Determinants of the Spin State of the Resting State of Cytochrome $P450_{cam}$

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Abstract: The origin of the low-spin ground state of the substrate-free ferric resting state of cytochrome $P450_{cam}$ has been investigated with use of the combined techniques of restricted open-shell Hartree–Fock INDO/S calculations together with molecular dynamics. The presence of water as the heme-iron ligand, while resulting in a small energy separation of the $S = 5/2$ and $1/2$ ferric heme spin states, is by itself insufficient to explain the experimentally observed low-spin resting state. However, the inclusion of the electrostatic field of the protein in the INDO/S Hamiltonian, using the optimized X-ray coordinates and electrostatic potential-derived partial charges found in AMBER, results in a low-spin resting state of cytochrome $P\text{-}450_{cam}$. Molecular dynamic simulations of the optimized X-ray structure further support the role of the protein in modulating the spin state equilibrium. The dynamic motion of the heme unit is not sufficient to account for the predominance of the low-spin state, while the dynamic effect of the field of the protein favors the low-spin state. By contrast, in the camphor-bound cytochrome $P450_{cam}$, the field of the protein does not reverse the high-spin state found for the optimized X-ray structure of the heme unit. These results have revealed a heretofore unidentified role of the protein in modulating spin equilibrium, a property of the ferric heme unit that is central to the maintenance of the enzymatic function of the cytochrome P450s.

Introduction

Cytochrome P450s are a family of ubiquitous metabolizing heme proteins involved in a variety of functions including biosynthesis of steroid hormones and solubilization of nonpolar foreign substances. Although the isozymes differ in substrate and product specificity, the first four steps of the enzymatic cycle leading to the catalytically active species are common to all P450s. They involve transformation of the ferric resting state, by substrate binding, one-electron reduction, addition of molecular oxygen, and a second electron reduction to the putative enzymatically active species of a ferryl (Fe=O) complex that transfers a single oxygen atom to a variety of substrates. Although more than 200 P450 isozymes from many sources including plant, fungii, bacterial, and mammalian species have now been cloned and sequenced,[1] cytochrome $P450_{cam}$, from the bacterium *Pseudomonas putida*, is still perhaps the best characterized of the P450 family[2,3] and the only P450 isozyme for which there is a reported X-ray structure.

The 2.2 Å resolution crystal structure of the substrate-free resting state of cytochrome $P450_{cam}$ definitely shows that the substrate cavity is filled with a network of waters.[4] One of these waters is a ligand of the heme iron and has a relatively small thermal factor (16.5 $Å^2$). The B factors for the remaining cavity waters are larger than the average B factors of even surface waters, suggesting a mobile internal water network.

Electron spin resonance and temperature-dependent magnetic susceptibility measurements indicate that the resting state of cytochrome $P450_{cam}$ is a mixture of low and high spin forms, but it is predominantly, i.e. 96%, low spin at low temperatures (ca. 273 K).[5] This result is surprising since, in both model ferric heme complexes and other heme proteins with water as a 6th ligand, the doublet state is not the ground state. The identification of water as an axial ligand thus poses the enigmatic question of the origin of the low-spin ground state in the resting state of $P450_{cam}$.

The factors that modulate the spin states of the cytochrome P450s are particularly important for understanding this family of metabolizing heme proteins because spin-state changes play an important role in the formation of the catalytically active species. Upon binding of camphor, the natural substrate, the spin-state equilibria shifts to predominantly high spin, leading to a change in reduction potential from −300 to −170 mV that is crucial for subsequent one-electron redution to occur. Factors that allow the ferric state of the enzyme to remain in the low-spin state could adversely affect its function. For example, the binding of different analogs may control the spin state and, hence, affect the redox potential.[3,6] Substrates such as camphor and adamantanone that bind in the distal pocket with the dissociation of the ligand water result in a high-spin pentacoordinated heme with relatively high redox potential. Substrates such as camphane,[6] norcamphor,[7] and thiocamphor,[6] which allow the ligand water to remain, lead to complexes with substantial low-spin character (e.g. 46% for norcamphor compared to ca. 3% for camphor and adamantanone)[6] and much lower redox potentials. The presence of a ligand water after such substrates binds may be one of several factors contributing to uncoupling, i.e. the production of hydrogen peroxide by the enzyme rather than hydroxylation of substrate. Finally, ligands that displace the water ligand and bind to the iron itself such as metapyrone remain in the low-spin state and are inhibitors of enzyme function. Therefore, it is important to understand the factors that determine spin-state equilibrium when H_2O is the 6th ligand.

