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The role of the proximal cysteine hydrogen bonding interaction in cytochrome P450 2B4 studied by cryoreduction/EPR/ENDOR spectroscopy

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Abstract

Crystallographic studies have shown that the F429H mutation of cytochrome P450 2B4 introduces an H-bond between His 429 and the proximal thiolate ligand, Cys 436, without altering the protein fold but sharply decreases the enzymatic activity and stabilizes the oxyferrous P450 2B4 complex. To characterize the influence of this hydrogen bond on the states of the catalytic cycle we have used radiolytic cryoreduction combined with EPR and ENDOR spectroscopy to study and compare their characteristics for wild type (WT) P450 2B4 and the F429H mutant. (i) The addition of an H-bond to the axial Cys436 thiolate significantly changes the EPR signals of both low-spin and high-spin heme-iron (III) and the hyperfine couplings of the heme-pyrrole ¹⁴N, but has relatively little effect on the ¹H ENDOR spectra of the water ligand in the six-coordinate low-spin ferriheme state. These changes indicate that the H-bond introduced between His and the proximal cysteine decreases the S→Fe electron donation and weakens the Fe(III)-S bond. (ii) The added H-bond changes the primary product of cryoreduction of the Fe(II) enzyme, which is trapped in the conformation of the parent Fe(II) state. In wild-type enzyme the added electron localizes on the porphyrin, generating an *S* = 3/2 state with the anion radical exchange-coupled to the Fe (II). In the mutant it localizes on the iron, generating a *S* = 1/2 Fe(I) state. (iii) The additional H-bond has little effect on *g*-values and ¹H, ¹⁴N hyperfine couplings of the cryogenerated, ferric hydroperoxy intermediate but noticeably slows its decay during cryoannealing. (iv) In both WT and mutant enzyme, this decay shows a significant solvent kinetic isotope effect, indicating that the decay reflects a proton-assisted conversion to Compound I (Cpd I). (v) We confirm that Cpd I formed during the annealing of the cryogenerated hydroperoxy intermediate and that it is the

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Supporting Information

EPR and ENDOR Figures (16) and one Table. This material is available free of charge via the Internet at <http://pubs.acs.org>.

NOTES

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active hydroxylating species in both WT P450 2B4 and the F429H mutant. (vi) Our data also indicate that the added H-bond of the mutation diminishes the reactivity of Cpd I.

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Cytochrome P450s are involved in a number of critical processes in biology that use O₂ and NADPH cytochrome P450 reductase to insert an oxygen atom into organic compounds including steroids, xenobiotics, and fatty acids. The presence of an axial thiolate ligand distinguishes these enzymes from other heme based oxygenases and oxidases.^{1, 2} Other members of the heme monooxygenases are nitric oxide synthases, which catalyze the biosynthesis of NO in mammals for signaling and immune defense.^{3, 4} The mechanism of dioxygen activation by heme monooxygenases has been discussed in detail in many reviews.^{1, 2, 5–8}

In general, the catalytic turnover of cytochromes P450s involves substrate binding to the ferric form of P450, reduction to the ferrous state and binding of O₂. Subsequent one-electron reduction of the ferrous dioxygen complex forms the ferric peroxy state, which is protonated to form a ferric hydroperoxy species, often named Compound 0. Heterolytic cleavage of the O-O bond in this intermediate is assisted by addition of a proton to the distal oxygen of the bound hydroperoxide ligand that results in formation of a catalytically active Fe(IV)=O porphyrin π cation radical intermediate (Compound I), which is believed to be responsible for the majority of P450 catalyzed oxidations.^{1, 2, 5–10} The cysteine thiolate that is the proximal ligand of the heme iron is believed to be important for formation and reactivity of Cpd I in cytochrome P450. Electron donation from the proximal ligand is thought to facilitate protonation of the distal oxygen of the hydroperoxy ligand (Compound 0), thereby facilitating heterolytic cleavage of the O-O bond to form Compound I. This electron donation can be significantly modulated by hydrogen bonding interactions between the thiolate proximal ligand and the surrounding polypeptide chain. The experimental data available to date show that formation of a H-bond between the thiolate ligand and the proximal hydrogen bonding network decreases the covalence of the cysteine-Fe(III) bond¹¹, which weakens the Fe(III)-S bond,^{12, 13} strengthens the Fe-OO bond, elevates the redox potential of the ferric state, modulates the reactivity of the heme monooxygenases^{12, 14, 15} and changes the electronic structure of heme iron as reflected in the optical spectrum.^{16–21}

In particular, computations and experiments suggest that varying the environment and electron density in the vicinity of the proximal ligand can modulate the redox potential of the heme iron and affect the pKa of the hydroperoxo (OOH) ligand in Compound 0. The redox potential and pKa of Compound 0 (Cpd 0) play an important role in controlling the alternative fates of the intermediate: 1) conversion to Cpd I; 2) homolytic O-O bond cleavage; or 3) heterolytic Fe-OOH bond cleavage. Changes in electron density on the thiolate can further modulate the reactivity and stability of Cpd I.^{7, 11, 22–26} In particular, recent computations suggest that the enhanced H-bond interaction in the mutant Phe 429 His of CYP2B4 between His429 and the proximal cysteine ligand can disrupt conversion of Cpd 0 to Cpd I and lead to heme oxygenase activity of the mutant.²⁷

To our knowledge, the majority of the experimental studies on the influence of an H-bond to the thiolate ligand on the states involved in catalysis have focused on the Fe(III)-S bond in the ferric state, and this influence has not been explored in other steps of the reaction cycle. The structure of ferric CYP2B4 F429H, in complex with 4-chlorophenyl imidazole¹⁹ shows that the replacement of Phe 429 with His results in formation of a strong H-bond between the δN of His 429 and the thiolate of Cys436 which severely weakens the Fe(III)-S bond.¹⁹ This contrasts with the behavior in cytochrome P450 BM3, where the corresponding mutation F393H does not H-bond with Cys400, the thiolate axial ligand.²⁰ The substrate free redox potential changes little with the mutation: for the F429H mutant it is –245 mV versus –300 mV for the wild type.¹⁹ A parameter that does (inversely) correlate with H-bonding strength to the thiolate is the trans-axial Fe- CO stretching frequency. Here, the addition of the H-bond to axial thiolate in the F429H mutant shifts the frequency for the trans-axial ν Fe-C and ν C-O linkage to a higher frequency, which is indicative of diminished donation by the proximal thiolate ligand. The mutationally shifted value is similar to that of chloroperoxidase (CPO), which also has two strong H-bonds to thiolate and forms a Cpd I that is much less reactive than that of P450s. It has been shown recently that the low reactivity of Cpd I of CPO relative to that of P450s correlates with a lengthening in the Fe-S distance caused by the additional H-bond to axial thiolate in CPO.²⁸ Functionally, the F429H mutation was shown to substantially slow the rate of autooxidation of oxy ferrous P4502B4 complex, and to decrease the activity by over 90%.¹⁹

Application of radiolytic cryoreduction combined with EPR and ENDOR spectroscopy has previously been shown to enable the generation, trapping, and spectroscopic characterization of all the intermediates arising after the addition of an electron to an oxy heme center. In the present study, this approach has been used to explore the role of H-bonding to the thiolate sulfur of the axial ligand, Cys436, at each stage of the catalytic reaction cycle, through comparative studies on WT CYP2B4 and its CYP2B4 mutant F429H, in which the highly conserved proximal Phe429 is replaced with histidine. These comparative studies yield detailed insights into the effects of H-bonding for: the ferric heme state; the ferrous heme state as revealed in the properties of its EPR-active cryoreduced state; the cryogenerated ferric heme-hydroperoxy (Cpd 0) intermediate. These studies show that Cpd I is the active species in substrate hydroxylation in both WT and mutant enzyme, but the conversion of Cpd 0 to Cpd I is greatly slowed by the mutation. Perhaps most importantly, this study indicates that the addition of an H-bond to the axial thiolate ligand of a P450 decreases the

intrinsic reactivity of Cpd I, in keeping with work comparing the reactivity of Cpd's I of P450 and chloroperoxidase²⁸.

Experimental Procedures

Chemicals

All organic solvents, butylated hydroxy toluene (BHT), hydroxymethylbutylated hydroxy toluene (BHTOH), benzphetamine (BP) and sodium dithionite were from Sigma-Aldrich. O₂ was obtained from Matheson Co. Wild type cytochrome 2B4 and F429H were expressed in *E. coli* and purified to homogeneity as described^{19, 29, 30} and stored at -70°C in 100 mM K phosphate buffer (pH 7.5) containing 20% glycerol. 4-(4-chlorophenyl) imidazole (CPI) was purchased from Oakwood Products, Inc., West Columbia, South Carolina. Cambridge Isotope Laboratories of Andover, MA was the supplier for d3-glycerol [Glycerol (OD)3] and deuterated BHT (2,6-di-tert-butyl-4-methylphenol-d24) was purchased from CDN, Quebec, Canada.

Determination of Activity and NADPH Consumption by WT and F429H

CYP2B4—The activity of full-length CYP2B4 was examined in a purified reconstituted system by measuring the amount of formaldehyde formed by benzphetamine (BP) N-demethylation using Nash's reagent as previously described.³¹ Briefly the final reaction mixture contained 0.3 μM either WT CYP 2B4 or F429H, and cyt P450 reductase (molar ratio P450:reductase 1), 68 μM dilauroyl phosphatidyl choline, 50 mM potassium buffer at pH 7.4, 1 mM benzphetamine HCl, 300 μM NADPH. NADPH consumption was followed at 30° C for 5 minutes. The reaction was quenched after 5 minutes with trichloroacetate (final concentration 7%) and CH₂O was measured. In calculations of NADPH consumption, the NADPH consumed by the reductase alone was subtracted from that consumed in the complete reaction mixture.

The full-length wild type cyt P450 and cyt P450 reductase (NO His tags) were purified as previously described.^{19, 29} The reductase was purified without a 2'-5' ADP affinity column. The F429H mutant was purified in the same manner as the wild type except that BHT was added to the buffers during purification to prevent conversion to cyt P420, an inactive form of cyt P450. NADPH consumption was measured by recording the decrease in absorbance at 340 nm using an extinction coefficient of 6.22 mM⁻¹cm⁻¹ in the course of the reaction. BHT activity was measured as described previously with a LC/MS/MS assay²⁹

Sample preparation

Complexes of ferrous WT P450 2B4 and its F429H mutant with O₂ were prepared using a modified procedure described in Reference 28.²⁹ The ferrous P450 2B4 samples were prepared by incubating the ferric protein in aqueous 0.1M K phosphate buffer (pH 8.0) containing 1–2 mM benzphetamine (BP) or 1–2 mM BHT and 20% glycerol in an anaerobic glovebox overnight at 3°C to remove oxygen from the protein solution. An aliquot of a standardized solution of sodium dithionite was added to reduce the ferric P450, using a 10% molar excess of dithionite to cyt P450. An extinction coefficient of 8000 M⁻¹ cm⁻¹ at 315 nm was used to determine the concentration of dithionite.³²

The reduced protein solution was then mixed with oxygen-free 100% glycerol. Complete reduction of protein was confirmed spectrophotometrically. The sample of reduced P450 2B4 containing 1–2 mM BP or 1–2 mM BHT in 60% glycerol/buffer (0.3 M NaCl for BHT sample) was transferred into EPR tubes. Ferrous oxy P450 2B4-substrate complexes were made by bubbling the Fe(II) P450 sample at -25°C with 20 mL of cold oxygen gas for 30–60 s followed by rapid freezing in liquid nitrogen.

