Role of the Axial Ligand in Determining the Spin State of Resting Cytochrome P450

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The role played by axial ligands in determining the chemistry of heme proteins is a subject of debate.[1] Several intermediates in the catalytic cycle of cytochrome P450 have been suggested based in large part upon comparisons with horseradish peroxidase Compound I (**HRP-I**).[2] However, recent Xα calculations performed on a P450 Compound I analogue (**P450-I**) suggest that the electronic ground state of this species is very different from that found in the peroxidase system,[3] the main difference lying in the location of the radical−cation character. The Xα calculations predict, as is generally accepted, that HRP-I possesses a porphyrin radical−cation, but in the P450 analogue they suggest that the radical character is localized mainly upon the axial sulfur ligand. These results are in direct contrast to those obtained in a previous semiempirical investigation where, using the INDO method, investigators found both compounds to contain porphyrin radical−cations.[4] Since P450-I has never been experimentally observed and it is not known if the species exists at all, it is difficult to judge which of these descriptions is correct and the role, if any, played by the axial ligand in determining the chemistry of these heme proteins.

Comparisons of experimentally observable species have been made. The resting state of cytochrome P450 and the aqua compounds of the oxidized forms of hemoglobin (**metHb**) and myoglobin (**metMb**) all possess water coordinated to what is formally an Fe^{III} porphyrin center. The globin species are high-spin,[5] and it has been suggested by analogy that P450 should be as well.[6,7] This assumption, which is based upon the idea that water is a weak-field ligand, implies that the thiolate (cysteine) axial ligand of P450 and the imidazole (histidine) units of metHb and metMb play a similar role in determining the spin states of these heme proteins. Since the resting state of cytochrome P450 is decidedly low-spin, it would appear necessary, from this point of view, that there be some intrinsic (non-heme related) character of the enzyme which favors the low-spin state. Calculations by Harris and Loew seem to support this argument.[7] They performed semiempirical INDO calculations on the resting state of P450 in an attempt to discern the nature of its low-spin ground state and found that electronic structure calculations on the active site alone were incapable of accounting for the observed spin multiplicity. To obtain the correct ground state, they found it necessary to incorporate effects of the enzyme's electric field into their model. As a result of this study, they concluded that neither water ligation nor the enzyme's electric field was capable of independently producing the experimentally observed ground state but that both of these effects working in concert could.

The importance of the spin states of cytochrome P450 lies in their link to enzymatic activity.[8] When substrate binds, water is displaced from the heme, and the system shifts from low- to high-spin. This change in spin is accompanied by a 130-mV increase in reduction potential, which allows thermodynamic reduction by the native reductase, putidaredoxin. The calculations by Harris and Loew would seem to suggest that the protein, by controlling the spin state, plays an important and previously unidentified role in determining the active-site redox potential. While this possibility is interesting, it is also chemically unsettling in that the obvious chemical difference between the two systems plays no role in determining the characteristics observed. Thus, one is left wondering if a more accurate treatment of the species involved might reveal a different origin of the low-spin ground state.

To investigate the possibility that the thiolate axial ligand is responsible for the low-spin form of the resting state of cytochrome P450, calculations have been performed using a mixture of density functional and unrestricted Hartree−Fock methods (UB3LYP),[9,10] which has been shown to give accurate results for many transition-metal complexes and biochemical systems.[11,12] This study allows for an examination of the role played by the axial ligand in determining the chemistry of an experimentally observable species and, as a result, for an evaluation of comparisons made between heme proteins containing different axial ligands.

Using GAUSSIAN94,[10] calculations were performed on a 45 (42)-atom active-site model with distances taken from the experimentally observed crystal structure of substrate-free (bound) cytochrome P450.[13] The cysteine axial ligand was replaced with a methyl mercaptide unit, and hydrogen atoms were substituted for the eight carbons directly attached to porphyrin ring, yielding the $Fe(N_4C_{20}H_{12})(SCH_3)(H_2O)$ 45-atom species.

Although the correct ground states were predicted for both forms of the enzyme, it was found that obtaining a low-spin ground state for the resting form of cytochrome P450 was dependent upon the amount of sulfur character in the high-spin wave function, and that this in turn was dependent upon a more accurate (i.e., larger basis) description of the system.[14] Table 1 shows some results of this study. Here it can be seen that as the size of the basis set is increased the low-spin ($S = {}^1/_2$) state is stabilized with respect to the high-spin ($S = {}^5/_2$) state. (The energy of the intermediate-spin ($S = {}^3/_2$) state was also determined, but it was never found to be the ground state.) A key point to notice is the growth of sulfur spin density (a measure of unpaired electrons on sulfur) as the system is more accurately represented, while the other ligands show little change in this quantity. This increase in spin density represents a growth in the sulfur character of the molecular orbitals which would formally be assigned as metal d. In the simplest ligand field description, these metal orbitals are split into pseudo "t_{2g}" (metal nonbonding) and pseudo "e_g" (metal−ligand antibonding) sets. An increase in the ligand character of these wave functions leads to larger metal−ligand

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Table 1. Spin Densities, Fe−S Overlap Populations, and Energy Splittings for Sextet and Doublet States of Substrate-Free Cytochrome P450

basis	Mulliken spin density sextet/doublet				Fe−S Mulliken overlap population sextet/doublet	$E_{ls} - E_{hs}$ (cm^{-1})
	Fe	N[b]	O	S		
STO-3G	4.84/1.15	0.01/0.05	0.01/0.0	0.10/0.12	0.232/0.191	33010
3-21G	4.29/1.12	0.08/−0.03	0.02/0.0	0.31/−0.03	0.241/0.229	4643
6-311G	4.15/1.13	0.09/−0.03	0.01/0.0	0.41/−0.05	0.189/0.202	−287
6-311G(d)	4.12/1.10	0.09/−0.02	0.01/0.0	0.43/−0.03	0.183/0.201	−1054
6-311+G(d)[a,c]	4.00/1.06	0.10/−0.03	0.02/0.0	0.52/0.03		−1489
6-311+G(2d)[a,c]	4.03/1.05	0.10/−0.03	0.02/0.0	0.51/0.02		−1483

[a] Diffuse functions were not included on carbon. [b] N atom with the greatest magnitude. [c] See the text for a discussion of the Fe−S overlap populations.

antibonding interactions and a destabilization of the e_g set. Since the e_g set is only occupied in the high-spin form, this results in a relative destabilization of the high-spin case with respect to the low-spin one.

