (P450), horseradish peroxidase (HRP) or hemoglobin are all known to bind and/or reduce (activate) dioxygen at their heme active sites, in the ferrous form.[1-11] The thiolate-ligated ferrous heme of P450 binds dioxygen and promotes proton-dependent O-O bond cleavage following a one-electron reduction.[1] By contrast, the ferrous histidine-ligated heme of hemoglobin only binds dioxygen in a reversible manner.[12] Hemoprotein ferric-hydroperoxo complexes decay via proton-assisted heterolytic cleavage of the O-O bond to yield water and a $[Fe=O]^{3+}$ unit (known as Compound I), where the iron is considered to be in the formal oxidation state +4 and a further oxidizing equivalent is proposed to be delocalized onto the porphyrin. Two active-site "distal" residues, a histidine and an arginine, are crucial in promoting O-O bond cleavage in the ferric-hydroperoxo complex of canonical (histidine-ligated) peroxidases. The hemoglobin active site lacks the distal arginine and is much less efficient than HRP at activating peroxide.[1-10] The heme-thiolate active site of P450 does not contain the histidine-arginine catalytic pair of HRP, yet still cleaves the O-O bond of its ferric-hydroperoxo complex

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(using water molecules as the source of protons). These differences between thiolate and histidine-ligated hemes are traditionally rationalized in terms of the thiolate ligand being able to push electron density more efficiently onto the iron-dioxygen moiety, thereby facilitating O-O bond cleavage ("the push effect").[10, 11, 13]

Notable exceptions to the thiolate/histidine rule also exist. Cytochrome oxidases, which accomplish four-electron reduction of dioxygen to water, feature histidine and not thiolate ligands.[14] Heme oxygenase, which reduces dioxygen to hydroperoxide, also features a histidine ligand.[15]

Nitric oxide reduction by thiolate-ligated heme (in cytochrome P450 nitric oxide reductase, P450nor) and by lysine-ligated heme (in cytochrome c nitrite reductase, CcNIR) may either involve two one-electron reduction steps, $[FeNO]^{3+} \rightarrow [FeNO]^{2+} \rightarrow [FeNO]^{+}$, or one two-electron reduction step, $[FeNO]^{3+} \rightarrow [FeNO]^{+}$; both mechanisms involve concomitant or subsequent protonation of the NO ligand.[16] The one-step mechanism is now generally accepted for P450nor (where subsequent addition of a second NO molecule results in generation of N_2O), whereas the two-step mechanism is active with CcNIR (where the NO ligand is subsequently further reduced to NH_3 and H_2O).[16, 17] The choice for a one-step, 2-electron mechanism in P450nor was rationalized by differences in thermodynamics: the thiolate heme-NO adduct is more electron rich and therefore a second one-electron reduction would be harder to accomplish than with a lysine-ligated heme. Indeed, this second one-electron reduction was found to be, at least in gas-phase calculations, energetically uphill with thiolate and energetically downhill with lysine. Thus, although the "thiolate push" dogma[1, 13] would link thiolate axial ligands to more facile activation of diatomics, we found the opposite to be true for nitric oxide activation ("thiolate obstruction"). Accordingly, the electrons required for nitric oxide reduction in thiolate-ligated P450nor are directly supplied by the hydride (2-electron) donor NADH, whereas in CcNIR the electrons are supplied by neighboring hemes, in a one-electron fashion.[16, 17]

The "thiolate obstruction" theory further developed upon comparing one-electron reduction of formally ferrous-dioxygen species to formally ferric-peroxo.[11, 16] There too, gas-phase reduction was thermodynamically uphill with anionic ligands (thiolate, phenoxide, imidazolate), but thermodynamically downhill with a neutral ligand (imidazole, or no ligand at all). When the same calculations were performed in a solvent of $\varepsilon \sim 4.3$ rather than in gas-phase, thus mimicking an enzyme active site, the differences between histidine and anionic models were diminished to some extent, but the histidine still remained clearly more adept at favoring one-electron reduction of the ferrous-dioxygen adduct.[11] P450, which features an anionic thiolate ligand to the heme, activates dioxygen specifically for oxygen atom insertion into an organic substrate (monooxygenase chemistry). It is then desirable that dioxygen reduction only occur in the presence of substrate, to avoid the wasteful/toxic reduction of dioxygen known as uncoupling.[1] The thiolate, and anionic ligands in general, thus offers the possibility of an extra safety switch, that ensures maximum catalytic activity in systems fighting entropy (i.e., relying on the concomitant presence of more than one substrate at the active site).

