

Evidence for Sulfur-Based Radicals in Thiolate Compound I Intermediates

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Compound I species are believed to be the active intermediates in the catalytic cycles of a number of oxidative heme enzymes. With one exception,¹ these reactive complexes are thought to be best formulated as ferryl porphyrin radical cations. Other formulations have been suggested,² but results from experimental and theoretical investigations have generally supported the porphyrin radical cation model.^{3,4} Recent findings, however, indicate that the electronic structure of compound I may depend dramatically upon the nature of the axial ligand^{5,6} and suggest that in some cases (thiolate-ligated heme proteins in particular) an alternative formulation of the compound I species may be more appropriate.

Thiolate-heme proteins play critical roles in a number of important physiological processes (e.g., the metabolism of xenobiotics, neurotransmission, blood pressure control, and immune defense against tumor cells). Cytochrome P450 (P450), chloroperoxidase (CPO), and nitric oxide synthase (NOS) all possess an iron protoporphyrin with a cysteinate axial ligand. Compound I species are believed to be the reactive intermediates in the catalytic cycles of all three enzymes, but so far only CPO-I has been observed. Like other peroxidases, CPO-I is generally considered to possess a porphyrin radical, even though the spectra of CPO-I obtained by EPR and UV/vis techniques differ markedly from those obtained for horseradish peroxidase compound I,^{3b,d,7} the prototypical intermediate.

A few researchers have suggested that, in thiolate-heme proteins, the thiolate ligand (rather than the porphyrin) may give up an electron to stabilize the Fe(V)-oxo species, thereby generating a sulfur radical.^{2a,b,d} In support of this hypothesis, X α calculations on a thiolate compound I complex do show significant spin density on sulfur.⁵ However, these X α calculations yield a quartet ground state, while CPO-I is known to be a doublet.^{3d} This failure to predict the correct spin state is a problem since strong antiferromagnetic coupling between the iron-oxo unit and the radical species is one of the key differences between CPO-I and other known compound I systems.

More recent calculations have added to the uncertainty, as different groups using nonlocal functionals have found porphyrin-based radicals.^{4a,b} Since these functionals should provide a more accurate representation of the compound I intermediate than the X α method, the location of the radical center would seem to be resolved. However, these researchers also had trouble describing the CPO-I ground state correctly. Shaik and co-workers (using the BP86 functional and a mixed basis set) found a quartet ground state, while Harris and Loew (using the BPW91 functional and DZVP basis set) obtained a doublet ground state, with a coupling parameter, J , which is 2 orders of magnitude larger than the experimental value.^{3d} Thus, the true nature of this reactive intermediate remains open to question.

Recently the author showed that using the B3LYP hybrid functional,⁸ in conjunction with a large basis set, one could obtain a doublet ground state for an active-site model of substrate-free P450.⁶ This is of interest, because prior to this finding it was believed that sulfur coordination was incapable of producing the low-spin ground state observed for this ferric-aquo form of the enzyme, and it had been argued that the protein's electric field was responsible for the low-spin configuration.⁹ However, two things were immediately apparent from our investigation: (i) the thiolate ligand is responsible for the low-spin ground state of the ferric-aquo species, and (ii) large basis sets are crucial to obtaining an accurate description of the Fe-S bond. We found that as the size of the basis set in the calculations increased so did the amount of sulfur character in the high-spin wave function. This growth in sulfur character destabilized the high-spin configuration, leaving a doublet ground state. In light of our finding that the thiolate ligand plays such a critical role in determining the ground state of the resting enzyme, we wondered what role this ligand might play in determining the electronic structure of the compound I intermediate.

To investigate the possibility that thiolate compound I species possess sulfur-based radicals, calculations have been performed using the B3LYP functional, which is generally superior to the nonhybrid functionals used in prior investigations.¹⁰ These calculations also employ larger basis sets than previously used. As a result, this study should provide an accurate description of the electronic structure of a thiolate compound I intermediate.

