Mammalian cytochrome P450 enzymes often metabolize many pharmaceuticals and other xenobiotics, a feature that is valuable in a biotechnology setting. However, extant P450 enzymes are typically relatively unstable, with $T_{50}$ values of $\sim 30–40$ °C. Reconstructed ancestral cytochrome P450 enzymes tend to have variable substrate selectivity compared with related extant forms, but they also have higher thermostability and therefore may be excellent tools for commercial biosynthesis of important intermediates, final drug molecules, or drug metabolites. The mammalian ancestor of the cytochrome P450 1B subfamily was herein characterized structurally and functionally, revealing differences from the extant human CYP1B1 in ligand binding, metabolism, and potential molecular contributors to its thermostability. Whereas extant human CYP1B1 has one molecule of $\alpha$-naphthoflavone in a closed active site, we observed that subtle amino acid substitutions outside the active site in the ancestor CYP1B1 enzyme yielded an open active site with four ligand copies. A structure of the ancestor with $17\beta$-estradiol revealed only one molecule in the active site, which still had the same open conformation. Detailed comparisons between the extant and ancestor forms revealed increases in electrostatic and aromatic interactions between distinct secondary structure elements in the ancestral forms that may contribute to their thermostability. To the best of our knowledge, this represents the first structural evaluation of a reconstructed ancestral cytochrome P450, revealing key features that appear to contribute to its thermostability.

Cytochrome P450 enzymes are a diverse superfamily of hemoproteins, which carry out a wide variety of important physiological functions in organisms ranging from bacteria to higher animals and plants. In humans, the cytochrome P450 1 (CYP1) family consists of three genes (CYP1A1, CYP1A2, and CYP1B1). The corresponding proteins each metabolize a broad but overlapping set of endogenous and environmental chemicals, including steroids, pharmaceutical compounds, and dietary pro-carcinogens. CYP1 gene expression is regulated by the aryl hydrocarbon receptor, a transcriptional regulator with roles in both development and the response to exposure to environmental toxins, such as dioxins and polycyclic aromatic hydrocarbons. Many CYP1 substrates are aryl hydrocarbon receptor agonists, inducing the up-regulation of CYP1 gene expression and thereby their own clearance (reviewed in Refs. 1–3).

CYP1B1, the most recently identified of the human forms, metabolizes a wide range of compounds including xenobiotics, such as 7-ethoxyresorufin, caffeine, and theophylline (4), as well as endobiotics, including testosterone, estradiol, retinol, arachidonic acid, and melatonin (4–9). CYP1B1 is expressed in numerous extrahepatic tissues, including eye, brain, kidney, and breast (reviewed in Ref. 10), and is overexpressed in various cancer cell types (11, 12). This distribution is of interest, given the ability of CYP1B1 to bioactivate environmental carcinogens, including polycyclic aromatic hydrocarbons and estradiol, to DNA-reactive products (13–15). CYP1B1 is essential for the immune response in mice (16, 17), and CYP1B1 polymorphisms have been associated with congenital glaucoma (18, 19), hinting at an important but as yet unidentified physiological role.

The physiological relevance of CYP1B1 is also reflected in its relatively high degree of conservation in vertebrates, compared with the CYP1A subfamily forms. The CYP1A and CYP1B lineages were created by a gene duplication event that took place in early deuterostomes, resulting in the CYP1A/CYP1D and CYP1B/CYP1C clades. The CYP1C and CYP1D subfamilies diverged from the CYP1B and CYP1A lineages, respectively, after a second duplication event in fish (20–22). CYP1C forms are present in amphibian, fish, reptiles, and some avian species (20), whereas CYP1D forms are present in fish, amphibian, reptiles, and some mammals but are pseudogenes in humans (23).

Little is known about the historical function of the CYP1B/CYP1C clade aside from what has been gleaned from observing the activities of modern-day forms. In an effort to explore the evolution of the CYP1 forms, we recently reconstructed a series of ancestral P450 enzymes.
of CYP1 ancestors from key nodes of the evolutionary tree. In line with studies on other ancestral P450s, many of the CYP1 ancestors showed increased thermal tolerance compared with extant CYP1 human enzymes. N98_CYP1B1_Mammal, the predicted ancestor of mammalian CYP1B1 forms, was closest to the extant human mammalian CYP1B1 forms, sharing 81.8% sequence identity to human CYP1B1, yet had a $T_{50}$ (the temperature at which 50% of the protein remains folded after heating for 60 min) of 43 °C, which is 6 °C higher than that of extant human CYP1B1. Whereas P450 enzymes are of interest for use in biotechnology, the extant forms are not stable enough for such applications, but engineered forms with increased thermostability are more likely candidates.