Cyoreduction was performed at 77 K using samples prepared with 0.3–0.5mM oxy-2B4 in a solution containing 60% glycerol (v/v), 0.3 M NaCl, 100mM K phosphate buffer (pH 8), and 1mM BHT or BP. When needed, the protein was exchanged into buffers made using D₂O and glycerol-d₃. In D₂O samples, the pH was adjusted to pH 7.6.(pD8)³³

Gamma irradiation of the frozen hemoprotein solutions at 77 K typically was performed for ~30 h (dose rate of 0.1 Mrad/h, total dose 3Mrad) using a Gamma cell 220 ⁶⁰Co. Annealing at temperatures over the range 77–270 K was performed by placing the EPR sample in the appropriate bath (e.g., n-pentane or methanol cooled with liquid nitrogen) and then refreezing in liquid nitrogen.

Spectroscopic Techniques

EPR/ENDOR measurements of the samples were conducted as previously described.^{34, 35} X-Band continuous wave EPR spectra were recorded on a Bruker ESP 300 spectrometer equipped with an Oxford Instruments ESR 910 continuous He flow cryostat. All CW Q-band EPR/ ENDOR spectra were recorded at 2 K in dispersion mode, under “rapid passage” conditions, which gives an absorption line shape.³⁶ UV–vis spectra of the samples in 4 mm o.d. quartz tubes were measured at 77 K through immersion in a liquid N₂ finger dewar with a USB 2000 spectrophotometer (Ocean Optics, Inc.).³⁴

Results

Effect of F429H mutation on mechanism of hydroxylation BP by cyt P450 2B4

As mentioned above, Waskell and coworkers recently showed that the change of F429 to His significantly lowers the rate of formation of formaldehyde by N-demethylation of BP¹⁹. This lowered activity could result either from diminished catalytic activity as a result of the mutation,²⁹ or from reduced efficiency of the reaction caused by decoupling induced by the mutation.²⁷ In the present study, we have found that, although the activity and the rate of NADPH consumption by the mutant were decreased, the mutation had little effect on the amount of NADPH consumed relative to product generated during N-demethylation of BP (Table S1) For both WT and the mutant, the coupling of NADPH consumption to product formation was determined to be similar, 41% and 38% respectively. The latter data thus indicate that as in the case for WT P450 2B4, N-demethylated benzphetamine is the main product of the metabolism of BP catalyzed by the mutant and that the effect of the mutation on demethylation is to lower the rate of the reaction, not its efficiency.

It has been suggested that the F429H mutation might induce the Fe(III)-OOH[−] intermediate to undergo homolytic cleavage and heme self-hydroxylation²⁷ and this has been tested. In heme oxygenase, hydroxylation of the heme to α -hydroxyheme was shown to strongly affect

its EPR and UV-VIS spectra.^{37–39} In addition, α -hydroxyheme is very easily oxidized by molecular oxygen to verdoheme at ambient temperature with a further strong change in the optical spectrum.³⁷ Therefore, one would expect that significant hydroxylation of heme in the mutant F429H P450 2B4 should decrease the amount of P450 following catalytic turnover. To check if the mutant has heme oxygenase activity in the presence of the substrate BP, we measured the amount of P450 2B4 present at the end of a five hour reaction period under the usual turnover conditions. During this lengthy reaction time only a 5% decrease in the total amount of heme protein, which included cyt P450 and P420, was observed, ruling out significant amounts of heme hydroxylation and destruction in the mutant during enzymatic reaction.

EPR and ENDOR of WT and F429H ferric P450 2B4 complexes with BHT and BP

Optical spectroscopy shows that WT P450 2B4 is $\sim 90\%$ in the 5-coordinate, high-spin state at ambient temperature in the presence of either 1mM BP or 2 mM BHT, while the F429H variant is only $\sim 60\%$ high spin under similar conditions. Since the crystal structure reveals that the mutation does not significantly perturb the structure of the active site, it suggests that water, like oxygen and CO, may bind more tightly to the iron^{12,19}. As shown previously, in the presence of BP, the high-spin form of the WT enzyme essentially vanishes upon cooling to temperatures of 77 K, a change that is assigned to a repositioning of substrate in the heme pocket so that H₂O binds as a sixth ligand.²⁹ For the F429H variant in the presence of BP, the EPR signal of high spin 5-coordinate ferric conformer also significantly decreases upon cooling, indicating that BP likewise moves away from the heme, allowing H₂O to bind as a sixth ligand.

In contrast, in the presence of BHT the population of the high-spin form remains relatively high at temperatures of 77 K and below in both WT and mutant ferric P450. As shown in Fig. 1A, the low-spin form of WT P450 2B4 that does appear in the presence of BP shows a rhombic EPR spectrum with $g = [2.424, 2.25, 1.924]$ (Table 1) characteristic of heme iron(III) with a water molecule as the sixth, distal ligand trans to the proximal Cys.^{29, 40}

The substitution of F429 for His in ferric P450 2B4 in the presence of BHT does not change noticeably the relative populations of high- and low-spin ferriheme forms in the frozen solution, but the H429-axial cysteine H-bonding interaction causes significant changes in the orbital energies of both high-spin and low-spin ferriheme states of P450 2B4, whereas mutations in the distal pocket cause no essential perturbation of the ferriheme in either spin state. The mutation increases the g -value spread for the low-spin heme iron (III) (Figs. 1A, S1; Table 1); for example, $g_1(\text{F429H}) = 2.52$ as opposed to $g_1(\text{WT}) = 2.42$. The broadening of the g -value spread in the mutant represents a decrease in the axial crystal-field splitting between the $[xz, yz]$ orbital pairs and $[xy]$ orbital, which comprise the occupied T_2 d-orbital triplet (Table 1).⁴¹ Low spin ferriheme complexes of both WT P450 2B4 and F429H variant with the inhibitor 4-chlorophenyl imidazole (CPI) bound to the Fe(III) ion again show a larger g -spread for the mutant (Table 1). In contrast, although mutations of the conserved alcohol, acid pair, T302A and E301Q, in the distal pocket of the active site cause a 94% and 85% decrease in enzymatic activity, they cause minimal effect on the EPR signal of the low-spin aquo-ferriheme. (Table 1, Fig. S1)

Likewise, the F429H mutation causes a significant change in the electronic structure of the high-spin ferriheme, as seen in the EPR signal in the presence of BHT. The high-spin ferriheme EPR spectra of WT P450 2B4 is highly rhombic, $g = [8.12, 3.52, 1.68]$, $\delta g_{\text{perp}} = g_1 - g_2 = 4.6$. Fig. 1B, Table 2. The H-bond between His429 and Cys436 causes a remarkable decrease in the rhombicity of the high-spin EPR signal, to $\delta g_{\text{perp}} = 3.4$ in the mutant (Table 2, Fig. 1B)⁴². The $g_1 - g_2$ splitting of the $S = 5/2$ EPR spectra^{43,44} is determined by the ratio of the rhombic and axial zero-field splitting parameters, E/D , which in turn can be related to ligand-induced splitting of the ferric d orbitals.⁴⁵ The mutation causes a 25% decrease in E/D , from 0.11 to 0.082 (Fig. 1B, Table 2). In contrast, mutations T302A and E301Q in the distal pocket of the active site have a minimal effect on the EPR signal of the high-spin ferriheme (Table 2).

ENDOR of low-spin ferric WT and F429H P450 2B4 with BP

To further characterize electronic and structural changes in the heme center caused by introduction of the H-bonding interaction between His429 and the proximal thiolate ligand, we carried out ENDOR studies of the low-spin aquo-ferriheme forms of WT and F429H P450 2B4. The low-spin WT form shows a ^1H ENDOR signal, $A_{\text{max}} = 9.2$ MHz, that disappears in deuterated solvent, and can be assigned to the iron-coordinated H_2O .⁴⁶ (Fig. S2, S3) The low-spin form of the mutant F429H shows similar ^1H ENDOR pattern (Fig. S4, S5). Simulations of the ^1H ENDOR patterns for these two low-spin conformers confirm that H-bonding to the proximal ligand of F429H has a negligible effect on the almost completely dipolar hyperfine coupling to the protons of the distal water ligand, indicating that the geometry of this distal ligand is not modified by influencing the proximal one (Table 3, Fig. S3, S5).

In contrast, CW 35 GHz ^{14}N spectra show that the introduction of the H-bond between the proximal cysteinyl ligand and H429 sufficiently modifies the electronic/geometric properties of the ferriheme to noticeably alter the interaction between the Fe(III) ion and the ligating in-plane pyrrole nitrogens of the porphyrin macrocycle. Fig. 2 shows the ν_+ -branch of ^{14}N ENDOR spectra for WT P450 2B4 and F429H mutant taken at g_1 . These spectra exhibit ^{14}N features from two nonequivalent pairs of pyrrole nitrogens.^{46, 47} The hyperfine and quadrupole coupling constant along g_1 for these nitrogen pairs in WT P450 2B4 are $A_1 = 5.14, 5.72$ MHz, average value $\bar{A}_1 = 5.43$ MHz, with $3P_1 \approx 0.9$ MHz for both. The F429H mutation causes a distinct but differential increase in couplings for the two pairs, to $A_1 = 5.35, 6.19$ MHz, $\bar{A}_1 = 5.93$ MHz, without change in $3P_1$ (Fig. 2).

Simulation of 2D field-frequency patterns of ^{14}N ENDOR spectra collected across the EPR envelopes for WT P450 2B4 and F429H mutant (Fig. S6 and S7) correspondingly show that the mutation causes a distinct increase in the isotropic coupling to one of the pairs of pyrrole nitrogens, by $\sim 10\%$ (Table 3). This difference is similar to the difference in the ^{14}N ENDOR spectra of the pyrrole nitrogens caused by replacing the H_2O distal ligand by the CPI ligand (Fig. S8). Again, mutation of the conserved acid-alcohol pair of residues on the distal side of active site (T302A, E301Q) has little effect on ^{14}N ENDOR of pyrrole nitrogen (not shown).

EPR and ENDOR spectroscopy of ferric P450 with the BHT metabolite hydroxy methyl BHT (BHTOH)

To characterize the binding of BHTOH, the major metabolite of BHT by P450 2B4 and to generate reference spectra for the assignment of the ferric species in the EPR spectra of the irradiated samples of oxy P450 2B4 during annealing, the effect of BHTOH on the EPR and absorption spectra of ferric P450 2B4 and its mutant were studied.