Using GAUSSIAN94,¹¹ unrestricted calculations were performed on a 43 atom active-site model of a thiolate compound I intermediate. The cysteinate axial ligand was replaced with a methyl mercaptide unit, and hydrogen atoms were substituted for the eight carbons directly attached to the porphyrin ring, yielding the Fe(N₄C₂₀H₁₂)(SCH₃)O 43 atom species. Geometry optimizations were carried out at the 6-311+G level (diffuse functions were not included on carbon), and polarization functions were added to all heavy atoms when single-point calculations were performed at the optimized geometries.

Some results are shown in Table 1. Our calculations predict a doublet ground state, in agreement with EPR experiments that show CPO-I to display strong antiferromagnetic coupling ($J = -35 \text{ cm}^{-1}$).^{3d} Since CPO-I is the only known compound I system to show this sort of coupling, our calculated value of $J = -77 \text{ cm}^{-1}$ is an important indicator of the quality of our calculations. Previous attempts to model the thiolate-heme system have resulted in either ferromagnetic coupling^{4a} or antiferromagnetic coupling that is 2 orders of magnitude larger than experiment.^{4b}

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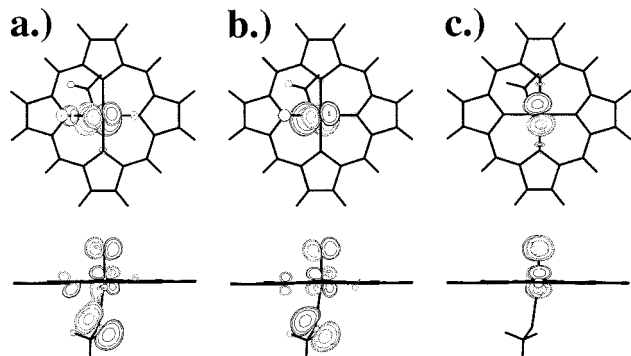
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Table 1. Ground State, Coupling Constant, Bond Distances, and NMO (SCF) Spin Densities of Thiolate-Ligated Heme Species

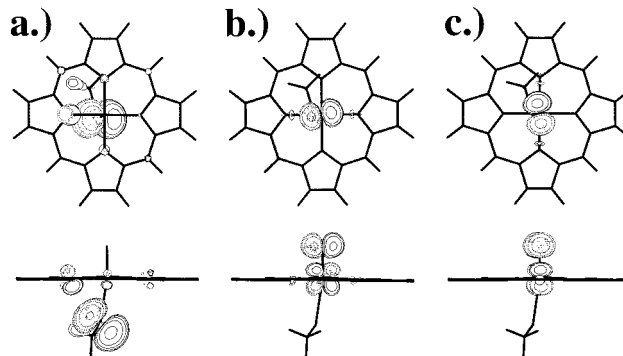
species	ground state	$J \text{ cm}^{-1}$	distance			spin density ^a			average spin density ^a			
			Fe-L	Fe-O	Fe-N _{avg}	L	Por	FeO	N	C _m	C _α	C _β
compound I	$S = 1/2$	-77	2.69	1.65	2.02	-0.78 (-0.82)	-0.16 (-0.31)	1.94 (2.13)	-0.020 (-0.059)	-0.022 (-0.047)	0.000 (0.013)	0.000 (0.001)
resting state			2.26	2.15	2.02							

^a NMO (SCF) spin densities.**Figure 1.** Natural orbitals of $S = 1/2$ thiolate compound I intermediate with occupation numbers of (a) 1.10, (b) 0.90, (c) 1.00. Top and side views are shown at a contour value of 0.05.

For comparison, the structure of a ferric-aquo model of a thiolate-heme protein was also optimized using the same functional and basis set employed for the compound I system. This resulted in an Fe-S bond length of 2.26 Å, which is in good agreement with the value of 2.25 Å obtained from the P450 crystal structure.¹² With this in mind, it seems clear from examining the optimized bond lengths in Table 1 that something dramatic has happened to the thiolate ligand during compound I formation. The optimized Fe-S bond length of 2.69 Å is much longer than the 2.26 Å of the resting state. This change in Fe-S bond length indicates that sulfur participates actively in forming the compound I intermediate.

