Role of the Heme Active Site and Protein Environment in Structure, Spectra, and Function of the Cytochrome P450s

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I. Introduction and Background

Heme proteins are a class of biologically important macromolecules that have a unique, common active site: an iron−protoporphyrin-IX complex shown in Figure 1. This "heme" unit is a nearly independent planar entity linked to the remainder of the globular protein by only one or two amino acids that serve as axial ligands to the heme iron. Thus, heme complexes consist of three covalently linked components: the iron, the porphyrin macrocycle, and axial ligands binding directly to the iron. There is a great deal of experimental and computational evidence that both the observed spectroscopic properties and the function of heme proteins center on this heme unit itself.

One unique property of heme units in both intact heme proteins and model heme systems is that there is significant electron and spin delocalization among and between its three components. In particular, the porphyrin has an electron density characteristic of a highly delocalized system, allowing facile electron and spin delocalization.[1−5] In addition, significant charge transfer is found between the metal and the porphyrin due to covalent interactions, resulting in a reduction of the formal charge on the metal ion and on the porphyrin. This delocalization is manifest in the nature of the molecular orbitals as well as in the net atomic charges calculated from them.

The ability of the porphyrin macrocycle to participate in efficient redistribution of net atomic charges and unpaired spin density can play a key role in the formation and stabilization of the various heme species needed for function. Changes in the formal oxidation state of the heme iron occur during many heme protein functions. For example, the enzymatically active heme species in the metabolizing heme proteins, peroxidases, and cytochrome P450s (CYP450s) corresponds to a formal Fe(V) oxidation state, two oxidation states above the ferric form. Such a high oxidation state is too unstable to occur in any

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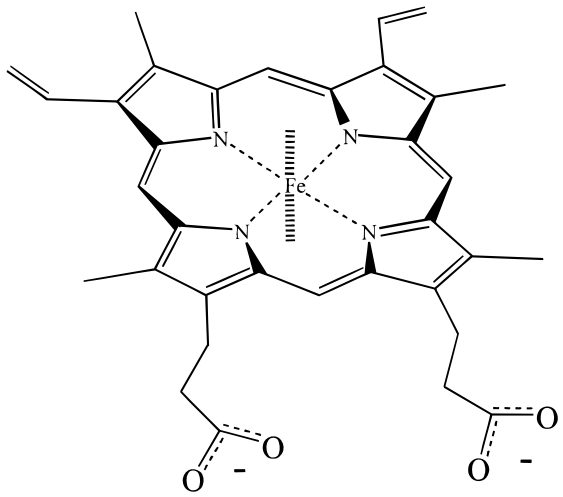

Figure 1. Common active site of heme proteins: a [Fe−Protoporphyrin IX] complex. It is linked to the globular protein by one or two axial ligands of the heme iron.

other iron-containing systems. However, the formation of such a heme species can be attributed to extensive electron redistribution that greatly reduces the net charge on the formal Fe(V) iron. The d electron distribution around the iron corresponds to an Fe(IV) d^4 species, and the charge distribution of the porphyrin ring is that of a π cation radical.

In addition to extensive electron and spin delocalization, another characteristic property of heme sites is the presence of low-energy states with different spin multiplicities. Specifically, ferrous porphyrins have quintet, triplet, and singlet state species and ferric porphyrins have sextet, quartet, and doublet state species in close energetic proximity. This behavior is in marked contrast to inorganic ferrous- and ferric-containing compounds with very well-defined ground states and large energy separations between states of different spin multiplicities. The presence of low-lying states of differing spin multiplicity is manifest in a number of signature spectroscopic properties of heme sites such as large values of zero-field splittings, large anisotropy in g values in electron spin resonance, and deviations from spin-only values of magnetic moments. In addition, because of the small energy separation of states of different spin multiplicity, the relative spin-state energy ordering is very sensitive to the nature of the metal ion and the number and nature of axial ligands.

Changes in spin state can be an integral part of heme protein function. One of the most striking biological manifestations of this modulation is the binding of molecular oxygen to the five-coordinated ferrous heme site of globins. The addition of O_2 and other small ligands such as CO transforms a high-spin, quintet species to a singlet species. Another significant effect of spin-state change on function is the transformation of the low-spin (doublet) ferric resting form of many CYP450s to a five-coordinated high-spin (sextet) system upon substrate binding. The change in spin state facilitates the next step in their enzymatic cycle, one-electron reduction.

While various spectroscopic techniques are helpful in identifying the spin multiplicity of the ground state, it is not always possible to clearly determine this property from experiment alone. It is even more difficult to obtain the rank order of energy of states of different multiplicities for a given heme species by either experimental or computational methods. A number of computational studies have addressed this problem. For example, the relative spin-state ordering in a four-coordinated unsubstituted iron porphyrin [Fe(II)−P] complex has been studied by ab initio methods.[4−11] In all of these studies, a pair of nearly degenerate triplet states, a $^3A_{2g}$ state and a ^3E_g state, were found, with one or the other predicted to be the ground state. Mossbauer resonance spectroscopy indicates that the [Fe(II)−P] complex should be a quintet state,[12−14] while proton NMR spectra suggest that [Fe(II)−P] has a triplet ground state,[15,16] as found in these calculations.

An all valence electron semiempirical INDO/ROHF/CI method, which includes both open and closed shell transition metals and extensive configuration interaction,[17−19] has also been used to calculate the relative energy ordering of states of different multiplicities in heme sites. Its reliability has been systematically tested by studies of a series of 18 ferric porphyrin complexes with varying axial ligands and known ground-state spins. Among these were ferric heme complexes known to have high- (sextet), intermediate- (quartet), and low- (doublet) spin ground states that could be inferred from the observed electromagnetic properties.[20] The consistency between calculated and observed properties obtained for all complexes studied provided strong support for the reliability of the INDO/ROHF/CI method to obtain relative energy ordering of states of different multiplicity for heme systems with known geometries.

Another signature property of heme sites in both intact proteins and model compounds is the common features observed in their electronic spectra. All heme complexes have two characteristic $\pi-\pi^*$ absorption regions, the so-called Q bands, weak visible transitions (Q_o, Q_v) at 550−570 nm and intense UV Soret transitions (B) at 408−450 nm. The higher UV bands (L and N) appear in a third region and are broad and diffuse. An important first step in understanding the origin of these common spectroscopic features was the identification of the $a_{1u}(\pi)$ and $a_{2u}(\pi)$ orbitals as the highest occupied orbitals and $e_g(\pi^*)$ orbitals as the lowest empty ones in diverse metal porphyrins by both computational[3−6,21−24] and a variety of spectroscopic investigations.[25−45]

The persistence of the $a_{1u}(\pi)$ and $a_{2u}(\pi)$ orbitals as the highest occupied orbitals and $e_g(\pi^*)$ orbitals as the lowest empty ones in diverse metal porphyrins can account for the facile formation of porphyrin π cation radicals that play an important role in the biological and catalytic function of porphyrins. For example, oxidative metabolism involving heme proteins and model heme sites proceeds via formation of the highly oxidized porphyrin−iron−oxo (compound **I**) π cation radical heme species. In addition, π cation radical formation is critical to the photooxidation of chlorophyll, in which Mg−porphyrins play a central role.