Previous explanations for the observation of a low-spin resting state of cytochrome $P450_{cam}$ identified two possible causes: an in-plane iron with a water ligand or the presence of a hydroxide ligand at a short distance of 1.75 Å in the substrate cavity. However, while demonstrating that these two factors could in

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principle lead to a low-spin state, these preliminary studies did not attempt to provide evidence that they were in fact present in the resting state of the enzyme.[8] The crystal structure is now of sufficiently high resolution to discern that the iron is decidedly out-of-plane. The second possible origin, the hydroxide character of the ligands, was inferred because of the hydrogen-bonding network of water molecules involving the ligand water in the substrate binding site.[7,8] However, the iron–ligand distance of 1.75 Å chosen was much shorter than that observed in the X-ray structure in the presence of this network. Also, the likelihood that hydroxide is the sixth ligand in the low-spin state is difficult to reconcile with the fact that the experiments conducted to determine spin state were done at ca. pH 7.[5]

Given the importance of the spin-state change in the initial steps in the enzymatic cycle to P450 enzymatic efficiency and the lack of a firm theoretical explanation, we have used the combined techniques of molecular dynamic simulations and semiempirical quantum mechanical calculations to continue to probe the likely origin of the experimentally observed low-spin state of the resting state of $P450_{cam}$ and the factors that modulate its spin-state equilibrium. In addition, we have also addressed the origin of the high-spin state in the substrate bound ferric $P450_{cam}$. While not as enigmatic, since five-coordinated ferric heme complexes are in general sextet states, if the results obtained can explain the observed differences in both forms of the enzyme, their credibility is strengthened and additional insight is provided into the factors that modulate spin-state equilibrium in the P450s.

Computational Methodology

The crystal structures of cytochrome $P450_{cam}$ in the substrate-free and camphor-bound forms, including crystallographic waters, were used as the starting structures in this study. The structures were energy minimized for 2000 steps (100 steepest descents and 1900 conjugate gradient) with use of AMBER 3.0a[9] with the polar hydrogen representation.

In the first study, heme and ligand coordinates were extracted from the optimized resting state and camphor-bound crystal structures. These coordinates were used as input for INDO/ROHF semiempirical calculations of the electronic structures and the relative energies of the high ($S = 5/2$), intermediate ($S = 3/2$), and low-spin states ($S = 1/2$). The explicit heme model used consisted of the entire protoporphyrin IX unit with neutral propionic acid substituents to simulate the proton transfer to these substituents in the protein from nearby cationic residues. The cysteine ligand was modeled by a methyl mercaptide. For the resting state two different water models were investigated. The first included the ligand water and five additional waters associated with it in the distal binding pocket. The second model included only the ligand water. In each model, the geometry was taken from the optimized crystal structure. For the camphor-bound species, camphor was included explicitly; the geometry was that of the optimized camphor-bound crystal structure.

In a second study, the INDO/ROHF/S method was used, together with the optimized crystal structure of the entire protein, including all crystallographic waters, in the substrate-free and substrate-bound states to investigate the effect of the electrostatic field of the protein on the relative energies of the high-, intermediate-, and low-spin states. In these calculations, each atom of the protein was represented in the INDO/ROHF/S calculations as a point charge modifying the one-electron potential in the INDO Hamiltonian. The point charges used were those derived for the AMBER potential function set that reproduce the quantum mechanical molecular electrostatic potential of the isolated amino acids but do not incorporate polarization effects that should not significantly affect this study. In addition, counterions were added to those surface charged residues not present in salt bridges by using the CION option of AMBER. These charges were included in the point charge representation input to INDO/ROHF/S. This step was used to simulate the effect of an ionic atmosphere present in solution-based measurements.

