Cytochrome P450 is a vital enzyme in oxidative biotransformations, responsible for the detoxification of biological systems and for the synthesis of sex hormones. Recent experimental results demonstrate that, despite previous reports, the active species of the enzyme, compound I (Cpd I), is still undetected. The calculations project the unusual nature of this Cpd I that might be derived by mimetic systems, mutation studies, and X-ray crystal structures of P450 enzymes were taken into account as follows: a) Embedding of 2 in a polarizing medium of a low dielectric constant ($\varepsilon = 5.7$) serves to mimic the effect of polarization by the dipoles of the protein pocket near Cys 357 (using the numbering system in P450$_{cam}$). b) An internal NH...S hydrogen bond exists, but eludes detection.

Our benchmark system 2 (Scheme I) involves octamethyl porphyrin and an axial cysteinato ligand. From an electronic point of view, methyl substituents are good representations of the side chains in 1, while avoiding complications due to internal rotations of the long side chains. Noncovalent interactions revealed by mimic systems, mutation studies, and X-ray crystal structures of P450 enzymes were taken into account as follows: a) Embedding of 2 in a polarizing medium of a low dielectric constant ($\varepsilon = 5.7$) serves to mimic the effect of polarization by the dipoles of the protein pocket near Cys 357 (using the numbering system in P450$_{cam}$).

**KEYWORDS:**
- cytochromes
- density functional calculations
- hydrogen bonds
- porphyrins
- solvent effects

The Experimentally Elusive Oxidant of Cytochrome P450: A Theoretical “Trapping” Defining More Closely the “Real” Species

François Ogliaro, Samuél P. de Visser, Shimrit Cohen, Jose Kaneti, and Sason Shaik* [a]

---

Received: March 8, 2001
Revised version: July 9, 2001 [Z 240]
hydrogen bond of cysteinate was augmented by two external NH$_3$ molecules (Scheme 2), thus accounting for the three hydrogen bonds observed in the native enzyme (with Leu 358, Gly 359, and Glu 360).[3, 11] c) Interactions of amino acid residues with the porphyrin and the NH$_2$Cys group in the native enzyme[11] were internally accommodated by the cysteine itself (see below).

\[
\begin{align*}
\text{Fe} & \quad \text{O} \\
\text{S} & \quad \text{HN} \\
\text{CH}_2 & \quad \text{H} (i) \\
\text{CH}_3 & \quad \text{H} (e) -\text{NH}_2 \\
\text{H} (e) -\text{NH}_2 & \quad \text{H} (e) -\text{S} = 2.66 \, \text{Å} \\
\text{Fe} -\text{S} -\text{H} (e) & = 101.9 \\
\text{H} (e) -\text{S} -\text{H} (e) & = 104.0
\end{align*}
\]

Scheme 2. Model 2 of compound I, with internal (i) and external (e) NH ··· S hydrogen bonds. The external distances and angles were taken from ref. [8].

All calculations on 2 used the hybrid density functional U3LYP, coupled with the LACVP double-ζ quality basis set and the iterative solvent code in the JAGUAR package.[12] All geometries were fully optimized and double-checked with the Gaussian 98 package[13] that has a superior optimizer. Additional comparative calculations were carried out on 3, 4, and on 4 containing a pristine porphine. Both 3 and 4 were calculated also with polarized double- and triple-ζ basis sets (LACVP* and LACV3P*) in JAGUAR[12] and characterized by full frequency calculations. Only the key results will be discussed here.

The located conformation of 2 (2a – 2c) are shown in Figure 1. In 2a the ligand establishes NH···N$_{	ext{Por}}$ hydrogen bonds and CH····N$_{	ext{Por}}$ interactions with the porphyrin (Por). Due to the partial positive charge on the C-H hydrogen atoms, these latter interactions are of similar nature to NH····N$_{	ext{Por}}$ hydrogen bonds. In 2b, the cysteine couples with the heme by CH····N$_{	ext{Por}}$ contacts and with the side alkyl groups by C=O····H$_3$C interactions. In 2c, the cysteinato ligand is extended upright and its CH$_2$ group maintains CH····N$_{	ext{Por}}$ interactions. All the conformations possess internal NH····S and CO$_2$H····N$_{	ext{Cys}}$ hydrogen bonds within the cysteine. In the isolated molecular state, 2b is the most stable, whereas with medium polarization it is 2a. Screening of the protein database reveals that the cysteinato ligand in P450 species is invariably oriented in a folded conformation, akin to 2a. The NH$_{\text{Cys}}$ group in the native enzyme is coordinated by hydrogen bonding to a few residues (e.g., Phe 350, His 355), which exert an electron-withdrawing effect that contributes to stabilize the sulfur anion. The internal OH····N$_{\text{Cys}}$ hydrogen bond provides this feature. In the native enzyme, the heme nitrogen atoms are ligated by CH····N$_{\text{Por}}$ interactions (with residues 357 and 359) and NH····N$_{\text{Por}}$ hydrogen bonds (with residues 356 and 359). In 2a, the NH$_2$ and CH$_2$ groups of the cysteine interact with the porphyrin in an analogous manner to the native interactions.