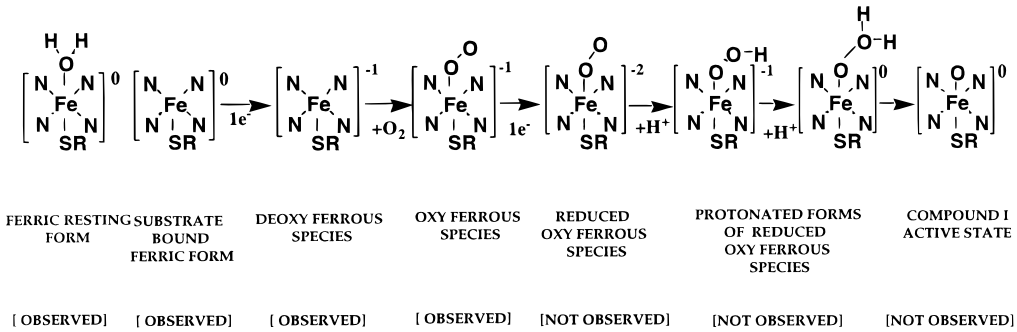

Figure 2. Stable and transient heme species thought to be involved in the enzymatic cycle common to all CYP450s.

The identification of the $a_{1u}(\pi)$ and $a_{2u}(\pi)$ orbitals as the highest occupied orbitals and $e_g(\pi^*)$ orbitals as the lowest empty ones also led to an explanation of the origin of the common features of heme spectra. Specifically, it was demonstrated that when these four orbitals are allowed to mix by configuration interaction, the resulting transitions have the observed properties, a weak Q-band in the visible and a very strong B or Soret band in the near UV.

A number of reviews have described the success of the four-orbital model based primarily on simple Hückel molecular orbital models as well as π-electron-only methods such as the SCF−PPP−CI method and the all valence electron CNDO/S methods to account for these regular $\pi-\pi^*$ transitions.[46−48] Additional early alternative approaches based on semiempirical quantum chemical methods applied to the calculation of the spectra of iron−porphyrins were the IEHT,[47,49−52] PPP/CI,[53] and Xα/CI[54] methods. While these methods have proven to be extremely useful in qualitative interpretation of electronic absorption spectra, quantitative assignment of both the typical Q and B bands and extra features often present in heme spectra and the effect of changes in spin state, ligands, and oxidation state on heme spectra requires more accurate methods.

Ab initio calculations of the electronic spectra of transition-metal porphyrin complexes have been limited to the electronic spectra of free base porphyrin[55,56] and of a closed shell singlet [Fe(II)−P−Im-CO] complex.[57] These studies clearly indicate that the use of split valence-type basis sets and the inclusion of correlation effects of both π and σ electrons are important to obtain good agreement with the observed Q and B band energies. However, these methods are very difficult to reliably extend to complex systems containing metal ions and unpaired spin density and for calculation of other than Q- or B-type excitations.

The INDO/SCF/CI method which includes both open and closed shell transition metals and extensive configuration interaction[17−19] fills a very important gap between the IEHT and ab initio methods for the calculation of spectra. This method has been successfully used for the calculation of transition frequencies, oscillator strengths, and the $x-y-z$ components of transition moments and the assignment of the electronic excitations contributing to each observed absorption for a variety of heme models for intact proteins. It allows the assignment not only of $\pi-\pi^*$ but of d−d* and all types of charge-transfer transitions that appear as extra features in many iron porphyrin compounds. First tested for ferrocene,[58] it was then successfully used for a variety of heme sites of intact proteins. Among the studies of closed shell systems that have been made are calculations of the spectra of oxy[59] and carbonyl ferrous[60] Hb complexes and oxy, carbonyl,[61] and carbene[62] ferrous P450 complexes. Each of these spectra presented very interesting unresolved questions, which these calculations were able to help resolve.

The extension of the INDO method to include restricted open shell, i.e., INDO/ROHF/CI capabilities, allowed for the first time the calculation of the spectra of radical transition-metal species, a capability that remains unique. This method can now be used to accurately predict the effect of changes in spin state, axial ligands, and oxidation state on the spectra of heme sites of stable and transient species. However, the optimum use of INDO/ROHF/CI methods requires a reliable geometry for the heme species under investigation. The use of nonlocal density functional (DFT) methods to obtain optimized geometries of heme sites is now possible. Thus, this review focuses on studies of one family of metabolizing heme proteins, the CYP450s, using the semiempirical INDO/ROHF/CI and DFT methods, which is a promising combination to continue to probe the relation between structure, spectra, and function of heme proteins.

The cytochrome P450s (CYP450s) are a ubiquitous superfamily of metabolizing heme enzymes with more that 500 isozymes cloned and sequenced. In all of these enzymes, a sulfur atom of a conserved proximal cysteine is the axial ligand to the heme iron. The CYP450s are present in a broad range of species including plants, fungi, bacteria, insects, and mammals. Mammalian CYP450s participate in metabolism of many exogenous and endogenous compounds as well as in every step of steroid hormone biosynthesis. The CYP450s function as monoxygenases, transferring a single iron-bound oxygen atom to a variety of largely nonpolar substituents, typically leading to aromatic and aliphatic hydroxylations, epoxidation, and heteroatom oxide formation.

As shown schematically in Figure 2, the heme site of the CYP450s undergo complex transformations during its enzymatic cycle. This complex pathway from the inactive resting species to the putative catalytically active ferryl Fe=O compound **I** species involves sequential changes in the oxidation state, distal ligands, and spin state of the heme, forming

both known and postulated transient species. The techniques of computational chemistry are ideally suited to assess whether the properties of the heme species implicated in this function are consistent with their postulated role and observed properties.

The first question to be addressed in this review is the origin of the low-spin ground state of the ferric resting form of the heme site of CYP450s.[63] The finding of such a ground state by ESR and temperature-dependent magnetic susceptibility studies of CYP450cam[64] was unexpected since the X-ray structure[65] revealed that a water molecule at a long distance (2.4 Å) was the other axial ligand of the iron. Ferric heme complexes with such a weak sixth ligand typically have a high-spin, sextet ground state. Thus, it was challenging to address the origin of this unusual finding.