Each of the INDO/ROHF/S calculations with and without the field of the protein were performed for the unique high-spin ($S = 5/2$) configuration, for a $d_{xy}^2 d_{xz}^{1.5} d_{yz}^{1.5}$ low spin ($S = 1/2$) assignment to eliminate "symmetry breaking" effects due to the neglect of configuration interaction,[11] and for a $d_{xy}^2 d_{xz}^1 d_{yz}^1 d_{z2}^1$ configuration for the quartet state ($S = 3/2$).[12]

In a third study, molecular dynamics simulations were performed for the resting state, beginning with the minimized resting state crystal structure, to assess the effects of the dynamic behavior of the protein on the relative energies of the different heme spin states. Both local effects involving changes in the heme unit alone and global effects involving changes in the protein structure were investigated. The principal questions addressed in the MD simulations were the following: (i) is the dynamic behavior of the heme unit alone enough to account for the preference for a low-spin state and (ii) does the qualitative effect of the field of the protein in its optimized X-ray structure on the spin-state equilibrium persist when the dynamic behavior of the protein is considered.

For molecular dynamic simulations of the substrate-free resting state, the optimized structure of the protein was equilibrated at 300 K for 30 ps by coupling to a heat bath with a relaxation time of 0.1 ps. This equilibration was followed by 40 ps of dynamics using a 15 Å belly option region centered on iron and containing 1159 atoms. Heavy-atom hydrogen bond lengths were constrained by using the SHAKE[10] algorithm, and an integration time step of 1 fs was used. Trajectory coordinate information was saved every 0.02 ps for subsequent analysis. Of the 200 coordinate sets saved, 60 were selected as input for INDO/ROHF calculation of the electronic structure and relative energies of the high- and low-spin states with and without the field of the protein. The sampling scheme was designed to elucidate representive effects of both heme and protein environmental fluctuations on "heme vibrational" and residue reorientational time scales. Time points were selected on (1) a coarse/residue reorientational time scale at 1–2-ps intervals and (2) a "vibrational" time scale at 0.02-ps intervals.

To investigate the effect of dynamic changes in the heme unit alone on spin state, heme coordinates were extracted from each of 60 sets selected from those saved. Each coordinate set was used as input for an INDO/ROHF calculation of the electronic structure and relative energies of the high- and low-spin states. In addition, in order to investigate the effect of dynamic changes in the electric field of the protein on spin-state energies, the INDO/RHF calculations were repeated including the electrostatic field of the protein in each of these 60 instantaneous geometries.

Although the AMBER parameters used were independent of the spin state of the heme unit, the optimized and X-ray structures of the substrate-free and substrate-bound states were very similar, indicating that fine tuning the empirical energy based parameters for spin-state changes, while desirable, is not necessary to obtain reliable results for the properties addressed. It should also be noted that the use of INDO/S calculations has been remarkably successful in predicting the spin-state ordering of the low-lying multiplets of numerous ferric porphyrins.[12] In another context, this same semiempirical methodology already has been used in conjunction with molecular dynamics to reliably predict polar environmental effects on spectra and excited-state relaxation (dipolar relaxation) of chromophores.[13] Thus, although it is still a novel approach, there is precedence for exploring the effects of a fluctuating polar environment on electronic states by using a hybrid MD-INDO/S procedure.

