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

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

Cytochrome P450 is a vital enzyme in oxidative biotransformations, responsible for the detoxification of biological systems and for the synthesis of sex hormones.[1] Recent experimental results[2] demonstrate that, despite previous reports,[3, 4] the active species of the enzyme, compound I (Cpd I, **1**; Scheme 1), might have never been detected since it does not seem to accumulate during the catalytic cycle even at low temperature. To date, the only characterized Cpd I of a cysteinate enzyme belongs to chloroperoxidase (CPO).[5] However, here too the geometry of the species is unknown, and the precise identity of the ground state is still debated.[5, 6] Thus, a key species of one of the most important enzymes of biological systems is known to exist, but eludes detection.

We present here theoretical calculations of the so far most extensive and most realistic Cpd I model,[7, 8] with an account of the interaction types exerted by the apoprotein environment. We assign the ground state of Cpd I (**1**) as $^2A_{2u}$, thereby settling previous theoretical disagreements and hopefully contributing toward an eventual resolution of the experimental controversy. The calculations project the unusual nature of this Cpd I that behaves as a chameleon species by adopting its electronic and geometric features to the protein environment to which it has to accomodate.

Our benchmark system **2** (Scheme 1) 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 mimetic systems,[9] mutation studies,[10] and X-ray crystal structures of P450 enzymes[3, 11] 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$_{cam}$).[11] b) An internal NH $\cdots$ S

[a] Prof. Dr. S. Shaik, Dr. F. Ogliaro, Dr. S. P. de Visser, S. Cohen, Dr. J. Kaneti
Department of Organic Chemistry
and
Lise Meitner-Minerva Center for Computational Quantum Chemistry The Hebrew University
Givat Ram Campus, 91904 Jerusalem (Israel)
Fax: (+ 972) 2-6584680
E-mail: sason@yfaat.ch.huji.ac.il
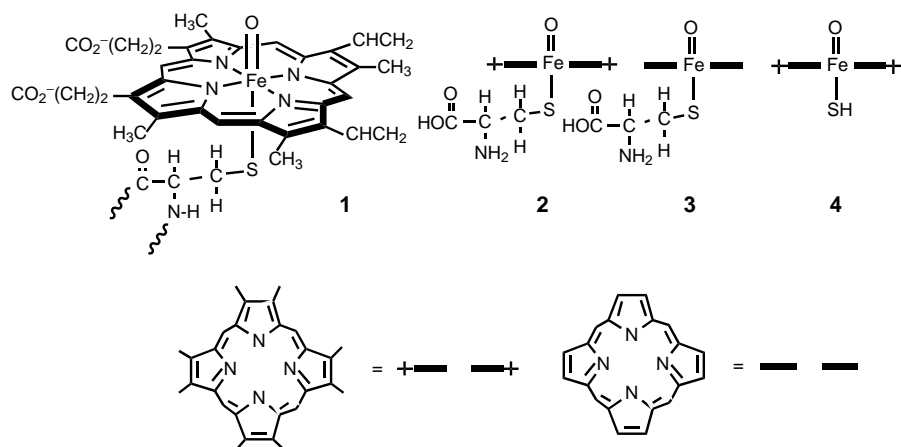
Scheme 1. *The native compound I (1) and its models (2 – 4) studied herein.*

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_{Cys} group in the native enzyme[11] were internally accommodated by the cysteine itself (see below).

$$H^{(e)}\text{-S} = 2.66 \text{ Å}$$
$$\text{Fe-S-}H^{(e)} = 101.9°$$
$$H^{(e)}\text{-S-}H^{(e)} = 104.0°$$
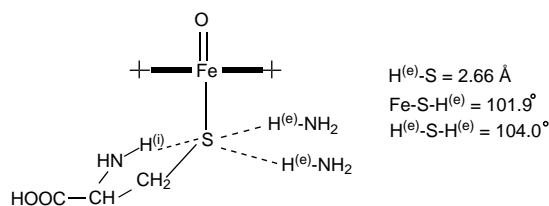
Scheme 2. *Model 2 of compound I, with internal (i) and external (e) NH···S hydrogen bonds. The external distances and angles were taken from ref. [8].*

