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How do metal ions modulate the rate-determining electron transfer step in Cytochrome P450 reactions?

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Abstract: Cytochrome P450 (CYP450) enzymes have important functions related to human health and their reaction rate is dependent on the first electron transfer from the reduction partner. Interestingly, experimental work showed that this step is highly affected by the addition of metal ions. To understand the effect of external perturbations on the CYP450 first reduction step, we performed a computational study with model complexes in the presence of metal and organic ions, solvent molecules and an electric field effect. The work shows that these medium-range interactions affect the driving force as well as electron transfer rates dramatically. Based on the location, distance and direction of the ions/electric field the reaction rate values are improved or impaired for catalysis. Calculations on a large crystal structure with alkali metal ions bound implicated inhibition patterns of the ions; therefore, we predict that active forms of the natural CYP450 isozymes will not have more than one alkali metal ions bound in the second-coordination sphere. As such the work gives insight into the activity of CYP450 enzymes and the effect of ions and electric field perturbations on its activity.

Introduction

Cytochrome P450 (CYP450) mono-oxygenases play a major role in the metabolism of a wide variety of endogenous molecules, xenobiotics and drugs in humans.^[1–3] These enzymes are heme containing enzymes, whereby the central iron atom is linked to the protein through a proximal cysteinate bond from the axial ligand, while the distal site is the oxygen and substrate binding site.^[4,5] Understanding of the CYP450 catalytic cycle and the identity and role of reactive intermediates in the rate determining step has increased tremendously over the last two decades through a combination of computational and experimental efforts.^{[1,4,5],[8,9]} Structure and ligand based models (classical and deep learning) predict sites of metabolism and potential isoforms with accuracies converging to 85 – 90%.^[10,11]

Most of the work in the literature focused on the catalytic role of the iron(IV)-oxo heme cation radical species (Compound I, **Cpd I**)^[6,12,13] and ignored the fact that for the majority of the CYP450s the rate-determining step in the catalytic cycle is the first electron transfer from the CYP450-reductase (CPR) that reduces the pentacoordinated iron(III)-heme (**2**, Scheme. 1).^[6] MD simulations have given insights into the dynamical interactions between CYP450 and CPR, its redox partner.^[14–16] A close

approach between the CPR Flavin mononucleotide (FMN) and CYP450 heme (8.8 Å) triggers the first electron transfer.^[14]

Scheme. 1. The CYP450 catalytic cycle. The rate determining reduction of the substrate bound oxidized ferric state to the reduced ferrous state is highlighted in red color.

Often the second-coordination sphere in heme and non-heme iron enzymes plays a crucial role in determining selectivity and activities of enzymes through either substrate and oxidant positioning or long-range electrostatic perturbations.^[17] As the substrate-binding pocket in CYP450 isozymes often is tight, a straightforward Hammett analysis with analogous substrates is challenging. In particular, the active site interactions and dynamics may alter the binding affinity and/or reorientation of a substrate, thus modulating the site that is oxidized by **Cpd I**. This argument is often invoked to explain the experimentally observed product ratios, which cannot be accounted for by calculated C–H activation barriers only. Nevertheless, it has been established in many cases that amino acids far from the active site do influence the substrate-binding, and selectivity in CYP450 reactions.^[18] The resting state redox potential (E°) and spin state ratios are controlled by a combination of ligand (and/or water) binding^[19] and

the local electrostatic environment around the heme iron center.^[6] Allosteric regulation of monomeric proteins and CYP101 activity has been reviewed in the recent past.^[20] The role of auxiliary ligands and transition metal ions in the CYP450 activation and inhibition has also been studied. Considering the tight regulation of intracellular alkali (Na^+ , K^+), alkaline-earth (Ca^{2+} , Mg^{2+}) and

transition metal ion (Cu^{2+} , Zn^{2+}) concentrations, and their physiological, pathophysiological roles, the effect of these ions on the CYP450 rate-determining step cannot be ignored. Monovalent metal ions, namely Na^+ and K^+ , have been shown to activate CYP450s,^[21] induce conformational changes and increase the α -helix content in CYP1A2.^[22]

Figure 1. Extracts of the CYP101 PDB files 1DZ4 and 3L63 with heme, axial cysteinate and K^+ binding sites highlighted.

The presence of Na^+ (at concentrations of 80 nM) and K^+ at physiological concentrations (100 – 150 mM) increases the high spin content and the ligand affinity for CYP1A2^[22] and CYP101.^[20] Yun et al. have also reported an increase in rat CYP2B1 activities and shift towards high-spin spectral characteristics upon addition of Na^+ (0 – 100 mM) and K^+ (0 – 20 mM).^[23] The CYP2B1 activities (both reconstituted and cumene peroxide supported) decreased upon further increases in the monovalent ion concentrations. The authors ascribed these observations mostly to the modulation of CYP450 structure (increase in the helical content) and CPR-CYP450 interactions.

Divalent alkaline-earth metal ions, such as Mg^{2+} and Ca^{2+} , on the contrary were found to reversibly inhibit camphor binding, decrease the high-spin content and induce conversion of P450 to P420 UV-visible spectral signatures in CYP101.^[24] Many CYP101 structures, i.e. P450_{cam}: see for instance the protein databank (PDB)^[25] structures (e.g. 1DZ4, 3L63 and for others see Table S1) contain K^+ ions bound near the active site, see Figure 1. In the majority of these structures the K^+ ion interacts with a surface cysteinate residue (Cys₈₅) and the backbone of Tyr₉₆, which forms a backbone H-bonding interaction with the camphor carbonyl. The heme iron to K^+ distances are about 18 Å. An additional variable site is often observed near the N-terminus of the protein. A double mutant (4L4G: L358P/K178G, Table S1) contains an additional K^+ ion in the active site bound to the backbone of Gly₁₇₈ (I helix) at a distance of 12.9 Å from the heme iron. Although allosteric interactions have been invoked, the mechanisms for activity and spectral characteristic changes induced by the divalent cations remain unclear and cannot be explained solely based on the long-distance interactions.^[24]

The effect of divalent transition metal ions on the CYP1A2 and CYP3A4 activities has been studied^[26] and it was found that only Cu^{2+} ions induce conformational changes in the protein and inhibit the electron transfer from the CPR to CYP450 and consequently the product formation. However, the mechanisms of Cu^{2+}

mediated CYP1A2 and CYP3A4 inhibition remains unresolved.^[26,27] The lack of CYP450 inhibition by Mg^{2+} and Ca^{2+} ions reported by Yun et al.^[26] and Kim^[28] is in agreement with the observations of Manna and Mazumdar^[24] that the P450 to P420 conversion induced by these ions is reversed upon the addition of K^+ ions (100 mM). Investigations of the role of K^+ ions in controlling the structure and reactivity of redox active heme sites in peroxidases shows similar effect although the cations were found to be located at a smaller (13.5 Å) distance from the heme.^[29] Mutations of the K^+ binding sites induces a bis-His coordination, loss of high-spin spectral signatures and reduced peroxidase activity. Interestingly, K^+ binding site in heme peroxidases is proximal in contrast to the distal allosteric site in CYP101. However, the cation binding site in cytochrome c peroxidase was shown to affect the electronic ground state of Cpd I and gives it a protein-based radical (on an axial Trp residue) rather than a heme radical as typically seen in peroxidases and P450 Cpd I.^[30–33]

The studies discussed above show that ionic strengths have significant influence on the activities of CYP450s. With this background in mind, our manuscript addresses the following questions: i) what is the influence of water coordination and hydronium binding on the resting state heme redox potential (E°), its driving force (ΔE°), electron transfer barriers (E_{act}) and rates ($\log K_e$)? ii) How are these factors influenced by the physiological alkali, alkaline-earth and transition metal ions (and by bridging waters)? iii) Is there a difference in the stabilization, ΔE° , E_{act} and $\log K_e$ values between these metal ions? iv) Can Marcus theory predict the activation or inhibition of CYP450 electron transfer steps observed with different metals? v) How does the metal-heme distance influence these parameters? vi) What is influence of the proximal heme ligand on these predictions? Can an external electric field (EEF) and point charges model the effect of metal ions?

