Cytochrome P450 4B1 (4B1) functions in both xenobiotic and endobiotic metabolism. An ester linkage between Glu-310 in 4B1 and the 5-methyl group of heme facilitates preferential hydroxylation of terminal (ω) methyl groups of hydrocarbons (HCs) and fatty acids compared with ω–1 sites bearing weaker C–H bonds. This preference is retained albeit diminished 4-fold for the E310A mutant, but the reason for this is unclear. Here, a crystal structure of the E310A–octane complex disclosed that noncovalent interactions maintain heme deformation in the absence of the ester linkage. Consistent with the lower symmetry of the heme, resonance Raman (RR) spectroscopy revealed large enhancements of RR peaks for high-spin HC complexes of 4B1 and the E310A mutant relative to P450 3A4. Whereas these enhancements were diminished in RR spectra of a low-spin 4B1–N-hydroxy-N′-(4-butyl-2-methylphenyl)formamidine complex, a crystal structure indicated that this inhibitor does not alter heme ruffling. RR spectra of Fe^{2+}–CO HC complexes revealed larger effects of HC length in E310A than in 4B1, suggesting that reduced rigidity probably underlies increased E310A-catalyzed (ω–1)-hydroxylation. Diminished effects of the HC on the position of the Fe–CO stretching mode in 4B1 suggested that the ester linkage limits substrate access to the CO. Heme ruffling probably facilitates autocatalytic ester formation by reducing inhibitory coordination of Glu-310 with the heme iron. This also positions the 5-methyl for a reaction with the proposed glutamyl radical intermediate and potentially enhances oxo-ferryl intermediate reactivity for generation of the glutamyl radical to initiate ester bond formation and ω-hydroxylation.

Cytochrome P450 4B1 (4B1) maintains functional niches in both xenobiotic disposition and endobiotic metabolism (1–3). In the xenobiotic realm, 4B1 transforms the anticonvulsant valproic acid (VPA) to 5-hydroxy-VPA and to a lesser extent 4-hydroxy-VPA and 4-ene-VPA (4). The latter metabolite is a potent inhibitor of fatty acid β-oxidation and a hepatotoxin (5–8). 4B1 is also involved in the bioactivation of the pro-pnemotoxins 4-ipomeanol (9) and 4-methylindole (10) as well as aromatic amines tied to bladder cancers (11, 12).

4B1 has the remarkable ability to selectively target the terminal (ω) carbon atom of aliphatic substrates. This is intriguing because hydrogen atom abstraction from the penultimate (ω–1) carbon atoms is thermodynamically more facile (1). Thus, to obtain high ω/ω–1 ratios, the enzyme active site must restrain the substrate to restrict access of the oxo-ferryl intermediate (Compound I) to less reactive primary carbon–hydrogen bonds. The most unusual feature that has apparently evolved to confer regioselectivity is an ester bond between the heme 5-methyl and a glutamic acid (Glu-310 in 4B1; Fig. 1A), likewise conserved in other ω-hydroxylases of the CYP4 family (13, 14). Family 4 P450s contribute physiologic processes that are important in formation of the water barrier in skin (15), generation of signaling molecules (16, 17), and elimination of drugs and excess nutrients (18, 19).

A crystal structure of the rabbit 4B1–octane complex recently reported by two of authors (M.-H. H. and E. F. J.) revealed an active site optimized to accommodate octane in an extended all-trans-conformation with the terminal methyl group closest to the heme iron (20). The Glu-310 side chain forms part of the octane-binding surface, consistent with the notion that this feature restricts access of the reactive intermediate to the ω position. The heme is highly ruffled in the 4B1 structure, which may reflect the ester linkage, as seen in mammalian peroxidases. Heme ruffling contributes to the capacity of mammalian peroxidases to oxidize halide ions (21) and may also contribute to the capacity of family 4 P450s to oxidize ω-halogen atoms (22). Accordingly, the glutamic acid residue is normally an alanine or glycine in most P450s as well as for human family 4 P450s that do not exhibit capacities for ω-hy-
Heme ruffling in CYP4B1

Figure 1. Structures of the 5-methyl-esterified heme (A) and HET0016 (B).

hydroxylation. Surprisingly, the 4B1 E310A mutant retains a preference for octane \( \omega \)-hydroxylation, albeit with a 4-fold loss in \( \omega/\omega-1 \) regioselectivity compared with the WT enzyme while maintaining a similar overall rate of metabolite formation. These observations suggest that other aspects of the active site contribute to preferences for \( \omega \)-hydroxylation (20).

To identify features that contribute to \( \omega \)-hydroxylation of octane in the absence of the ester linkage, the structure of E310A mutant complexed with octane was determined and compared with the WT 4B1 structure. In contrast to peroxides, significant ruffling of the heme was observed in the E310A mutant. Resonance Raman (RR) spectroscopy, which permits highly selective measurement of vibrational spectra of the heme chromophore with little or no background from solvent or protein, provided corroboratory evidence for heme distortion in both proteins. Additionally, carbon monoxide is exploited as an environmental probe of HC-excluded volume near the heme iron in 4B1 and the E310A mutant. Together with the previously determined X-ray crystal structure of the 4B1–octane complex, the RR studies and newly determined structures of the 4B1–HET0016 and 4B1–E310A octane complexes reported here contribute to our understanding of the structural adaptations evolved to enable \( \omega \)-regioselectivity and selective inhibition by HET0016.