Substrate-free WT ferric P450 2B4 essentially contains only six-coordinate (6c) low-spin aqua ferric heme with α - and β -bands at 533nm and 569 nm, respectively and with an EPR signal described by the g-tensor, $g = [2.42; 2.25; 1.92]$. In the presence of a saturating concentration of BHTOH at ambient temperatures, a new band appears at 645 nm that is due to high spin 5c ferric P450 2B4 with a slight shift of the maxima of the α - and β - bands of low-spin form of ferric P450 2B4 to 539 and 567 nm, respectively (Fig. S9).

In the presence of a saturating amount of BHTOH, the low temperature EPR spectrum of WT ferric P450 exhibits a rhombic high-spin signal, confirming that the BHT metabolite, BHTOH, induces a partial loss of the H₂O ligand to form the high-spin 5c state (Table 1,2). Accompanying this signal are two low-spin ferriheme signals that can be attributed to slightly perturbed 6c aquo ferric heme, $g = [2.41, 2.27, 1.99]$ and $[2.44, 2.26, 1.94]$. ¹H ENDOR spectra for g_1 2.41 and g_1 2.44 conformers taken at g_1 show signals from H₂O protons that are equivalent to these for wild-type low-spin aqua ferriheme P450 2B4 (not shown), confirming that in these conformers the binding of metabolite in the distal heme pocket causes only minor perturbations in geometry of the water ligand, and not its loss. However, the addition of BHTOH also generates a more perturbed minor low-spin conformer with $g = [2.55, 2.27, 1.88]$ (minor) (Table 1). The ¹H ENDOR spectrum taken at g_1 2.56 for this conformer does not exhibit a strongly-coupled proton signal characteristic of coordinated water, suggesting that the water has been replaced by bound BHTOH.

The ferric F429H variant in the presence of BHTOH likewise shows the presence of high and low-spin EPR signals, indicating partial loss of H₂O. The low-spin signal exhibits major and minor components associated with a minimally perturbed aquo-ferric heme, with $g_1 = 2.50$ and 2.55, respectively; in addition, there is a minor component with $g_1 = 2.46$ that may be from water or the heme-bound BHTOH; g-values for the various high and low-spin conformers of the mutant are listed in Tables 1,2.

Ferrous P450 2B4

Irradiation of frozen solutions of EPR-silent high spin ferrous hemoproteins (S=2) has been shown to generate both one-electron oxidized and one-electron reduced Fe(I) species.⁴⁸ Such paramagnetic species produced and trapped at 77 K retain the conformation of the ferrous precursor and can be used as a sensitive EPR probe of the structure of the EPR-silent ferrous state.

The EPR spectrum of γ -irradiated ferrous WT P450 2B4 in the presence of BP, presented in Fig. 3, exhibits three distinct EPR signals from the cryoirradiated ferrous heme. The major signal is characterized by a rhombic g-tensor, $g = [4.17; 3.88, \sim 2]$ (Fig. 3) and can be

assigned to the $S=3/2$ state of the cryoreduced ferroheme form in which a porphyrin-centered π -anion radical antiferromagnetically couples to high-spin Fe(II) ($S=2$).⁴⁸

A second signal, characterized by $\mathbf{g} = [2.39; 2.24; 1.93]$, can be assigned to low-spin ferriheme generated by cryooxidation of the minority low-spin 6c ferrous P450 2B4 parent, which was shown to be present in the ferrous cytochrome P450 2B4 samples.¹⁸ The third signal with $g_{\perp} = 8.09$ (the presence of the strong $S=3/2$ signal hinders identification of the g component within the $g \sim 4$ region) belongs to a high-spin pentacoordinated ferric P450 2B4 formed during cryooxidation of the ferrous state.⁴⁸ The cryoreduced heme of A is trapped in a nonequilibrium state at 77 K, and upon annealing at $T > 175$ K undergoes structural relaxation leading to intra-heme electron transfer and converts to an Fe(I) state with $g_{\perp} = 2.22$ and $g_{\parallel} = 1.944$.⁴⁸ (Fig. 3B).

The F429H mutation totally changes the cryoreduction and annealing pattern of the ferroheme state of the enzyme: the primary site of cryoreduction is the Fe ion, not the porphyrin as seen with the WT enzyme. Cryoreduction of the Fe(II) F429H P450 mutant directly yields a Fe(I) species (Fig. 3C) showing an axial EPR signal with $g_{\perp} = 2.26$ and $g_{\parallel} \approx 1.94$, which then disappears upon annealing at $T > 200$ K. Of particular interest, the generation of a Fe (I) primary product upon cryoreduction of F429H matches the cryoreduction behavior of ferrous P450cam (Fig. 3E). Once again, the T302A mutation on the distal side of ferrous P450 2B4 has relatively little influence on the properties of the heme and its interactions, as the cryoreduction and annealing pattern is similar to that of the WT. (Fig. 3D).

Cryoreduction and Annealing of Oxyferrous WT and F429H P450 2B4 Substrate Complexes

Wild type

BHT: Fig. 4 presents the EPR spectra of ternary oxy-WT P450-BHT complex cryoreduced at 77 K and annealed at higher temperatures. The EPR signal from the one-electron reduced ternary oxyferrous substrate complex trapped at 77K has $\mathbf{g} = [2.32, 2.18, \text{nd}]$ (Fig. 4, Table 4), characteristic of the ferric-hydroperoxy state.²⁹

The generation of this species at 77 K indicates that the enzyme has an efficient proton delivery network that can support protonation of the cryogenerated peroxo ligand at 77 K.²⁹ Previously we showed that during annealing at $T = 175$ K the hydroperoxo-ferric P450 2B4-BHT gradually decays, and that this process is accompanied by a parallel increase in the magnitude of the high-spin ferri-P450 2B4 signal, $\mathbf{g} = [8.11, 3.55, \sim 1.7]$ assigned to high-spin ferri WT P450 2B4 in the presence of the reaction product, BHTOH (Table 2). The decay of the WT hydroperoxo intermediate is more than two-fold slower in deuterated solvent (Fig. S10). This solvent kinetic isotope effect (sKIE) is close to that reported for decay of the cryogenerated ferri P450cam-OOH intermediate in the presence of camphor,⁴⁹ a process in which this intermediate converts to Cpd I, which is the active species in the hydroxylation of camphor.⁵⁰ Note that deuteration of BHT does not slow the decay of the hydroperoxo WT protein complex with BHT. This is the expected if conversion of ferric hydroperoxo intermediate in Cpd I is rate limiting step. (Fig. S10).

More fine-grained annealing measurements now show that during decay of the hydroperoxo species a new low-spin intermediate with $g = [2.47; 2.22; 1.90]$ arises. This intermediate may be assigned to the primary product of hydroxylation BHTOH bound to ferriheme (Fig. 4). Upon further annealing at 195–200 K this species relaxes to an intermediate with $g_1 = 2.55$, spectroscopically similar to the state attributed above to the equilibrium conformer of the BHTOH bound to the ferric heme of P450 2B4. As we have discussed, the occurrence of a ferriheme complex in which the hydroxylation product is bound to Fe (III) implies that the WT P450 2B4 hydroperoxo intermediate converts to Cpd I, which promptly hydroxylates BHT.²⁹

The g 2.55 equilibrium product-bound form completely decays during annealing at 215 K (Fig. 4), a process accompanied by a further increase of the high-spin ferric P450 signal^{29,51} (not shown). At temperatures below 210 K, where molecular movements are restricted, this high-spin form of P450 is unlikely to result from the autooxidation of the unreduced ferrous P450 2B4, or from replacement by BHT of BHTOH formed during enzymatic turnover.⁵¹ These observations may be accounted for by the scheme proposed for hydroxylation of camphor by cryoreduced oxy P450cam.⁵² In this context it is worth noting that the relative intensity of the g 2.55 intermediate is noticeably higher than that in the equilibrium product complex, as expected for a high occupancy in the distal heme pocket of BHTOH formed during hydroxylation of BHT at low temperature, as opposed to a low occupancy in the equilibrium state.

In this scheme heterolytic, proton-assisted cleavage of the O-O bond of the hydroperoxide ligand leads to formation of an ferryl porphyrin π -cation radical (Cpd I) which then rapidly hydroxylates substrate to form product BHTOH bound to heme iron(III) in a non-equilibrium conformation (species 2.47) which then relaxes to the equilibrium state showing an EPR signal with a $g_1 = 2.55$, seen for the ferric P450-BHTOH complex. Because of the low affinity of BHTOH for the heme iron (III), this complex dissociates and the g 2.55 intermediate decays within the temperature range 176–215 K, forming the high-spin 5c BHTOH bound ferric P450 state with adjacent BHTOH. This observation of product-bound states during cryoreduction and annealing of the enzyme with bound BHT, which is hydroxylated during this process, contrasts nicely with the data on cryoreduced oxy WT P450 2B4 prepared in the presence of BP, as is now presented.

BP: It was shown previously that cryoreduced oxy WT P450 2B4 cannot hydroxylate BP because the substrate shifts away from the heme at low temperatures.²⁹ As implied by this finding, the cryoreduction and annealing patterns of oxy WT P450 2B4 in the presence of BP and BHT as substrates are quite different.

Cryoreduction of oxy P450 2B4 in the presence of BP generates two states whose g -tensor components and ¹H ENDOR spectra identify them as ferric-hydroperoxy EPR signals (Fig. 5, Table 4): major signal A with $g = [2.28, 2.17, \text{nd}]$ and minor signal B with $g = [2.30, 2.17, \text{nd}]$ (Fig. 5); ¹H ENDOR of species A is characteristic of a hydroperoxy proton, with $A_{\text{max}} = +10$ MHz, exchangeable in D₂O, (Fig. 6, Fig. S11).

During progressive annealing from 77 K to 185 K the ferric-hydroperoxy heme signal A undergoes conformational relaxation to signal C, a hydroperoxo-ferriheme conformer with $g = [2.32, 2.17, 1.93]$ (Fig. 5), a process completed by annealing at 200 K (Fig. 5). Signal C vanishes by 235 K (not shown), a process accompanied by an increase in the resting low-spin aquo-ferriheme signal ($g = [2.42, 2.24, 1.92]$). (Fig. 5). The overall decay of the hydroperoxo intermediate at 176 K as measured by the loss of the $g_2 \sim 2.17$ feature in the spectra, slows by a factor of ~ 5 in deuterated solvent (not shown). This solvent kinetic isotope effect (sKIE) indicates that, as in case of BHT, the hydroperoxo intermediates generated in the presence of BP decays with a rate-limiting step of heterolytic, proton-assisted cleavage of the O-O bond of the hydroperoxide ligand, leading to formation of Cpd I. However, in contrast to the behavior with BHT, cryoreduction and annealing in the presence of BP produces no signal from product complexes and no high-spin product signal. The aquo ferriheme P450 2B4 generated during decay of the hydroperoxy intermediates is possibly due to two-electron reduction of Cpd I by radiolytically generated radicals.⁵²

F429H mutant

BHT—Cryoreduction of the ternary oxyferrous F429H P450-BHT complex produces a major species with $g = [2.30, 2.18, \text{nd}]$ (Fig. 7, Table 4) and minor one with $g = [2.28; 2.175, \text{nd}]$, both g -tensors characteristic of ferri-hydroperoxy intermediates. The ^1H ENDOR spectrum at g_1 for the dominant intermediate supports this assignment: the spectrum exhibits a strongly coupled proton signal with $A \sim 9$ MHz which disappears in deuterated solvent, (Fig. 6), as expected for a During annealing at 176 K the cryogenerated hydroperoxy intermediate decays (Fig. 7), accompanied by a small increase of high-spin signal (Fig. S12) and appearance of relaxed hydroperoxy conformers with $g_1 = 2.34$ and 2.37 (Fig. 7). In addition, this annealing generates two signals whose g -tensor components, $g = [2.53, 2.27, 1.88]$ and $g = [2.48, 2.27, 1.90]$, and ^1H ENDOR spectra (not shown) identify them as the resting aqua ferric state of the mutant, plus a third minor signal whose g -values resemble those for the minor signal in the resting ferric state of the F429H P450 2B4 (Table 1). Interestingly, the F429H mutation slows the decay of the iron(III) hydroperoxy intermediate compared to WT by a factor of 4–5 at 176 K (Fig S.10). Furthermore, this decay of the Fe(III) hydroperoxy intermediates shows a stronger sKIE > 4 (Fig. S13) than the WT enzyme. The two main aquo-ferric EPR signals are strongly increased by annealing up to 281 K through auto oxidation of the oxy complex that was not reduced during 77 K irradiation of the mutant (Fig. 7).