The second question addressed here involves the properties of the heme site of the substrate-bound ferric species of CYP450s. The optimum function of these enzymes involves a change to a high-spin state that is triggered by the binding of the substrate. The goals of these calculations were to understand (i) the origin of the spin-state change upon substrate binding and (ii) the relationship between the spin-state change and observed changes in the electronic spectra used to monitor substrate binding.

The remaining questions addressed in this review focus on the nature of the putative transient heme species thought to be involved in the formation of the enzymatically active form of the CYP450s. As shown in Figure 2, the ferric resting form and substrate-bound forms of CYP450s to be discussed are the first two species in the complex path to the putative enzymatically active form. As also shown in this figure, one-electron reduction and then binding of molecular oxygen to the ferrous heme site follow. This oxyferrous species is the last stable one in the enzymatic cycle.

The subsequent steps in the enzymatic cycle after formation of the oxyferrous species namely from the reduced oxyferrous species to the enzymatically active compound **I**, ferryl species are among the least resolved aspects of CYP450s enzymology. While it is known that a second electron reduction of the oxyferrous species is required, the resulting reduced oxyferrous species formed is too transient to have been isolated. A transient spectrum attributed to this species has recently been reported, but no independent evidence exists that confirms that this species is the origin of the observed spectra. Thus, in the third study described, the combined techniques of DFT and semiempirical INDO methods have been used to further identify the species that is the origin of the observed spectrum.

In the fourth study, DFT methods have been used to probe the validity of the widely assumed pathway to formation of the enzymatically active form of the CYP450s from the reduced oxyferrous species. As seen in Figure 2, the proposed pathway involves proton-assisted dioxygen bond cleavage of this species to form the enzymatically active oxyferryl, Fe═O, compound **I** species that transfers the active oxygen atom to a variety of substrates. While there is indirect evidence for such a mechanism, one important role of computational methods is to help to further elucidate the steps in the activation of these important heme enzymes.

In a fifth study, density functional theory has been used to characterize the electronic structure of the putative enzymatically active compound **I** using the complete protoporphyrin-IX heme unit found in the intact protein. One of the main goals of this study was to determine how similar the optimized geometry and electronic structure of this more realistic heme model is to a simple porphyrin model previously studied by several investigators using DFT-based methods with different geometries functions and basis sets.[66−68]

The final study described probes the role of the protein environment itself in compound **I** formation. Specifically, an intact CYP450s protein is examined for a plausible mechanism of proton donation to the distal oxygen of the reduced ferrous dioxygen heme site and to help identify the ultimate source of these protons.

II. The Heme Site of the Resting Form of CYP450s

The resting form of the substrate-free CYP450s in isozymes with a known X-ray structure[69−72] is a ferric aquo heme complex with a mercaptide ligand from a nearby conserved cysteine. In an attempt to independently verify the presence of a water as the second axial ligand, the ESR spectrum of the substrate-free resting form of P450cam in 65% ^{17}O enriched H_2O has been determined.[73] In this initial study, no hyperfine splittings of the electron spin resonance due to nuclear−electron spin interactions were observed. These negative results were in apparent conflict with the X-ray structure that indicated that CYP450cam has a water molecule as a sixth ligand. The question of whether negative results in such ESR experiments need always be interpreted as the absence of water was investigated by computational studies of the expected unpaired spin density on the oxygen atom of the water ligand in the six-coordinated ferric aquo P450 resting form in the high-spin and low-spin states using the INDO/ROHF method.[74] The results of these studies predicted negligible unpaired spin density on the water oxygen in both of these spin states. The calculated value was greatly reduced by a factor of over 100 compared to the calculated value for MetMb, for which broadening of the ESR is signal is barely observed. Thus, these results resolved the conflict between the ESR and X-ray spectra by illustrating that the absence of broadening or splitting of the ESR signal is not a priori evidence for the lack of a water ligand. More recently, the ESR spectrum of the substrate-free resting form of P450cam in 65% ^{17}O enriched H_2O has been reexamined using a more sensitive electron spin−echo spectroscopic technique that was able to better detect some residual broadening that confirmed the presence of water.[75]

However, another enigma remained, the origin of the low-spin resting state so unusual for a ferric aquo heme complex. The observed temperature dependence of magnetic susceptibility[76,77] of quadrupole

splitting in Mossbauer resonance,[78−80] electron spin resonance,[81−84] resonance Raman,[85−89] and electronic spectra[83,90] of horseradish peroxidase, cytochrome *c* peroxidase, and MetMb indicate either a sextet or quartet ground state albeit with subtle variations of populations of different spin states. By contrast, the preferred ground-state spin of the substrate-free ferric resting form of CYP450s is low spin. This preference for a low-spin state in ferric aquo heme complexes in intact proteins is unprecedented. Moreover, in two known model structures, a ferric diaquo−porphyrin complex[91] and a very recent aquo thiolate heme model for the resting state of CYP450s,[92] a high-spin state is the ground state.

Computational studies have thus been performed to determine the origin of the low-spin ground state in the ferric resting form of intact CYP450s. In the first study, a heme model of the substrate-free ferric resting form of cytochrome P450cam was used together with the combined techniques of restricted open shell Hartree−Fock INDO/ROHF methods and molecular dynamic simulations.[63] For the heme site by itself, [Fe(III)−Protoporphyrin-IX−H_2O−SCH_3^-], a sextet ground state was found albeit with a very small energy separation of 3.8 kcal/mol to the doublet state and 5.80 kcal/mol to the quartet state. Thus, the presence of the two axial ligands alone, water and mercaptide, did not by themselves appear sufficient to explain the experimentally observed low-spin state of the intact P450cam protein based on ESR and temperature-dependent magnetic susceptibility measurements.[64]

To further probe the origin of the low-spin state observed only in the ferric resting form of the intact protein, the role of the protein environment as a modulator of the relative spin-state energies of the [Fe(III)−Protoporphyrin-IX−H_2O−SCH_3^-] heme site was investigated. To this end, the energies of the sextet, quartet, and doublet states were calculated using the INDO/ROHF method with the addition of the electrostatic field due to the protein to the 1e-portion of the Hamiltonian.[63] The field of the protein was included using the net atomic charges for every atom in P450cam in the position occupied by that atom in the optimized X-ray structure of substrate-free P450cam. For this purpose, the electrostatic potential derived partial charges found in the AMBER suite of programs[93] were used. The inclusion of the electrostatic field of the protein in the INDO/ROHF Hamiltonian resulted in a low-spin resting form of cytochrome P450cam in agreement with experiment. The high-spin state was 2.35 kcal/mol higher in energy. Molecular dynamic simulations of the optimized X-ray structure using the Amber suite of programs[93] further supported the role of the protein in modulating the spin-state equilibrium. The dynamic motion of the heme unit alone 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 sate. 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.