Results

Structural characterization of the octane complex of the CYP4B1 E310A mutant

To better understand the effects of the E310A mutation on the active site architecture, heme conformation, and substrate binding, the structure of the octane complex of the E310A mutant was determined by X-ray crystallography to a limiting resolution of 2.675 Å with an \( R/R_{\text{free}} \) of 18.9%/23.8% (Table 1) using conditions like those used for WT 4B1 (20). The structure of the E310A mutant is highly similar to that of the WT 4B1 octane complex with an RMSD of 0.2 Å using the “align” command in PyMOL for alignment of 3295 of the 3845 atoms after five cycles of refinement to exclude atoms exhibiting \( >2\sigma \) RMSD. As shown in Fig. 2A, most of the amino acid residues that form the substrate-binding cavity are unaffected by alanine substitution when the WT 4B1 structure (green carbons) is superimposed on the structure of the E310A mutant (yellow carbons). In the absence of Glu-310 and the ester linkage with the 5-methyl of the heme, the Ala-310 C\( ^{8} \) is positioned differently relative to the C\( ^{6} \) of the esterified glutamic acid, and Leu-122 shifts into a portion of the space that would otherwise be occupied by the glutamic acid ester. Octane, which contacts the Ala-310 C\( ^{8} \) and Thr-314 C\( ^{\gamma} \), moves outward in the cavity, whereas Thr-314 adopts a more ideal rotamer that is closer to the Ala-310 C\( ^{\delta} \). The position of the octane is well-defined by a substrate omit map contoured at 1\( \sigma \) around the substrate (Fig. 2A), and the terminal methyl near the heme iron where Compound I is formed is positioned 3.5 Å from the iron. The conformation of octane in the E310A mutant exhibits the low-energy, all-trans-conformation seen in the WT enzyme (20), but there is a greater out-of-plane deflection of the terminal methyl group near the distal end of the binding cavity resulting from the displacement of octane by positioning of Ala-310. The repositioning of Leu-485 is likely to reflect the change in octane’s location. Additionally, there is a consistent shift in position of the tetrapyrrrole core (RMSD 0.3 Å) that is likely to reflect the loss of the ester linkage. Observed differences in the vinyl groups and the two propionates on the periphery are attributable to adaptation to their protein environments. These differences are lower than estimates of the upper limit for coordinate error (0.37 Å) based on \( R_{\text{free}} \) but they are larger than the RMSD of 0.19 Å observed for superposition of the protein atoms and the minimal estimate of the coordinate error of 0.137 Å. Additionally, the \( B \)-factors for the heme, the substrate, and nearby residues are significantly lower than average values for the protein (Table 1), which would reduce coordinate error for this portion of the structure.

Like the WT enzyme, the pyrrole rings of the heme are rotated out of the plane defined by the iron and the four nitrogens, which is supported by the heme omit map contoured at 1\( \sigma \) around the heme in Fig. 2B. To refine the structure of the distorted heme, the DivCon 6 Phenix plugin (23) was employed.
for a priori restraint of the geometry during refinement against the structure factors as described previously for the octane complex of the WT 4B1 (20). This uses the semi-empirical PM6 Hamiltonian (24), which requires closed shells. For this purpose, the high-spin (HS) ferric heme iron was modeled as a low-spin (LS) ferrous ion, which might impart a bias to iron–nitrogen bond lengths, which would be larger for the ferric HS pose, the high-spin (HS) ferric heme iron was modeled as a complex of the WT 4B1 (20). This uses the semi-empirical PM6 superimposed rather than the hemes. These corresponding atoms of the hemes when the E310A mutant and the placement of the 5-methyl within reach of Glu-310. There may also be a small effect of the covalent linkage by 1.0 Å in the E310A mutant (Fig. 2D). This effectively positions the methyl group for reaction with the proposed glutamate radical intermediate for formation of the ester bond (25). The juxtaposition of contacts between residues on helix I and the upper surface of the heme and with the axial cysteine ligand and adjacent residues below the heme surface also counter the effects of the 6-propionate (Fig. 2B). The bulky Ile-449 resides underneath the 5-methyl carbon and the β-meso-carbon, which exhibit the largest displacements from an idealized model of a planar heme, and the Ile-449 Cγ2 contacts the 5-methyl carbon and β-meso-carbon.

When the heme of the 4B1 structure is superimposed on the heme of the E310A structure, the RMSD differences between all 43 atoms is 0.15 Å (Fig. 2D). The ruffling of the hemes in the two structures is highly similar, with the largest difference of 0.3 Å evident for 5-methyl carbon that is linked to Glu-310 by the ester bond, and this difference is like the differences between corresponding atoms of the hemes when the E310A mutant and 4B1 structures are superimposed rather than the hemes. These considerations indicate that the ruffling of the heme is induced by noncovalent interactions between the protein and the heme, and the ruffling is likely to facilitate the formation of the ester by the placement of the 5-methyl within reach of Glu-310. There may also be a small effect of the covalent linkage on the out-of-plane distortion and on the positioning of the heme relative to the protein that alters the relationship of the propionates with hydrogen-bonded partners (Fig. 2, A, C, and D).

### Table 1

Data processing and model refinement statistics

| Protein complex                   | 4B1 E310A–octane complex | 4B1–HET0016 complex |
|-----------------------------------|--------------------------|---------------------|
| PDB code                          | 6C93                     | 6C94                |
| Data reduction                    |                          |                     |
| Space group                       | P 32 2 1                 | P 32 2 1            |
| Unit cell dimensions              | a, b, c (Å)              | 109.435, 109.435, 126.443 | 109.891, 109.891, 126.659 |
| Nominal resolution range (Å)      | 38.51 to 2.674 (2.80 to 2.674) | 38.59 to 2.72 (2.85 to 2.72) |
| Unique reflections                | 24,818 (3162)            | 24,218 (3145)       |
| Resolution range (Å)              | 38.51 to 2.674 (2.781 to 2.674) | 38.59 to 2.72 (2.8291 to 2.7202) |
| Completeness (%)                  | 98.17 (94.0)             | 99.8 (99.0)         |
| Test set (%)                      | 4.93                     | 4.97                |
| Rwork (%)                         | 18.94 (26.33)            | 19.3 (25.06)        |
| Rfree (%)                         | 6.93                     | 7.25               |
| Mean B factors (Å²)               | 24,776 (2609)            | 24,175 (2632)       |
| Complete (%)                      | 98.17 (94.0)             | 99.8 (99.0)         |
| Mean B factors (Å²)               | 19.94 (26.33)            | 19.3 (25.06)        |
| Heavy atoms                       | 23.78 (29.55)            | 24.97 (29.77)       |
| Mean B factors (Å²)               | 3951                     | 4018               |
| Protein                           | 40.2                     | 41.3               |
| Heme                              | 51.2                     | 38.62              |
| Ligand                            | 57.2                     | 39.18              |
| Solvent                           | 54.9                     | 41.3               |
| Model refinement                  |                          |                     |
| Resolution range (Å)              | 38.51 to 2.674 (2.781 to 2.674) | 38.59 to 2.72 (2.8291 to 2.7202) |
| Unique reflections                | 24,818 (3162)            | 24,218 (3145)       |
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| Mean B factors (Å²)               | 3951                     | 4018               |
| Protein                           | 40.2                     | 41.3               |
| Heme                              | 51.2                     | 38.62              |
| Ligand                            | 57.2                     | 39.18              |
| Solvent                           | 54.9                     | 41.3               |
| Model quality                     |                          |                     |
| RMSD bond lengths (Å)             | 0.013                    | 0.014              |
| RMSD bond angles (degrees)        | 1.055                    | 1.16               |
| Ramachandran plot Molprobity      |                          |                     |
| Favored (%)                       | 96.2                     | 95.83              |
| Allowed (%)                       | 3.80                     | 3.00               |
| Outliers (%)                      | 0.00                     | 1.17               |