Taken together, these observations lead us to infer that the hydroperoxy state of the mutant generates Cpd I during annealing, and this is reduced to the 6c-aqua form. We neither observe nor expect to detect signals from product-bound ferriheme in the case of the mutant, given that the yield of BHTOH during cryoreduction and annealing of oxyferrous F429 H P450 2B4-BHT is a factor of ~ 10 less than that for WT.²⁹ However a small increase of the high-spin signal after decay of the hydroperoxo intermediate at 176–185 K (Fig. S12) may be assigned to formation of BHTOH.

BP—As with the WT enzyme, the primary product of 77 K cryoreduction of oxyferrous F429H P450 2B4 BP trapped at 77 K has g -values characteristic of the ferric hydroperoxy

intermediate (Table 4, Fig. 8); this is confirmed by ^1H ENDOR spectroscopy (see below). The cryoannealing pattern of this intermediate is similar to that observed during annealing of the cryoreduced oxyferrous F429H P450 2B4-BHT complex, yielding two stable low spin aqua ferric states with $g = [2.52, 2.27, 1.88]$ and $g = [2.48, 2.27, 1.90]$ at temperatures below 210 K. However, unlike the case of BHT, this process generates no hs 5c ferriheme (not shown).

Upon annealing the irradiated sample at 273 K the nonreduced oxy complex is completely autoxidized, and the intensity of the aquo ferriheme 2.53/2.47 signals increases significantly without change of their relative ratio. Taken together, these observations indicate that the 2.53/2.47 signals are aqua-ferriheme conformers; as expected, neither is a product complex, nor is it cyt P420 as shown by optical measurements upon warming to ambient temperatures, reduction, and addition of CO.

As mentioned above, generation of low-spin 6c aqua ferric heme as a primary product of the decay of hydroperoxy intermediate of the mutant rather than the 5c high-spin state, is expected during reduction of Cpd I byproducts of radiolysis, because dissociation of H_2O_2 should result in formation of the 5c high-spin ferric state in the frozen matrix.

ENDOR studies of cryogenerated hydroperoxy ferric WT and mutant P450-BP/BHT intermediates

We applied ENDOR spectroscopy to examine in detail the effects of the F429H mutation on the cryogenerated Fe(III)-OOH precursors of the catalytically functional active-oxygen intermediates.

^1H ENDOR— ^1H ENDOR spectra of the cryogenerated Fe(III) hydroperoxy WT P450 2B4 and the F429H variant show strongly coupled ^1H ENDOR signals with $A_{\text{max}} \approx 11.5$ MHz, characteristic of a proton of a hydroperoxide ligand (Figs 6, Fig S11, S14–16).^{34, 38, 52} Simulation of the 2D frequency-field of the ^1H ENDOR pattern of the hydroperoxy intermediates of WT and mutant P450 2B4, each with BP and BHT bound, and the fitting parameters are presented in Figs. S11, S14–16 and Table 5. The F429 to His mutation has only a relatively weak effect on the 2D field-frequency ^1H ENDOR pattern of the hydroperoxide ligand and consequently its geometry in the active site (Table 5 and Figs. S11, S14–16); there is also no significant difference in either case between the ^1H ENDOR in the presence of BP or BHT (Table 5).

^{14}N ENDOR—To gain insight into the effect of the mutation on the electronic properties of the hydroperoxy ferriheme center, we have compared ^{14}N ENDOR spectra taken at the g_{max} turning point of the WT enzyme in the presence of BP and BHT.

The intermediates of both mutant and WT enzyme, with BP and BHT present, show more complex ^{14}N ENDOR spectra than that of the aquo-ferric P450 2B4 in the absence of substrate and, indeed, more complicated spectra than typically seen for hydroperoxy intermediates. The ^{14}N ENDOR spectra at g_{max} of the WT aquo-ferric form (Fig. 9), like those seen previously for hydroperoxo ferric intermediates, indicate that the ^{14}N pyrroles form two slightly different pairs of equivalent ^{14}N . The spectra of the WT hydroperoxo

ferric hemes with both BHT and BP present (Fig. 9), show responses from four distinct ^{14}N . The spectra for the Fe(III)hydroperoxy F429H enzyme with BHT and BP present show responses from three types of ^{14}N , two unique and one essentially equivalent pair which gives two signals (Fig. 9). One possible interpretation of this observation is that the spectra are “superpositions” from multiple conformers with slightly differing ^{14}N properties; another would be a further desymmetrization of the heme from two slightly different pairs to one pair and two unique ^{14}Ns .⁽¹⁾

Discussion

This cryoreduction/EPR/ENDOR study demonstrates that the addition of an H-bond to the proximal cysteine through mutation of Phe 429 to His has: (i) a substantial effect on both the physical and chemical properties of the ferric and ferrous states of cytochrome P450 2B4, (ii) but little effect on the spectroscopic properties of the ferric-hydroperoxy intermediate (Cpd 0) that is the precursor of Cpd I, while (iii) strongly slowing the decay of the ferric-hydroperoxo state and reducing the catalytic activity of Cpd I.

These observations extend earlier work on mutation-induced properties. The enhanced H-bonding to the proximal cysteine in the F429H P450 2B4 causes a positive shift in the redox potential (E_m) and slows the rate of auto oxidation of the oxyferrous complex ~ 40 -fold.¹⁹ A similar effect on E_m from enhanced H-bonding interaction with the proximal cysteinyl was seen in several NOS enzymes.^{14, 54} This was computationally explained by a diminished Fe-S covalency induced by strengthened H-bonding to the cysteinyl sulfur.^{11, 55} As mentioned above, a similar study of the F393H variant of cytochrome P450 BM3 gave a puzzling result.^{21, 56} This mutation caused similar changes in redox potential and reactivity yet the crystal structure, resonance Raman, and EPR spectroscopies did not show an H-bond interaction between H393 and the thiolate ligand in this mutant.

We first discuss the changes in the properties and reactivities of individual states, then consider possible implications of this work for enzymatic function.

Ferric state of cyt P450 2B4

The formation of the strong His $\cdots$ S – hydrogen bond in F429H variant, causes a sharp increase of the rhombicity of the EPR signal of 6c low-spin aquo ferriheme center (Table 1) while reducing the rhombicity (E/D) of the S = 5/2 5c hs EPR signal from substrate bound enzyme (Table 2). Similar phenomena are seen with some NOS variants.¹⁶ These have been shown⁴⁵ to correlate with reduced covalence and lengthening of the Fe-S bond to the ferriheme caused by enhanced H-bonding to the thiolate. The most direct observation of the consequences of reducing electronegativity of the proximal ligand through introduction of an H-bond by the mutation is the considerable increase in the hyperfine coupling to the pyrrole ^{14}N (Table 3), which can be attributed to weakening the Fe-S π -bond covalence.¹¹ As the ^1H ENDOR from distal bound water is controlled by the through-space dipolar

(1)The presence of ferric contaminants as well as a strong radical signal makes it difficult to compare the full 2D frequency-field ^{14}N ENDOR pattern for the cryogenerated hydroperoxy ferric intermediates.

interaction and thus coordination geometry, not spin delocalization ($a_{\text{iso}} \sim 0$, Table 3), it does not change with the proximal mutation.

These changes in ferriheme electronic structure caused by the F429H mutation correlate with changes in the redox-potential of the heme iron and with the crystallographic data for cytochrome P450 model (porphyrin)(thiolate) Fe(III) complexes with single and double $\text{NH}\cdots\text{S}$ hydrogen bonds.⁵⁷ Sulfur K-Edge XAS and DFT calculations on P450 model complexes and P450cam showed that strengthening the $\text{NH}\cdots\text{S}$ - hydrogen bond leads to reduced electronegativity of the thiolate ligand and weakening the Fe (d)—S (p) π - bond covalency, which in turn causes a positive shift of midpoint redox potential of E_m and weakening of the Fe-S bond.^{11, 55} Indeed, $\nu(\text{Fe(III)-S})$ in 5c high-spin WT P450 2B4 was recently shown to shift to lower frequency upon substitution of F429 to His.¹² Taken together, these data imply that the observed decreased E/D for the 5c-hs Fe(III) F429H P450 2B4 variant is due to weakening of the Fe-S- bond induced by strong H-bonding interaction between His 429 and the proximal ligand.