Results and Discussion

Spin-State Equilibrium in Energy-Optimized Crystal Structures of the Resting and Camphor-Bound States of Cytochrome $P-450_{cam}$. Figure 1 shows the AMBER 3.0a energy-optimized heme unit of the substrate-free cytochrome $P450_{cam}$ with the proximal cysteine ligand of the Fe(III) and the network of six waters that fills the substrate binding cavity in the absence of substrate. One of these waters constitutes the sixth ligand of the ferric heme iron in the substrate-free form of this enzyme. The active site shown in Figure 1 is buried in the protein and has no direct access to bulk solvent. Upon binding of camphor the ligand and cavity waters are displaced from the binding site cavity.

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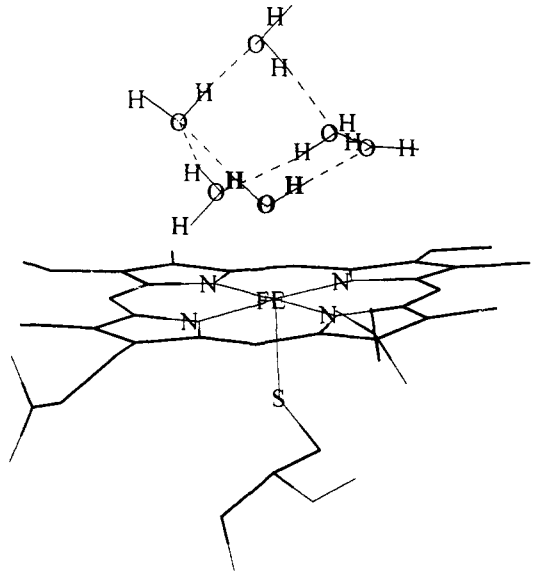

Figure 1. The cysteinate ferric heme and associated water network from the optimized resting state crystal structure of cytochrome P450cam (surrounding protein not shown).

Table I. Relative Energies of Different Spin States of the Substrate-Free Resting State of Cytochrome P-450cam

spin state	active site complex[a]	active site complex in the field of full protein
	A. Optimized X-ray Structure	
5/2	0	0
3/2	+5.80 (+6.64)[b]	+1.03
1/2	+3.84 (+3.98)[b]	−1.59
	B. MD Average Behavior	
5/2	0	0
3/2	+5.62[c] (+6.64)[d]	+0.37[c]
1/2	+4.9[c] (+4.9)[d]	−2.35[c] (−0.43)[d]

[a] The active site complex includes ferric-heme with SCH_3^- and ligand water. All energies (kcal/mol) are relative to that of the $S = 5/2$ spin state. [b] Figures in parentheses are for the active site complex model including the ligand water and 5-associated substrate binding site waters. [c] Results from calculation on the MD average structure. [d] Results from 60 ZINDO calculations sampled during the 40 ps with and without the field of the protein.

The geometries of the energy-minimized substrate-free and substrate-bound heme structures are virtually identical with the corresponding crystal structures. The average Fe–N distances in the substrate-free crystal structure and energy-minimized crystal structures are both 2.03 Å. The average Fe–N distance is 2.04 Å in the substrate-bound crystal structure and 2.02 Å in the optimized crystal structure. The iron–sulfur distances in the substrate-free crystal structure and energy-minimized crystal structure are both 2.25 Å. The iron–sulfur distances are 2.20 Å in the camphor-bound crystal structure and 2.22 Å in the energy-optimized structure. Although model ferric heme complexes in a high-spin state frequently have longer Fe–N distances, on the order of 2.05 Å, than those in a low-spin state, typically 2 Å or less, the cytochrome P-450 substrate-free and substrate-bound crystal structure and energy-minimized crystal structures do not have these differences in the heme iron–nitrogen distances, mainly because the iron remains out-of-plane in both states.