Heme ruffling in CYP4B1

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11435
Resonance Raman spectroscopy of Fe(III) 4B1 and the E310A mutant

RR spectroscopy is very sensitive to out-of-plane heme deformations and the effects of covalent linkages on heme dynamics that may be too small to document by X-ray crystallography. RR spectra of the two 4B1 proteins were examined to identify similarities and differences in the presence of HC substrates and HET0016. RR spectra were also obtained for P450 3A4 as an example of a P450 with a planar heme. Assignments are based on those made in other heme proteins and model compounds (26). The spectra at high frequencies were normalized to the height of the band at 1375–1376 cm\(^{-1}\), whose position is consistent with the ferric oxidation state of the proteins (Fig. 3). The high-frequency region contains vibrational normal modes of the porphyrin core that are sensitive to the oxidation state (\(\nu_4\)) and spin state (\(\nu_3\)) as well as those that are both sensitive to the spin state and coordination environment (\(\nu_5\) and \(\nu_{10}\)). The \(\nu_3\), \(\nu_5\), and \(\nu_{10}\) bands in the spectra of ligand-free WT 4B1 occurred at 1505, 1587, and 1639 cm\(^{-1}\) and for 3A4 at 1503, 1584, and 1638 cm\(^{-1}\), indicating that the LS state dominates in these samples. RR spectra of the complex with HET0016 are indicative of a six-coordinate, LS complex arising from coordination of a formamidine nitrogen (Fig. 1B) to the heme iron. In agreement with UV-visible experiments, downward shifts of the \(\nu_3\), \(\nu_5\), and \(\nu_{10}\) bands to 1490–1491, 1572, and 1627 cm\(^{-1}\) following the addition of HCs to 4B1 and to 1489, 1572, and 1626 cm\(^{-1}\) following testosterone addition to 3A4 reflect the HS conversion due to displacement of the heme-coordinated water. For the ligand-free E310A mutant, the \(\nu_3\), \(\nu_5\), and \(\nu_{10}\) bands in the spectra occur at 1504, 1585, and 1642 cm\(^{-1}\), with a 1490 cm\(^{-1}\) shoulder on \(\nu_3\) due to a minor HS population (Fig. 3B). As expected from the ligand-free results, bands arising from the HS enzyme disappear in the presence of HET0016.

Whereas the shifts observed in the 3A4, WT 4B1 and E310A mutant spectra were similar, there were remarkable differences in the intensities (relative to \(\nu_4\)) of the porphyrin core bands. The intensities in the 3A4-testosterone and 4B1-heptane spectra were similar; however, the intensities of the HS components

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**Figure 2.** A, a view of the active site showing a superposition of the WT 4B1–octane complex structure (PDB code 5T6Q; yellow carbons) with that determined for the E310A mutant structure (green carbons). Active-site amino acid side chains, the backbone for helix I, the heme, and octane are depicted. The distance from octane to the heme iron is shown for the E310A complex. Distances are shown in Å. A 2DFo–mFc omit map contoured at 1\(\sigma\) around the E310A octane at 1σ. The Glu-310 ester bond with the heme 5-methyl is associated with a 0.3-Å RMSD shift of the heme. B, a side view of the heme in the E310A mutant showing out-of-plane deformations of the tetrapyrrole core and a heme omit 2DFo–mFc map contoured at 1\(\sigma\) around the heme. The Fischer numbering scheme for the methyl, vinyl, and propionate groups is shown. Cys-448 coordinates with the heme iron in the axial position and is shown with adjacent residues below the heme and a portion of helix I above that contribute to the out-of-plane distortion of the heme. C, a view of the propionate interactions with protein neutral and charge-stabilized hydrogen bond donors (dotted lines) for the E310A mutant. These interactions contribute to asymmetric effects on heme distortion in both the WT and E310A mutant. Intervening water molecules are shown as red spheres, which are slightly displaced due to the shift of the heme elicited by the ester linkage. D, the heme of the 5T6Q WT structure and an idealized planar heme from the PDB Ligand Expo (black carbons) are superimposed on the E310A mutant heme using the align function of PyMOL using 32 and 36 of 43 atoms, respectively. PyMOL was also used to generate the figures. The relative displacement of the \(\alpha\), \(\beta\), \(\gamma\), and \(\delta\) meso-carbons are similar for WT 4B1 and the E310A mutant and shown in the figure. The 5-methyl is displaced by 1 Å in the E310A mutant and by 1.3 Å in WT 4B1, where it is esterified to Glu-310.
in the spectra of 4B1 with octane, nonane, and decane were dramatically enhanced. These enhancements were most pronounced in the 4B1-nonane sample, where the intensity of ν₅ (HS) approached that of ν₄. Enhancements of these intensities by HCs were even greater for the E310A mutant (relative to ν₄), where the intensity of ν₅ (HS) exceeded that of ν₄ for octane, nonane, and decane (Fig. 3B).