Ferrous state

Previous studies of the influence of His 429 H-bonding with the proximal thiolate ligand in the deoxyferrous state of the P450 2B4 revealed a noticeable red shift of the Soret band in the mutant in comparison with WT P450 2B4.¹⁸ Here we have examined the EPR-active cryoreduced ferrous P450 intermediate trapped at 77 K as a sensitive EPR probe of the effects of the mutation on the parent 5c high-spin ($S=2$) Fe(II) heme.⁴⁸ Cryoreduction of ferrous WT P450 2B4 primarily creates an $S=3/2$ state in which a porphyrin-centered cryogenerated anion radical is antiferromagnetically coupled to the hs Fe(II) ($S=2$) ion.⁴⁸ This spin-coupled species is trapped in a nonequilibrium conformation characteristic of the ferrous precursor, and relaxes with internal electron transfer during annealing at $T > 175$ K to the $S=1/2$ Fe(I) center. In contrast, the Fe(I) state is the primary product trapped during the 77 K cryoreduction of ferrous F429H P450 2B4. This difference in electronic structures of the cryoreduced WT and mutant ferrous states trapped at 77 K reflects structural differences between heme centers in the ferrous precursors and/or electronic-structural differences in the cryoreduced state. DFT computations suggested that the different states may result because of a shorter Fe-S bond distance in $S=3/2$ spin state.⁵⁸

Oxyferrous State

Cryoreduction of the oxy-P450 substrate complex at 77 K generates EPR-active states that retain the conformation of the oxy-precursor^{9, 50,29, 34, 59} and thus provides a sensitive EPR and ENDOR probe of the diamagnetic oxy-ferrous precursor. EPR spectra show that the primary species trapped by 77 K cryoreduction of both WT and mutant oxy-2B4 is the hydroperoxy-ferriheme state. Previous cryoreduction studies with a variety of oxy-hemeproteins, including cytochromes P450^{50, 29,59} NOS,³⁴ heme oxygenase,³⁸ IDO⁶⁰ and oxy-globins^{35,61–63} showed that such proton transfer to the initial product of cryoreduction, the basic peroxo-ligand trapped at 77 K or below, requires the presence in the parent oxy-hemoprotein of an extended hydrogen-bonded proton delivery network that includes an ordered water molecule in the active site that is hydrogen bonded to the terminal oxygen of the bound dioxygen ligand, and that shuttles the proton to the peroxide ligand generated by

cryoreduction.^{64, 65} This situation has been shown crystallographically for the oxy-P450cam-camphor complex, in which a water molecule forms an H-bond to the distal oxygen of O₂.⁶⁶ Thus, comparison with previous measurements indicates that the ternary oxy-P450 2B4-substrate complex contains an active-site water molecule that H-bonds to the terminal oxygen of the dioxygen ligand. Previous studies have shown that the transferred proton can originate either from acid/base groups provided by amino acid residues within the active site,^{29, 50, 62} from bound substrate,³⁴ or from water clusters connected to the active site by a proton delivery network.³⁸

Properties of Ferric hydroperoxy intermediates

Although the F429H mutation slows the decay of this intermediate through protonation and formation of Cpd I, it has relatively little effect on the electronic structure of the hydroperoxo-ferri state, as shown by the absence of significant effects on the g-tensor and ¹H/¹⁴N ENDOR parameters for this state (Table 4). This correlates with electronic absorption and magnetic circular dichroism (MCD) studies for WT and the mutant oxyferrous P450 2B4 complexes, which show that the F429H mutation causes only a 2–3nm red shift of the absorption and MCD spectra.¹⁸ This is consistent with results from various cryogenerated ferric hydroperoxy intermediates of hemoproteins, which showed that the g-tensor components depend significantly on structure and geometry of the distal axial oxygenic ligands (O²⁻, OOH⁻), but relatively weakly on the nature of the proximal ligand (histidine, cysteine) (Table S1). In turn, this is in agreement with the recently reported results of theoretical calculations accomplished by Shaik and collaborators for cytochrome P450 2B4 and F429H mutant²⁷ and correlates with the reported weak influence of the electron donating ability of an aryl thiolate ligand on EPR properties of alkylperoxo-iron (III) species [Fe(III)([15]aneN4)(SR)(OOR).⁶⁷ The enhanced stability of the hydroperoxo-ferri state induced by the mutation may have its origin in an effect on the transient state formed by proton delivery to it.

Hydroxylation mechanism

Relaxation of the cryogenerated hydroperoxy intermediate of WT enzyme leads to quantitative hydroxylation of BHT, forming BHTOH which was quantified by a HPLC/MS/MS assay as previously described.²⁹ The data presented above for the WT enzyme, in particular, the characterization of the primary product of the decay of the hydroperoxo-ferric intermediate as a product-bound ferriheme state, indicates that the active hydroxylating species is Cpd I.

The yield of BHTOH upon cryoannealing the hydroperoxy intermediate of the F429H mutant is small (~4%). Neither WT nor mutant hydroxylates BP during cryoannealing, presumably because this substrate moves away from the heme at low temperatures, behavior that is independent of the mutation.²⁹ Nonetheless, the above data indicate that Cpd I forms during cryoannealing in these cases too. We propose that in these cases the majority of Cpd I is reduced by radiolytically generated radicals to the 1s aquaferric state.^{9,53} The alternative decay mode, protonation of the proximal oxygen of the hydroperoxy ligand to generate H₂O₂ which dissociates, would leave behind the 5c high-spin ferric form under these conditions, contrary to observation, not the low-spin aquo-ferric form, as observed.

Computations have suggested that the dramatic loss of the activity and NADPH consumption of the mutant in the hydroxylation of BHT and BP occurs because the mutation impairs formation of Cpd I. QM/MM calculations recently reported by Shaik and collaborators suggests that the F429H mutation may retard heterolytic cleavage in BP bound protein and thereby enhance homolytic O-OH cleavage to generate the active hydroxyl radical OH*, with the Thr302 water cluster and bound substrate structurally orienting the nascent radical OH*, so as to enforce meso-hydroxylation of the porphyrin.²⁷ However, as described above, even five hours of enzymatic reaction did not induce significant amounts of heme hydroxylation in the mutant. Moreover, the theoretical explanation implies a strong increase of uncoupling in the hydroxylation of BP by the mutant in comparison with WT P450 2B4. Instead, the kinetic studies on enzymatic hydroxylation of BP at ambient temperature presented above showed that the mutation F429H does not significantly change the extent of coupling (55% and 45% for WT and F429H mutant, respectively). These findings rule out a mutation-induced change in mechanism: Cpd I is the active species.

Although the mutation does not change the active species, it does change the reactivity of Cpd I. If the mutation had no effect on the intrinsic reactivity of Cpd I, then the mutation-induced decrease in the rate of cryo-conversion of the hydroperoxy intermediate into Cpd I would be expected to decrease the rate *but not yield* of product formation during relaxation of cryoreduced oxy ferrous P450 2B4. Thus, the observed ~ 4% cryo-yield of BHTOH for the F429 H mutant, compared to wt product cryo-yield of approximately 80%, may be explained by strongly diminished intrinsic reactivity of the Cpd I active species, which in turn results in an increased destruction of Cpd I through side reactions of Cpd I with radiolytically generated radicals.^{53,29} In support of this view, it is worth noting that the D251N mutation in P450cam, which slows the rate of hydroxylation of camphor at ambient temperatures, has no effect on the yield of product during annealing the cryoreduced ternary oxy P450cam-camphor complex²⁸.

The conclusion that an additional H-bond decreases the reactivity of Cpd I is in excellent agreement with the recent report by Green et al., who compared the properties of Cpd I of P450s, which have a single H-bond to axial thiolate, and chloroperoxidase which has two, as in the mutant enzyme studied here.²⁸ They showed that the lower reactivity of Cpd I in CPO, compared to that in P450, is associated with a decrease- in the strength of the Fe-S bond induced by the additional H-bond to thiolate. Such an influence of the added H-bond is also seen in the change in C-O stretching frequency of the Fe(II)C-O upon addition of the second thiolate H-bond in F429H 2B4. This frequency has been shown to correlate with the intrinsic reactivity of Cpd I.

The fact that uncoupling is high (~50%) in ambient-temperature hydroxylation of BP by both WT and mutant enzyme may be explained by dissociation of hydroperoxy intermediate through protonation of the proximal oxygen of the hydroperoxy ligand, leading to formation H₂O₂.^{1, 6} This high level of uncoupling is typical of microsomal cytochromes P450 and likely reflects a poor ability of the substrate to bind in the active site in a conformation consistent with substrate oxidation. In fact BHT hydroxylation by cryoreduced oxy WT P450 2B4 is characterized by higher percent coupling (~90%)²⁹ in comparison with BHT

under physiological conditions, presumably a result of decreased active site dynamics of the substrate and protein at cryogenic temperatures.

Reactivity of Cpd I

The above results are most consistent with the notion that Cpd I of the F429H mutant has much lower reactivity towards bound substrate than that of WT Cpd I. Cpd I abstracts a hydrogen atom from the substrate forming a Fe(IV)-OH intermediate, which rapidly recombines with the substrate radical to yield a hydroxylated product.⁶⁸ The ability of Fe(IV)=O (Cpd II) to abstract hydrogen scales with the strength of the O-H bond formed during H-bond abstraction. The energy of this bond is determined by the one-electron reduction potential of compound I and the pKa of compound II.⁶⁹ In other words, greater electron donation from the proximal ligand results in a higher pKa of Cpd II and enzymatic activity. The lower activity of F429H thus may be associated with decreased electron donation to the heme iron from the axial thiolate. DFT calculations indicate that the strength of the C-H bond of a substrate that can be abstracted by Cpd I with different axial ligands is largely determined by the pKa of the resultant Fe(IV)=O species. The ionization potential of Fe(IV)=O depends weakly on the proximal ligand,^{11, 22} whereas the pKa of Cpd II varies dramatically with the proximal ligand, reaching a value of ~ 12 when the proximal ligand is the P450 thiolate, but ~ 3.5 when His is the proximal ligand.^{69, 70}

For completeness, we note it has also been proposed that the proximal axial ligand involved in the hydrogen bond can also fine-tune the reactivity of Cpd I by varying the radical character of the oxo ligand in the catalytically active intermediate by the extent of delocalization of one oxidizing equivalent over the heme and the axial ligand.^{71–73} Valence tautomerism of the thiolate ligated oxoiron(IV)/porphyrin π -cation radical form with the oxoiron(V)/porphyrin species may likewise be proposed to play some role in cytochrome P450 reactions.^{72, 74, 7576, 77}

Implications of cryoreduction measurements for ambient-temperature kinetics

These findings reveal the influence of the added H-bond in P450 2B4 on the properties of the individual states that form during the activation of the oxy-ferrous enzyme for hydroxylation, but do not determine the cause of the lower activity of the mutant in catalyzing the hydroxylation of BP at ambient temperature. The F429H mutation both lowers the reactivity of Cpd I, and also strongly slows the decay of the hydroperoxoferric P450 2B4 at 176 K, by a factor of ~ 5 (Fig. S13). In principle, either one of these effects could lower the observed rate constant of the mutant at ambient temperature; the measurements reported here do not allow us to estimate the relative contribution of these factors.