Figure 2 shows the key geometric parameters and energy separation of the ferro- and antiferromagnetic states for 2a – c in the isolated state (ε = 1) and in a polarizing environment (ε = 5.7). The average Fe–N bond (2.013 – 2.018 Å) exhibits no sensitivity to the polarization and is not shown. It is seen that the Fe–O bond is marginally elongated by the polarization, while the protrusion of the iron above the ring ($\Delta_{\text{Fe-Por}}$) generally decreases. In contrast, the Fe–S bond length is very sensitive to all interactions. It is shortest for the upright conformation, 2c.
and gets elongated by hydrogen bonding to the porphyrin ring in 2a and 2b. The polarization shortens the Fe–S bond for all the conformations, and for the antiferromagnetic state of 2ab by as much as 0.132 Å. The two conformations of the simpler model 3ab exhibit very close features to 2a–c. Very similar ones are found for compound 4 containing porphine instead of octamethyl porphine (4(porphine)) and for 4. Thus, the polarizing environment unequivocally shortens the Fe–S bond. In contrast, the specific hydrogen bonding to porphyrin acts in opposition and lengthens the Fe–S bond, since this interaction disfavors the thiolate (S⁻) form.[8]

For 4(porphine), NH···S hydrogen bonding causes by itself a small shortening of ca. 0.022 and 0.013 Å, respectively, on the ferro- and antiferromagnetic states, in general accord with experimental data for mimetic complexes.[9] The choice of the basis set had an effect on the geometry of complexes (2.2 Å),[14] but shorter than that in the isolated molecule, increases with the polarization effect.[17] and this is expected to be further augmented by the NH···S hydrogen bonding. Thus, in accord with experimental observation, the polarity of the proximal pocket of the protein and its hydrogen bonding capability are needed to sustain the Fe–S bond.[10]

The considerable conformational freedom of the cysteinato ligand in 2 (Figure 1) and 3 indicates that the conformational choice of the native enzyme is a consequence of the structure of the cysteine helix in the proximal pocket and not of any strong inherent preference of the cysteinato moiety itself. All conformers share a common electronic structure with an antiferromagnetic (A) ground state, possessing three unpaired electrons, two in dπ(pz) and dπ(π*) orbitals of the Fe=O moiety and the third one in an orbital that has a mixed porphyrin/sulfur character. The antiferromagnetic state (A) is further stabilized by the polarization effect (Figure 2).

To appreciate the cooperativity of polarization and NH···S hydrogen bonding on the stability of the cysteinato group against oxidation,[10] we show in Figure 3A the group density distribution for the two extreme conformations 2a and 2c in the various environments. In the isolated molecule, the cysteinato ligand carries 62–68% of this unpaired spin density, that is, sulfur is almost fully oxidized (S⁰). Medium polarization reduces state (which is the Fe³⁺–water complex[15]) to the Fe₂⁺–water complex that typifies the Fe–S linkage of Cpd I, the net bond shortening elicited by the noncovalent interactions is associated with bond strengthening.[8] In 3 (3a, b), the bond energy, which is $D = 6.9$ (5.8, 6.3) kcal mol⁻¹ for the isolated molecule, increases with the polarization effect and this is expected to be further augmented by the NH···S hydrogen bonding. Thus, in accord with experimental observation, the polarity of the proximal pocket of the protein and its hydrogen bonding capability are needed to sustain the Fe–S bond.[10]

Figure 2. Key geometric parameters for model compounds 2a–c and 3a,b. The parameters are shown for the pair of ferromagnetic (A) and antiferromagnetic (A) states; in each line, the first data pair refers to the isolated molecule (e = 1), and the second pair in square brackets to the molecule in a polarizing environment (e = 5.7). At the bottom, energy differences (in cm⁻¹) for the ferromagnetic and antiferromagnetic states are shown (negative values indicate a more stable A).