The intermediate-frequency regions of the WT 4B1, E310A mutant, and 3A4 RR spectra (Fig. 4) were likewise normalized to ν₄. The spectra of both ligand-free and HET0016-bound 4B1 and E310A mutant were characterized by a few very weak bands, typical of P₄₅₀ RR spectra. Whereas no enhancements were observed with the addition of testosterone to 3A4, the addition of all HCs to 4B1 or E310A enhanced the intensities of several bands. The most intense band occurred at 1048–1050 cm⁻¹ for 4B1 and the E310A mutant, respectively, which was tentatively assigned as an asymmetric stretch (δ(CH₂)) of one of the vinyl substituents (27). These changes in intensity are probably the result of perturbations induced by the local protein environment, as seen in the structures (Fig. 2). The remaining bands correspond to the skeletal porphyrin modes ν₄₆ (~931 cm⁻¹), ν₅ (~1123 cm⁻¹), ν₄₄ (~1132 cm⁻¹), ν₈ (~1173 cm⁻¹), and ν₃₀ (~1183 cm⁻¹). Normal mode analyses of model systems assign these to breathing and asymmetric stretching modes of the pyrrole(s). Furthermore, two of these modes (ν₄₆ and ν₃₄) belong to the E₂ irreducible representation of the D₄₅₆ point group. If the unperturbed heme macrocycle has pseudo-D₄₅₆ symmetry, the observation of these otherwise forbidden modes supports the ruffling seen in structures of both WT 4B1 and the E310A mutant and the possibility that HC binding contributes to distortion, or breaking, of the heme symmetry.

Figure 3. High-frequency regions of 4B1 and 3A4 (A) and the E310A mutant (B) RR spectra obtained with 406.7-nm laser excitation. 4B1 and E310A samples contained ~10 μM protein, with or without 500 μM HC or with or without 150 μM HET0016. 3A4 samples contained ~10 μM protein with or without 500 μM testosterone.

The low-frequency regions of the RR spectra are illustrated in Fig. 5, wherein each has been normalized to the height of the ν₁ band at ~678 cm⁻¹. Changes in the ~360–395 cm⁻¹ region of the spectra reflect changes in the local environments that impact the propionate heme substituents. The so-called propionate bonding modes are sensitive to changes in hydrogen-bonding partners (28, 29) anchoring these to the protein, although studies using deuterated hemes support the possibility that out-of-plane distortions of the heme C and D pyrroles also contribute to these modes (30, 31). The 363 cm⁻¹ and 373 cm⁻¹ δ(C₉₋₁₀₋₁₁₋₁₂) bands observed in WT 4B1 coalesce into a single visible band at 381 cm⁻¹ in the E310A mutant. A similar upshift from 362 and 372 cm⁻¹ in the WT 4B1–HET0016 complex to 384 cm⁻¹ is also observed. In both cases, these upshifts are consistent with rigidification of the interactions between the propionates and the protein environment with loss of the heme ester linkage.

Upon the addition of HCs to WT 4B1 and the E310A mutant, enhancements in several bands were observed, although not when testosterone was added to 3A4. The most remarkable enhancement in the 4B1–HC spectra is for the δ(C₉₋₁₀₋₁₁₋₁₂) mode at 363 cm⁻¹. The most dramatic change compared with 4B1 is the disappearance of the single, strong δ(C₉₋₁₀₋₁₁₋₁₂) band at 363 cm⁻¹ that is replaced with two δ(C₉₋₁₀₋₁₁₋₁₂) bands at 368 and 384 cm⁻¹ in the E310A mutant (Fig. 5). The splitting and shift to
higher frequency of the propionate bands in the E310A mutant signals that these groups experience environmental changes that differentially enhance the propionate interaction strength with the protein matrix (32, 33). Illustrated in Fig. 6, the \( \nu_4/\delta(C_{PCC}C_{6}) \) pair in the 4B1 spectra and the \( \nu_4 \) regions of both spectra were each fit to pairs of Lorentzian functions, and the \( \nu_4/\delta(C_{PCC}C_{6}) \) region of the E310A spectra was fit to three Lorentzians. In every case, the \( \delta(C_{PCC}C_{6}) \) and \( \nu_4 \) (HS) intensities directly correspond and generally increase with HC size. Insofar as the enhancement of these bands is representative of those broadly observed, it appears that larger HCs induce greater distortion in the heme macrocycle. However, this does not imply that distortion is absent in the HET0016-bound and ligand-free enzymes. Indeed, consistent upshifts in the \( \delta(C_{PCC}C_{6}) \) envelopes confirm active-site adaptions to both loss of the ester linkage and accommodation of HET0016. It is plausible that different energetic spacings between the Soret bands of the LS systems and the laser excitation wavelength could preclude observation of enhancements attributable to heme deformation.

**UV-visible spectroscopy**

For comparison, the extent of LS to HS state conversion of WT 4B1 and the E310A mutant following saturation with HC or HET0016 was determined by UV-visible absorption spectroscopy. UV-visible spectra of the RR samples containing \( \sim 10 \mu M \) WT 4B1 or the E310A mutant with 500 \( \mu M \) HC or 150 \( \mu M \) HET0016 were estimated using a variant of the method described by Jung et al. (34). Unlike the original method that fit the LS and HS components of the Soret band and overlapping \( \delta \)-band to three Gaussian basis functions, it was determined that improved fits are attainable using Lorentzian functions. The fit of ligand-free 4B1 yielded bands centered at 350 nm (\( \delta \)-band), 395 nm (Soret band, HS), and 418 nm (Soret band, LS). Subsequent measurements made in the presence of HCs used Lorentzian functions centered on these wavelengths to ensure that the same bands were being fitted. Extinction coefficients \( (e) \) of 102 \( \text{mM}^{-1} \text{cm}^{-1} \) and 110 \( \text{mM}^{-1} \text{cm}^{-1} \) were used to calculate the respective concentrations of the HS and LS enzyme at their respective wavelengths. The fractions of WT 4B1 and the E310A mutant in the HS state \( (f_{\text{HS}}) \) were calculated using the equation,

\[
\frac{f_{\text{HS}}}{h_{\text{HS}}} = \frac{h_{395 \text{ nm}}}{h_{395 \text{ nm}}} + \frac{h_{418 \text{ nm}}}{h_{418 \text{ nm}}} \quad \text{(Eq. 1)}
\]

where the \( h \) variables represent the heights of the Lorentzian fits centered at 395 and 418 nm.