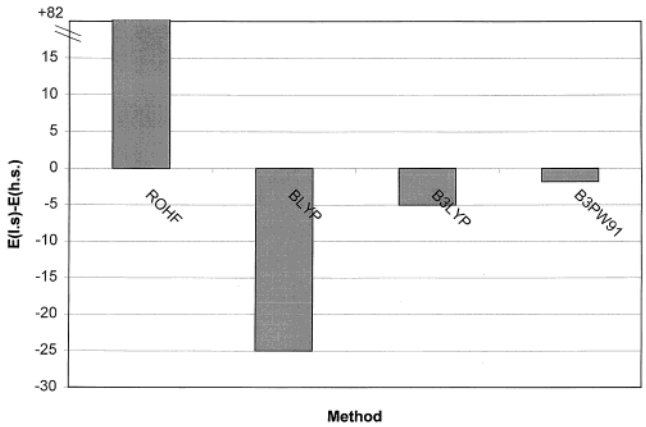

Figure 3. Relative energies of the high-spin (sextet) and low-spin (doublet) states of a porphine model heme site for ferric aquo resting of CYP450s, calculated using the same basis set but different ab initio methods.

In several subsequent studies the question of the origin of the low-spin state of the resting state has been reexamined using both local DFT[94,95] and nonlocal DFT[96] density functional theoretical methods using idealized porphine models of the heme. The results of the local DFT studies were not clear, since the energy difference between the high- and low-spin states was not reported, while the results found from the nonlocal DFT calculation were that the doublet state was the ground state. This result clearly indicates that the extent to which exchange and correlation effects are included in the calculation is an important factor in the relative energy of states of different multiplicity obtained. However, in the prior studies, different heme models, different geometries, and different basis set were used.

To more even handedly examine the effect of the balance between electron and exchange on the calculated energy ordering of states of different multiplicities, calculations for the same heme model of the ferric resting form of Cyp450s, a simple iron porphine with a water and methyl mercaptide axial ligands and the same geometry, were performed using the same basis set (ATZVP Fe; 6-311+g* S; 6−311 g* all other atoms) but with ab initio methods differing in the balance of correlation and exchange effects included. Specifically, the methods used were (i) restricted open shell Hartree−Fock (ROHF) with no electron correlation effects, (ii) nonlocal BLYP with both exchange and correlation effects introduced via energy functionals, and (iii) hybrid B3LYP or B3PW91 which contain correlation functionals and both Hartree−Fock and density functional exchange contributions. The results are summarized in Figure 3. As seen in this figure, the relative energy of the high- and low-spin states differs markedly among these calculations, reflecting a different balance between exchange and correlation effects. These results clearly indicate the challenge to computational methods of obtaining the energy ordering of two nearly degenerate electronic states of different multiplicity for open shell transition-metal complexes.

When the computation studies described above were reported for the isolated heme site, it was not possible to decide which was correct, because there

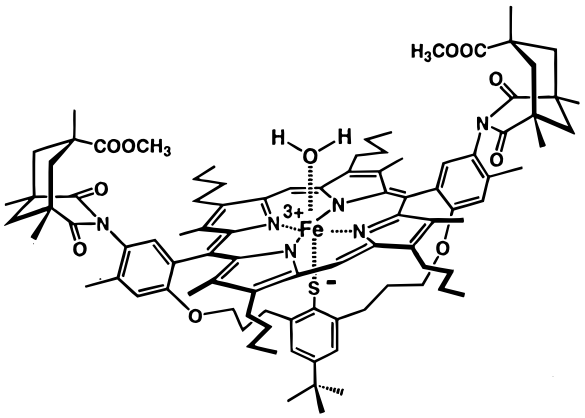

Figure 4. Structure of the model heme site the ferric aquo resting of CYP450s recently synthesized and spectroscopically characterized[92] for which calculations were made.

were no experimental results of a heme model for the resting form of CYP450 with which to compare them. Since then, a heme model shown in Figure 4 for the ferric aquo thiolate resting state of CYP450s was synthesized and the electron spin resonance and other spectroscopic properties determined.[92] These results clearly demonstrated that a sextet state is the ground state for this ferric aquo thiolate heme complex, consistent with the results found for the INDO/ROHF methods. However, although this direct comparison between calculated and experimental results for a ferric aquo thiolate heme model is promising, the heme complex for which the calculation was performed and that for which the experimental study was made differed significantly in substituents on the porphyrin ring. Thus, to further explore the reliability of the INDO/ROHF method, it was used to calculate the relative energies of the high- and low-spin states for the exact heme model studied experimentally. Consistent with experiment, the results led to a sextet ground state, with the doublet about 10 kcal/mol higher in energy. These combined experimental and computational results provide convincing evidence that the heme unit alone is not sufficient to account for the low-spin state observed in the intact CYP450 proteins in their ferric aquo resting states.

III. Heme Site of the Substrate Bound Ferric CYP450s

The goals of these calculations were to understand the origin of the spin-state change upon substrate binding and the relationship between this change and observed changes in the electronic spectra used to monitor substrate binding.

Substrate binding to the distal binding pocket of intact P450 isozymes appears to result in a five-coordinated species with the heme iron bound only to the proximal cysteine ligand. The X-ray structures of two substrate-bound CYP450s, CYP450cam[70] and CYP450-ery,[69] confirm this trend. INDO/ROHF calculations made for the heme unit of this species, a [Fe(III)−Protoporphyrin-IX−SCH_3^-] complex,[63] using the X-ray coordinates from the crystal structure of CYP450cam resulted in a high-spin ground state with the quartet and doublet states 3 and 18 kcal/mol above the sextet state. In contrast to the resting form, the high-spin state remains the ground state even in the field of the camphor-bound CYP450cam. These results are consistent with the high-spin ground state inferred from a number of spectroscopic measurements of substrate-bound CYP450cam including ESR and optical spectra.

When substrates bind to the ferric resting form of the CYP450s, a small shift to higher frequency is observed in the maximum of the Soret B band.[64,97−100] This shift is used to monitor the binding of substrates to the CYP450s. Moreover, this shift has been attributed to the spin-state change from a low-spin to a high-spin ferric species, which generally occurs upon substrate binding to the P450s. Thus, for many years now, a shift in frequency of the maximum in the Soret band in the UV−vis spectra has been routinely used to quantify the amounts of high- and low-spin forms of ferriheme proteins. Moreover, since this spin-state change has been shown to be functionally important in the enzymatic cycle of this family of metabolizing heme proteins, characterization of the percentage of high-spin form in this manner resulting from binding of different substrates or from mutations of the enzyme has become a commonly used indicator of enzymatic efficiency.