All calculations on **2** used the hybrid density functional UB3LYP, 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 conformations for **2** (**2 a** – **2 c**) are shown in Figure 1. In **2 a** the ligand establishes NH···N_{Por} hydrogen bonds and CH···N_{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_{Por} hydrogen bonds. In **2 b**, the cysteine couples with the heme by CH···N_{Por} contacts and with the side alkyl groups by C=O···H_3C interactions. In **2 c**, the cysteinato ligand is extended upright and its CH_2 group maintains CH···N_{Por} interactions. All the conformations possess internal NH···S and CO_2H···N_{Cys} hydrogen bonds within the cysteine. In the isolated molecular state, **2 b** is the most stable, whereas with medium polarization it is **2 a**. Screening of the protein database reveals that the cysteinato ligand in P450 species is invariably oriented in a folded conformation, akin to **2 a**. The NH_{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_{Cys} hydrogen bond provides this feature. In the native enzyme, the heme nitrogen atoms are ligated by CH···N_{Por} interactions (with residues 357 and 359) and NH···N_{Por} hydrogen bonds (with residues 356 and 359). In **2 a**, 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 **2 a – c** in the isolated state ($\varepsilon = 1$) and in a polarizing environment ($\varepsilon = 5.7$). 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 ($\Delta_{Fe\text{-}Por}$) generally decreases. In contrast, the Fe-S bond length is very sensitive to all interactions. It is shortest for the upright conformation, **2 c**,

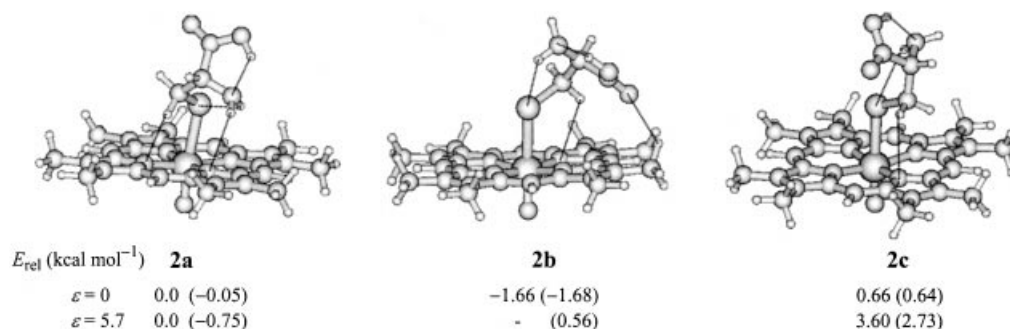| E_{rel} (kcal mol^{-1})	**2a**	**2b**	**2c**
$\varepsilon = 0$	0.0 (−0.05)	−1.66 (−1.68)	0.66 (0.64)
$\varepsilon = 5.7$	0.0 (−0.75)	- (0.56)	3.60 (2.73)

Figure 1. *Conformations of 2 and their relative energies in kcal mol^{-1} (values for ferromagnetic states are given without parentheses, for antiferromagnetic states in parentheses). Dotted lines indicate the various interactions discussed in the text.*
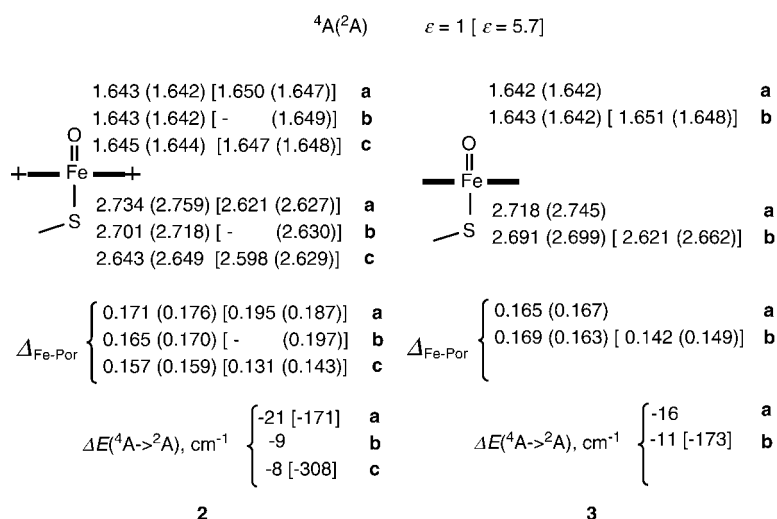
$^4A(^2A)$ $\varepsilon = 1\ [\ \varepsilon = 5.7]$