Following removal of co-purified heptane from 4B1, less than half of the enzyme remained in the HS state. Consistent with the RR results, a 50-fold excess of HCs consistently drove 4B1 to a predominately HS state (Fig. S1). Spanning heptane to decane, there is a small increase (from 82 to 90%) in the extent of the high-spin induction. In contrast, binding of HET0016 (Fig. 1B) to the enzyme produced an LS complex, indicative of coordina-
tion of one of the nitrogens of the formamidine moiety to the heme iron.

UV-visible spectra of the E310A mutant are illustrated in Fig. S2. As observed in WT 4B1, removal of co-purified heptane resulted in a predominately LS population (65%). Unlike WT 4B1, however, a graded increase in the extent of HS conversion was not observed with increasing HC chain length. An LS component could not be fit, thereby supporting a nearly complete HS conversion in these HC complexes. The UV-visible spectra of the HET0016 complex was identical to that obtained with 4B1. Although small changes in spin state might have contributed to the HC enhancements for 4B1, the results for the E310A mutant suggest that the HC enhancements are not likely to reflect differential conversion to the HS state with HC length.

**Characterization of the structure of the HET0016 complex**

The structure of WT 4B1–HET0016 complex was determined to identify potential effects of the larger molecule (15 heavy atoms compared with 8 for octane) on the small active site and heme conformation as well as its mode of binding to the enzyme (Fig. 7). The binding of HET0016 in the enzyme has little effect on the positions of amino acid side chains or the conformation of the heme relative to the 5T6Q structure of the octane complex, except for Val-486, which is displaced by the benzyl moiety of the ligand (Fig. 7A). The observed position of the inhibitor and the conformation of the heme are supported by the 2mFo−DFc omit-map density depicted in Fig. 7A. HET0016 can potentially exist as cis- or trans-isomers and tautomers that can be interconverted by protonation of the imine nitrogen under acidic conditions (Fig. 1B). The structure of the 4B1 complex reveals that the nitrogen of the N-hydroxyl group is positioned above the heme iron at 2.0 Å (consistent with the shift of the Soret band), and the hydroxyl group is positioned to accept a hydrogen bond from Thr-314. The hydroxyl group can donate a hydrogen bond to the adjacent pyrrole nitrogen (Fig. 7B). The coordination of the lone pair of the nitrogen to the iron implies that the nitrogen is linked by an unsaturated bond to the adjacent carbon in a z conformation relative to positions of the aniline nitrogen and the hydroxyl group (Fig. 7B). The terminal butyl chain of the inhibitor adopts a high-energy cis-conformation with hydrogen atoms eclipsed (white atoms in Fig. 7B). In contrast, the extended conformation of octane exhibits a low-energy conformation. This comparison suggests that HCs longer than octane are likely to adopt higher-energy conformations to fit within the relatively rigid active site cavity, which may contribute to the relative increase of (ω−1) hydroxylation.

The formation of the coordinate covalent bond of HET0016 with the heme iron greatly favors the LS ferric state, which, in turn, shifts the Soret band away from the laser excitation frequency in the RR experiment. It also increases the degeneracy of the three-occupied d-orbitals and degeneracy of the two-unoccupied d-orbitals. As the ruffling and orientation of the heme of the 4B1 octane complex is retained (RMSD 0.17 Å) in the HET0016 complex (Fig. 7A), HC binding is likely to increase...
the scattering cross-section and the loss of degeneracy of the d-orbitals in the HS state that contribute to the enhanced intensity of RR spectra of the HC complexes.

**Resonance Raman spectroscopy of Fe\textsuperscript{2+}–CO 4B1 and E310A**

Bands corresponding to the normal modes of the Fe\textsuperscript{2+}–CO unit can provide useful insight into the sterically and polar interactions experienced by the diatomic ligand in the active-site milieu through changes in the Fe–CO stretching (ν(Fe–CO)), Fe–C–O bending δ(Fe–C–O), and C–O stretching (ν(CO)) frequencies (35). The Fe\textsuperscript{2+}–CO complexes were stable throughout the RR experiments, as evidenced by the dominant Soret band at 446 nm in the UV-visible spectrum before and after collection of the RR spectra. The 446 nm band confirms the stable coordination of CO and cysteine thiolate to the heme iron. Conversely, contributions of P420 to these spectra were minor and changed negligibly during the experiments.

RR spectra of Fe\textsuperscript{2+}–CO 4B1 and E310A are illustrated in Fig. 8 (A–E and F–J, respectively). Envelopes encompassing the ν(Fe–CO) (450–510 cm\textsuperscript{−1}) and δ(Fe–C–O) frequencies (540–556 cm\textsuperscript{−1}) are illustrated in the main body of the plots, whereas the weaker ν(CO) frequencies are shown in the insets. ν(Fe–CO) and δ(Fe–C–O) bands were simultaneously fit to a basis set of five Lorentzian functions, allowing both the frequencies and bandwidth to vary. Attempts to fit the data with fewer Lorentzian functions consistently failed to account for all of the underlying bands, whereas fits using a greater number resulted in components with negligible coefficients. An r\textsuperscript{2} > 0.99 was achieved in all of the fitting procedures.

The ν(Fe–CO) envelope in the spectra of WT 4B1 has obvious asymmetry and a larger bandwidth than the corresponding HC complexes. ν(Fe–CO) was found to fit to three bands consisting of low-, medium-, and high-frequency components at 464, 474, and 486 cm\textsuperscript{−1}, respectively. The three components of the ν(Fe–CO) are nearly equally represented. In the absence of any interactions, the nearly linear configuration of the Fe–C–O unit maximizes back-bonding and thereby lowers ν(Fe–CO). Conversely, steric crowding and environmental polarity, such as through hydrogen-bonding to the Fe–C–O unit, attenuates the back-bonding and thereby increases ν(Fe–CO). Hence the three bands of the deconvoluted ν(Fe–CO) envelope represent populations of three conformers in distinct environments. When heptane and more so octane bind, selective depopulation of the high-frequency component is observed with corresponding increases in the lower-frequency populations. When nonane is bound, the low and high frequency contributions are nearly abolished, indicating that nonane limits the Fe–C–O unit to a population of very similar conformations. In the presence of decane, some of the conformational heterogeneity reemerges, as evidenced by the small increases in the low- and high-frequency contributions and a decrease in the dominant medium-frequency component.