Figure 2. DFT cluster models investigated in this work: D is a distal bound (coordinated) ligand, while M^+ is a noncovalent bound cation.

Results and Discussion

Although the majority of the CYP450 substrates/inhibitors tend to be hydrophobic, the ligand recognition and positioning is a result of complex interplay between ionic and non-polar interactions within and at the periphery of the CYP450 active site. These interactions are mostly with residues from the G, F, F' and I helices,^[34] but we anticipate that long-range electrostatic interactions could affect the E° and hence the catalytic ability of the CYP450. Considering the active site volumes, sizes of the entry and exit channels,^[35,36] and charged amino acid residues at the active site roof and periphery, it is possible that monovalent and divalent ions in their (partially) hydrated forms will get within interacting distance to the heme iron center. We will not go into detail of the CYP450 structure and the dynamical nature of water-heme interaction; however, a literature discussion is given in the Supporting Information. Past and recent results on water residence times (picoseconds) in Na^+ and K^+ first hydrations shells suggest the possibility that these ions can possibly interact with heme-porphyrin nitrogen atoms.^[37,38] Such interactions can potentially stabilize the reduced (negatively charged) ferrous quintet spin states, thus modulating the redox potential and rate of CPR-CYP450 electron transfers.

To gain insight into the long-range electrostatic interactions on the heme reduction potential of CYP450 enzymes, we set up density functional theory (DFT) cluster models, see Figure 2, and investigated the E° and how it is affected by external perturbations. Firstly, we investigated two minimal heme models designated **A1** and **A2**, which represent either $[\text{Fe}^{\text{III}}(\text{Por})\text{SMe}]$ or $[\text{Fe}^{\text{III}}(\text{Por})\text{SH}]$ with Por = porphyrin and methylmercaptate or thiolate as axial ligand. Secondly, a large CYP450 CAM active site structure was investigated that includes the heme with propionate groups replaced by hydrogen atoms, substrate camphor and several protein residues in the distal and axial sites. In addition, model **B** has two K^+ binding sites and four water molecules and is based on the crystal structure coordinates of the 4L4G PDB file.^[39] It includes the heme with propionate groups replaced by hydrogen atoms, an axial ligand loop of three amino acids, the substrate (camphor) and the protein residues within 5 Å of the substrate. In addition, two K^+ ions and their coordinated ligands and water molecules are added. The **A1** and **A2** models were optimized for the pentacoordinated heme complexes in the oxidized (**2**) and reduced (**3**) states with overall multiplicity sextet for structure **2** and quintet for structure **3**. Other spin states were also tested for model **A1** and **A2** but found to be higher in energy, namely $^2\mathbf{2A}_1$ and $^4\mathbf{2A}_1$ are 1.9 and 1.4 kcal mol⁻¹ higher in energy than $^6\mathbf{2A}_1$, whereas $^1\mathbf{3A}_1$ and $^3\mathbf{3A}_1$ were 17.4 and 9.5 kcal mol⁻¹ above the $^5\mathbf{3A}_1$ ground state, respectively. Our calculated spin-state orderings and ground spin assignments are in line with previous

calculations using similar cluster model complexes or QM/MM calculations as well as experimental electron paramagnetic resonance studies.^{[6,40,41][42][43][9,44]}

Although the absolute CYP450 redox potentials (E° , -0.4 to -0.3 V) are known to be isoform specific^[45] and dependent on the active site interactions,^[19] the relative change in free energy and redox potentials (ΔE° with reference to the redox partner, CPR)

are more relevant (since this appears in the Marcus equation, as ΔG°). Therefore, the focus of this work will be mostly on model **A1** and how external perturbations on the bare system affects the E° of structure **2**. Furthermore, the differences in the redox potentials (ΔE°) w.r.t. CPR are then used to estimate the activation barriers (E_{act})^[46] and log rates ($\log K_e$)^[47] for the first electron transfer step (**2** $\rightarrow$ **3**).

Table 1. Stabilization of heme (A1 model) redox states (2 and 3) with coordinating (C) or hydrogen-bonded (HB) water molecules, hydronium, ammonium, ethylenediamine, trimethylammonium, methylammonium or methylguanidinium ions.^d

Distal group	N ^e	HB/C ^f	Stabilization energy ^g		Relative stabilization of reduced state (3)
			2, (Oxidized)	3, (Reduced)	
H ₂ O	1	HB	6.37	5.52	-0.85
	1 ^a	C	8.72	8.26	-0.46
	2	HB	13.09	11.59	-1.50
	3	HB	19.45	17.02	-2.43
	4	HB	20.59	19.73	-0.86
H ₃ O ⁺	1 ^b	HB	-25.71	-54.80	-29.08
	2 ^b	HB	-31.59	-87.70	-56.10
H ₃ O ⁺ --H ₂ O ^b		HB	-15.61	-50.32	-34.71
(NH ₄ ⁺) ^c	1	HB	0.35	-18.67	-19.02
	2	HB	21.78	-11.49	-33.26
(NH ₃ Me ⁺) ^c	1	HB	4.90	-15.78	-20.68
(NHMe ₃ ⁺) ^c	1	HB	8.65	-5.35	-14.00
Methylguanidinium ^c	1	HB	8.29	-7.66	-15.95

^a The coordinating water binds with **2** and **3** in low spin state (doublet and singlet respectively).

^b The proton gets transferred to the porphyrin N atoms during optimization as reported by others.

^c The protons formed H-bonds with Heme (distance 1.7 Å) and remained bounded to the ammonium group (H₃N-H distance = 1.08 Å).

^d Free energies at 298 K with entropic, solvent and zero-point corrections included. Values in kcal mol⁻¹.

^e Number of molecules or ions in the model.

^f HB = H-bonding, C = heme coordinating.

^g The stabilization energies were calculated using the equations:

- 1) **2** + distal group $\rightarrow$ **2**-distal group complex; stabilization energies upon distal group binding to **2** ΔG_1°
- 2) **3** + distal group $\rightarrow$ **3**-distal group complex; stabilization energies upon distal group binding to **3** ΔG_2°
- 3) Relative stabilization of **3** = $\Delta G_2^\circ - \Delta G_1^\circ$

The minimal models **A1** and **A2** are calculated to have redox potentials of 1.99 and 1.82 V in the gas-phase, which decreases to 0.59 V and 0.46 V when a polarized continuum model ($\epsilon = 4.24$) is added.