1.643 (1.642) [1.650 (1.647)]		a
1.643 (1.642) [- (1.649)]		b
1.645 (1.644) [1.647 (1.648)]		c

O‖Fe (+—Fe—+, |, S)

2.734 (2.759) [2.621 (2.627)]		a
2.701 (2.718) [- (2.630)]		b
2.643 (2.649 [2.598 (2.629)]		c

$\Delta_{\text{Fe-Por}}$
0.171 (0.176) [0.195 (0.187)]	a
0.165 (0.170) [- (0.197)]	b
0.157 (0.159) [0.131 (0.143)]	c

$\Delta E(^4A\text{-}>^2A),\ cm^{-1}$
-21 [-171]	a
-9	b
-8 [-308]	c

2

$^4A(^2A)$

1.642 (1.642)		a
1.643 (1.642) [1.651 (1.648)]		b

O‖Fe (—Fe—, |, S)

2.718 (2.745)		a
2.691 (2.699) [2.621 (2.662)]		b

$\Delta_{\text{Fe-Por}}$
0.165 (0.167)	a
0.169 (0.163) [0.142 (0.149)]	b

$\Delta E(^4A\text{-}>^2A),\ cm^{-1}$
-16	a
-11 [-173]	b

3

Figure 2. Key geometric parameters for model compounds 2a–c and 3a, b. The parameters are shown for the pair of ferromagnetic (^4A) and antiferromagnetic (^2A) states; in each line, the first data pair refers to the isolated molecule ($\varepsilon = 1$), and the second pair in square brackets to the molecule in a polarizing environment ($\varepsilon = 5.7$). At the bottom, energy differences (in cm^{-1}) for the ferromagnetic and antiferromagnetic states are shown (negative values indicate a more stable ^2A).

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 **2a** by as much as 0.132 Å. The two conformations of the simpler model **3a, b** exhibit very close features to **2a–c**. Very similar ones are found for compound **4** containing porphine instead of octamethyl porphine (**4**(porphine))[8] 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 **3**: mproving the basis set to a polarized triple-ζ quality caused a small Fe—S shortening (of 0.03 and 0.034 Å for the ferro- and antiferromagentic states, respectively). Assuming additivity of all these effects leads to an estimate of the Fe—S bond length, such that for the ferromagnetic (antiferromagnetic) states of the three conformations of **2**, $r_{\text{Fe-S}}$ ranges between 2.569 (2.580) Å for **2a** to 2.546 (2.582) for **2c**. Thus, the Fe—S bond is predicted to be significantly longer than in Fe^{III}-S complexes (2.2 Å),[14] but shorter than that in the isolated molecular state. In accord with these findings, the computed IR frequency of the Fe—S bond in the isolated molecular state of **3** is 241 cm^{-1}, substantially lower than our calculated value of 347 cm^{-1} for the resting

state (which is the Fe^{III} – water complex[1]). Due to the redox mesomerism[1, 14–16] 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 **4** (**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,[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 (^2A) ground state, possessing three unpaired electrons, two in $d_{xz}(\pi^*)$ and $d_{yz}(\pi^*)$ orbitals of the Fe=O moiety and the third one in an orbital that has a mixed porphyrin/sulfur character. The antiferromagnetic state (^2A) 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 (S⁺). Medium polarization reduces
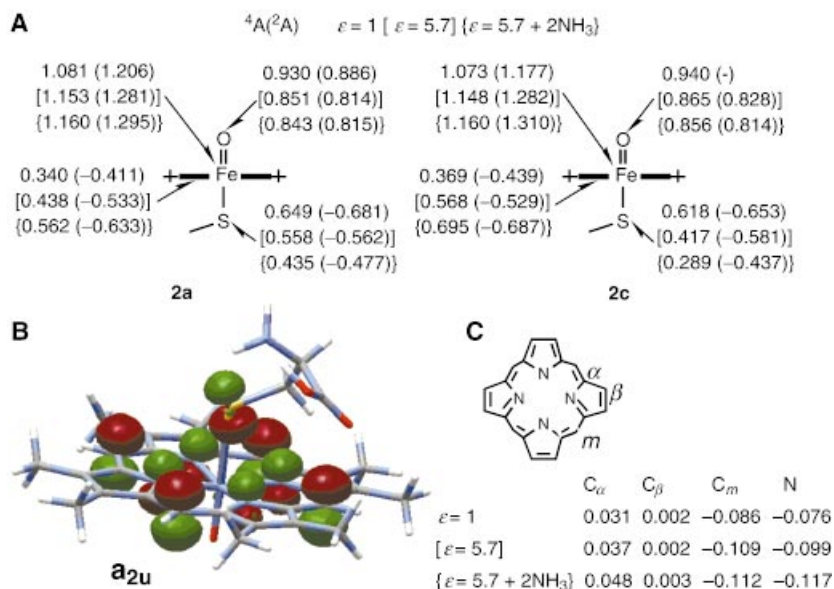
A $^4A(^2A)$ $\varepsilon = 1\ [\ \varepsilon = 5.7]\ \{\varepsilon = 5.7 + 2NH_3\}$