The δ(Fe–C–O) frequencies at 555 cm\textsuperscript{−1} were invariant in the spectra of Fe\textsuperscript{2+}–CO WT 4B1. In ligand-free 4B1, ν(CO) bands were observed at 1925 and 1952 cm\textsuperscript{−1}. Based on previous analyses of the inverse correlation between ν(Fe–CO) and ν(CO) (35), it is estimated that the 1925 and 1952 cm\textsuperscript{−1} components correspond to the high- and medium-frequency components of the ν(Fe–CO) envelope. This assignment is further supported by the simultaneous disappearance of the 1925 cm\textsuperscript{−1} and high-frequency ν(Fe–CO) components in the presence of HCs.

The ligand-free E310A mutant likewise displays the most asymmetry in the ν(Fe–CO) envelope. The two dominant components were centered at 469 and 480 cm\textsuperscript{−1}. The addition of HCs suppressed the low-frequency component, resulting in a population represented by one dominant conformer population that underwent upward shifts in 2 cm\textsuperscript{−1} increments with longer HCs. Whereas additional conformational heterogeneity is permitted in the E310A mutant with nonane, decane selects essentially a single population that represents 93% of the intensity. Hence, it is possible for the E310A mutant to accommodate more ligand bulk near the heme iron. Comparison of the dominant ν(Fe–CO) bands of the WT 4B1 and E310A mutant decane complexes indicates that this ligand imparts different environmental conditions to the Fe–C–O unit in these systems. The 7 cm\textsuperscript{−1} blue shift of ν(Fe–CO) in the E310A mutant spectra relative to WT 4B1 is indicative of enhanced polarity through hydrogen bonding and/or steric compression of the Fe–C–O unit. The δ(Fe–C–O) in E310A 4B1 was fit to a single Lorentzian at 555 cm\textsuperscript{−1} in the ligand-free enzyme and at 556–557 cm\textsuperscript{−1} in the
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Figure 7. View of the active site showing the binding of HET0016 (violet carbons) to the heme by coordination of the nitrogen lone pair to the ferric iron of the heme. The protein carbons are colored from blue at the N terminus to red at the C terminus with heme carbons colored brown. A, a ZDF, mF, ligand omit map is contoured at 1σ around the HET0016. The 3T6Q structure (gray carbons) of the WT 4B1 octane complex is superimposed on structure of the HET0016 complex. A small shift of Val-486 rotamer is evident to accommodate HET0016, but the binding of the HET0016 does not elicit significant effects on other residues or the ruffling of the heme. B, a different view of the active site showing the implied location of the double bond that defines the tautomer and the cis orientation of the N-hydroxyl group. The black and orange dashed lines show potential hydrogen bonding and coordinate covalent bond to the heme, respectively, with the distances shown in Å. The gray atoms on HET0016 depict hydrogens and show how the cis-conformation of the butyl side chain that allows it to fit in the cavity but leads to hydrogen overlaps. Larger HCs may adopt similar conformations to fit in the cavity, which can increase the likelihood for hydrogen abstraction from secondary carbons.

Discussion

Heme protein capacity to execute a wide range of functions stems from the highly tunable nature of the heme by the surrounding protein environment (36, 37). In the absence of environmental perturbations, the heme is generally planar; however, substantial deviations from planarity are observed to be enforced in various protein active sites. Whereas other means of tuning the heme reactivity are possible, such as strong local electric fields (38, 39), heme distortion is well-known to affect both redox potentials and spectroscopic characteristics as well as catalytic activity.

Esters involving the heme methyl and acidic amino acid residues are not unique to members of the CYP4 family. In mammalian peroxidases, the heme 1- and 5-methyl groups are esterified by nearby acidic residues (25, 40). These covalent links protect the heme vinyls from modification during halide oxidation (41) and appear to tune the reactivity of the Compound I reactive intermediate (42, 43). In lactoperoxidase (LPO), only one of the heme covalent links is necessary to protect against heme modification (44). In myeloperoxidase (MPO), loss of one linkage substantially attenuates halide oxidation compared with the WT enzyme (42). A cyanobacterial peroxidase from Lyngbya sp. (LspPOX) that bears two heme ester linkages and oxidizes halides with similar rates to LPO has proven to be a valuable model to investigate the functional relevance of the heme covalent links (43). The extent of LspPOX heme modification can be controlled without site-directed mutagenesis, affording direct evaluation of the impact of heme covalent links on Compound I reactivity. Although elimination of the ester linkages did not impact rates of Compound I formation, they remarkably enhance rates of bromide, iodide, and thiocyanate oxidation. The high reactivity of P450 Compound I precludes analogous experimental comparisons afforded to peroxidases, although it is plausible that similar enhancements in Compound I reactivity are conferred by heme-covalent links in 4B1.

Heme out-of-plane distortions associated with covalent linkages are evident in both crystal structures and RR spectra of peroxidases. Remarkably strong enhancements in LPO RR spectra were first reported by Kimura et al. (45) and later by Manthey et al. (46). Zbylut and Kincaid (47) convincingly demonstrated that the low-frequency enhancements were attributable to protein-induced heme distortion. In their work, comparison of RR spectra of LPO proteolytic fragments that maintained 1- and 5-methyl esters with native LPO revealed that the low-frequency enhancements required that the heme be enveloped by the full-length LPO polypeptide. MPO also bears both the 1- and 5-methyl esters as well as a vinyl-sulfonium linkage. Accordingly, MPO RR spectra are likewise extremely rich due to activation of all of the heme skeletal modes by distortion and lowering of the heme symmetry (48). Eliminating the sulfonium linkage results in RR spectra like LPO and loss of chloride oxidation capacity. Elimination of one of the heme ester linkages abolishes all enhancements in the RR spectra and reduces chloride and bromide oxidation to 2 and 24% of the WT capacity, respectively (48). Considering that elimination of the heme links does not affect the rates of Compound I formation, it appears that heme distortion con-
Figure 8. RR spectra of Fe\(^{3+}\)–CO 4B1 (A–E) and the E310A mutant (F–J) obtained with 441.6-nm laser excitation. The low-frequency v(Fe–CO) and \(\delta\)(Fe–C–O) bands appear in the main plots, whereas the v(Fe) bands are shown as insets. The raw data and sum of the five-Lorentzian deconvolution are represented by gray circles and a solid black line, respectively. \(^{13}\)CO substitution confirmed that the 506–507 cm\(^{-1}\) bands appearing in the E310A mutant spectra were not attributable to CO; therefore, they were omitted from the calculations of v(Fe–CO) envelope fractional areas.