Figure 3. Electronic structures of 2a and 2c. A: Group spin densities (data are given for a pair of ferromagnetic and antiferromagnetic states; the first pair refers to the isolated molecule (e = 1), the second (in square brackets) to the molecule in a polarizing environment (e = 5.7), and the third pair (in curly brackets) includes the effect of two external hydrogen bonds). B: The singly occupied dπ orbital. C: Averaged atomic spin densities on porphyrin positions for the antiferromagnetic state of 2a.
this spin density to 42–58%. Hydrogen bonding exhibits two effects. The minor one is caused by the hydrogen bonding to the porphyrin, as in 2a, while the major effect is due to the hydrogen bonding to the cysteine as in 2c. The hydrogen bond in 2a drives spin density away from the ring toward sulfur. The NH···S bonds in 2c exert the more dramatic effect. These interactions shift the spin density to 56–69% on the porphyrin moiety, thereby restoring the S\textsuperscript{2} nature of the ligand. Thus, under conditions which mimic critical interactions in the protein pocket, Cpd I has an antiferromagnetic ground state and an unpaired electron located more heavily on the porphyrin ring, but has also a significant spin density on sulfur, as predicted first by Moesbauer spectroscopy\cite{15}. Figure 3B shows the Kohn–Sham orbital\cite{16} that contains this unpaired electron, and it is apparent that this orbital is an antibonding combination of the porphyrin a\textsubscript{1u} and the α hybrid of the sulfur ligand. In this respect, the antiferro- and ferromagnetic pair of states of Cpd I of P450 is unequivocally \( {^{1}A_{1u}^{0}} \), a la Groves’ original “green” Cpd I model.\cite{15} However, the significant sulfur contribution, its variability and sensitivity to the NH···N\textsubscript{por} and NH···S hydrogen bonds, certainly marks this as unique among the known Cpd I species.

2a–c have C\textsubscript{2v} symmetry where no restriction exists on orbital mixing.\cite{15,16} Thus, while the singly occupied orbital in Figure 3B conserves its local a\textsubscript{1u} character in the UB3LYP calculation, a mixed-orbital character manifests itself in lower lying orbitals of the porphyrin. Figure 3C shows the spin density distribution only on the porphyrin moiety and for the antiferromagnetic state of 2a (the same applies to the ferromagnetic state whose spin density is polarized, too). Thus, even though the large spin densities on the nitrogen atoms and the C\textsubscript{m} densities on the sulfur atoms and the C\textsubscript{2a} in MHz are: \( C_{m} = -12.90; C_{m} = +5.53; C_{g} = +0.06; N = -5.85 \) might be taken as an underground \( {^{1}A_{1u}^{0}} \) character. This contention is supported by recent INDO/S/Cl calculations on an extensive model of compound 1.\cite{24} An experimental probe that focuses on the cation radical situation of the porphyrin may yield information as though the species has a mixed a\textsubscript{1u}–a\textsubscript{1u} character. This perhaps explains the resonance Raman assignment of CP0(I) as a “gray” a\textsubscript{1u} state.\cite{25} However, the proper a\textsubscript{1u} states, with an unpaired electron located in an a\textsubscript{1u} orbital, were computed\cite{10} to be ca. 21 kcal mol\textsuperscript{-1} higher in energy than the a\textsubscript{1u} states.

The discussion of the interplay of the NH···S and NH···N\textsubscript{por} hydrogen bonding and the Fe–S bond flexibility projects the unusual nature of Cpd I, whose molecular features are shaped by the host environment. This implies, in turn, that Cpd I of different cysteinate enzymes should exhibit somewhat different characteristics, and presumably also different reactivities, depending on the structure of the protein pocket and its specific side chains. However, in all environments the ground state will be an antiferromagnetic \( {^{3}A_{1u}^{0}} \) type.

This research was sponsored in part by the Israel Science Foundation (ISF) and the Binational German Israeli Foundation (GIF). F.O. thanks the EU for a Marie Curie Fellowship.

References:

17. It is not possible to calculate the actual Fe–S bond energy in solution, since the solvent calculation is not size-consistent due to the employed grid surface method.\cite{24} In ref. [8], it was assumed that the fragments (thiolo radical and iron–oxo porphyrin) are only weakly solvated relative to Cpd I, such that the Fe–S bond energy is given by the gas-phase bond energy plus the total solvation energy of Cpd I (\( D_{excp} = -28 \) kcal mol\textsuperscript{-1}). Strictly speaking, however, the bond energy would be augmented by the difference in solvation energies of Cpd I and its fragments (i.e. \( D_{excp} < -28 \) kcal mol\textsuperscript{-1}).

Received: May 11, 2001 [Z 242]