Summary

Comparative studies on the influence of the hydrogen bond between the proximal Cys436 and His429 on the electronic structure of the heme iron in ferric and in cryoreduced ferrous forms and cryogenerated ferric hydroperoxy intermediates of WT cytochrome P450 2B4 and the mutant using EPR and ENDOR spectroscopy indicate that the introduction of an H-bond between His and the proximal cysteine decreases $S \rightarrow Fe$ electron donation and weakens the

Fe-S bond. This change has little effect on the electronic structure of the cryogenerated ferric hydroperoxo intermediate, but noticeably slows its decay during cryoannealing. In both the WT and the mutant enzyme this decay shows a significant solvent kinetic isotope effect associated with the proton-assisted conversion to Compound I. We confirm that Compound I is the catalytically active intermediate in both mutant and wild-type enzyme. However, we show that the intrinsic reactivity of Cpd I is decreased by the enhanced H-bonding to the cysteinyl sulfur, which diminishes Fe-S covalency and S→Fe electron donation. It is plausible that this change in electronic structure diminishes the reactivity of Cpd I by changing the pKa of the Fe(IV)=O moiety.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

P450	cytochrome P450
2B4	cytochrome P450 2B4
F429H	P450 2B4 F429H cytochrome P450 2B4
Am	amplitude modulation
BHT	butylated hydroxytoluene
BP	benzphetamine
BHTOH	hydroxymethylbutylated hydroxy toluene
CPI	(4-chlorophenyl) imidazole
Cpd I	compound I
Cpd 0	compound 0
CW	continuous wave
DFT	density functional theory
EPR	electron paramagnetic resonance
ENDOR	electron-nuclear double resonance
F	microwave frequency
KPi	potassium phosphate
P	microwave power

T	temperature
WT	wild type)

References

1. Sono M, Roach MP, Coulter ED, Dawson JH. Heme-containing oxygenases. *Chem. Rev.* 1996; 96:2841–2887. [PubMed: 11848843]
2. Denisov IG, Makris TM, Sligar SG, Schlichting I. Structure and chemistry of cytochrome P450. *Chem. Rev.* 2005; 105:2253–2277. [PubMed: 15941214]
3. Marletta MA, Hurshman AR, Rusche KM. Catalysis by nitric oxide synthase. *Curr. Opin. Chem. Biol.* 1998; 2:656–663. [PubMed: 9818193]
4. Zhu Y, Silverman RB. Revisiting heme mechanisms. A perspective on the mechanisms of nitric oxide synthase (NOS), heme oxygenase (HO), and cytochrome P450s (CYP450s). *Biochemistry.* 2008; 47:2231–2243. [PubMed: 18237198]
5. Ortiz de Montellano PR, De Voss JJ. Oxidizing species in the mechanism of cytochrome P450. *Natural Product Reports.* 2002; 19:477–493. [PubMed: 12195813]
6. Jung C. The mystery of cytochrome P450 compound I: A mini-review dedicated to Klaus Ruckpaul. *Biochim. Biophys. Acta.* 2011; 1814:46–57. [PubMed: 20558327]
7. Shaik S, Cohen S, Wang Y, Chen H, Kumar D, Thiel W. P 450 enzymes: Their structure, reactivity, and selectivity - modeled by QM/MM calculations. *Chem. Rev.* 2010; 110:949–1017. [PubMed: 19813749]
8. Hlavica P. Models and mechanisms of O-O bond activation by cytochrome P450. A critical assessment of the potential role of multiple active intermediates in oxidative catalysis. *Eur. J. Biochem.* 2004; 271:4335–4360. [PubMed: 15560776]
9. Davydov R, Hoffman BM. Active intermediates in heme monooxygenase reactions as revealed by cryoreduction/annealing, EPR/ENDOR studies. *Arch Biochem Biophys.* 2011; 507:36–43. [PubMed: 20854788]
10. Sivaramakrishnan S, Ouellet H, Matsumura H, Guan SH, Moenne-Loccoz P, Burlingame AL, Ortiz de Montellano PR. Proximal ligand electron donation and reactivity of the cytochrome P450 ferric-peroxo anion. *J. Am. Chem. Soc.* 2012; 134:6673–6684. [PubMed: 22444582]
11. Dey A, Jiang Y, Ortiz de Montellano P, Hodgson KO, Hedman B, Solomon EI. S K-edge XAS and DFT calculations on cytochrome P450: Covalent and ionic contributions to the cysteine-Fe bond and their contribution to reactivity. *J. Am. Chem. Soc.* 2009; 131:7869–7878. [PubMed: 19438234]
12. Mak PJ, Yang Y, Im S, Waskell LA, Kincaid JR. Experimental documentation of the structural consequences of hydrogen-bonding interactions to the proximal cysteine of a cytochrome P450. *Angew Chem Int Ed Engl.* 2012; 51:10403–10407. [PubMed: 22968976]
13. Galinato MG, Spolidakis T, Ballou DP, Lehnert N. Elucidating the role of the proximal cysteine hydrogen-bonding network in ferric cytochrome P450cam and corresponding mutants using magnetic circular dichroism spectroscopy. *Biochemistry.* 2011; 50:1053–1069. [PubMed: 21158478]
14. Lang J, Santolini J, Couture M. The conserved Trp-Cys hydrogen bond dampens the “push effect” of the heme cysteinyl proximal ligand during the first catalytic cycle of nitric oxide synthase. *Biochemistry.* 2011; 50:10069–10081. [PubMed: 22023145]
15. Matsumura H, Wakatabi M, Omi S, Ohtaki A, Nakamura N, Yohda M, Ohno H. Modulation of redox potential and alteration in reactivity via the peroxide shunt pathway by mutation of cytochrome P450 around the proximal heme ligand. *Biochemistry.* 2008; 47:4834–4842. [PubMed: 18363338]
16. Brunel A, Wilson A, Henry L, Dorlet P, Santolini J. The proximal hydrogen bond network modulates *Bacillus subtilis* nitric-oxide synthase electronic and structural properties. *J. Biol. Chem.* 2011; 286:11997–12005. [PubMed: 21310962]

17. Yoshioka S, Tosha T, Takahashi S, Ishimori K, Hori H, Morishima I. Roles of the proximal hydrogen bonding network in cytochrome P450cam-catalyzed oxygenation. *J. Am. Chem. Soc.* 2002; 124:14571–14579. [PubMed: 12465966]
18. Perera R, Sono M, Kinloch R, Zhang H, Tarasev M, Im SC, Waskell L, Dawson JH. Stabilization and spectroscopic characterization of the dioxygen complex of wild-type cytochrome P450 2B4 (CYP2B4) and its distal side E301Q, T302A and proximal side F429H mutants at subzero temperatures. *Biochim. Biophys. Acta.* 2011; 1814:69–75. [PubMed: 20637316]
19. Yang Y, Zhang H, Usharani D, Bu W, Im S, Tarasev M, Rwere F, Pearl NM, Meagher J, Sun C, Stuckey J, Shaik S, Waskell L. Structural and functional characterization of a cytochrome P450 2B4 F429H mutant with an axial thiolate-histidine hydrogen bond. *Biochemistry.* 2014; 53:5080–5091. [PubMed: 25029089]
20. Ost TWB, Munro AW, Mowat CG, Taylor PR, Pessegueiro A, Fulco AJ, Cho AK, Cheesman MA, Walkinshaw MD, Chapman SK. Structural and spectroscopic analysis of the F393H mutant of flavocytochrome P450 BM3. *Biochemistry.* 2001; 40:13430–13438. [PubMed: 11695889]
21. Ost TWB, Miles CS, Munro AW, Murdoch J, Reid GA, Chapman SK. Phenylalanine 393 exerts thermodynamic control over the heme of flavocytochrome P450 BM3. *Biochemistry.* 2001; 40:13421–13429. [PubMed: 11695888]
22. Kumar D, Sastry GN, de Visser SP. Axial ligand effect on the rate constant of aromatic hydroxylation by iron(IV)-oxo complexes mimicking cytochrome P450 enzymes. *J. Phys. Chem. B.* 2012; 116:718–730. [PubMed: 22132821]
23. Kang Y, Chen H, Jeong YJ, Lai W, Bae EH, Shaik S, Nam W. Enhanced reactivities of iron(IV)-oxo porphyrin π -cation radicals in oxygenation reactions by electron-donating axial ligands. *Chemistry A European Journal.* 2009; 15:10039–10046. S10039/10031-S10039/10046.
24. Takahashi A, Yamaki D, Ikemura K, Kurahashi T, Ogura T, Hada M, Fujii H. Effect of the axial ligand on the reactivity of the oxoiron(IV) porphyrin π -cation radical complex: Higher stabilization of the product state relative to the reactant state. *Inorg. Chem.* 2012; 51:7296–7305. [PubMed: 22716193]
25. Ogliaro F, de Visser SP, Shaik S. The ‘push’ effect of the thiolate ligand in cytochrome P450: A theoretical gauging. *J. Inorg. Biochem.* 2002; 91:554–567. [PubMed: 12237222]
26. Das PK, Chatterjee S, Samanta S, Dey A. EPR, resonance Raman, and DFT calculations on thiolate- and imidazole-bound iron(III) porphyrin complexes: Role of the axial ligand in tuning the electronic structure. *Inorg. Chem.* 2012; 51:10704–10714. [PubMed: 23013308]
27. Usharani D, Zazza C, Lai WZ, Chourasia M, Waskell L, Shaik S. A single-site mutation (F429H) converts the enzyme CYP 2B4 into a heme oxygenase: A QM/MM study. *J. Am. Chem. Soc.* 2012; 134:4053–4056. [PubMed: 22356576]
28. Krest CM, Silakov A, Rittle J, Yosca TH, Onderko EL, Calixto JC, Green MT. Significantly shorter Fe–S bond in cytochrome P450-I is consistent with greater reactivity relative to chloroperoxidase. *Nat Chem.* 2015; 7:696–702. PMID: PMC4580274 (available 2016-03-01). [PubMed: 26291940]
29. Davydov R, Razeghifard R, Im SC, Waskell L, Hoffman BM. Characterization of the microsomal cytochrome P450 2B4 O₂-activation intermediates by cryoreduction/EPR. *Biochemistry.* 2008; 47:9661–9666. [PubMed: 18700729]
30. Sheng X, Zhang H, Im SC, Horner JH, Waskell L, Hollenberg PF, Newcomb M. Kinetics of oxidation of benzphetamine by compounds I of cytochrome P450 2B4 and its mutants. *J. Am. Chem. Soc.* 2009; 131:2971–2976. [PubMed: 19209859]
31. Bridges A, Gruenke L, Chang YT, Vakser IA, Loew G, Waskell L. Identification of the binding site on cytochrome P450 2B4 for cytochrome b5 and cytochrome P450 reductase. *J. Biol. Chem.* 1998; 273:17036–17049. [PubMed: 9642268]
32. McKenna CE, Gutheil WG, Song W. A method for preparing analytically pure sodium dithionite. Dithionite quality and observed nitrogenase-specific activities. *Biochim. Biophys. Acta.* 1991; 1075:109–117. [PubMed: 1892862]
33. Glasoe PK, Long FA. Use of glass electrodes to measure acidities in deuterium oxide. *J. Phys. Chem.* 1960; 64:188–190.