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4B1 is a prototypical example of the members of the CYP4 family that contain a heme–protein covalent bond, and the 4B1–octane crystal structure reveals several structural adaptations that afford regioselective \(\omega\)-hydroxylation. Illustrated in Fig. 2 (B and C), the ester involving Glu-310 visibly distorts the 5-methyl, but the effect is small relative to the out-of-plane distortions of the heme seen in the structure of the E310A mutant reported here. The loss of heme ester in the mutant is also associated with a 0.3-Å shift of the heme relative to its position in WT 4B1. The out-of-plane distortion evident for both 4B1 and the E310A mutant is likely to underlie pronounced activation of the same vibrational and bending modes seen for 4B1 relative to 3A4, which has a more planar tetrapyrrole ring. The presence of the ester linkage is also associated with a large difference in RR spectra for the propionate/pyrrole bending mode.

The E310A mutant retains almost all of the additional out-of-plane distortions of the heme seen in the native protein, 0.2 Å RMSD. This observation suggests that the heme binding site is tuned to facilitate formation of the covalent bond by positioning the 5-methyl for esterification with the Glu-310 mt-10 rotamer. The deformations may also limit inhibition of the enzyme by coordination of the Glu-310 tp10 rotamer to the heme iron because of steric interference by the out-of-plane deformation pyrrole C and the \(\delta\)-meso-carbon. This mode of binding competes with substrate binding and alters the redox potential, which inhibits reduction for oxygen binding. The binding and inhibition were observed for both the P450cam G248E and the P450 BM3 A264E mutants (49–51). Attempts to generate covalent linkages with the heme were not successful for BM3 A264E, and only 10% cross-linking was obtained for the P450cam G248E mutant after a 5-h reaction with the enzyme reconstituted with putaredoxin, putaredoxin reductase, and NADH. In 4B1, the Glu-310 mm-40 rotamer also projects into the active site where octane resides, and formation of the ester bond reduces the configurational entropy of the glutamate and anchors the heme in position to support selective \(\omega\)-hydroxylation of alkanes and fatty acids. The prevailing mechanism (52) posits a role for Glu-310 that involves hydrogen abstraction by P450 Compound I from the mm-40 rotamer and subsequent hydrogen abstraction from the heme 5-methyl when the Glu-310 radical adopts the mt-10 rotamer, leading to the formation of a 5-methylene carbocation and Compound II. This is followed by reaction of Glu-310 with the carbocation to form the ester bond. The overall mechanism is consistent with evidence that ester oxygen is derived exclusively from Glu-310 (53, 54). The shorter 4B1 Asp-310 mutant can initiate the formation of the carbocation but cannot effectively compete with water to form ester product, which leads to hydroxylation of the 5-methyl group. If there is a correspondence between heme distortion and increased Compound I reactivity in 4B1 as in peroxidases, conservation of the out-of-plane distortion in the E310A mutant is consistent with its conserved capacity for \(\omega\)-hydroxylation and, potentially, abstraction of a hydrogen from the carboxyl group of Glu-310.
Interestingly, the remaining out-of-plane distortions in the absence of the ester linkage relate to the interaction of pyrrole B where the heme is sandwiched between helix I and Gly-450 on the loop between Cys-448 (the axial ligand to the heme) and the beginning of helix L, where Ile-449 pushes the heme upward. Additionally, the interaction of the 6-propionate with Trp-129, Arg-133, and Arg-446 below the heme surface pulls downward to force a large out-of-plane orientation of pyrrole C. In contrast, the 7-propionate on pyrrole D interacts with Tyr-110 above the surface of the heme, creating an offsetting out-of-plane orientation. This trans-orientation of the 7-propionate relative to the 6-propionate is unusual for a mammalian P450, where most, such as 3A4, place both propionates below the heme plane. The trans-orientation of the 7-propionate contributes to a narrow channel near the heme where octane packs against C_p and C_y of Glu-310 and C_b of the 7-propionate.

Heptane, octane, nonane, and decane induce progressively larger enhancements of the intensity of the RR bands. In 4B1, the Glu-310 side chain is restrained by its attachment to the heme, and the shorter HCs are permitted to bind in a predominately extended conformation. We speculate that the intensity enhancements result from accommodation of the additional methylene units of the larger HCs above the 6-pyrrole and 7-propionate, which alter the dynamics of the distorted heme by counteracting the upward interaction of the 7-propionate with Tyr-110. This scenario is consistent with the strong activation of the 363 cm⁻¹ band in the RR spectrum of WT 4B1. Although the structure of the HET0016 complex did not reveal significant changes for the conformation of the heme, the RR spectra of hexavalent inhibitor complex were weak. In contrast, significant changes for the conformation of the heme, the RR spectroscopy of the Fe³⁺–CO enzymes clearly demonstrates an HC length–dependent restriction of the conformational heterogeneity of coordinated CO. Spectra obtained for both experiments agree that the additional methylene units of longer HCs are accommodated near the heme and the CO-binding site. Owing to the unusual ester linkage, the crystal structure reveals a narrow, confined active site near the heme. Accordingly, comparisons of the Fe²⁺–CO WT 4B1 and E310A mutant RR spectra suggest that the latter permits greater conformational heterogeneity of the CO, reflecting a less confined active site in the mutant that is not evident from the structure but is likely to reflect increased plasticity due to the loss of ester linkage. In the structure of the E310A mutant, Leu-122 moves into the space occupied by the ester linkage, and this change is likely to be reversed when larger substrates bind, leading to the observed increase in ω–1 hydroxylation. Most remarkable are the HC-induced enhancements observed in Fe³⁺–CO RR of spectra that unveil an underappreciated function of the heme, in that it can also constitute a deformable boundary of the active site. Whereas the heme undergoes HC-dependent heme distortion in WT 4B1, studies of the E310A mutant show that the deformation modes available to the heme are comparatively limited in the enzyme. We conclude that the interplay of noncovalent protein-directed heme deformation and a con-

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finned and rigidified active site due to covalent linkage of the heme to the protein are the primary features that confer α-regioselectivity in 4B1 oxidations.