Effect of water, and hydronium ion binding on heme redox states, ΔE° , E_{act} and $\log K_e$ for the reaction from **2** $\rightarrow$ **3**

Next, we added small molecules to the distal position and recalculated the E° . **Table 1** shows heme model redox state (**2** and **3**) binding free energies for the addition of water, ammonium and selected organic ions to the distal position. H-bonding (HB) interactions of water molecules with the redox states (**2** and **3**) increase the endothermicity of the reaction but the relative stabilization of **3** increases with up to three water molecules bound, while addition of a fourth water molecule does not increasingly stabilize **3**. This suggests that the number of water molecules surrounding the heme redox states have little influence on the electron transfer. In the heme-iron coordinating (C) geometry, water stabilizes the reduced (**3**) singlet spin state by only 0.5 kcal mol⁻¹. Similar behavior is seen with other well-known Type II inhibitors known to coordinate with heme-iron, such as pyridine and imidazole.^[48,49]

Hydronium ions, by contrast, have strikingly opposite influence and significantly stabilize **3** (29 and 56 kcal mol⁻¹ for 1 and 2 hydronium ions respectively). However, their effect is not innocent as during the geometry optimization a proton transfer

takes place from H₃O⁺ to the porphyrin nitrogen atoms. The Fe-S distance decreases significantly from 2.44 to 2.38 Å (Figure S7) upon hydronium binding. H-bonding to the ammonium ion (an important intracellular ion) stabilizes **3** via H-bonding (19 and 33.3 kcal mol⁻¹ for 1 and 2 ions respectively). A calculation was also attempted with three ammonium ions included in the model; however, due to large repulsive forces between the positive charges this calculation failed to converge. The stabilization due to trimethylammonium, methylammonium, and methylguanidinium ions was studied to model the influence of lysine, and arginine residues that are often present within CYP450 active sites. These ions stabilize state **3** by 14, 21, and 16 kcal mol⁻¹ respectively hence leading to a larger driving force for the electron transfer reaction and thereby decrease the redox potential. **Table 2** shows the free energies for the redox reaction (**2** $\rightarrow$ **3**), estimated redox potential (E°), and the driving force for the electron-transfer reaction between the heme model and CPR (ΔE°), estimated electron transfer log rates ($\log K_e$) and barriers (E_{act}). The ΔE° , E_{act} and $\log K_e$ for the free heme model are 0.665 V, 15.4 kcal mol⁻¹ and -1.2 respectively. Coordination of a water molecule to heme iron (known to stabilize low spin states)^[6] indeed raises E° to 1.25 V, E_{act} to 32.8 kcal mol⁻¹ and decreases $\log K_e$ to -13.4. This is in agreement with the fact that the heme resting state does not usually undergo reduction in the absence of a substrate ligand. When water is in an H-bonding geometry (via porphyrin N-atoms) the unbound values change only

FULL PAPER

marginally (entry 3, 4, 5, and 6 in Table 2). H-bonding with additional water molecules lowers the barrier by 1.3 and 2.2 kcal mol⁻¹ for the addition of two and three water molecules, respectively, while a fourth water molecule raises it back to 14.6 kcal mol⁻¹. Nonetheless, log K_e remains lower and becomes a small positive number only with the addition of three water molecules.

Since proton exchange and shuttling are important events in the CYP450 catalytic cycle,^[6,50] we investigated the effect of hydronium ion binding on the predicted ΔE° , E_{act} and log K_e values.

The hydronium ions reduce the E° and the driving force (ΔE°) significantly for one and two hydronium ion bound states: -0.596 and -1.768 V, respectively. The influence on the E_{act} and log K_e predicted using the Marcus equation are interesting and worth notice. Stabilization by one hydronium ion lowers the E_{act} to almost zero and thus the predicted log K_e is higher than for the unligated heme. When the hydronium ion is H-bonded to an additional water molecule (Table 2, entry 9) the ΔE° increases 0.2 V but E_{act} decreases, nevertheless the log K_e is small suggesting that possibility of electron transfer is low.

Table 2. Free energies for the single electron transfer step (2 → 3 in Scheme. 1), estimated redox potentials (E°), and redox potential difference (ΔE°) between the CPR and ligand bound Heme (Flavin $\Delta E^\circ = -0.006$ V). For Type II ligands energies for the most stable Heme states where calculated (doublet oxidized and singlet reduced states) for estimating E° values. Type II ligands are known to inhibit the CYP450 catalytic cycle.