With the aid of natural orbitals (NOs), we can begin to understand how thiolate ligation affects the electronic structure of compound I intermediates. The three NOs shown in Figure 1, which are needed for the multideterminant description of the doublet ground state, have occupations of 1.10, 0.90, and 1.00 (the rest are > 1.99 or < 0.01). Clearly these are not the orbitals one would expect based on the porphyrin radical cation model. First and foremost, they have little porphyrin content, and there is only one orbital which is solely FeO π^* in character (1c). The other two natural orbitals (1a and 1b) are in- and out-of-phase combinations of sulfur p and the other orbital of the FeO π^* set. These two natural orbitals are typical of those found in antiferromagnetically coupled diradical systems. This coupling makes itself apparent in the occupation numbers of these orbitals ($1.10 + 0.90 = 2.0$). Significantly, the uncoupled orbital (1c) has an occupation number of 1.00.

The construction of natural magnetic orbitals (NMOs) illustrates the electronic interactions more clearly. This approach has been used to explain spin-spin couplings in numerous systems,¹³ and is justified by the weak π interaction between the FeO unit and sulfur p orbital (the Fe-S bond length is 2.69 Å). Taking linear combinations of the coupled natural orbitals shown in Figure 1 yields the natural magnetic orbitals shown in Figure 2. This operation recovers the two FeO π^* orbitals shown in Figure 2. The electrons in these two orbitals couple ferromagnetically due to exchange (Hund's rule) giving the well characterized $S = 1$ iron-oxo unit. The other natural magnetic orbital (2a) corresponds to the radical center. Again, the character and location of the radical

**Figure 2.** Natural magnetic orbitals. Top and side views are shown at a contour value of 0.05.

center in the thiolate complex are very different than that predicted by the porphyrin radical cation model. That antiferromagnetic coupling is a consequence of this radical character is apparent from the NMOs shown in Figure 2. The sulfur-based radical (2a) created during the formation of the compound I intermediate has a nonzero overlap integral with the FeO π^* orbital (2b), which results in a very modest π interaction. This bonding interaction favors spin pairing, producing the $S = 1/2$ ground state.

The spin densities obtained from the self-consistent field (SCF) calculation and the NMOs of the thiolate complex are shown in Table 1. Using only the three NOs shown in Figure 1 results in the removal of spin polarization effects. Thus, the NMO spin densities shown in Table 1 (which are smaller than the SCF values) can be attributed solely to the unpaired electrons in the $S = 1$ iron-oxo unit and the $S = 1/2$ radical center. As one might guess from examination of Figure 2a, almost 80% of the radical's spin density resides on the thiolate ligand. This obvious departure from the porphyrin radical cation model results in porphyrin meso-carbon SCF spin densities that are reduced by a factor of 4.6 from the values obtained for an imidazole-ligated compound I intermediate.^{4c} Figure 2a shows the porphyrin spin density ($\sim 20\%$) to be distributed with roughly a_{2u} symmetry. Fujii has shown experimentally that porphyrin substituents can alter the symmetry character of porphyrin radical compound I systems (i.e., A_{1u} vs A_{2u}).¹⁴ The effect porphyrin substituents have on thiolate-radical compound I systems is unknown.

In conclusion, the results obtained indicate that thiolate compound I species possess sulfur-based radicals. The methods employed predict antiferromagnetic coupling ($J = -77 \text{ cm}^{-1}$), which is in good agreement with experiment. A significant lengthening of the Fe-S bond was found upon compound I formation, and greatly diminished porphyrin spin densities were predicted relative to imidazole-ligated compound I intermediates. Presently no structural or magnetic data exists to test the validity of these findings. Once such data becomes available, it can be used with the results of this study to elucidate the true nature of thiolate compound I intermediates.

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