34. Davydov R, Sudhamsu J, Lees NS, Crane BR, Hoffman BM. EPR and endor characterization of the reactive intermediates in the generation of NO by cryoreduced oxy-nitric oxide synthase from *G. stearothermophilus*. *J. Am. Chem. Soc.* 2009; 131:14493–14507. [PubMed: 19754116]
35. Davydov R, Osborne R, Shanmugam M, Du J, Dawson J, Hoffman B. Probing the oxyferrous and catalytically active ferryl states of *Amphitrite ornata* dehaloperoxidase by cryoreduction and EPR/ENDOR spectroscopy. Detection of compound I. *J. Am. Chem. Soc.* 2010; 132:14995–15004. [PubMed: 20925340]
36. Hoffman, BM.; Gurbel, RJ.; Werst, MM.; Sivaraja, M. Advanced EPR Applications in Biology and Biochem. Hoff, AJ., editor. Amsterdam: Elsevier; 1989. p. 541-591.
37. Sakamoto H, Omata Y, Palmer G, Noguchi M. Ferric alpha-hydroxyheme bound to heme oxygenase can be converted to verdoheme by dioxygen in the absence of added reducing equivalents. *J. Biol. Chem.* 1999; 274:18196–18200. [PubMed: 10373419]
38. Davydov R, Kofman V, Fujii H, Yoshida T, Ikeda-Saito M, Hoffman B. Catalytic mechanism of heme oxygenase through EPR and ENDOR of cryoreduced oxy-heme oxygenase and its Asp140 mutants. *J. Am. Chem. Soc.* 2002; 124:1798–1808. [PubMed: 11853459]
39. Ortiz de Montellano PR. Heme oxygenase mechanism: Evidence for an electrophilic, ferric peroxide species. *Acc. Chem. Res.* 1998; 31:543–549.
40. LeLean JE, Moon N, Dunham WR, Coon MJ. EPR spectrometry of cytochrome P450 2B4: Effects of mutations and substrate binding. *Biochem. Biophys. Res. Commun.* 2000; 276:762–766. [PubMed: 11027544]
41. Taylor CPS. The EPR of low spin heme complexes. *Biochim. Biophys. Acta.* 1977; 491:137–149. [PubMed: 191085]
42. Peisach J, Blumberg WE. Electron paramagnetic resonance study of the high-and low-spin forms of cytochrome p-450 in liver and in liver microsomes from a methylcholanthrene-treated rabbit. *Proc .Nat. Acad. Sci.* 1970; 67:172–179. [PubMed: 4318775]
43. Salerno JC, Frey C, McMillan K, Williams RF, Masters BSS, Griffith OW. Characterization by electron paramagnetic resonance of the interactions of L-arginine and L-thiocitrulline with the heme cofactor region of nitric oxide synthase. *J. Biol. Chem.* 1995; 270:27423–27428. [PubMed: 7499198]
44. Salerno JC, McMillan K, Masters BSS. Binding of intermediate, product, and substrate analogs to neuronal nitric oxide synthase: Ferriheme is sensitive to ligand-specific effects in the l-arginine binding site. *Biochemistry.* 1996; 35:11839–11845. [PubMed: 8794766]
45. Solomon EI, Zhou J, Neese F, Pavel EG. New insights from spectroscopy into the structure/function relationships of lipoxygenases. *Chem. Biol.* 1997; 4:795–808. [PubMed: 9384534]
46. Fann Y-C, Gerber NC, Osmulski PA, Hager LP, Sligar SG, Hoffman BM. ENDOR determination of heme ligation in chloroperoxidase and comparison with cytochrome P-450_{cam}. *J. Am. Chem. Soc.* 1994; 116:5989–5990.
47. LoBrutto R, Scholes CP, Wagner GC, Gunsalus IC, Debrunner PG. Electron nuclear double resonance of ferric cytochrome P450_{cam}. *J. Am. Chem. Soc.* 1980; 102:1167–1170.
48. Davydov R, Hoffman BM. EPR and ENDOR studies of Fe(II) hemoproteins reduced and oxidized at 77K. *J. Biol. Inorg. Chem.* 2008; 13:357–369. [PubMed: 18058139]
49. Kim SH, Yang T-C, Perera R, Jin S, Bryson TA, Sono M, Davydov R, Dawson JH, Hoffman BM. Cryoreduction EPR and ¹³C, ¹⁹F ENDOR study of substrate-bound substrates and solvent kinetic isotope effects in the catalytic cycle of cytochrome P450_{cam} and its T252A mutant. *Dalton Transactions.* 2005; 21:3464–3469. [PubMed: 16234926]
50. Davydov R, Dawson JH, Perera R, Hoffman BM. The use of deuterated camphor as a substrate in ¹H ENDOR studies of hydroxylation by cryoreduced oxy P450_{cam} provides new evidence of the involvement of compound I. *Biochemistry.* 2013; 52:667–671. [PubMed: 23215047]
51. Davydov R, Perera R, Jin S, Yang T-C, Bryson TA, Sono M, Dawson JH, Hoffman BM. Substrate modulation of the properties and reactivity of the oxy-ferrous and hydroperoxo-ferric intermediates of cytochrome P450_{cam} as shown by cryoreduction-EPR/ENDOR spectroscopy. *J. Amer. Chem. Soc.* 2005; 127:1403–1413. [PubMed: 15686372]
52. Davydov R, Makris TM, Kofman V, Werst DW, Sligar SG, Hoffman BM. Hydroxylation of camphor by reduced oxy-cytochrome P450_{cam}: Mechanistic implications of EPR and ENDOR of

- catalytic intermediates in native and mutant enzymes. *J. Am. Chem. Soc.* 2001; 123:1403–1415. [PubMed: 11456714]
53. Davydov R, Laryukhin M, Ledbetter-Rogers A, Sono M, Dawson JH, Hoffman BM. Electron paramagnetic resonance and electron-nuclear double resonance studies of the reactions of cryogenerated hydroperoxoferric-hemoprotein intermediates. *Biochemistry.* 2014; 53:4894–4903. [PubMed: 25046203]
54. Tejero J, Biswas A, Wang Z-Q, Page RC, Haque MM, Hemann C, Zweier JL, Misra S, Stuehr DJ. Stabilization and characterization of a heme-oxy reaction intermediate in inducible nitric-oxide synthase. *J. Biol. Chem.* 2008; 283:33498–33507. [PubMed: 18815130]
55. Dey A, Okamura T, Ueyama N, Hedman B, Hodgson KO, Solomon EI. Sulfur K-edge XAS and DFT calculations on P450 model complexes: Effects of hydrogen bonding on electronic structure and redox potentials. *J. Am. Chem. Soc.* 2005; 127:12046–12053. [PubMed: 16117545]
56. Ost TW, Clark J, Mowat CG, Miles CS, Walkinshaw MD, Reid GA, Chapman SK, Daff S. Oxygen activation and electron transfer in flavocytochrome P450 BM3. *J. Am. Chem. Soc.* 2003; 125:15010–15020. [PubMed: 14653735]
57. Ueyama N, Nishikawa N, Yamada Y, Okamura T, Nakamura A. Cytochrome P450 Model (Porphinato)(thiolato)iron(III) Complexes with Single and Double NH S Hydrogen Bonds at the Thiolate Site. *J. Am. Chem. Soc.* 1996; 118:12826–12827.
58. Porro CS, Kumar D, de Visser SP. Electronic properties of pentacoordinated heme complexes in cytochrome P450 enzymes: Search for an Fe(I) oxidation state. *Phys. Chem. Chem. Phys.* 2009; 11:10219–10226. [PubMed: 19865780]
59. Davydov R, Perera R, Jin S, Yang T-C, Bryson TA, Sono M, Dawson JH, Hoffman BM. Substrate modulation of the properties and reactivity of the oxy-ferrous and hydroperoxoferric intermediates of cytochrome P450cam as shown by cryoreduction-EPR/ENDOR spectroscopy. *J. Am. Chem. Soc.* 2005; 127:1403–1413. [PubMed: 15686372]
60. Davydov RM, Chauhan N, Thackray SJ, Anderson JLR, Papadopoulou ND, Mowat CG, Chapman SK, Raven EL, Hoffman BM. Probing the ternary complexes of indoleamine and tryptophan 2,3-dioxygenases by cryoreduction EPR and ENDOR spectroscopy. *J. Am. Chem. Soc.* 2010; 132:5494–5500. [PubMed: 20353179]
61. Davydov R, Kofman V, Nocek J, Noble RW, Hui H, Hoffman BM. Conformational substates of the oxyheme centers in a and b subunits of hemoglobin as disclosed by EPR and ENDOR studies of cryoreduced protein. *Biochemistry.* 2004; 43:6330–6338. [PubMed: 15147217]
62. Davydov R, Satterlee JD, Fujii H, Sauer-Masarwa A, Busch DH, Hoffman BM. A superoxo-ferrous state in a reduced oxy-ferrous hemoprotein and model compounds. *J. Am. Chem. Soc.* 2003; 125:16340–16346. [PubMed: 14692776]
63. Kappl R, Höhn-Berlage M, Hüttermann J, Bartlett N, Symons MCR. Electron spin and electron nuclear-double resonance of the [FeO₂] centre from irradiated oxyhemo- and oxymyoglobin. *Biochim. Biophys. Acta.* 1985; 827:327–343.
64. Guallar V, Harris DL, Batista VS, Miller WH. Proton-transfer dynamics in the activation of cytochrome P450ERYF. *J. Am. Chem. Soc.* 2002; 124:1430–1437. [PubMed: 11841312]
65. Kumar D, Altun A, Shaikh S, Thiel W. Water as biocatalyst in cytochrome P450. *Faraday Discuss.* 2011; 148:373–383. discussion 421–341. [PubMed: 21322494]
66. Schlichting I, Berendzen J, Chu K, Stock AM, Maves SA, Benson DE, Sweet BM, Ringe D, Petsko GA, Sligar SG. The catalytic pathway of cytochrome P450cam at atomic resolution. *Science.* 2000; 287:1615–1622. [PubMed: 10698731]
67. Namuswe F, Kasper GD, Sarjeant AA, Hayashi T, Krest CM, Green MT, Moenne-Loccoz P, Goldberg DP. Rational Tuning of the Thiolate Donor in Model Complexes of Superoxide Reductase: Direct Evidence for a trans influence in Fe^{III}-OOR Complexes. *J. Am. Chem. Soc.* 2008; 130:14189–14200. [PubMed: 18837497]
68. Groves JT, McCluskey GA. Aliphatic hydroxylation via oxygen rebound. Oxygen transfer catalyzed by iron. *J. Am. Chem. Soc.* 1976; 98:859–861.
69. Behan RK, Green MT. On the status of ferryl protonation. *J. Inorg. Biochem.* 2006; 100:448–459. [PubMed: 16500711]

70. Stone KL, Behan RK, Green MT. Resonance Raman spectroscopy of chloroperoxidase compound II provides direct evidence for the existence of an iron(IV)-hydroxide. *Proc. Nat. Acad. Sci. USA*. 2006; 103:12307–12310. [PubMed: 16895990]
71. Weiss R, Mandon D, Wolter T, Trautwein AX, Muther M, Bill E, Gold A, Jayaraj K, Turner J. Delocalization over the heme and the axial ligands of one of the two oxidizing equivalents stored above the ferric state in the peroxidase and catalase Compound-I intermediates: Indirect participation of the proximal axial ligand of iron in the oxidation reactions catalyzed by heme-based peroxidases and catalases? *J. Biol. Inorg. Chem.* 1996; 1:377–383.
72. Hryciay EG, Bandiera SM. The monooxygenase, peroxidase, and peroxygenase properties of cytochrome P450. *Arch Biochem Biophys.* 2012; 522:71–89. [PubMed: 22266245]
73. Champion PM. Elementary electronic excitations and the mechanism of cytochrome-P450. *J. Am. Chem. Soc.* 1989; 111:3433–3434.
74. Weiss R, Bulach V, Gold A, Turner J, Trautwein AX. Valence-tautomerism in high-valent iron and manganese porphyrins. *J. Biol. Inorg. Chem.* 2001; 6:831–845. [PubMed: 11713691]
75. Zhang R, Newcomb M. Laser flash photolysis generation of high-valent transition metal-oxo species: Insights from kinetic studies in real time. *Acc. Chem. Res.* 2008; 41:468–477. [PubMed: 18278877]
76. Isobe H, Yamanaka S, Okumura M, Yamaguchi K, Shimada J. Unique structural and electronic features of perferryl-oxo oxidant in cytochrome P450. *J. Phys. Chem. B.* 2011; 115:10730–10738. [PubMed: 21812482]
77. Isobe H, Yamaguchi K, Okumura M, Shimada J. Role of perferryl-oxo oxidant in alkane hydroxylation catalyzed by cytochrome P450: A hybrid density functional study. *J. Phys. Chem. B.* 2012; 116:4713–4730. [PubMed: 22510212]
78. Blumberg, WE.; Peisch, J. *Probes of Structure and Function of Macromolecules and Membranes*. Chance, B.; Yonetani, T.; Mildvan, SA., editors. New York: Academic Press; 1971. p. 215-229.