Cytochrome *cd*$_1$ nitrite reductase (cd1NIR) is known to efficiently reduce dioxygen to water at its heme *d*$_1$ active site, thus acting as a soluble cytochrome oxidase.[18] The axial heme ligand in cd1NIR is a histidine. In line with our previous findings on heme *b* models, we expect one–electron reduction of the cd1NIR ferrous-

dioxygen adduct to be energetically very favorable. To verify this, and to investigate the extent to which the heme d_1 may alter the properties of the ferrous-dioxygen adduct and of its reduced congeners, we report here DFT geometry optimization results on models of the cd1NIR ferrous-dioxygen, reduced ferrous-dioxygen, and ferric-hydroperoxo adducts.

Also elaborating on our "thiolate obstruction" theory, we report DFT geometry optimization results for models consisting of a histidine-ligated *b* heme coordinated by nitric oxide, in three oxidation states: Fe(III)-NO, Fe(II)-NO, and Fe(II)-NO^-; for the latter model, the two protonated states, Fe(II)-N(H)-O and Fe(II)-N-O-H are also investigated. These heme *b* models mimic the nitric oxide adducts of myoglobin, and, in addition to corroborating the "thiolate obstruction" theory, they provide insight into myoglobin's ability to perform nitric oxide reductase chemistry.

METHODS

Geometries for all models were optimized without any constraints with the UBP86 functional, which uses the gradient-corrected exchange functional proposed by Becke (1988),[19] the correlation functional by Perdew (1986),[20] and the DN** numerical basis set (comparable in size to 6-31G**) as implemented in Spartan.[21] For the SCF calculations, a fine grid was used, and the convergence criteria were set to 10^{-6} (for the root-mean square of electron density) and 10^{-8} (energy), respectively. For geometry optimization, convergence criteria were set to 0.001 au (maximum gradient criterion) and 0.0003 (maximum displacement criterion). Partial atomic charges and spin densities were derived from Mulliken population analyses.

Low-spin states (S=0, S=1/2) were assumed for all models, in line with previous experimental and theoretical findings.[11, 16, 18, 22]

RESULTS AND DISCUSSION

Table 1 and Figure 1 show geometry optimization results for cd1NIR models. Overall, the optimized geometries are consistent with crystal structure of the ferrous-dioxygen cd1NIR.

The models in Figure 1 feature a non-planar heme. This is consistent with our previous findings[11] on dioxygen/peroxo heme *b* models. Conversely, the heme was calculated to be essentially planar in the nitric oxide and nitrite adducts of cd1NIR.[18, 23] Data in Table 1 are very similar to those previously reported on equivalent heme *b* models,[11] thus suggesting that the unusual heme d_1 at the active site of cd1NIR does not alter the heme-iron-dioxygen/peroxo chemistry to any significant extent. Importantly, one-electron reduction of the formally ferrous-dioxygen species is still significantly "exothermic", confirming our "thiolate obstruction" theory. Consistent with previous findings[11] on related heme systems, $[FeO_2]^{2+}$ cd1NIR appears as a ferrous-dioxygen/ferric-superoxo hybrid. $[FeO_2]^{+}$ cd1NIR features one unpaired electron on the oxygen atoms and in this respect is best described as a superoxo adduct rather than peroxo. Assigning the iron in $[FeO_2]^{+}$ as ferrous may at this stage be tempting; however, a ferric-superoxo description (with one extra electron delocalized onto the porphyrin, and with the iron and porphyrin unpaired electrons strongly covalently coupled) is more likely.[11]