Entry	Ligands bound to Heme (model A1)	ΔG	E° (V) 2 → 3	ΔE° (V)	log K _e	E_{act} 2 → 3
1	Unbound A1	88.58	0.60	0.67	-1.2	15.4
2	H ₂ O-A1*	73.54	1.25	1.32	-13.4	32.8
3	H ₂ O-A1 ^a	89.43	0.56	0.63	-0.6	14.6
4	(H ₂ O) ₂ -A1 ^a	90.08	0.53	0.60	-0.2	14.0
5	(H ₂ O) ₃ -A1 ^a	91.02	0.49	0.56	0.5	13.2
6	(H ₂ O) ₄ -A1	88.98	0.56	0.63	-0.6	14.6
7	(H ₃ O ⁺)-A1 ^a	117.67	-0.66	-0.60	-0.1	0.2
8	(H ₃ O ⁺) ₂ -A1 ^a	144.69	-1.83	-1.77	-23.8	8.0
9	(H ₂ O)(H ₃ O ⁺)-A1 ^a	123.29	-0.91	-0.84	-4.1	0.1
10	Li ⁺ -A1	110.84	-0.37	-0.30	4.1	1.6
11	Li ⁺ (H ₂ O)-A1	110.42	-0.35	-0.28	4.3	1.7
12	Li ⁺ (H ₂ O) ₂ -A1 ^a	107.94	-0.24	-0.17	5.6	2.5
13	Li ⁺ (H ₂ O) ₂ -A1 ^b	106.18	-0.16	-0.10	6.5	3.3
14	Li ⁺ (H ₂ O) ₃ -A1 ^b	105.57	-0.14	-0.07	6.8	3.5
15	(Li ⁺) ₂ -A1	129.12	-1.16	-1.09	-8.8	0.9
16	(Li ⁺) ₂ (H ₂ O)-A1	127.31	-1.08	-1.02	-7.3	0.5
17	(Li ⁺) ₃ (H ₂ O)-A1	145.25	-1.86	-1.79	-24.4	8.3
18	Na ⁺ -A1	107.62	-0.23	-0.16	5.8	2.7
19	Na ⁺ (H ₂ O)-A1 ^a	107.66	-0.23	-0.16	5.8	2.7
20	Na ⁺ (H ₂ O) ₂ -A1 ^a	106.16	-0.16	-0.10	6.5	3.3
21	Na ⁺ (H ₂ O) ₂ -A1 ^b	103.85	-0.06	0.00	7.6	4.4
22	Na ⁺ (H ₂ O) ₃ -A1 ^b	104.06	-0.07	-0.01	7.5	4.3
23	(Na ⁺) ₂ -A1	122.08	-0.85	-0.79	-3.2	0.0
24	(Na ⁺) ₂ (H ₂ O)-A1	122.64	-0.88	-0.81	-3.6	0.0
25	K ⁺ -A1	106.41	-0.17	-0.11	6.4	3.2
26	K ⁺ (H ₂ O)-A1 ^a	106.81	-0.19	-0.13	6.2	3.0
27	K ⁺ (H ₂ O) ₂ -A1 ^a	105.02	-0.11	-0.05	7.1	3.8
28	K ⁺ (H ₂ O) ₃ -A1 ^b	103.06	-0.03	0.04	7.2	4.8
29	(K ⁺) ₂ -A1	115.10	-0.55	-0.49	1.5	0.5
30	(K ⁺) ₂ (H ₂ O)-A1	119.18	-0.73	-0.66	-1.1	0.1
31	Cu ²⁺ -A1	156.63	-2.35	-2.29	-37.9	18.1
32	Cu ²⁺ (H ₂ O)-A1	153.00	-2.19	-2.13	-33.4	14.6
33	Cu ²⁺ (H ₂ O) ₂ -A1	146.61	-1.92	-1.85	-25.9	9.3
34	Ca ²⁺ -A1	129.72	-1.19	-1.12	-9.3	1.0
35	Ca ²⁺ (H ₂ O)-A1	128.66	-1.14	-1.07	-8.4	0.8
36	Mg ²⁺ -A1	158.71	-2.44	-2.38	-40.6	20.3
37	Mg ²⁺ (H ₂ O)-A1	137.72	-1.53	-1.47	-16.6	3.9
38	Zn ²⁺ -A1	119.05	-0.72	-0.66	-1.1	0.1
39	Zn ²⁺ (H ₂ O)-A1	107.32	-0.21	-0.15	6.0	2.8
40	Zn ²⁺ (H ₂ O) ₂ -A1	117.92	-0.67	-0.61	-0.3	0.2
41	NH ₄ ⁺ -A1	107.60	-0.23	-0.16	5.8	2.7
42	(NH ₄ ⁺) ₂ -A1	121.85	-0.84	-0.78	-3.0	0.0
43	(EtNH ₃ ⁺) ₂ -A1	123.95	-0.94	-0.87	-4.6	0.1
44	(MeNH ₃ ⁺)-A1	109.26	-0.30	-0.23	4.9	2.1
45	(GuaniMe ⁺)-A1	104.53	-0.09	-0.03	7.3	4.0
46	(MeH)-A1	88.79	0.59	0.66	-1.0	15.2
47	(PhH)-A1	88.97	0.58	0.65	-0.9	15.0
48	(NMe ₃)-A1	89.31	0.57	0.63	-0.7	14.7
49	(NHMe ₃ ⁺)-A1	102.58	-0.01	0.06	7.0	5.0
50	(NHMe ₃ ⁺) _{inv} -A1	104.92	-0.11	-0.04	7.1	3.8
51	Imd-A1*	71.19	1.35	1.42	-15.6	36.2
52	Pyridine-A1*	72.44	1.30	1.37	-14.4	34.4
53	(Cu ²⁺) ₂ (H ₂ O)-A1	200.86	-4.27	-4.20	-109.4	91.7

On the contrary, two hydronium ions increase the driving force far too much, thus entering into the inverted Marcus region where the E_{act} for electron transfer is higher (at 8 kcal mol⁻¹) and log K_e (-23.83) significantly lower. This suggests that (over)stabilization of the reduced state (**3**) equivalent to the perturbation by two positively charged ions/species has the potential to inhibit the rate-determining first electron transfer. As such a delicate charge-balance and pH will be needed in the protein. Indeed, most of the CYP450s are known to operate in a small pH window of 7.0 to 7.4.^[51] The analysis of these parameters for the N containing ions follows a similar pattern, thus implying that interaction with an ammonium ion, lysine or arginine residue side chains can potentially activate the electron transfer step while interaction with 2 such residues can inhibit the CYP450s catalytic cycle. In some pathological conditions e.g. hyperammonemia, high ammonia levels have been implicated in the modulation of activities of many CYP450 (mouse CYP3A4, CYP2E1 increased, CYP2A decreased and CYP4B1 unaffected).^[52] E_{act} and log K_e values for ammonium and N-containing ions (Table 2) suggest possible interactions with the heme center in CYP450 active sites especially for isoforms with larger distribution of negatively charged residues (e.g. CYP2 family and CYP101). This can potentially explain the mechanisms of modulation of CYP450 activities in such pathological conditions, and experimental studies should be conducted for further exploration and potential applications. As seen in Table 2, binding of methane (MeH), benzene (PhH) and trimethylamine which represent typical sites of CYP450 metabolic reactions do not significantly alter these parameters for the unligated heme. For the protonated trimethylammonium and methylammonium ions the corresponding ΔE° , E_{act} and log K_e imply a significantly lower barrier and higher electron transfer rates and potential for CYP450 activation.

Effect of alkali metal binding on heme redox states, ΔE° , E_{act} and log K_e for **2** → **3**

Next, the influence of alkali metal ions on the redox potential (E°) and electron transfer rates was investigated since many crystal structures (see Table S1) and the observations reported in the literature (see above) suggest that these ions might get access into the CYP450 active sites. Especially structures with polar amino acid residues such as the isozymes CYP101 and CYP2D6 appear to undergo changes in reactivity. Thus, heme model redox states were optimized in the presence of one and two alkali (Li^+ , Na^+ , K^+), alkaline-earth (Mg^{2+} , Ca^{2+}) and with 0 – 3 water molecules bound to the metal ion(s). All monovalent ions (M) interacted with porphyrin nitrogen atoms in the reduced state (**3**) and showed M—N distances of 2.17, 2.52, 2.98 Å, for Li^+ , Na^+ , and K^+ respectively suggesting progressively weaker binding. Direct metal-heme-N interaction reduces the Fe-S distance from 2.45 to 2.38, 2.39, 2.42 Å for Li^+ , Na^+ and K^+ , respectively, see Figure S5 – S8. With two monovalent ions bonded to the heme, Li^+ showed the strongest interactions with the porphyrin nitrogen atoms: Li—N and Li—Li (2.12, 2.95 Å), Na—N and Na—Na (2.52, 3.35 Å) and K—N and K—K (3.09, 4.19 Å, see Figure S1, Supporting Information). This suggests that interaction of the metal ions with the heme becomes increasingly electrostatic with an increase in the ionic radius. These observations and explanation are in agreement with general chemical intuition and literature reports of an increase in the electrostatic contribution to binding energy in alkali metal and nucleophile complexes with an

increase in corresponding M—N distance.^[53] The two metal ions align with a line joining the two C—H groups connecting the heme pyrrole rings (Figure S1). Coordination of these metals with a water molecule further increased the M—N distances (Figure S7–S10). Attempts to optimize three metal ion bound structures, expectedly, failed due to very large repulsive interactions between the positively charged ions. A bridging alkali metal-coordinating water molecule stabilized the $(\text{Li}^+)_3$ system but was unable to stabilize the excess positive charges on 3Na^+ and 3K^+ bound systems.