While monitoring a small change in the frequency of the Soret band is a very efficient way of inferring a spin-state change in heme proteins, a mechanistic link between the observed blue shift in the Soret band and a change in the spin state of the iron had yet to be established, since optical spectra are not a priori a probe of the spin states of a chromophore. Another confounding aspect is that, in all cases, Soret band shifts are monitored between two heme proteins that differ in some respect in addition to a spin-state change, for example, in axial ligand, in substrate, inhibitor, or a site-specific mutation of the protein. Subtle changes in the heme unit such as different orientations of the vinyl substituents have also been shown to affect the position of the Soret maximum in other heme protein families.[101,102] Few of the many heme systems thus studied have had both their magnetic and optical properties characterized. Thus, it was possible that the observed shift in the B band maximum was a direct result of other differences between the two protein species being monitored without the obligatory involvement of a spin-state change.

The techniques of computational chemistry are ideally suited to address both of these concerns. Thus, the INDO/ROHF/CI method has been used[103] to determine whether there is an underlying mechanism coupling the spin-state change with the observed shifts in the Soret band of the optical spectra of heme proteins. The calculations were made for the identical heme complex in both a high- and low-spin state without changing any other feature of the heme unit. The model used in the study was the active site of substrate-free CYP450cam, an [Fe(III)−Protoporphyrin-IX−aquo mercaptide] complex, as found in the 2.2 Å resolution crystal structure.[70] Such an idealized comparison is possible only in a computational ap-

proach, since in a real system some change has to occur to cause the spin-state change and one can never completely decouple the two possible sources of the effect on the Soret band.

Comparing the calculated spectra of the high- and low-spin states revealed that upon change in the Fe(III) from a low- to a high-spin reference state, without any other change, in the heme complex, a blue shift does indeed occur in the strong Soret (B) band absorptions found in the 25 000 cm^{-1} region that corresponds to the observed shift in the Soret band. The calculations also elucidated the origin of the observed shift. It was shown to be a direct consequence of the spin-state change. In the low-spin state, there is enhanced mixing of the iron d and porphyrin $4e_g(\pi^*)$ orbitals resulting in a lowering of the $e_g(\pi^*)$ states and a consequent shift to the red of the Soret band. These results clearly demonstrate an underlying physical basis for the observed correlation between ferric heme spin states and measured spectral shifts associated with ($\pi \rightarrow \pi^*$) heme transitions.

IV. The Heme Site of the Ferrous Dioxygen Species and the Putative Transient Reduced Ferrous Dioxygen Species in CYP450s

The oxyferrous P450 species is the last intermediate in the enzymatic cycle of P450 shown in Figure 2 that is stable enough to have been characterized by a variety of spectroscopic properties. The second electron reduction of the oxyferrous P450 species that follows results in the formation of a reduced oxyferrous species, a very transient species two oxidation states below the ferric resting form. This species rapidly forms water and compound **I**, the putative catalytically active species that in turn rapidly oxidized substrates by transfer of the active oxygen atom to them. There have been no X-ray structures reported for the ferrous dioxygen species, the reduced ferrous dioxygen species, or the compound **I** species of any of the CYP450 enzymes.

Of these three oxygen-containing intermediates in the CYP450 enzymatic cycle, most is known about the ferrous dioxygen species because it is stable enough to have been characterized by a number of spectroscopic methods. An EXAFS study of the ferrous dioxygen intermediate in CYP450cam[104,105] as well as a X-ray structure of a model dioxygen CYP450 heme complex[106] have been reported. In the crystal structure of this model compound, the dioxygen molecule is bound in an asymmetric "end-on" fashion to the heme iron. In addition to structural information, a number of spectroscopic properties have been measured for this heme model and for the intact ferrous P450cam dioxygen species that clearly indicate that both are in a diamagnetic singlet state.

Because of its transient nature, the effect of the second electron reduction of the ferrous dioxygen heme site of CYP450s on the geometry of the dioxygen ligand and on the electronic structure and spin state of the resulting reduced oxyferrous species is unknown. Although both end-on and bridged structures have been proposed,[107−109] it has not been possible to establish the preferred binding mode from experiment alone.

An electronic spectra thought to originate from this reduced dioxygen P450cam has recently been reported.[110] Although plausible, the electronic spectra reported provided no independent evidence that the species from which it originated is indeed the elusive twice reduced ferrous dioxygen form nor can the geometry or mode of binding of the dioxygen species be deduced from these studies. To further characterize this species and to determine if the observed transient spectrum originated from it, computational studies involving the combined use of nonlocal DFT and INDO/ROHF/CI methods have been performed.[111]

Nonlocal DFT has been applied to a range of transition-metal complexes such as iron chlorides[112] as well as a number systems of biochemical interest. Among these are determination of the optimized geometries for a five-coordinate model of the ferryl heme complex[113] and iron sulfur clusters relevant to these centers in proteins.[114] It has also recently been applied to the calculation of the properties of compound **I** in peroxidases at experimentally determined geometries[115] and to the calculation of the equilibrium geometries and electronic structure of a number of five- and six-coordinated heme sites of globins with CO, O_2, and NO as ligands.[116] In a number of other studies, nonlocal DFT has also been shown to perform well for open-shell systems with unpaired spin density.[117−119]

To continue to assess the reliability of the nonlocal DFT methods, calculations were made of the optimized geometries ground-state spin distributions and electronic structure of the ferrous dioxygen CYP450 heme site. The calculated geometries and diamagnetic ground-state spin were in excellent agreement with the experimentally observed results for the model compound.

After this successful validation, the same methods and procedures were used to further probe the existence and nature of the more elusive reduced ferrous dioxygen heme species. The calculated ground state of the reduced ferrous dioxygen heme species is a low-spin doublet state, in agreement with the reported ESR signature of this species in CYP450cam.[120] The dioxygen ligand in the reduced form is shown to preferentially bind to the heme iron in an asymmetric end-on geometry, similar to the ferrous dioxygen CYP450 heme site before one-electron reduction. There is no stable minimum corresponding to a bridged structure. There is no weakening of the O−O bond of the dioxygen ligand. These results strongly suggest that one-electron reduction alone is not sufficient to cleave this bond, leading to the oxyferryl Fe═O active species. The most pronounced structural effects of the reduction of the ferrous dioxygen species are the elongation of the Fe−O and Fe−S axial ligand bonds. This bond lengthening is due to the addition of an electron into a molecular orbital of significant antibonding character in the S−Fe−O bonds upon reduction of the ferrous dioxygen heme species.