Table IA summarizes the relative energies of the optimal ferric heme unit in different spin states. Shown in this table are results obtained for the heme complex with the distal axial ligand water, the effect of including the remaining five binding site cavity waters, and the effect of the entire protein on the spin-state energetics. The results in this table illustrate that the sextet state is the ground state for the model complex with only the ligand water included and remains so when the hydrogen-bonded network of waters are included. This result clearly shows that including the network of hydrogen-bonded cavity waters does not result in sufficient hydroxide-like character to the ligand water to produce a low-spin ground state, a possibility that had been previously suggested.[3,8] Thus, the active site complex alone with or without the associated distal water cluster does not account for the predominance of the doublet state. Strikingly, when the electrostatic field of the protein is included in the calculation, the doublet state becomes the ground state. In contrast, as seen in Table II, for the camphor-bound form, the sextet state is the ground state of the active site complex and is even further stabilized by the field of the protein.

Table II. Relative Energies of Different Spin States of Substrate-Bound Ferric Cytochrome P-450cam

spin state	active site complex[a]	active site complex in the field of full protein
	Optimized X-ray Structure	
5/2	0	0
3/2	+3.18	+1.60
1/2	+18.12	+20.40

[a] The active site complex includes ferric-heme with SCH_3^- and camphor. All energies (kcal/mol) are relative to that of the $S = 5/2$ spin state.

These results taken together are consistent with experimental observation for both the substrate-free and the substrate-bound forms. They also elucidate the origin of the enigmatic low-spin state of the substrate-free enzyme. The results suggest that two factors lead to the low-spin state: (1) the presence of the aquo ligand and (2) the specific protein environment of P450cam. The presence of water as the sixth ligand is essential to reduce the sextet–doublet state energy splitting to a point where field effects due to the protein environment may additionally stabilize the doublet, making it the ground state of the enzyme. The importance of the specific protein environment is exemplified by the fact that most model ferric heme aquo complexes are high spin.

Dynamic Behavior of the Resting State of Cytochrome P450cam

A molecular dynamics simulation was performed to probe the effects of changes in both the heme geometry and amino acid residues in the protein environment on the ferric heme spin states. The main questions were (1) whether fluctuations in the heme geometry alone were sufficient to account for a low-spin ground state and (2) whether the fluctuating field of the protein, as well as the energy-optimized one, favored the low-spin state.

Heme Unit Distortions and Their Effect on Relative Spin-State Energetics. During the course of the molecular dynamic simulations of the substrate-free form of cytochrome P450cam, the ligand water remains closely associated with the heme iron, while the remainder of the waters in the distal binding site are quite mobile. Two of the five waters shown in Figure 1 remain hydrogen bonded to the ligand water throughout the 40-ps simulation. In the simulations, the heme iron moves in and out of the plane and the porphyrin undergoes vibrational distortions that result in variations in all the iron–ligand distances. The iron–water distance varies from 1.90 to 2.4 Å, with the MD average of 2.1 ± 0.17 Å, very similar to the iron–oxygen distance of 2.05 Å in the minimized crystal structure. Similarly, the average Fe–N distance of 2.03 ± 0.12 Å and the average Fe–S distance of 2.23 ± 0.09 Å are in excellent agreement with those of the optimized crystal structure.

The magnitude of the root-mean-square (rms) fluctuations of the heme residue atoms from the simulation and in the X-ray crystal structure are comparable (0.60 Å in the simulation and 0.69 Å in the crystal structure). A comparison of the rms fluctuations of the substrate cavity waters deduced from X-ray

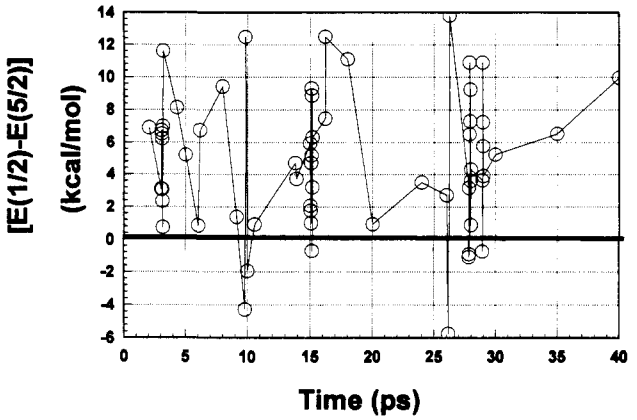

Figure 2. The effect of variation in the heme unit geometry alone on the relative energy of the high-spin and low-spin states $[E(1/2) - E(5/2)]$ (in kcal/mol). These calculations were made for the active site consisting of an aquo methyl mercaptide–ferric–heme complex in the absence of the field of the protein. Results are shown for all heme geometries samples during the MD simulations. Points above the base line indicate a high-spin ground state.