Table 3 shows the relative stabilization of heme oxidized (**2**) and reduced (**3**) states due to alkali metal ion binding. The oxidized (**2**) state is stabilized by one metal ion binding but destabilized by two metal ions. The reduced quintet state (**3**) is consistently more stabilized than **2** with the extent of stabilization decreasing with the number of bound ions. This suggests that binding with more than one alkali metal ion is less favorable especially for Li^+ and K^+ ions. Inclusion of alkali metal coordinating waters has a small influence on the relative stabilization of the reduced state, **3** (1 – 3 kcal mol⁻¹).

The nature of the electrostatic interactions (especially related to the addition of Na^+ and K^+) bound systems prompted us to investigate the stabilization of **3** in configurations where alkali metal-ions interact with the heme only via bridging water molecules. Such configurations seem more realistic considering the presence of amino acids with hydrophobic side chains near the heme center in the active sites of some CYP450 isoforms. Loss of direct contact decreases the relative stabilization of the reduced state (**3**) marginally (< 2, 0.5 kcal mol⁻¹ for Na^+ , and K^+ respectively). For Li^+ ions, due to its stronger interaction with heme, loss of direct contact decreases the stabilization energy by ~5 kcal mol⁻¹.

Table 3. Heme (model A1) in the oxidized (**2**) and reduced (**3**) state stabilization due to alkali, alkaline-earth and transition metal ion binding.

	M ^a	Heme redox states (Model A1)		Relative stabilization
		2	3	
Lithium	Li ⁺	-0.33	-22.58	-22.25
	Li ⁺ (H ₂ O)	0.71	-21.13	-21.84
	Li ⁺ (H ₂ O) ₂	2.08	-17.27	-19.35
	(Li ⁺) ₂	21.00	-19.54	-40.53
	(Li ⁺) ₂ (H ₂ O)	17.93	-20.80	-38.73
	(Li ⁺) ₃ (H ₂ O)	63.31	06.65	-55.66
	Li ⁺ (H ₂ O) ₂ (Li not directly bound to Heme)	0.28	-17.31	-17.59
	Li ⁺ (H ₂ O) ₃ (Li not directly bound to Heme)	-3.72	-20.70	-16.98
	Na ⁺	-0.42	-19.45	-19.04
	Na ⁺ (H ₂ O)	1.31	-17.76	-19.07
	Na ⁺ (H ₂ O) ₂	5.18	-12.40	-17.58
	(Na ⁺) ₂	19.18	-14.32	-33.50
	(Na ⁺) ₂ (H ₂ O)	19.86	-14.20	-34.06
Sodium	Na ⁺ (H ₂ O) ₂ (Na not directly bound to Iron- Por-SMe)	4.48	-10.78	-15.27
	Na ⁺ (H ₂ O) ₃ (Na not directly bound to Iron- Por-SMe)	8.66	-06.82	-15.48
	K ⁺	2.02	-15.81	-17.83
Potassium	K ⁺ (H ₂ O)	5.12	-13.11	-18.23
	K ⁺ (H ₂ O) ₂	8.65	-07.78	-16.43

	(K ⁺) ₂	17.34	-09.17	-26.52
	(K ⁺) ₂ (H ₂ O)	23.27	-07.32	-30.60
	K ⁺ (H ₂ O) ₂ (K not directly bound to Heme)	8.74	-7.45	-16.19
	K ⁺ (H ₂ O) ₃ (K not directly bonded to Heme)	13.61	-00.86	-14.47
Calcium	Ca ²⁺	-08.24	-49.38	-41.14
	Ca ²⁺ (H ₂ O)	-12.01	-52.09	-40.08
Magnesium	Mg ²⁺	-31.60	-101.73	-70.13
	Mg ²⁺ (H ₂ O)	-43.45	-92.59	-49.13
	Cu ²⁺	-152.29	-220.34	-68.05
	Cu ²⁺ (H ₂ O)	-167.92	-232.34	-64.41
Copper	Cu ²⁺ (H ₂ O) ₂ (Cu not directly bonded to Heme)	-165.39	-223.42	-58.03
	Zn ²⁺	-39.03	-69.50	-30.47
	Zn ²⁺ (H ₂ O)	-46.51	-65.25	-18.74
Zinc	Zn ²⁺ (H ₂ O) ₂ (Zn not directly bonded to Heme)	-39.78	-69.12	-29.34

^a Metal ion and number coordinating water (coordination with alkali metals).

All the alkali metal ions reduce the unbound heme redox potential (E° : Li⁺; -0.366, -1.16, Na⁺; -0.227, -0.854, and K⁺; -0.174, -0.55 V for 1 and 2 ions, respectively) in accordance with their electropositive character and ionic radii. The electron transfer barriers (E_{act} , **2** → **3**) are reduced considerably by these monovalent ion binding to heme redox states (Li⁺: 1.5 – 0.54, Na⁺: 2.7 – 0.03 and K⁺: 3.2 – 0.06 kcal mol⁻¹). There is an increase in the predicted electron transfer rates (log K_e) with one metal ion binding (Li⁺: 4.1 – 5.6, Na⁺: 5.8 – 7.5, K⁺: 6.4 – 1.5), while two metal ions takes the heme into inverted Marcus region and are predicted to decrease log K_e for Li⁺ and Na⁺ ions (-8.8 to -7.9 and -3.2 to -3.6). The effect of two K⁺ ions is comparatively smaller. These predictions are in agreement with the experimental observations that 0 – 100 mM Na⁺ and K⁺ increase the high/low spin ratios (spectral signatures), electron transfer from CPR to CYP450 and the rate of CYP450 catalyzed oxidative reactions. However, higher concentrations have been reported to inhibit CYP450 spectral spin state changes and activities.^[23]

Coordination of water with these alkali metal ions (while maintaining direct alkali metal-heme N interactions) has little influence on ΔE° and log K_e . When the alkali metal ions are only coordinated with water molecules, remarkably these values remain similar (Table 2 rows with b-superscript). The Na-Fe distance in the two and three water coordinated structures is 4.82 Å. For two and three water coordinated K⁺ ion complexes the K-Fe distances are 4.23 and 5.38 Å (Figure S7 – S10). This suggests that the stabilizing influence of the alkali-metal ions is not dependent on direct contact with the heme. Thus, charge transfer interactions with the alkali-metal ions can take place via bridging waters (and possibly through space and protein backbone/side chain atoms) in CYP450 active sites.