INDO/ROHF/CI calculations of spectra were performed for both ferrous dioxygen and the reduced ferrous dioxygen heme sites at the computed density functional optimized geometries. For comparison, the

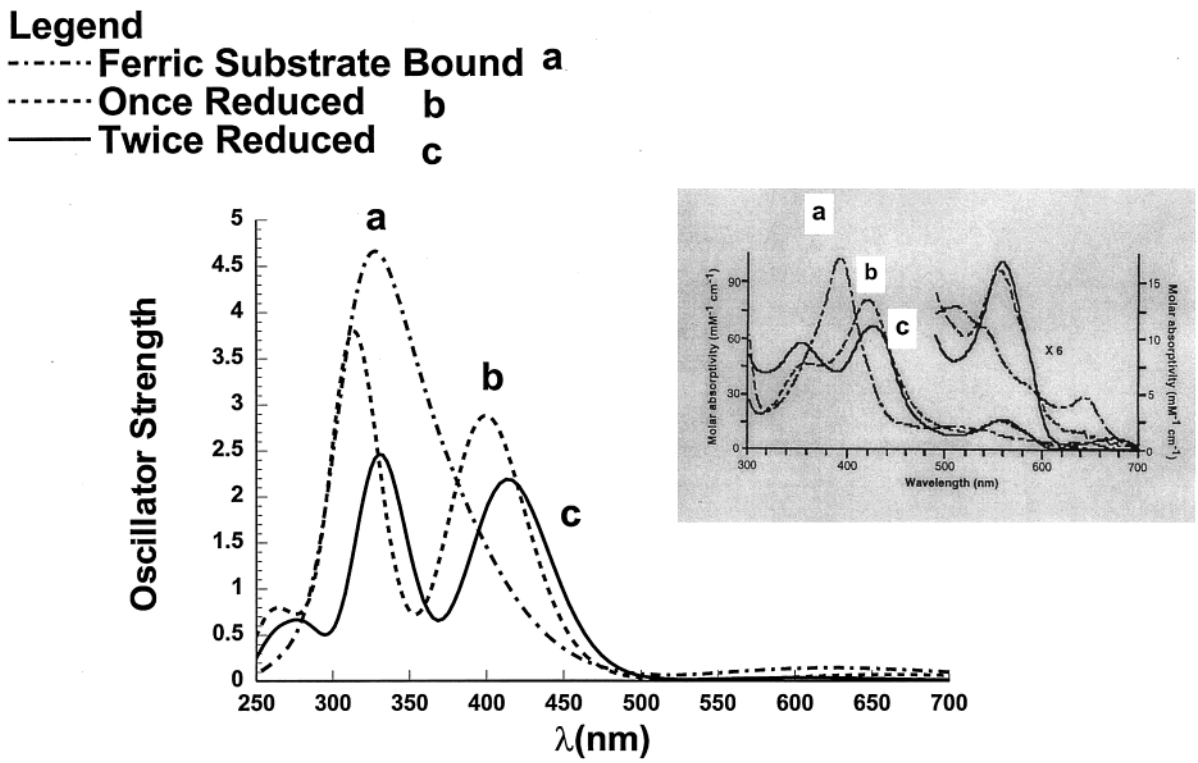

Figure 5. INDO/ROHF/CI calculated spectra of the ferrous dioxygen and reduced ferrous dioxygen heme sites of CYP450s juxtaposed with the calculated spectrum for the ferric high-spin substrate-bound CYP450 heme site. The insert in this figure gives the corresponding experimental spectra reported.[110]

spectrum of the substrate-bound ferric heme species was also calculated using the known X-ray structure of substrate-bound CYP450cam. The computed spectra of these species together with the reported experimental spectra are shown in Figure 5. As seen in this figure, the calculated spectrum of the ferric substrate-bound form has a single intense Soret band while those of both dioxygen species have a split Soret band. This splitting is due to mixing of the sulfur π orbitals with the porphyrin π orbitals modulated by the dioxygen ligands. Comparing the calculated spectra of the two ferrous dioxygen species, it is seen that, after reduction, the lower energy component of the split Soret band is shifted to the red by 30 nm. The experimental spectra attributed to these three species, shown in the insert of Figure 5, exhibit trends in spectra shifts very similar to the calculated spectrum of each species. This similarity between the calculated spectra of the two ferrous dioxygen species and the observed spectra attributed to these species provides additional independent evidence that the origin of these experimental spectra are indeed the ferrous dioxygen and transient reduced ferrous dioxygen intermediates of CYP450.

V. Investigation of a Proposed Pathway to Formation of Compound I from the Twice Reduced Dioxygen Species of CYP450s

The steps in the common enzymatic cycle of all P450s leading to the formation of compound **I** from the reduced ferrous dioxygen P450 species are the least understood aspects of P450 metabolism because they occur very rapidly and involve extremely transient intermediates. The proposed pathway shown in Figure 2 involving a proton-assisted mechanism of dioxygen bond cleavage has only been inferred from indirect experimental evidence. This evidence includes kinetic isotope effect measurement that has definitively demonstrated the requirement of two protons to form compound **I** from the reduced ferrous dioxygen species.[121] In addition, earlier studies using isotopically enriched molecular oxygen established that the oxygen that is transferred to substrates is the "proximal" one directly bound to the heme iron and that the other "distal" oxygen is involved in the concurrent formation of water.[122,123] Although consistent with these observations, it is difficult from experiment alone to directly assess the validity of the proposed pathway shown in Figure 2.

To address this question, recent studies were made to determine whether the intrinsic properties of the putative heme intermediates are consistent with their proposed role in compound **I** formation.[124] To this end, nonlocal density functional methods were used with extended basis sets to obtain the optimized geometries, relative energies, and electronic structures of each of the putative heme species in the pathway from the ferrous dioxygen form to compound **I**. The results are summarized in Figure 6. Shown in this figure are the optimized structures of each of these species with the calculated bond lengths and bond order indicated for the crucial Fe−O and O−O bonds.

As already discussed, the optimized geometry of the ferrous dioxygen form shown in this figure is in good agreement with the experimental structure of a model compound of this species and the calculated diamagnetic singlet ground state is consistent with