and from MD simulation is also similar (ligand water 0.80 Å (X-ray)/0.60 Å (simulation); remaining cavity waters ca. 1.2 Å (X-ray)/1–2 Å (simulation)). Although the simulation interval used is relatively short and fluctuation properties, such as B factors, converge slowly,[14] these results indicate that the librations of the ferric heme and associated waters are reasonably described.

Figure 2 shows the effect of the heme geometry variation on the INDO/RHF calculated energy difference between the S = 1/2 and 5/2 spin states for all 60 geometries sampled during the molecular dynamics trajectory of the substrate free protein. The energy difference $[E(1/2) - E(5/2)]$ is positive for nearly all of the sampled points, indicating that heme unit geometry fluctuations during the MD simulations favor a sextet ground state and cannot by themselves account for the predominance of the low-spin ground state.

This result is confirmed by the MD average behavior of the heme unit shown in Table IB, considered in two different ways. In one INDO/RHF calculation, the MD average heme structure obtained from all of the transient structures saved during the trajectory was used. As seen in Table IB, in this structure, the sextet state is the ground state by 4.9 kcal/mol. The same results are obtained when the MD average is taken of the difference in energy of the sextet and doublet state over the 60 points calculated.

Effect of Protein Electrostatic Field Fluctuations on Relative Spin-State Energetics. During the course of the MD simulation, there is significant fluctuation in the protein structure. This fluctuation is manifest in the electrostatic field that the protein creates in each of the instantaneous conformations sampled by the protein, as shown in Figure 3. This figure shows the electrostatic potential at the ferric heme iron due to the protein charge distribution included in the INDO/S calculation. It excludes the portion of the system treated quantum mechanically, i.e. the cysteine ferric heme plus ligand water. It is thus an indication of the time variation of the electrostatic potential energy ($U = q\varphi$) felt by a charge in the quantum mechanical system due to temporal variation in the position of all residues. Note that, while the average electrostatic potential at the iron does not vary much for any 10-ps segment, significant short time scale fluctuations in the magnitude of the potential do occur. In addition, the geometry of the heme unit used explicitly in the INDO/ROHF calculation is also changing. Thus, the effect of the electric field of the protein on the relative energies of the sextet and doublet states calculated for the active site should vary during the course of the MD trajectory.

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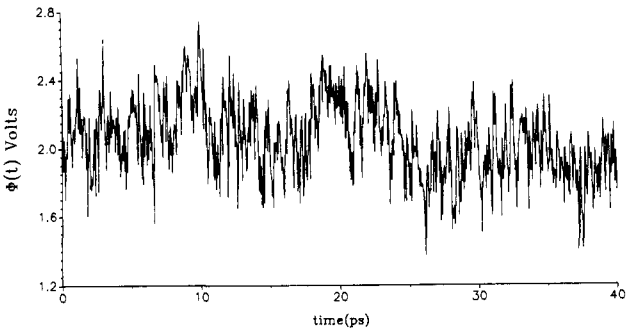

Figure 3. The time evolution of the electrostatic potential due to the protein environment surrounding the heme unit during the MD simulation time period.

