

Characterization of a Resting State Model of Peroxidases by *ab Initio* Methods: Optimized Geometries, Electronic Structures, and Relative Energies of the Sextet, Quartet, and Doublet Spin States

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Abstract: Ferric aquo heme complexes comprise the active site of three major families of heme proteins, the peroxidases, the cytochrome P450s, and metmyoglobin. There is ample evidence from a variety of spectroscopic studies of wild type (wt) and mutant enzymes that in these resting state complexes, the sextet, quartet, and doublet states are very close in energy and the predominant spin state observed is a sensitive function of the number, nature, and geometry of the axial ligands. One correlation that is very difficult to determine experimentally is the relationship between spin state and geometry in the same heme complex. This coupling of geometry and spin state in the same complex can be functionally important since spin state changes are often a key part of function, for example, in the enzymatic cycle of the cytochrome P450s. To further explore the relationship between geometry and spin state, we report here for the first time the use of *ab initio* methods to calculate optimized geometries and electronic structure of a model for the resting state of peroxidases in its sextet, quartet, and doublet states. The sextet state is found to be the lowest energy state in agreement with experimental results reported for a model diaquo heme compound. Although a longer Fe–water distance was obtained in the model compound, the unique feature of these calculations is their ability to monitor changes in geometry in the various spin states in the same complex. While the optimized quartet geometry is similar to the sextet geometry, the doublet state has a considerably shorter Fe–water distance. These results suggest that the environment of the protein can modulate spin state changes by imposing geometric changes in this mobile Fe–ligand interaction by interaction from both the proximal and distal sides. Experimental determination of spin state populations by a number of spectroscopic methods in wt and mutants of the Fe(III) resting form of cytochrome-C peroxidases (CCP) with known Fe–water distances from crystal structures provide strong support for this hypothesis.

Introduction

A ferric aquo heme complex is the common active site motif in the resting state of two major families of metabolizing heme proteins, peroxidases and cytochrome P450s, as well as in the oxidized form of the globins. Extensive spectroscopic studies of wild type (wt) and mutants of these enzymes show a wide range of behavior with respect to spin state populations in this Fe(III) species. For example, horseradish peroxidase (HRP-C), cytochrome C peroxidase (CCP), and metmyoglobin (Mb) all share an imidazole and water as axial ligands. Yet the observed temperature dependence of magnetic susceptibility^{1–3} of quadrupole splittings in Mossbauer resonance,^{4–6} the electron spin resonance spectra,^{7–10} the resonance Raman spectra^{11–15}

and the electronic spectra^{9,14,15,16,17} all indicate differing populations of the sextet, quartet, and doublet states. Similar subtle variations in distribution of populations of different spin states are observed among the isozymes of the P450s that all share a common mercaptide and water as axial ligands. The origin of these variations among complexes that have the same axial ligands is not clearly understood and can be of mechanistic relevance to the function of the protein. For example, among the P450 isozymes, the extent to which the high or low spin state is favored in the ferric state mediates the ease of reduction that is a crucial step in the enzymatic cycle.

There are a number of ways that the environment of a heme complex can modulate the relative energies of its spin states. In previous studies,¹⁸ we have determined that the electrostatic field of the protein can modulate differences in spin state energies. These studies were performed for the resting state of P450cam with a known structure, using the techniques of

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semiempirical quantum chemistry, together with molecular dynamics simulations of the full protein.

Another possible way in which the environment of the protein could modulate relative spin state energies is by causing a change in the geometry of the heme iron ligands by forces exerted on the axial ligands by interaction from both the proximal and distal sides of the protein. It is extremely difficult, both experimentally and theoretically, to explore the relationship between geometry and spin state in the same heme complex. Although changes in spin states and coordination numbers in different proteins and under different conditions have been detected by Resonance Raman, electronic spectra, and electron spin resonance methods,^{7–17} there is no direct way to experimentally determine the extent of geometry relaxation in different spin states of the same complex. Computationally, the difficulty has been the lack of efficient and reliable procedures for obtaining geometries of such challenging systems as open shell iron–porphyrin complexes.

The INDO/SCF method developed over many years in the laboratory of Prof. Zerner¹⁹ has proven very useful and reliable for determining the relative energies of states of different spin states for example, in model compounds with known X-ray structures and different ground states spins²⁰ and in the calculation of electronic spectra of model heme complexes.^{21,22} However, this method has not proven as reliable for geometry optimization and is best used to characterize the electronic structure and relative spin state energies of heme complexes with known geometries.