Experimental procedures

Materials

HET0016 was purchased from Cayman Chemical Co. (Ann Arbor, MI). Testosterone was from Steraloids (Newport, RI). All other reagents of the highest available grade were purchased from Sigma-Aldrich or Fisher.

Protein expression and purification

3A4 was expressed and purified as described previously (55). Rabbit 4B1 was expressed in *Escherichia coli* strain DH5α transformed with pCW-4B1#7 (54) and purified as described previously (20). The E310A mutant was introduced into pCW-4B1#7 plasmid using the QuikChange® II site-directed mutagenesis kit (Agilent). The forward mutagenic primer sequence is 5'-GACAGCTTCATGGTTCAGGTCATGA-CACCC-3' with the altered nucleotide underlined. The mutagenic reaction was performed according to the manual using the following cycling parameters: one cycle at 95 °C for 1.5 min; 19 cycles of 1 min at 95 °C and 18 min at 68 °C; and final extension at 68 °C for 7 min. Following PCR, the PCR mixture was digested with DpnI to remove the plasmid template and then transformed into *E. coli* XL1-Blue supercompetent cells (Agilent). The sequence of the mutant construct was confirmed by DNA sequencing, and the coding sequence was excised with Ndel and Sall and subcloned into pCW expression vector. The E310A mutant was expressed and purified using the same procedures used for the WT protein (20).

UV-visible spectroscopy

UV-visible spectra of RR samples were measured with a Cary-14 spectrophotometer conversion (Olis, Bogart, GA) in 5-mm NMR tubes using a custom adaptor. To remove heme present in the purification procedure and glycerol from the 5-mm NMR tubes using a custom adaptor. To remove heptane Cary-14 spectrophotometer conversion (Olis, Bogart, GA) in UV-visible spectroscopy procedures used for the WT protein (20).

Structure determination

For structure determination, the 4B1–HET0016 and E310A–octane complexes were prepared and crystallized as described previously for the 4B1–octane complex (20). The 4B1–HET0016 complex was crystallized by sitting-drop vapor diffusion. The drop contained 1 μl of protein solution with 0.2 μl of 192 mM n-decyl-N,N-dimethylglycine, 0.1 μl of 2 mM Façade-EM (Avanti Polar Lipids), and 1 μl of 25% PEG 3350 in 0.2 M trisodium citrate. X-ray diffraction data were obtained from a single crystal at 100 K on Stanford Synchrotron Radiation Lightsource Beamline 9-2 using BLUE-ICE. XDS (57) was used for integration, and Aimless (58) was used for data reduction to an apparent dmin of 2.78 Å. Data statistics are shown in Table 1.

The E310A–octane complex was crystallized by hanging-drop vapor diffusion. The drop contained 1 μl of protein solution, 0.2 μl of 275 mM dimethyl-4-heptyl-β-D-maltopyranoside, and 1 μl of 27% PEG 3350 in 0.2 M trisodium citrate, 80 mM NaCl, and 10 mM lithium sulfate. X-ray diffraction data were collected for the E310A–octane complex from a single crystal at 100 K on Stanford Synchrotron Radiation Lightsource Beamline 9-2 using BLUE-ICE. Intensities were integrated using XDS, and data reduction employed Aimless to a limiting dmin of 2.67 Å.

Initial phasing was obtained by molecular replacement in PHENIX (59) with Phaser (60) using the PDB 5T6Q structure of the WT 4B1–octane complex as the search model. One molecule was found in asymmetric unit in the P3221 space group in each case. Refinement of the models with riding hydrogens against the reflections utilized PHENIX. The DIVICON 6.1 plugin (23) with PM6 Hamiltonian (24) was used to calculate energy gradients for the heme geometry during refinement. Fitting the model to electron density maps was performed using COOT (61). Stereochemical restraints for alternative tautomers and stereoisomers of HET0016 were generated from isomeric SMILE strings using GRADE (http://grade.globalphasing.org/cgi-bin/grade/server.cgi). Refinement statistics are re-

Resonance Raman spectroscopy

RR measurements of the Fe³⁺ and Fe²⁺–CO enzymes were made with a f/9.7 single grating monochromator (Acton SP2750, Princeton Instruments) with a 100-μm slit width using 2400-groove/mm gratings and imaged using a 1340 × 400-pixel back-illuminated CCD camera with UV-optimized coatings (PyLoN 400BR eXcelon, Princeton Instruments). Fe³⁺ enzyme samples were illuminated with the 406.7-nm line from a Kr⁺ (Coherent Innova 302C) laser with a power of ~30 milliwatts at the sample. Fe²⁺–CO samples were prepared by multiple rounds of evacuation of the NMR tube and flushing with argon using a gas manifold. Samples were subsequently reduced with a few crystals of sodium dithionite, evacuated, flushed with CO, and then sealed. The Fe²⁺–CO samples were illuminated with the 441.6-nm line of a HeCd laser (IK series, Kimmon Koha, Tokyo, Japan) with a power of ~25 milliwatts at the sample. All samples were kept spinning during data collection to avoid photolysis and local heating. Reference calibrations were performed with respect to a mercury vapor lamp. The nonlinear fluorescence background of RR spectra were removed using asymmetric least squares smoothing (56) in MATLAB.
ported in Table 1. The maximum likelihood estimate of the upper limit for the coordinate error was generated by Phenix using the test reflections. The estimate for the minimum coordinate error was computed using SFCheck (62) in ccp4.

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