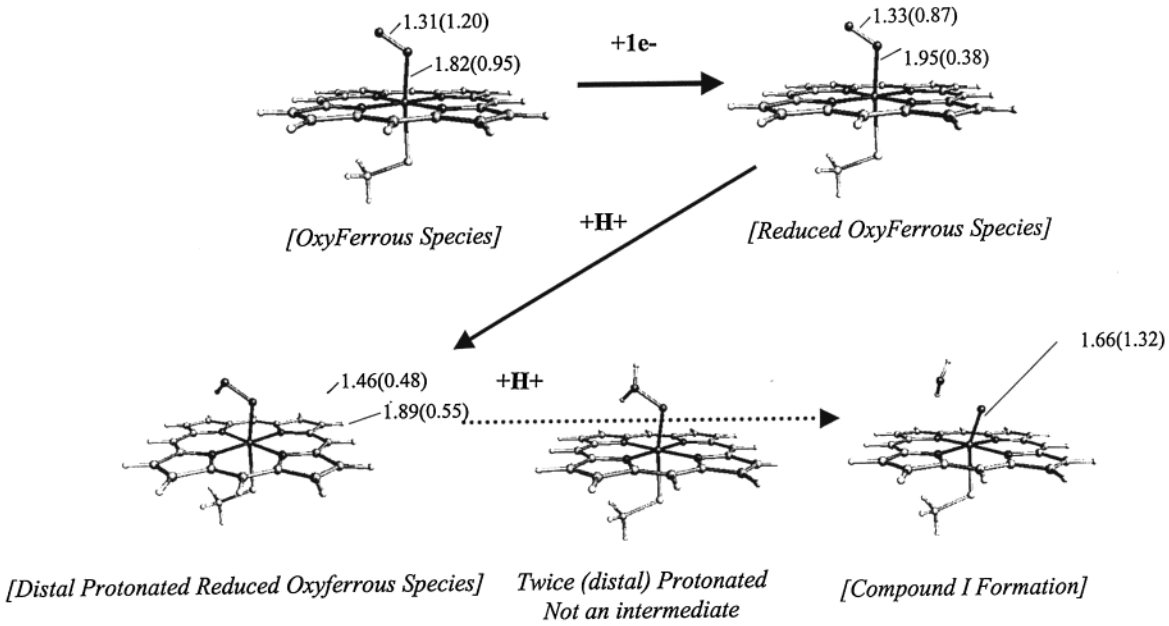

Figure 6. Optimized structures of each putative transient heme species in the pathway to formation of the catalytically active compound **I** explicitly indicating for each one Fe−O + O−O bond lengths and bond orders in parentheses. These results were obtained using nonlocal density functional theory methods with an DZVP basis set.

the reported lack of ESR spectrum.[106] Addition of an electron to the ferrous dioxygen form results in an energy-optimized species 37 kcal/mol higher in energy. This energy difference corresponds to the electron affinity of the ferrous heme species. It is not possible to calculate the energy of the total redox reaction that occurs, because the other half of the reaction, the ionization potential of the protein donating the electron, cannot be determined. The finding of an energy-optimized structure for the reduced oxyferrous species is further evidence that it is a viable intermediate, albeit highly reactive and therefore extremely transient. The main structural effect of second electron reduction is seen to be elongation and weakening of the Fe−O bond with no appreciable change in the O−O bond. Thus, second electron reduction alone is not sufficient to lead to facile dioxygen bond cleavage.

Distal protonation of the reduced ferrous dioxygen form does, however, lead to significant O−O bond weakening. As shown in Figure 6, this weakening is manifest in both an increase in O−O bond length from 1.33 to 1.46 Å and a decrease in bond order from 0.87 to 0.48. This significant O−O bond weakening upon even single protonation of the distal oxygen and the very large calculated proton affinity of −442.1 kcal/mol for the reduced oxyferrous species provide direct support for the proposed mechanism of proton-assisted dioxygen bond cleavage to formation of compound **I**. However, this protonated species corresponds to a stable minimum that does not spontaneously dissociate. Thus, neither the reduction step alone nor single protonation results in a species which spontaneously converts to the oxyferryl, compound **I**, species.

However, addition of a second proton to the distal oxygen does result in spontaneous dissociation to the compound **I** species. Specifically, no minimum was found corresponding to this twice protonated species. Rather, the addition of a second proton led directly and without any apparent energy barrier to the formation of compound **I** and water. The products, compound **I** and water, are much more stable than the reactants, the singly protonated distal species, and the second proton. These results, taken together, provide strong support for either consecutive or simultaneous double protonation of the distal oxygen atom of the reduced ferrous dioxygen CYP450 as a very favorable pathway to compound **I** formation.

VI. Electronic Structure of a P450 Compound I Heme Species

In this study, density functional theory has been used to characterize the electronic structure of the putative enzymatically active compound **I**. The heme model used included the complete protoporphyrin-IX with an oxygen atom and methyl mercaptide (SCH_3^-) as the axial ligands. The results were compared to previous DFT-based studies using a simple porphine model with different geometries, functions, and basis sets.[66−68] Full geometry optimization of the lowest quartet and doublet states led to the finding of an antiferromagnetic double ground state. In this state, there are two parallel unpaired spins on the Fe=O moiety and an antiparallel unpaired spin distributed between the $a_{2u}(\pi)$ orbital of the porphyrin ring and the π orbital of the ligand sulfur atom. The unpaired spin density on the sulfur ligand is the result of mixing of the a_{2u} porphyrin π orbitals and the S π orbitals in the highest occupied molecular orbital (HOMO). This same mixing was found in earlier studies in our laboratory[60] to be the origin of the split Soret that is the signature spectra of CYP450 enzymes. Previous calculations using the simple porphine model[66,67] also led to the finding of an antiferromagnetic doublet ground state with two parallel unpaired spins on the Fe=O moiety but with the third antiparallel radical center described as a sulfur radical. This result was obtained because the

HOMO in the ground state doublet is a S−Fe−O π orbital. These orbitals were also present in the studies made in our laboratory, but they are of lower energy, corresponding to HOMO-2 and HOMO-3. Differences in the energy ordering of the molecular orbitals are the consequence of different heme models and different geometries used for compound **I** and different levels of DFT theory and basis sets. The results reported here used the full protoporphyrin-IX heme model for compound **I** compared to the simple porphine used in prior studies. In addition, one of these studies[66] used an early very approximate Xα DFT method with a fixed geometry. In another,[67] a very long Fe−S bond of 2.69 Å was found on the optimized geometry, compared to 2.26 Å in the resting state X-ray structure of CYP450cam. Despite these differences, it is encouraging that the main characteristics calculated for the putative enzymatically active form common to the CYP450s are similar.

VII. Role of the Protein in Compound I Formation

While the heme unit is the primary center of both the spectroscopic properties and function of intact heme proteins, there is evidence for a key role of the protein in modulating both of these types of properties. The existence of X-ray structures of representatives of the major families of heme proteins, together with advances in computational capabilities, now allow the role of the protein in modulating structure, spectra, and function to be elucidated. An example of such modulation has already been described above. The anomalous low-spin ground state of the resting state of the CYP450s could only be explained by including the electrostatic field of the entire protein.[63] Another type of full protein studies related to the function of CYP450s is described below.