A number of pioneering *ab initio* calculations with the self consistent field Hartree–Fock (SCF/HF) procedures have been reported for model iron–porphyrin systems.^{23–25} These calculations were limited at first to closed shell systems and use of small basis sets and later expanded to include open shell systems. They focused mainly on electronic structures and spin distributions. Although useful in this respect, the methods used and computer capabilities available at that time did not allow geometry optimizations nor reliable characterization of excited states. More recently an alternative *ab initio* approach, based on density functional theory (DFT), has been actively explored as a more efficient means of obtaining optimized geometries of small molecules,²⁶ the active site of iron–sulfur proteins,²⁷ and model iron–porphyrin complexes.²⁸ In this latter study, calculations were made for a five-coordinated ferryl Fe=O species and did not address the problem of the relative energetics of states of different multiplicity or the extent of geometry relaxation associated with them.

In the studies made here, we report for the first time the use of *ab initio* quantum chemical methods to calculate the optimized geometries, electronic structure, and relative spin state energies of a model for the resting state of peroxidases in its sextet, quartet, and double states. Increases both in the efficiency of the computational methods used and in computer

capabilities combine to make these studies possible. In the studies reported here, the single configuration (HF) and multi-configuration complete active space (CAS) self-consistent-field (SCF) *ab initio* methods were used with extended basis sets. The results obtained indicate a significant change in Fe–water distances in the high and low spin forms of these complexes, suggesting that the environment of the protein could modulate spin state changes by imposing geometric changes on this mobile Fe–ligand interaction.

Methods and Procedures

In this study, an iron–porphyrin complex with an imidazole ligand and water ligand was used as the model of the 6-coordinated peroxidase resting state. The porphyrin ring is in the *xy* plane with the nitrogen atoms on the *x* and *y* axes, and the axial ligands, the oxygen of the water, and N of the imidazole are along the *z* axis. The imidazole is conserved as the distal axial ligand of the iron in all known peroxidase structures, with the exception of chloroperoxidase, and is considered the prototypical axial ligand of peroxidases.²⁹ Neutral forms of the imidazole and water ligand were used, resulting in a net charge on the complex of +1. The initial geometry of this six-coordinated species used was that of the corresponding heme complex in the resting state of CCP³⁰ with the substituents of the protoporphyrin IX ring replaced by H atoms. In this X-ray structure the iron–water distance is 2.4 Å. Geometry optimization of the sextet, quartet, and doublet states of this complex were then performed.

The calculations were performed by using a complete active space multi-configuration CAS SCF method with the following basis sets: 6-31G for the porphyrin atoms,³¹ 6-31G** for the imidazole and water ligands, and an effective core potential³² with triple- ζ d functions on the iron. Previous calculations by Almlof et al.²⁸ on the isolated porphyrin indicated that a double- ζ quality basis set gives a semiquantitative description of the porphyrin ring, in particular of the molecular orbital energy levels of the frontier orbitals. Such calculations, confirmed in the present study, indicated that among the orbitals of the porphyrin ring, the lowest unoccupied orbital is a π orbital with small negative orbital energy. The wave functions for the respective spin state were obtained by distributing (pairing and spin coupling) the electrons in what are essentially the d orbitals of iron in all possible ways consistent with spin and spatial symmetry of the system. For the sextet state, there is only 1 configuration, while for the quartet state there are 12 configurations and for the doublet state 75 configurations. Geometries for each spin state were fully optimized. No vibrational analyses were performed to ascertain the minimum energy character of the structures. However, the initial structures used for each spin state optimization provide confidence that these structures are indeed low-energy structures. Specifically, the sextet geometry was obtained by optimization starting from the experimental structure as described above. Both the quartet and the doublet spin state geometries were obtained by optimization starting from the sextet optimized geometry. Net atomic charges and spin distributions were calculated by using a Mulliken population analysis. All calculations were performed with the computer code HONDO.³³ The ability to converge on stable minima for these excited states represents a considerable increase in the usefulness of *ab initio* methods to describe iron porphyrin complexes, since one of the main limitations of prior *ab initio* calculations was the failure to obtain convergence or reasonable descriptions of such states.

Results and Discussion

The main features of the optimized geometries obtained for the resting state model for peroxidases in a sextet, quartet, and

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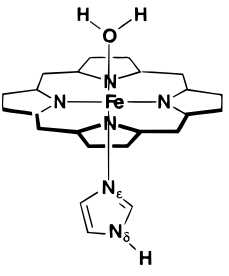
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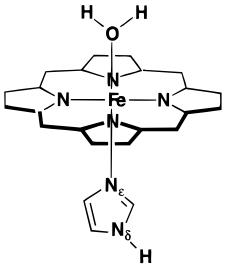
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Table 1. Resting State Model of Peroxidases: Optimized Geometries and Relative Energies of Different Spin States