Fig. 1.

A: CW X-band EPR spectra of 0.5mM low-spin ferric WT P450 2B4 and mutant F429H P450 2B4 in the presence of 1mM BP in 20% glycerol, 0.1M KPi buffer pH 8. Instrument settings: Amplitude modulation (Am) 10 G, microwave power (P) 2 mW, microwave frequency (F) 9.372 GHz, Temperature (T)=30 K.

B: CW X-band EPR spectra of 0.5mM high-spin ferric WT P450 2B4 and mutant F429H P450 2B4 in the presence of 2mM BHT in 20% glycerol, 0.1M KPi buffer pH 8 (black) and their simulations (red). The large signal next to the low spin gx band is from a contaminant which is visible at T 10K. Simulations were performed using the program *SpinCount* (www.chem.cmu.edu/groups/hendrich/facilities/index.html) using parameters: WT S=5/2; (g=2;2;2); D=7cm⁻¹; E/D=0.108; s E/D=0.005; line width=10 G; F429H S=5/2; (g=2;2;2); D=7cm⁻¹; E/D=0.108; s E/D=0.004; line width=10 G. Instrument settings: Amplitude modulation (Am) 10 G, microwave power (P) 2 mW, microwave frequency (F) 9.372 GHz, Temperature (T)=10 K.

Fig. 2.

^{14}N CW ENDOR spectra of low-spin ferric WT P450 2B4-BP and mutants T302A, E301Q and F429H P450 2B4 taken at g_{max} . Instrument settings: Amplitude Modulation (Am) 1 G, Temperature (T) 2 K, Radio frequency (rf) sweep rate 0.2 MHz/s, bandwidth broadening 20 kHz, 20 scans

Fig. 3. 35 GHz 2K EPR spectra of ferrous WT P450 2B4 (A), mutants F429H (C) and T302A P450 2B4 (D) and ferrous P450cam (E) exposed to γ -irradiation at 77 K with dose 2.5 Mr. Spectrum (B) is WT ferrous P450 2B4 irradiated at 77 K and then annealed at 180 K for 1 min. Instrument settings: Amplitude Modulation (Am) 1 G, Microwave frequency (F) 34.90 GHz, Temperature (T) 2 K.

Fig. 4. CW X-band EPR spectra of cryoreduced ternary complex of oxyferrous WT P450 2B4-BHT annealed at indicated temperatures. In the spectra strong signals at $g=2.0$ from radiolytically generated radicals have been deleted for clarity. Instrument settings as in Fig. 1

Fig. 5. 2 K 35 GHz CW EPR spectra of cryoreduced ternary complex of oxy WT P450 2B4- BP annealed at indicated temperatures for 2 min. Instrument settings: Amplitude modulation (Am) 1 G, Microwave frequency (F) 34.94 GHz, Temperature (T) 2 K.

Fig. 6. 35 GHz CW ^1H ENDOR spectra of cryogenerated iron(III) hydroperoxy intermediate of WT P450 2B4 and F429H P450 in the presence of BHT and BP 60 % glycerol, 0.1 M potassium phosphate buffer, 0.3M NaCl, in H_2O (pH 8) (solid line) and 60% d_3 -glycerol, 0.1M potassium phosphate, 0.3M NaCl in D_2O (pD8) (dotted line) taken at g_{max} . Instrument settings: Amplitude modulation (Am) 1 G, Temperature (T) 2 K, Radio frequency (rf) sweep rate 0.5MHz/s, bandwidth broadening 60 kHz, 20 scans.

Fig. 7.
 CW X-band EPR spectra of a cryoreduced ternary complex of oxy F429H P450 2B4-BHT annealed at the indicated temperatures. Instrument settings: Amplitude modulation (Am)10 G, microwave power (P)10 mW, microwave frequency (F) 9.372 GHz, Temperature (T) 30 K.

Fig. 8.
2K 35 GHz CW EPR spectra of cryoreduced ternary complex of oxy F429H P450 2B4 BP annealed at the indicated temperatures. Instrument settings: Amplitude modulation (Am)1 G, microwave frequency (F) 34.89 GHz, Temperature (T) 2 K.

Fig. 9. 35 GHz CW ^{14}N ENDOR spectra for cryogenerated Fe(III)-OOH intermediates of WT P450 2B4 and F429H P450 2B4 in the presence of BHT and BP taken at g_{max} . Instrument settings as in Fig. 2.

Scheme 1.

Table 1

EPR parameters for 6 coordinate low-spin ($S=1/2$) forms of ferric cytochrome P450 2B4, three mutants and complexes with 4-chlorophenyl imidazole (4-CPI).

P450 2B4	6th ligand	g-values	V/λ^b	λ^b
WT,	H ₂ O	2.42; 2.24; 1.926	3.0	6.4
T302A	H ₂ O	2.405; 2.246, 1.924		
E301Q ^a	H ₂ O	2.425; 2.245; 1.926		
F429H ^a	H ₂ O major	2.53; 2.27; 1.88	3.0	5.4
	H ₂ O minor	2.47; 2.27 ; 1.93		
WT+CPI	CPI	2.52; 2.26; 1.88	3.2	5.5
F429H+CPI	CPI	2.62; 2.27; 1.84	3.2	4.9
WT+BHTOH	BHTOH minor	2.55; 2.27; 1.88		
	H ₂ O major	2.41; 2.27; 1.99		
	H ₂ O minor	2.44, 2.26; 1.94		
F429H+BHTOH	H ₂ O major	2.50; 2.28; ~1.91		
	H ₂ O minor	2.55; 2.28; ~1.91		
	BHTOH or H ₂ O Minor	2.46; 2.28; ~1.91		

^aIn presence of BP

^bCrystal field parameters associated with splitting of the occupied T₂ orbitals of Fe^{78} is the axial splitting, V is the rhombic splitting, λ is the spin-orbit coupling constant. Parameters were calculated with the procedure originally used by Peisach and Blumberg,⁷⁸ i.e., $g_y > -g_x > -g_z > 0$

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Table 2
EPR parameters for high-spin forms of ferric WT cytochrome P450 2B4 and its F429H variant

P450	Substrate	g_x	g_y	g_z	E/D^a
WT ^a	BHT	8.11	3.52	1.68	0.11
T302A ^a	BHT	8.10	3.55	1.68	0.11
E301Q ^a	BHT	8.07	3.62	1.70	0.10
F429H ^a	BHT	7.71	4.03	1.79	0.08
WT ^b	BHTOH	8.11	3.55	-1.70	
F429H ^b	BHTOH	7.58	-4.20	1.81	

^a E/D values estimated from simulation of the EPR spectra

Table 3

Spin Hamiltonian parameters used for simulation of the 2D ENDOR patterns of ^1H and ^{14}N . Nuclei of ferri WT P450 2B4 and mutant F429H P450 2B4.

Hyperfine and quadrupolar tensors (MHz)						
Protein	WT ^b	F429H ^b	WT ^c	F429H ^c	WT ^d	F429H ^d
Nuclei	$^1\text{H}_A$	$^1\text{H}_A$	$^{14}\text{N}_A$; $\text{P}(^{14}\text{N}_A)$	$^{14}\text{N}_A$; $\text{P}(^{14}\text{N}_A)$	$^{14}\text{N}_B$; $\text{P}(^{14}\text{N}_B)$	$^{14}\text{N}_B$; $\text{P}(^{14}\text{N}_B)$
A ₁	-4.9	-5.2	5.7 (0.32)	6.1 (0.32)	5.1 (0.32)	5.3 (0.3)
A ₂	-4.9	-5.2	4.2 (0.64)	4.7 (0.61)	5.0 (0.64)	5.0 (0.61)
A ₃	+9.8	+9.8	5.1 (-0.96)	5.3 (-0.93)	5.0 (-0.96)	5.0 (-0.91)
<i>d_{iso}</i>	0	0.2	5.0	5.4	5.03	5.1

^aIn the simulations used $\mathbf{g} = [2.424; 2.24, 1.926]$ for WT and $\mathbf{g} = [2.425; 2.27, 1.88]$ for F429H

^bEuler angles for $^1\text{H}_A$ of WT and F429H are $\alpha = 0^\circ$, $\beta = 63^\circ$, $\gamma = 25^\circ$; EPR line width = 200 MHz, ENDOR line width = 0.4MHz.

^{c,d}For $^{14}\text{N}_A$ of WT and F429H the hyperfine and quadrupolar tensors are coaxial with $\mathbf{g}$, whereas for $^{14}\text{N}_B$ the quadrupolar tensor is rotated, $\alpha = 90^\circ$, $\beta = 0^\circ$, $\gamma = 0^\circ$. EPR line width = 200 MHz, ENDOR line width = 0.25 MHz.

Table 4

g- Values for the hydroperoxo-ferric WT P450 2B4 and F429H P450 2B4 complexes formed by cryoreduction of the corresponding ferrous oxy complexes in the presence BHT and BP.

Enzyme	Addition	Species	g ₁	g ₂	g ₃
WTP450 2B4	BHT		2.317	2.176	1.94
WTP450 2B4	BP major minor	A	2.28	2.17	nd
		B	2.30	2.17	nd
F429H	BHT major minor		2.30	2.175	nd
			2.28	2.17	nd
F429H	BP		2.29	2.17	nd

Table 5

Spin-Hamilton parameters used for simulation ^1H ENDOR spectra of cryogenerated hydroperoxy intermediates of WT and F429H ferric P450.

	^1H-Hyperfine Tensors of Cytochrome-P450 2B4/MHz			
Type	WT + BHT^b	F429H + BHT^b	WT + BP^b	F429H + BP^b
nuclei	^1H	^1H	^1H	^1H
A_1	1.8	2.0	2.0	2.0
A_2	10.6	10.6	10.2	10.6
A_3	10.0	9.8	9.6	9.8
a_{iso}	7.5	7.5	7.3	7.5

Simulations employed

$\mathbf{g} = [2.317, 2.176, 1.96]$ for WT + BHT

$\mathbf{g} = [2.302, 2.175, 1.94]$ for F429H + BHT

$\mathbf{g} = [2.29, 2.18, 1.97]$ for WT + BP.

$\mathbf{g} = [2.296, 2.181, 1.97]$ for F429H + BP

^bEuler angles for H of WT and F429H are, $\alpha = 0^\circ, \beta = 80^\circ, \gamma = 10^\circ$ (WT) 12° (mutants); EPR line width = 200 MHz, ENDOR line width = 0.8 MHz (WT) and 0.85 MHz (mutants).