Effect of alkaline-earth metal ion binding on heme redox states, ΔE° , E_{act} and log K_e for **2** → **3**

The divalent alkaline-earth metal ions have a larger relative stabilizing influence (Ca²⁺: 41.1 and Mg²⁺: 70.1 kcal mol⁻¹) on the heme reduced state (**3**) mostly due to two positive charges (Table 3). Inclusion of a coordinating water molecule has only a marginal influence on relative stabilization for Ca²⁺ bound complexes, whereas for Mg²⁺ ions the oxidized (**2**) state is stabilized

additionally thus reducing the relative stabilization of **3** by ~20 kcal mol⁻¹. Bare Mg²⁺ ion displaces the iron from the porphyrin ring during geometry optimization, while water coordination reduces Mg-N interactions. Mg²⁺ ions have the largest effect on heme redox potential (E° = -2.44 V) and the driving force (ΔE° = -2.38 V), thus giving the lowest predicted electron transfer rates (log K_e = -40.6). For Ca²⁺ ion and water coordinated Ca²⁺ and Mg²⁺ states, although the predicted E_{act} are smaller than in the unligated heme model, our predictions for log K_e suggest that these also inhibit the rate-determining electron transfer from CPR to CYP450. These predictions are in agreement with the experimental observations that alkaline-earth metal ions inhibit CYP450 metabolic reactions and give the characteristic spectrum of CYP420 species.^[24]

Effect of transition metal ions (Cu²⁺ and Zn²⁺) on heme redox states, ΔE° , E_{act} and log K_e for **2** → **3**

Since transition metals are known to inhibit electron transfer from CPR to CYP450, their interactions with the heme model **A1** were studied. Cu²⁺ and Zn²⁺ ions stabilize both the oxidized (**2**) and reduced (**3**) heme states, with a net stabilization for **3** (Table 3). Similar to the alkali and alkaline-earth metals, coordination with water leads to a decrease in the net stabilization of **3**. This effect is small for Cu²⁺ (4.4 kcal mol⁻¹), but larger for Zn²⁺ ions (~12 kcal mol⁻¹). Our predictions are in correspondence with the known covalent character of the Zn²⁺ ions and water interactions.^[54] Thus, Cu²⁺ ions are predicted to lead to a decrease in the ΔE° , comparable to Mg²⁺ (Table 2). Coordination with one water has a small effect. Furthermore, E_{act} and log K_e estimates suggest significant inhibition of the CPR to CYP450 electron transfer and the catalytic cycle in agreement with the literature. Zn²⁺ ions have a smaller impact, equivalent to 2 monovalent alkali cations and thus E_{act} and log K_e predictions imply inhibition of the CYP450 catalytic cycle. Water coordination raises the ΔE° , E_{act} and makes the log K_e positive. Therefore, we predict that Zn²⁺ ions are less likely to inhibit CYP450 spectral changes and the catalytic cycle. These predictions are in agreement with the observations that Zn²⁺ ion induced inhibition of CYP3A4 activities is not related to the rate-determining step but interactions with cytochrome b₅ (a CYP450 reductant).^[17]

Figure 3 displays a scatter plot of the driving force (ΔE°) for the model **A1** perturbed systems discussed (Table 2) versus log K_e and E_{act} . Both curves show a nicely quadratic correlation between ΔE° and the electron transfer activation energy (E_{act}), which is not surprising as both E_{act} and log K_e (Eqs 2 and 3, Methods section) are quadratically connected to ΔE° . Moreover, the plot shows that dramatic changes in electron transfer rate constant (log K_e) can be expected due to minor external perturbations, such as the addition of solvent molecules or alkali ions in the second-coordination sphere of the heme. The results predicted using Marcus theory shows the inverted region where activation barriers for electron transfer are higher in spite of larger driving force and thus the electron transfer rates (log K_e) are smaller.

Figure 3. Scatter plot of the driving force (ΔE°) vs E_{act} (in amber) and electron transfer rates ($\log K_e$, in green). The light blue region shows the range of ΔE° where electron transfer rates are expected to be positive and thus lead to CYP450 activation, whereas ligand/factors that move the ΔE° outside this range (light red/pink) are expected to inhibit CYP450 reactions. Experimental driving force (ΔE°) values for CYP3A4 and CYP450 BM3 are shown for comparison.

Nanodisc based experimental studies on modulation of CYP3A4 redox potentials (E°) by ligand binding have shown a typical range of -0.22 to -0.13 V.^[19] Similar E° values were reported with the use of gold-sputtered electrodes for testosterone binding to CYP3A4.^[55] For the bacterial CYP450 BM3 bound to sodium dodecyl sulfate (SDS) films adsorbed on graphite electrodes a E° value of -0.33 V has been reported.^[56] These equate to ΔE° values that ranges between -0.264 to -0.096 V (w.r.t. CPR) and are similar to our predictions for the influence of a single monovalent metal ion binding on the driving force for the electron transfer (i.e. ΔE°). Further the Dutton's model (Equation 3) gives positive electron transfer rates ($\log K_e$) values for these ΔE° values which are similar to those for CYP450 BM3.^[14] Thus our predictions of activation of CYP450 by alkali metal ions via increased electron transfer ($2 \rightarrow 3$) are in agreement with the experimental data.

To further test the effect of the protein we calculated a large active site model (**B**) of a substrate bound CYP450 camphor (CAM) structure and evaluated the E° . A single point calculation on the crystal structure coordinates gives E° of 1.57 V, while a full geometry optimization reduces it slightly to 1.40 V. These values are very high and well higher than the experimentally obtained E° of the wildtype systems. Moreover, the E° is well larger than that found for the small model complexes (**A1** and **A2**) reported above. A similar but smaller increase in the heme E° is observed when a single K^+ ion was kept at an increasing distance from the heme center (see next subsection and Table S3). Thus two K^+ ions at similar (12 Å) distance might amplify this effect, thus entering into the other Marcus inverted region (right side of **Figure 3**). Thus a E° of 1.57 V for two K^+ bound state would imply an inhibitory effect on the catalytic cycle and make the enzyme lesser active. Clearly, two K^+ ions in the second-coordination sphere in the model lead to major perturbations through long-range/medium-range electrostatic interactions that perturb the E° of the pentacoordinated heme and make the protein lesser active. In particular, the effect of these two K^+ ions is similar to that of inhibition of the enzyme. Consequently, the calculations predict

that the crystal structure represented by the 4L4G PDB file is an inactive form of the enzyme, where the two potassium ions bound in the second-coordination sphere perturb the chemical properties of the heme and hamper the electron transfer. It may; therefore, be that these ions were added to enhance the crystal growth of these substrate-bound complexes and assisted with the experimental efforts to generate structures. However, our modelling studies show that care should be taken with using PDB structures for computational modelling of enzyme reaction mechanisms as excess of bound ions might have a negative effect on catalysis and actually reduce the activity of the protein.

Relative independence of metal ion induced CYP450 activation or inhibition on heme to metal distance

As shown in the sections above, coordination with water and lack of direct metal-heme contacts has a small influence on the relative stabilization of the reduced (**3**) high spin state, change in the redox potential (ΔE°) and electron transfer rate ($\log K_e$) and barriers (E_{act}) predicted using Marcus theory. Thus, we investigated the dependence of these parameters on the metal-heme distance. For this one metal ion-heme (Li^+ , K^+ , and Cu^{2+}) binding energies for the oxidized (**2**) and reduced (**3**) states were calculated at an increasing metal-heme iron distance (incremented each time by 2 Å, see Figure S2 and Figure S3). The heme porphyrin ring structure was optimized while the metal-heme-iron distance was frozen. Table S2 shows that with increasing metal-heme distance the relative stabilization for the reduced (**3**) high spin state decreases. For Li^+ and K^+ ions the decrease is almost logarithmic suggesting that the alkali metal ions will retain the stabilizing influence up to 20 Å. The transition Cu^{2+} metal ion shows a complex relationship between metal-heme iron distance and the stabilization of **3**. Nevertheless, the Cu^{2+} ion retain significant stabilizing influence on the reduced (**3**) state up to 12.4 Å studied here.