	6A_1	4A_1	2A_1
$r(\text{Fe}-\text{OH}_2)$, Å	2.32	2.34	2.09
$r(\text{Fe}-\text{N}_\epsilon)$, Å	2.20	2.25	2.04
$r(\text{Fe}-\text{N}_{\text{pyr}})$, Å	2.05	2.00	2.01
$r(\text{Fe}-\text{N}_{\text{pyr}})$, Å	2.06	2.01	2.02
$\angle\text{FeOH}$, deg	125.5	125.6	124.7
$\tau\text{HO}-\text{Fe}-\text{N}_{\text{pyr}}$, deg	45.0	45.0	45.0
$\tau\text{HO}-\text{N}_\epsilon\text{C}$, deg	0	0	0
ΔE , eV	0.0	2.21	3.41

Table 2. Resting State Model of Peroxidases: Calculated Electronic Structure of Different Spin States^a


property	6A_1	4A_1	2A_1
net atomic charges			
q_{Fe}	+ 2.57	+2.49	+2.53
q_{O}	-0.67	-0.67	-0.70
q_{N_ϵ}	-0.79	-0.76	-0.83
$q_{\text{N}_{\text{pyr}}}$	-1.24	-1.21	-1.20
$q_{\text{H}_2\text{O}}$	+0.08	+0.05	+0.06
q_{porphyr}	-1.73	-1.61	-1.67
q_{Im}	+0.08	+0.07	+0.08
occupancy of CAS Orbitals			
$\rho(d_{x^2-y^2})$	1.00	1.97	1.94
ρd_{yz}	1.00	1.00	1.96
ρd_{xz}	1.00	1.00	1.00
ρd_{z^2}	1.00	1.00	0.07
ρd_{xy}	1.00	0.03	0.03
Fe unpaired spin	4.79	3.0	1.3
porphyrin unpaired spin	0.12		
Imh unpaired spin	0.07		
water unpaired spin	0.02		

^a The porphyrin ring is in the xy plane, with the nitrogen atoms on the x and y axes.

doublet state and the relative energies obtained for these states are given in Table 1. As seen from this table, the order of stability of the spin state energies is ${}^5/2 < {}^3/2 < {}^1/2$. Table 2 shows the net atomic charges and CAS orbital occupancies for the various states. These variable occupied CAS orbitals retained a very strong Fe(d) character. Also given in this table is the unpaired spin on the iron atom and, for the sextet state, on the remaining components of the complex.

The optimized structures obtained for each spin state are very similar. The most significant differences lie in the iron-axial ligand distances. Both the iron-water and the iron-imidazole distances are significantly shorter in the doublet state than in

the sextet and quartet states. For each spin state species, the four iron-porphyrin nitrogen distances are nearly equal, with a very small pairwise differentiation. These distances are somewhat longer in the sextet state than in the quartet and doublet states.

The variation of the Fe-axial ligand distances in the three spin states can be readily explain by examination of the occupation number of the natural orbitals of the CAS wave functions. As can be seen from Table 2, both the sextet and the quartet states, have a d_{z^2} orbital occupied, thus creating a repulsive interaction with the ligand lone pairs. This interaction results in the similar and longer Fe-water and Fe-imidazole distances in these two spin states compared to the doublet state in which the d_{z^2} orbital is unoccupied.

Also seen from the results in Table 2 is that the net atomic charges on the iron, the ligand atoms, the entire water and imidazole ligands and the porphyrin ring are very similar in the three spin states. While the ligand atoms themselves have substantial negative charge, -0.67 on the O atom of the water and -0.79 on the N_ϵ atom of the imidazole, there is very little net charge transfer to the axial ligands. Both water and imidazole remain essentially neutral, each with small positive, +0.08, charge. By contrast, there is a transfer of about half an electron from the formally dianionic porphyrin ring to the ferric iron. Also seen in Table 2, the iron atom carries most of the unpaired spins. Unpaired spins on the nitrogen atoms of the porphyrin ring, as well as on the imidazole ligand were found to be less than 0.03 in all cases. As already noted, the CAS orbitals with variable occupation numbers in all cases retained a very strong Fe d orbital character which resulted in this very limited spin transfer on the iron. Thus, the net atomic charge on the iron, the CAS orbital occupancy, and unpaired spin density in each state all retain the main features of a classical sextet, quartet, and doublet ferric ion.

No X-ray structure and no experimental determination of the ground state spin of a model six-coordinated ferric-porphyrin complex with a water and imidazole ligand have been reported. Thus the closest experimental comparisons that can be made of the computational results are with a model ferric-porphyrin diaquo complex.³⁴ The finding here of a sextet ground state for a ferric-porphyrin aquo imidazole complex is consistent with the experimental observation of a high-spin ground state in the model ferric-porphyrin diaquo complex. However, the optimized Fe-OH₂ distance found, 2.32 Å, is considerably longer than the value of 2.09 Å found in this model compound, and more consistent with the Fe-OH₂ distance of 2.4 Å found in the X-ray structure of CCP.³⁰ Given that results here are for an isolated heme unit, they should in principle be more comparable to the model compound.