Because of the antibonding nature of the e_g set, one would expect a transition from high- to low-spin to be accompanied by an increase in metal−ligand overlap population (an indicator of covalent bond strength).[15] Shown in Table 1 are the total Fe−S overlap populations for the resting form of the enzyme. Notice that the smallest basis sets (which predict a high-spin ground state) do not exhibit the anticipated behavior but the larger ones do. This trend is not observed for the other ligands, which as expected always show the low-spin form to have the larger value. This pattern in the Fe−S overlap population and the growth in S spin density seem to be key indicators that an accurate representation of the Fe−S interaction is critical to obtaining the correct ground state. The overlap populations for the largest basis sets were not included in Table 1 as these values (between −3.0 and −4.0 for all of the species examined) were deemed pathological. These unreasonable values (which reflect the limitations of Mulliken analysis but in no way indicate problems with the SCF wave function) appear to result from the very large orbital overlaps caused by the inclusion of an extra set of diffuse functions on Fe, N, O, and S.[16]

To investigate the mechanism outlined above, we attempted to examine the growth of S character into the molecular orbital (**MO**) formally labeled as Fe d_{z^2}. However, due to the relative energies of the Fe, S, and porphyrin-ring fragments, a significant amount of d_{z^2} character could be found in several MOs, making it difficult to definitively assign an individual MO as metal-based. Also, the up and down spin orbitals obtained from unrestricted calculations are not a matched set. They have different energies as well as spatial extents, and this leads to difficulties in determining which orbitals are responsible for the spin density. Both of these problems made understanding the growth in S spin density and its effect upon the sextet−doublet splitting a difficult task.

These problems were avoided by constructing natural orbitals (**NOs**).[17] For the high-spin case, this process yielded five orbitals with occupations of 1.0 (the rest were either >1.99 or <0.01). These five orbitals are almost textbook descriptions of the e_g and t_{2g} sets and show a clear growth in S character as the basis set is increased. This can be seen in Figure 1 which shows the natural orbital describing the $d_{z^2} - p_z$ σ^* interaction between Fe and S in the STO-3G and 6-311+G(d) basis sets. Table 2 shows the effect of this increase on the Fe−S overlap population. The Fe−S antibonding interaction increases as the system is more accurately represented, and this results in the relative destabilization of the high-spin case. So that comparisons could be made among the different basis sets, the results of all NO calculations were projected onto the 6-311G(d) basis set.

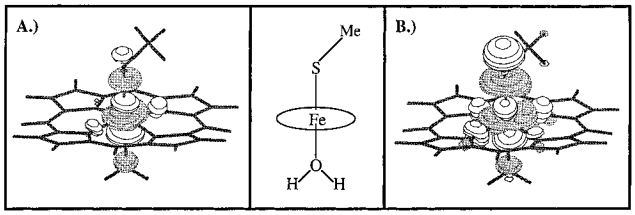

Figure 1. The Fe d_{z^2} natural orbital for the (A) STO-3G basis, (B) 6-311+G(d) basis. The contour value is 0.03.

Table 2. Population Analysis for the Five Singly-Occupied Sextet Natural Orbitals of Substrate-Free Cytochrome P450, Projected (Not Projected) onto 6-311G(d) Basis

basis	Mulliken spin density				Fe−S Mulliken overlap population
	Fe	N[b]	O	S	
STO-3G	4.770	0.025	0.013	0.052	−0.002
	(4.795)	(0.021)	(0.011)	(0.046)	(−0.001)
3-21G	4.230	0.082	0.018	0.301	−0.025
	(4.261)	(0.070)	(0.019)	(0.286)	(−0.022)
6-311G	4.127	0.083	0.015	0.373	−0.052
6-311G(d)	4.090	0.084	0.015	0.398	−0.053
6-311+G(d)[a]	4.075	0.083	0.014	0.408	−0.046
	(4.068)	(0.089)	(0.005)	(0.420)	(−0.097)

[a] Diffuse functions were not included on carbon. [b] N atom with the greatest magnitude.

In conclusion, it appears that the thiolate axial ligand is responsible for the low-spin form of the resting state of cytochrome P450 and that, as a result, the comparisons between this form of the enzyme and the histidine-containing globin species are not well-based. No need was found to invoke the enzyme's electric field in order to stabilize the low-spin state, indicating that INDO calculations are incapable of accurately representing the details of P450 (and most likely other thiolate-ligated heme systems as well).[18] Even the higher level of theory used in this study did not guarantee obtainment of a low-spin ground state as it was found necessary to use a basis set beyond the split valence (double-ζ) level in order to obtain qualitatively correct results. Due to the inadequacies of the INDO method, it seems that the recent Xα study gives the most reliable description to date of the proposed P450-I species. Taken together, the results of our study and those of the Xα investigation suggest that comparisons between P450 and the peroxidase system are tenuous. The electronic structures of the two species examined in these studies differ greatly from those of their imidazole-ligated counterparts, and the thiolate axial ligand plays a central role in determining this difference.

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