Figure S4 shows the influence of metal ion-heme iron distance on the predicted electron transfer rate ($\log K_e$), barrier (E_{act}) and driving force (ΔE°). For Li^+ and K^+ ion binding, the E_{act} and ΔE° increase with increasing Metal-heme iron distance, while $\log K_e$ decreases. For Cu^{2+} ion binding, the $\log K_e$ and ΔE° remain lower, while the E_{act} remains positive. This indicates that alkali metals would retain activating influence on the rate determining step of the CYP450 catalytic cycle, while the transition metal (Cu^{2+}) ions would have inhibitory influence even at a distance > 12 Å.

Effect of point charge on the heme redox states, ΔE° , E_{act} and $\log K_e$ for $2 \rightarrow 3$

To investigate if the influence of metal ions on the heme redox properties can be explained by electrostatic interactions, Li^+ , K^+ and Cu^{2+} ions were replaced with corresponding point charges (PC) with a magnitude of $Q = +1e$ for Li^+ and K^+ , while a value of $Q = +2e$ was used for the system with Cu^{2+} ions. Single point energy calculations were performed at the same level of theory and the effect of point charges on the $\log K_e$ and E_{act} were calculated at various distances from the heme iron ion and compared with the effect of metal ions.

Figure 4 shows the estimated $\log K_e$ and E_{act} for point charge and metal ions bound CYP450 heme model **A1**.

Figure 4. Comparison of effect of point charge and monovalent metal ion on the CYP450 heme redox electron transfer rates ($\log K_e$) and barriers (E_{act}) for the rate-determining step.

As can be seen from Figure 4, point charges lead to an increase of the electron transfer rate constant particularly between 5–12 Å. This implies that closely bound metals, such as alkali metal ions,

through electrostatic interactions can modulate the CYP450 electron transfer rate. Indeed, consistent with these point-charge calculations our results on large model **B** show an increase of the electron transfer activation barrier due to the addition of two potassium ions ($Q = +2e$) to the second-coordination sphere. The small model (**A1**) calculations with point charges added to the model indeed confirm that second-sphere ions, such as two K^+ ions, can be detrimental for CYP450 activity and show that their long-range electrostatic interactions interfere with the E° and thus electron transfer in the pentacoordinated heme. The trends are more consistent for the predicted barrier (E_{act}) for the electron transfer, than for the electron transfer rates ($\log K_e$). At distances smaller than 4 Å, covalent, charge transfer, exchange and geometry distortion based interactions are known to contribute significantly in addition to the electrostatics which tend to dominate at larger distances.^[57] Thus at smaller distances the point charge model is unable to simulate the influence of the metal ion on CYP450 redox properties (see Table S3 in the supporting information).

Figure 5. Effect of the external electric field (applied along the x, y and z-axes) on the CYP450 heme redox potential and definition of the x, y and z-axis in model A1.

For the divalent Cu^{2+} ions, the point charge model reproduces explicit metal ion trends of an increase in the barrier and the inhibition of electron transfer (negative $\log K_e$ values) step, but overestimates these parameters at short distances and underestimates at larger distances respectively (see Table S4 and Figure S5). This suggests that for transition metal ions the influence on heme redox properties is not a simple function of electrostatics.

Effect of oriented external electric field (EEF) on the heme redox states, E_{act} and $\log K_e$ for $2 \rightarrow 3$

Oriented external electric fields (EEF) are known to influence the reactivity in organic and biochemical reactions.^[58] The direction of the EEFs when oriented along the Fe–S bond was shown to alter the regioselectivity of CYP450 **Cpd I** (model) mediated activation of propene.^[59] In addition, computational studies on the nonheme iron halogenase HctB showed that a chemoselective halogenation was obtained with an external electric field along the Fe–Cl axis but hydroxylation became dominant with a reverse field. Hence external perturbations from an electric dipole field can affect structure, activity and selectivity, which encouraged us to

investigate its role on the first redox potential in the CYP450s.^[60] Thus we investigated the influence of EEF on the rate-determining first electron transfer step (i.e. **2** $\rightarrow$ **3**) in the CYP450 catalytic cycle. For this, the Fe–S bond was oriented along the z-axis and the porphyrin ring was contained within the xy plane with the axis through the meso-carbons of the porphyrin ring. The effect of EEF was evaluated with single point calculations on the optimized geometries and varied along the three axes between 0.02 to -0.02 au (equal to 1.0 to -1.0 V Å⁻¹) and the results are shown in Figure 5. These field values are typically used in theoretical mechanistic crossover studies^[61] and experimentally observed in vibrational Stark effect^[62] studies.

The redox potential (E°), electron transfer rates ($\log K_e$) and barrier (E_{act}) are fairly constant at moderate field strength along the y-axis and change only at relatively high field values (> 0.5 V Å⁻¹ Figure 5 and Figure S6). For the x-axis (oriented along the methylene bridge of the porphyrin ring), there is a gradual increase in the E° and corresponding decrease in $\log K_e$ values from -0.7 to $+0.7$ V Å⁻¹. In that window the E° changes by 0.5 V. Very large positive or negative field strengths applied in the xy

plane give negative $\log K_e$ and large E_{act} predictions suggesting an inhibition of the electron transfer from CPR to CYP450. This is most likely due to a sudden jump in the E° is caused by a cross-over between the ground-state and respectively the quintet/sextet state. This can be interpreted as lack of dependence of the CYP450 Heme redox properties on the EEF at moderate strengths along the xy plane. The relative redox state stabilization and possibly high/low spin ratios are affected only at high field strengths along the xy plane, which are unlikely in CYP450 proteins. Along the z-axis, the predicted E° , $\log K_e$ and E_{act} values show more systematic trends suggesting that CYP450 heme redox properties are more sensitive towards changes in EEF strength along the z-axis (i.e. Fe-S bond). Positive EEF along the z-axis are predicted to increase the $\log K_e$ and lower the E_{act} indicating activation of the CYP450. While negative EEF values along this direction predict an inhibition of the CYP450 rate-determining step. These findings are similar to earlier reports of the influence of EEF on the relative energies of spin-states involved in competing reactions, and thus regioselectivity of Cpd I catalyzed propene oxidations.^[59] In the present case the relative energies of two spin states (**62** and **53**) is influenced by the EEF along the z-axis. The electric field effect results on the small model complex **A1** show that external perturbations (particularly along the z-axis) can affect the E° dramatically and either make the enzyme more reactive or can work as an inhibitor to slow down the rate-determining electron transfer step in the catalytic cycle.