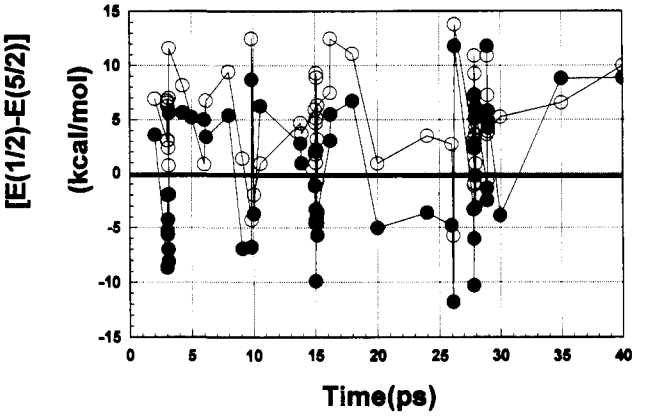

Figure 4. The relative energies of S = 1/2 and 5/2 states for the geometries sampled during the MD simulations calculated with use of INDO/S in the presence of the electric field of the protein at each point (filled circles). For comparison, the results obtained in the absence of the field, shown in Figure 2, are repeated here (open circles).

Figure 4 shows the doublet–sextet energy differences including the field of the protein calculated for each of the same points used in calculations without the field of the protein shown in Figure 2 and repeated here. The results in Figure 4 clearly demonstrate that the systematic effect of the electrostatic field is to stabilize the S = 1/2 state relative to the S = 5/2 state. In most of the cases, the calculation including the field of the protein shows increases in doublet state stabilization compared to the result excluding the field of the protein. Consideration of all the points sampled in our study indicates that active site complexes that are high spin by themselves with stabilization relative to the S = 1/2 state by as much as 10 kcal/mol are "transformed" into low-spin states due to the small, yet finite effect of the protein field.

These results are confirmed by the MD average value of relative energies of the high- and low-spin states in the presence of the protein field shown in Table IB. By using the molecular dynamic average structure in the INDO/ROHF calculations with the field of the protein, the doublet is lower by 2.35 kcal/mol. Also, the MD average value of $[E(1/2) - E(5/2)]$ leads to a low-spin state with an energy difference of –0.43 kcal/mol. Thus the results from the energy optimized structure and the inclusion of the dynamic behavior of the protein lead to the same conclusions. Neither the energy optimized nor the dynamic behavior of the active site complex by itself can explain the observed low-spin resting state. Rather the field of the protein considered from either static optimized crystal structure results or dynamic results leads to a low-spin ground state. These results, taken together, point to a significant role for the protein field effect as a modulator of spin-state equilibrium and indicate that it is the major determinant of the predominance of the low-spin state in the resting state of cytochrome $P450_{cam}$.

Conclusion

The electronic structures of the active site complex in its optimized crystal structure geometry cannot account for the low-spin resting state of cytochrome P-450$_{cam}$, even if the entire water cluster in the distal binding site is included. Moreover, the dynamic behavior of the active site complex still favors a high-spin ground state. The differential effect of the electric field of the protein on the spin multiplet energetics leads to a low-spin resting state. By comparing the results for the 6-coordinated substrate-free and 5-coordinated substrate-bound states, a role for the ligand water is also apparent. The presence of ligand water decreases the sextet–doublet splitting from 18.0 to 3.8 kcal/mol so that the differential stabilization due to the field of the protein results in a low-spin ground state. In contrast, without the water, in the substrate-bound state, not only is the sextet–doublet energy difference greater, but the field of the protein preferentially favors the sextet state. Thus there is a striking qualitative difference in the effect of the field of the protein in these two forms of the enzyme. Both the presence of water as a ligand and the field of the protein combine to form a low-spin resting state.

This delicate balance has functional implications. If the water ligand alone were sufficient to produce a low-spin ground state, then contrary to observation most aquo complexes of ferric heme protein[4,15,16] and model compounds[12] would have low-spin ground states. If the field effect were the sole determinant of the observed low-spin ground state, then one might not observe a significant shift to high spin upon camphor–substrate binding as is also verified by our calculations on the camphor-bound cytochrome P450. Our results indicate that both the ligand water and the protein field are determinants of the spin state of the ground state of cytochrome P450$_{cam}$. They also reveal a novel, heretofore unidentified, role of the globular protein in determining a crucial property of this family of metabolizing heme proteins.

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