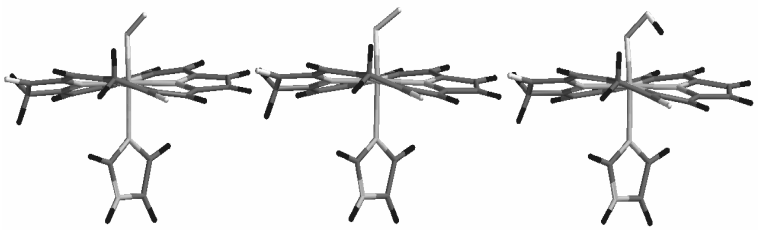

Figure 1. Geometry-optimized structures for (from left to right): formally ferrous-dioxygen ($[FeO_2]^{2+}$), ferric-peroxo ($[FeO_2]^{+}$]), and ferric-hydroperoxo ($[FeO_2H]^{2+}$) cd1NIR adducts.

Table 1.

Energies (a.u.), distances (Å), partial atomic charges and spin densities (the latter shown in parentheses) for formally ferrous-dioxygen ($[FeO_2]^{2+}$), ferric-peroxo ($[FeO_2]^{+}$]), and ferric-hydroperoxo ($[FeO_2H]^{2+}$) cd1NIR adducts.

	energy	Fe-O	O-O	Fe-N	Fe	O1	O2	OOH
$[FeO_2]^{2+}$	-2780.07420	1.78	1.28	2.11	0.48	-0.02	-0.15	-0.17
$[FeO_2]^{+}$	-2780.16461	1.90	1.31	2.15	0.46 (-0.07)	-0.13 (0.42)	-0.24 (0.52)	-0.37 (0.94)
$[FeO_2H]^{2+}$	-2780.69712	1.82	1.44	2.06	0.49 (0.56)	-0.19 (0.34)	-0.22 (0.13)	-0.07 (0.47)

Table 2 and Figure 2 show geometry optimization results for the myoglobin iron-nitric oxide adducts. These results are consistent with our previous reports on similar models with thiolate, lysine, or with no axial ligand instead of the imidazole.[16] Importantly, one-electron reduction of the formally Fe(II)-NO species is significantly "exothermic", similar to ammonia-ligated models but unlike thiolate-ligated models – in line with the "thiolate obstruction" theory.[16]

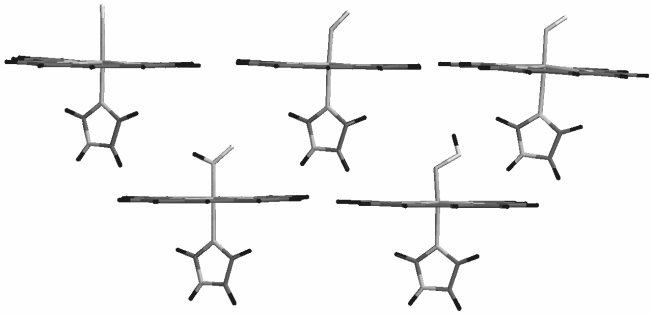

Figure 2. Optimized geometries for heme-histidine $[FeNO]^6$, $[FeNO]^7$, $[FeNO]^8$ (top row, from left to right), $[FeNHO]^8$, and $[FeNOH]^8$ adducts (bottom row, from left to right).

The $[FeNO]^6$ model features a significant amount of positive charge on the NO ligand and is thus best described as Fe^{2+}-NO^+, in line with previous assignments.[16] The $[FeNO]^7$ model features more electron density on the NO as well as a bent Fe-N-O unit, which is consistent with a Fe^{2+}-NO/Fe^{3+}-NO^- hybrid – again as previously

assigned in related models.[16] The $[FeNO]^8$, $[FeNHO]^8$, and $[FeNOH]^8$ models all clearly feature an NO^- ligand, implying a ferrous center as previously assumed.[16] However, we note that, as in all other heme complexes examined to date,[11, 16, 22, 24] the bulk of the two electrons upon going from $[FeNO]^6$ to $[FeNO]^8$ is in fact not found on the NO, even though formal changes in oxidation state of the NO ligand from +1 to -1 cannot be negated. There is thus a distinction between *formal oxidation state*, which changes for NO from +1 to -1 upon going from $[FeNO]^6$ to $[FeNO]^8$, and *electron density*, which in fact varies less on the NO ligand and more on the porphyrin – even though the latter cannot be claimed to undergo any formal change in oxidation state.[22]

Table 2.