Conclusion

In summary, the present work is the first application of Marcus theory to predict and explain well known but often confusing observations of changes in CYP450 spectral characteristics and activities induced by physiological metals and organic ions. Estimates of redox potential differences (the driving force ΔE°), electron transfer barriers (E_{act}) and rates ($\log K_e$) allow the prediction of alkali metal induced activation and (reversible) inhibition often seen with alkaline-earth and transition metal ions. Our results further provide tangible explanations to the observations of an increase in CYP450 activity by physiologically relevant concentrations of alkali metals, and inhibition by higher concentrations reported earlier. Considering the possibility that these ions can enter into CYP450 active sites, activation by one monovalent ion and inhibition by the interaction of two ions with heme center (via bridging waters) in the active site is probable. The potential of these metal ions to activate or inhibit CYP450 rate-determining steps is not dependent on direct metal-heme interactions and is retained at large distances (up to 14 Å tested here). Thus experimental studies should be designed to search for the presence of metal ions inside the CYP450 active sites. Proton transfer mechanisms have been investigated earlier for steps after dioxygen binding, but results presented here suggest that proton-exchange mechanism can also significantly influence the rate-determining electron transfer (one proton activating, but inhibition is likely with excess proton/positive charge flow into the CYP450 active sites). Point charges can be used to model the effect of monovalent metal ions between 4–12 Å distance from the heme-iron. The direction of the external electric field (EEF) oriented along the Fe–S bond can potentially activate or inhibit the rate determining electron transfer. These findings should

raise hopes for a coherent understanding and control of the factors influencing isoform specificities and selectivity in CYP450 catalyzed reactions for a variety of biotechnological and synthetic chemistry applications.

Experimental Section

Computational Details.

Models. The heme model (**A**) for structures **2**, **3** in the catalytic cycle had an iron bound to a truncated porphyrin-ring with all side chains replaced by hydrogen atoms and either SMe^- (Model **A1**) or SH^- (Model **A2**) to represent the axial cysteinate ligand. We tested the binding of metal ions (Li^+ / Na^+ / K^+ / Ca^{2+} / Mg^{2+} / Cu^{2+} and Zn^{2+}) at various distances from the heme. In addition, the influence of water coordination to these metal ions was studied by the addition of one, and two water molecules to the respective complexes (Figure S7–S10). In general, changing the model from **A1** to **A2** has little effect on the structure and energetics of the reduction reaction, see Table S6 and S7 in the Supporting Information. Model **B** was generated from the 4L4G PDB file, which is a substrate-bound crystal structure. Hydrogen atoms were added in Chimera.^[63] Positions where the protein was cut were filled with hydrogen atoms to complete their valency.

Methods. Density functional theory (DFT) calculations were performed with Gaussian 09.^[64] The unrestricted B3LYP functional and 6-31+G(d,p) basis set were used for C, N, H, O, S and/or any other non-transition metals, while LANL2DZ basis set and pseudo potentials were used for Fe, Zn and Cu atoms. The continuum polarized conductor model mimicking a diethylether solvent ($\epsilon = 4.24$) were chosen to approximate typical protein active site environments.^[65] Full geometry optimizations without constraints were performed (unless mentioned) and all stationary points were verified by the absence of an imaginary (negative) frequency with a frequency (Hessian) calculation. The predicted free energy changes (ΔG°), redox potentials (E°), electron transfer barriers (E_{act}) and rates ($\log K_e$) were not significantly affected by the choice of the DFT functionals. In particular, redox potential calculations with BP86, PBE0, and B3LYP-D3 compared well with those obtained with B3LYP, see supporting information.

The redox potentials (E°) were then estimated using the Nernst equation using solvation free energy differences between the oxidized and reduced states as described in Eq. 1.

$$E^\circ = -\Delta G^\circ / 23.061 + \text{SHE} (4.44 \text{ V}) \quad (1)$$

The redox potential for FMN (−0.006 V) of CPR was used to estimate the driving force ($\Delta G^\circ \propto \Delta E^\circ$) between the donor (FMN) and acceptor (heme) centers.^[66] The activation energy (E_{act}) for the electron transfer was estimated using the classical Marcus Equation (Eq. 2).^[46] Marcus theory explains and predicts the ranges for the driving force for which the electron transfer barriers are lower to facilitate effective redox reactions. This allows the prediction of a Marcus inverted region where despite an increase in the driving force (more exothermic reaction or larger redox potential difference, ΔE° in volts), the activation energy (E_{act}) becomes increasingly positive whereby E_{act} is described as a parabolic function of the driving force, ΔG° (kcal mol^{-1}) $\propto \Delta E^\circ$. Electron transfer rates ($\log K_e$) were predicted using eq. 3 suggested by Dutton et al., (R is radius in Å).^[47] Here λ represents the sum of the donor (CPR)-acceptor (CYP450) reorganization energies. ρ ($\rho = 0.76$) is a factor representing the protein atom packing density between the donor and acceptor.

$$E_{\text{act}} = (\lambda + \Delta G^\circ)^2 / (4 * \lambda) \quad (2)$$

FULL PAPER

$$\log(K_e) = 13.0 - (1.2 - 0.8 \rho) * (R - 3.6) - (3.1 * [\Delta G^\circ + \lambda]^2 / \lambda) - (\Delta G^\circ / 0.06) \quad (3)$$

In the present work, $\rho = 0.76$ recommended by Dutton et al.^[47] and $R = 8.8$ Å from Shaik and Dubey were used.^[14] An oxidant (acceptor)-reductant (donor) reorganization energy ($\lambda \sim 0.7$ eV) was suggested by Dutton *et al* based on an empirical approach.^[47]

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Supporting information details

Supporting information includes 1) A brief description of the heme water interactions observed in the CYP450 crystal structures found in Protein Data Bank (PDB). 2) Table S1 with a list of CYP2D6, CYP2C9, CYP1A1, CYP1A2, CYP2C8, CYP2E1, CYP2C19 and CYP101 crystal structures (PDB IDs) containing different metal ions. Table S2 contains the relative stabilization of reduced state (3) by metal ions at increasing distance from the heme for model A1. 3) Figures S1, S3 and S7-S10 show geometries for metal bound heme reduced states (A1). 4) Figure S2 shows stabilization of reduced state induced by metal ion binding and Figure S4 show the corresponding electrons transfer parameters for model A1, 5) Table S3, Table S4, and Figure S5 show the influence of point charges, while Table S5 and Figure S6 show the effect of EEF on model A1, 6) Table S6 and Table S7 show the effect of metal ion binding on the relative energies and redox parameters for the heme model A2, 7) A section on the influence of the choice of DFT functional and 8) Name, Free Energies and Coordinates of all the structures reported in this paper.

Author Contributions

VAD conceptualized the work and manuscript, performed calculations, analyzed the data, wrote the manuscript and participated in discussions. SPdV and JW analyzed the data, wrote the manuscript, and participated in discussions.

Keywords: CYP450 redox potential, Metal ions, electron transfer rates, density functional theory, second-coordination sphere.

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Entry for the Table of Contents

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Puzzling CYP450 activation by alkali and inhibiting by alkaline-earth and transition metal ions has been resolved. Marcus theory based electron transfer barriers (E_{act}) and log rates ($\log K_e$) estimates for the rate-determining CYP450 reaction (heme models) reveal the mechanistic differences for these metal ions and classic Type II inhibitors. These results open new options for modulating CYP450 reactivity and inhibitor design.

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