As just discussed, the most enigmatic part of the enzymatic cycle common to all CYP450s is the mechanism of formation of the putative compound **I** species after the second electron reduction of the substrate-bound ferrous dioxygen species. The DFT calculations described above provided support for a plausible mechanism involving the addition of two protons to the distal oxygen of the twice reduced ferrous dioxygen heme species, leading to facile proton-assisted cleavage of the O−O bond and formation of the Fe═O compound **I** species and water. This mechanism is consistent with current experimental evidence including isotope substitution kinetic data.[122,123]

Two important questions, however, remain that are best addressed by complimentary studies of the entire protein. These are the pathways by which the protons are delivered to the distal oxygen of the reduced ferrous dioxygen heme site in the intact protein and the ultimate source of these protons. In recent work,[125] molecular dynamic simulations have been performed for the reduced ferrous dioxygen species of substrate-bound CYP450eryF[69] using the recently determined X-ray structure to address these two aspects of the mechanism of formation of compound **I** from the reduced ferrous dioxygen species. The analysis of the molecular dynamics trajectories of this species revealed two dynamically stable hydrogen-bond networks to the distal oxygen atom of the dioxygen ligand, pointing to the substrate and a bound water as the nearby proton donors. The extensive, dynamical stable hydrogen-bond network identified led from the distal oxygen atom to the proximal proton donors and from there, via specific protein residues and bound waters, to the carboxylic oxygen of Glu360 in P450eryF. This Glu residue is in a channel accessible to solvent and could, hence, be the ultimate source of protons in CYP450eryF. To further explore this possibility, simulations were performed for the intact reduced ferrous dioxygen form of CYP450eryF with the Glu360 in both a protonated and unprotonated state. Complementary stable H-bonded networks were found between the immediate proton donors to the distal oxygen and the Glu residue in both of these forms. The combined results of these simulations provided further evidence for a proton-assisted pathway from the twice reduced dioxygen form of P450s to the catalytically active ferryl species and for a possible source of the protons.

Although these studies have clearly implicated a crucial role of the protein environment in the proton-assisted pathway to the catalytically active ferryl species, the specific manner in which the amino acids and bound water participate in compound **I** formation from the reduced dioxygen bound ligand should be isozyme dependent. This expected specificity is based on the low sequence homology among different families of CYP450 isozymes and the significant variability in the composition and architecture of the binding sites. For example, in P450cam a threonine residue in the structurally conserved I helix near the distal binding site is thought to be important in formation of compound **I**.[126−128] Moreover, mutation of this threonine to an alanine leads to a dysfunctional enzyme. However, by contrast, in the wild-type P450eryF, there is an alanine in this equivalent position and mutation to threonine leads to dysfunctional enzyme. Thus, neither the role of a specific amino acid nor the consequence of its mutation to another can be inferred for one CYP450 enzyme based on results from another isozyme.

Another confounding feature in the interpretation of the effect of site-specific mutations on the formation of compound **I** is the possibility of compensatory changes in the mutant protein conformation or position of bound waters that could alter the H-bonded network facilitating proton delivery. For example, in P450cam, in addition to the threonine residue an aspartate residue in the structurally conserved I helix is thought to be important in formation of compound **I**. Yet mutation of this residue to asparagine did not significantly effect the function of P450cam.[129] The authors offer as a possible explanation the compensatory utilization of bound waters in this mutant. Thus, the finding reported in this same paper, that the mutation of Glu366 in P450cam, corresponding to Glu360 in CYP450eryF, only moderately decreased the rate of NADH oxidation,[129] does not necessarily rule out a rule for Glu366 in P450eryf. It is possible that the intervening H-bonding network in the wild-type of the two isozymes would be significantly different and also there could be direct compensatory

effects of the mutation that alter the proton-donating pathway.

VIII. Conclusions

While the active site of most protein families is composed of a specific stereochemical arrangement of a few amino acids that are an integral part of the polypeptide chain, heme proteins belong to an unusual group of proteins in which the active site is a metal−organic complex, an iron−porphyrin "heme" unit that is a nearly independent entity with totally different properties than the rest of the protein. The electronic structure of this heme unit is well suited to perform the major functions of heme proteins and to facilitate the requisite changes that occur in this species during function. The heme unit also has unique spectroscopic properties that are sensitive indicators of the nature of the heme unit and changes in this species during function. Thus, while it is difficult to make small compounds that mimic the activity and spectroscopic properties of intact proteins in general, this is not the case for heme proteins and an enormous experimental effort has centered on the synthesis and characterization of model iron−porphyrin compounds and the extent to which they mimic the spectroscopic properties and function of intact heme proteins. In general, spectroscopic properties of the isolated heme complexes mimic those of the same heme species in the intact protein. It is much more difficult to mimic function with comparable efficiency.

Complementary computational studies focusing on the heme unit itself have elucidated the molecular origin of many observed properties. The fundamental origin of several of these properties were shown to be the presence of low-lying states of different spin multiplicities that in most inorganic iron-containing systems have large energy separations. In addition, these calculations identified a vital role of the porphyrin macrocycle in facilitating extensive and facile electron and spin delocalization favoring the unusual heme species, such as the ferryl form, involved in function. Electronic spectra calculations of putative transient species in the enzymatic cycle of the CYP450 enzymes provided the key link between observed spectra of these transient species and their molecular origin. Most recently, density functional calculations have allowed characterization of transient species and assessment of long-proposed mechanisms of activation of heme proteins to their enzymatically active form, providing crucial support for these species and pathways that could not be established by experiment alone. While all levels of theory over the past 30 years have made contributions to elucidation of structure, spectra, and function of heme species, advances in computational chemical methods and increasingly efficient software and hardware have allowed more and more reliable methods and realistic models to be used. It is expected that these improvements will continue and will further expand the scope, depth, and accuracy of computational studies of the structure, spectra, and function of heme sites.

In addition to continued studies of heme species themselves, the advent of known X-ray structures of heme proteins and the complementary development of computational methods that allow the characterization of full proteins have led to the use of these computational methods to probe the role of the protein environment in heme protein function. Although function centers on the heme unit, characterization of the heme unit associated with each type of heme function is not sufficient to explain differences in function. Results of full protein simulations have also shown that the pathway from inactive to enzymatically active species is greatly facilitated by key residues in the protein environment. The effect of mutation on function can only be addressed by such studies. A key advance still in progress is the combination of quantum mechanical and empirical energy methods that will allow simultaneous and enhanced insights into the role of both the heme site and the protein in function.

IX. Glossary of Abbreviations

INDO	intermediate neglect of differential overlap
ROHF	restricted open shell Hartree−Fock
DFT	density functional theory
CI	configuration interaction
SCF	self-consistent field
PPP	Parsier−Parr−Pople
CNDO/S	complete neglect of differential overlap method with spectroscopic parametrization
IEHT	iterative extended Hückel theory
Hb	Hemoglobin
MetMb	Metmyoglobin
ESR	electron spin resonance
CYP450	the cytochrome P450 superfamily

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