There are a number of possible reasons for this disparity. It could be that higher levels of theory are required to obtain improved agreement. There could also be some effect of the differences in the other axial ligand, an imidazole in the model peroxidase, and a second water in the complex with known structure. In addition, the X-ray structure was determined in an aqueous environment and the water ligands are particularly susceptible to interactions with the surrounding solvent, an interaction that could affect the Fe-water distance observed.

The unique feature of these calculations is the ability to monitor changes in geometry as a function of spin state change in the same complex. Although there are many X-ray structures of model heme complexes with different ground state spins, there is no direct experimental way to determine the extent of

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Table 3. Effect of CCP Mutation on Spin State and Measured Values of Fe–Water Distances from X-ray Structure

mutant	Fe→OH ₂ Å (ref)	predominant spin state	
		low <i>T</i> (ref)	high <i>T</i> (ref)
wtCCP (yeast)	2.4 (30)	hs (13)	hs (13, 14)
wtCCP (M ₁)	2.7 (35)	hs (13)	hs (13, 14)
D235A	1.87 (16)	ls (16)	ls (16)
W191G	1.98 (17)	ls (17)	ls (17)
D235N	2.03 (35)		ls (13, 14)
D235E	2.31 (16)		hs (16)
W191F	2.54 (35)	ls (13)	hs (13, 15)
W51F	2.59 (35)	hs (13)	hs (13, 14)
R48L	2.84 (36)		hs (14)
H175E	3.30 (15)	hs (15)	hs (15)

geometry relaxation in different spin states of the same complex. The present results provide this missing component, allowing further insight into the relationship between the iron–ligand geometry in a given complex and its spin state. The shorter Fe–OH₂ bond found for the doublet state is consistent with the experimental observation that strong interactions of the axial ligands favor the low spin state. This observation has been made for both model and intact six-coordinated ferric heme complexes with varying axial ligand. Ligands such as CN⁻, N₃⁻ and OH⁻, generally lead to low spin ground states, and ligands such as Cl⁻, F⁻ and H₂O generally lead to high spin ground states.²⁰

The results of the present study also suggest a mechanism that could explain the different spin state distributions commonly found for ferric aquo heme complexes with the same axial ligands in different protein environments. It is possible that such changes in the environment around the heme unit could induce a geometric change in the labile water position that in turn modulates the population of spin states of different multiplicities. Experimental determination of spin state populations by a number of spectroscopic methods, notably resonance Raman,^{13–15} electronic spectra,^{9,13–17} and electron spin resonance^{9,16,17} in wt and in a series of mutants of CCP Fe(III) resting forms with known Fe–water distances from crystal structures,^{16,17,30,35,36} provide strong support for this hypothesis.

Table 3 presents a summary of the experimental results of the effect of mutations on spin state preference of CCP Fe(III) resting forms and their Fe–water distances determined from X-ray structures. Seen in this table is the striking result that

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the three CCP mutants, D235A W191G and D235N, in which the experimental Fe–water distance is the same or less than the calculated value of 2.09 Å for the low spin, are found to be predominantly low spin. Also, consistent with the calculated results is the observation that the wt and six mutants with known Fe–water distances comparable or greater than the optimized calculated distance of 2.31 Å for the sextet state are all found to be predominantly high spin. This consistency between experimental and theoretical findings compliments the findings and reinforces the hypothesis that changes in the environment around the heme unit could induce a geometric change in the labile bound water position that in turn modulates the population of spin states of different multiplicities.

The experimental results alone, although compelling, have the disadvantage that the X-ray structure determinations and the spectroscopic measurements to obtain spin state preferences are not done under identical conditions and that variations in the conditions themselves influence the spin populations. For example, X-ray structure determinations are made at room temperature usually with 60% glycerol or 40% MPD (2-methyl-2,4-pentane diol) added to the solvent.¹⁷ It is well-known that solution or crystalline form, the presence or absence of these agents in the solvent, changes in temperature, and even the enzyme aging process^{9,13–17} all influence the relative spin state distribution. Because of these confounding factors, observed spin state distributions cannot be systematically related directly to known changes in the Fe–water distances. Single crystal resonance Raman spectra in 40% MPD obtained for three of these mutants,¹³ D235N, W191F, and W51F, come the closest to simulating the X-ray conditions. Given these ambiguities in the experimental correlation, the computational results directly linking different optimized Fe–water distances of the same species to different spin states and the remarkably good agreement between the observed and calculated distances associated with these spin states provide convincing additional evidence that changes in the environment around the heme unit could induce a geometric change in the labile water position that in turn modulates the population of different spin states.

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