Energies (a.u.), distances (Å), partial atomic charges and spin densities (the latter shown in parentheses) for heme-histidine $[FeNO]^6$, $[FeNO]^7$, $[FeNO]^8$, $[FeNHO]^8$, and $[FeNOH]^8$ adducts.

	energy	Fe-NO	N-O	Fe-N	Fe-N-O	Fe	N	O	NO(H)
$[FeNO]^6$	-2608.96248	1.65	1.15	2.05	179	0.48	0.28	0.01	0.29
$[FeNO]^7$	-2609.16404	1.75	1.19	2.15	140	0.49 (0.57)	0.13 (0.26)	-0.13 (0.15)	0.00 (0.41)
$[FeNO]^8$	-2609.21773	1.81	1.21	2.41	123	0.51	0.02	-0.22	-0.20
$[FeNHO]^8$	-2609.76451	1.79	1.24	2.13	131	0.52	0.07	-0.24	0.04
$[FeNOH]^8$	-2609.73346	1.72	1.38	2.20	116	0.55	-0.07	-0.22	0.00

Data on the protonated models, $[FeNHO]^8$ and $[FeNOH]^8$, is particularly important, since a protonated $[FeNO]^8$ species has indeed been isolated at the histidine-ligated *b* heme of myoglobin.[25] Table 2 confirms that, as previously proposed,[25] the proton must lie on the nitrogen rather than on the oxygen atom. Second, examining partial atomic charges and bond lengths in $[FeNHO]^8$, we find all parameters to be consistent with previously described thiolate-ligated heme $[FeNHO]^8$.[16] Based on knowledge on the reactivity of this latter thiolate system,[16] the myoglobin $[FeNHO]^8$ adduct should also react with a second NO molecule, thereby generating N_2O and H_2O. Indeed, evidence for such a reaction in myoglobin was available experimentally and has to this date not been rationalized.[25] This N_2O production by myoglobin appears however to be slow and incomplete; this deficiency is likely due to three factors. Firstly, myoglobin lacks good proton donors at the active site; two protons are in fact needed to convert $[FeNHO]^8$ + NO into N_2O and H_2O. Secondly, the myoglobin heme site is relatively small and designed to bind *one*, rather than two diatomics.[12] Thirdly, myoglobin is not designed to receive electrons from biological electron-transfer agents in a rapid and efficient manner.[12] By contrast, the P450nor active site is designed to allow unrestricted contact between the NADH hydride donor to the Fe-NO adduct, and is also large enough to accommodate entry of a second NO molecule – as required for N_2O formation.[16]

CcNIR, cd1NIR and respiratory cytochrome oxidases feature additional heme groups, which are located very close to the catalytic active site and deliver electrons immediately after initial binding of the diatomic substrate (NO or O_2).[16, 17]

The energetics of the two processes examined here, dioxygen reduction by cd1NIR and nitric oxide reduction by myoglobin, correlate well with previous findings on other hemoproteins, and allow us to better elaborate on our newly proposed “thiolate obstruction” theory. This theory, orthogonal if not opposed to the classical “thiolate push” dogma, argues that efficient reduction of diatomics such as dioxygen and nitric oxide is more readily accomplished by histidine-ligated hemes than by thiolate– ligated hemes. Generalizing, for reductive processes involving a small diatomic as the sole substrate, neutral ligands (e.g., histidine in cytochrome oxidases and heme oxygenase, lysine in cytochrome c nitrite reductase) are found to always be preferable over anionic ligands. By contrast, in enzymes designed to deal with more than one substrate, anionic ligands (e.g., the cysteine or tyrosine in heme-containing monooxygenases) are preferable, since they allow the safety switches needed to avoid uncoupling in their race against entropy.

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