2a

O: 1.081 (1.206) [1.153 (1.281)] {1.160 (1.295)}   0.930 (0.886) [0.851 (0.814)] {0.843 (0.815)}

Fe: 0.340 (−0.411) [0.438 (−0.533)] {0.562 (−0.633)}   S: 0.649 (−0.681) [0.558 (−0.562)] {0.435 (−0.477)}

2c

O: 1.073 (1.177) [1.148 (1.282)] {1.160 (1.310)}   0.940 (-) [0.865 (0.828)] {0.856 (0.814)}

Fe: 0.369 (−0.439) [0.568 (−0.529)] {0.695 (−0.687)}   S: 0.618 (−0.653) [0.417 (−0.581)] {0.289 (−0.437)}

B

a_{2u}

C

	C_α	C_β	C_m	N
$\varepsilon = 1$	0.031	0.002	−0.086	−0.076
$[\varepsilon = 5.7]$	0.037	0.002	−0.109	−0.099
$\{\varepsilon = 5.7 + 2NH_3\}$	0.048	0.003	−0.112	−0.117

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 ($\varepsilon = 1$), the second (in square brackets) to the molecule in a polarizing environment ($\varepsilon = 5.7$), and the third pair (in curly brackets) includes the effect of two external hydrogen bonds). B: The singly occupied a_{2u} 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:⁻ nature of the ligand. Thus, under conditions which mimic critical interactions in the protein pocket, Cpd I has an antiferromagentic 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 Moessbauer spectroscopy.[15] Figure 3B shows the Kohn–Sham orbital[16] that contains this unpaired electron, and it is apparent that this orbital is an antibonding combination of the porphyrin a_{2u} and the σ hybrid of the sulfur ligand. In this respect, the antiferro- and ferromagnetic pair of states of Cpd I of P450 is unequivocally $^{2,4}A_{2u}$, à la Groves' original "green" Cpd I model.[1] However, the significant sulfur contribution, its variability and sensitivity to the NH···N_{Por} and NH···S hydrogen bonds, certainly marks this as unique among the known Cpd I species.

2a–c have C_1 symmetry where no restriction exists on orbital mixing.[15, 16] Thus, while the singly occupied orbital in Figure 3B conserves its local a_{2u} 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_m positions mark a predominant $^2A_{2u}$ nature of the ground state, the significant contributions on C_α (hyperfine coupling constants for the bare **2a** in MHz are: $C_m = -12.90$; $C_\alpha = +5.53$; $C_\beta = +0.06$; N = -5.85) might be taken as an underground "A_{1u}" character. This contention is supported by recent INDO/S/CI calculations on an extensive model of compound I.[7b] An experimental probe that focuses on the cation radical situation of the porphyrin may yield information as though the species has a mixed A_{2u}–A_{1u} character. This perhaps explains the resonance Raman assignment of CPO(I) as a "gray" A_{1u} state.[6, 15] However, the proper A_{1u} states, with an unpaired electron located in an a_{1u} orbital, were computed[18] to be ca. 21 kcal mol⁻¹ higher in energy than the A_{2u} states.

Our discussion of the interplay of the NH···S and NH···N_{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 $^2A_{2u}$ type.

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