The N98_CYP1B1_Mammal ancestor also showed intriguing differences in the metabolism of representative substrates compared with the extant human CYP1B1 enzyme. For example, human CYP1B1 predominantly metabolizes estradiol to 4-hydroxyestradiol (6). However, whereas both N98_CYP1B1_Mammal and an older common ancestor of all CYP1B forms (N20_CYP1B) were less active than human CYP1B1, they produced both 4- and 2-hydroxyestradiol in similar amounts. However, these ancestors both showed slightly greater activity than human CYP1B1 toward testosterone. Both human CYP1B1 and the two ancestors produced similar, small amounts of 6β-hydroxytestosterone and 16α-hydroxytestosterone plus trace quantities of two unidentified metabolites (8). Thus, the reconstructed ancestors vary in both thermostability and metabolic capabilities, both important characteristics to understand for biotechnology applications.

To initiate an analysis of how CYP1 ancestral sequences have resulted in increased thermostability and varied substrate metabolism, structures of several ancestral forms are being pursued. Crystals of the N98_CYP1B1_Mammal ancestor were successfully produced with either 17β-estradiol or the polycyclic aromatic compound α-naphthoflavone, diffracting to limiting resolutions of 3.1 and 2.95 Å, respectively (Table 1). The structure of this ancestral CYP1B1 with the small, planar α-naphthoflavone permits comparison with structures of all three extant human CYP1B1, CYP1A1, and CYP1A2 enzymes generated with this same ligand (PDB: 3PM0 (26), 4I8V (27), and 2HI4 (28), respectively). This is particularly useful because many cytochrome P450 enzymes from families 1–3 demonstrate substantial reconfiguration of the active-site dimensions with different active-site ligands. To evaluate the ligand-specific effects on N98_CYP1B1_Mammal conformation, a structure with the endogenous steroid estradiol was subsequently solved. Both represent the first reported ancestral cytochrome P450 structures and suggest structural features that may modulate catalytic activity and thermostability.

### Results and discussion

#### Comparison of overall structures: Ancestral CYP1B1 versus extant human CYP1B1

Structures of the ancestral reconstructed mammalian CYP1B1 enzyme, N98_CYP1B1_Mammal, with α-naphthoflavone as the bound ligand, revealed that this enzyme maintains a canonical cytochrome P450 fold (Fig. 1A). Structural comparison with the extant human CYP1B1 (PDB: 3PM0) reveals that the mammalian ancestor has similar Co backbone positioning. The Co root mean square deviation (RMSD) between the two structures with α-naphthoflavone is 1.43 Å (Fig. 1A). Whereas most of the boundaries for helices and β-strand regions are also conserved, significant differences are observed in the conformation of the F helix (Fig. 1A). First, the ancestral CYP1B1 has its F helix extended by eight residues compared with the extant

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3 K. L. Harris, Y. Gumulya, G. Foley, P. Syed, T. Janosik, A. Sandinge, S. Andersson, U. Jurva, M. Bodén, and E. M. J. Gillam, unpublished observations.
human CYP1B1 (Fig. 1B). Second, whereas the extant human CYP1B1 and all of the other human CYP1 family enzymes have an unusual break near the middle of the F helix (26–28), the ancestral CYP1B1 F helix is intact throughout these residues (Fig. 1B). The net result is that the C terminus of the F helix is much farther away from the heme in the ancestral CYP1B1 structure than in the extant human structure, and this substantially changes the active-site accessibility and ligand binding (see below).

The residues that form the immediate central active-site cavity are largely conserved, as well as regions important for heme binding and proximal cysteine ligation, such as the L helix. Differences in the primary sequences of the CYP1B1 forms are distributed throughout the tertiary structure and generally occur farther from the heme and active site (Fig. 1A, bright blue). Examination of the amino acid differences in the linear alignment (Fig. 1B, red text) reveals some notable groupings of divergent amino acids compared with the human enzyme. Portions of the H helix and H/I loop have multiple substitutions, but there is considerable disorder in this region (Fig. 1A) for both the human structure (amino acids 308–311 of the H/I loop disordered) and the ancestral CYP1B1 structure (amino acids 300–311 of the H/I loop disordered), which precludes structural comparisons.

**Ancestral CYP1B1 structure with α-naphthoflavone**

Co-crystallization of the mammalian ancestral CYP1B1 with the flavonoid α-naphthoflavone was initially pursued to facilitate comparison with the human CYP1B1 structure containing the same ligand. Surprising substantial differences were first observed in ligand binding. Whereas the human CYP1B1 enzyme has a single copy of α-naphthoflavone (Fig. 2A) within a relatively small, planar, enclosed active-site cavity, the ancestral CYP1B1 structure displays clear evidence for binding four copies of α-naphthoflavone within a substantially expanded active site (Fig. 2B), largely generated by reconfigurations in helix F discussed below.

In the ancestral CYP1B1 structure, the benzochromenone core of the α-naphthoflavone closest to the heme essentially occupies the same space as it does in the human structure, tilted toward the central L helix. However, the ligand is flipped by 180° such that the phenyl ring is directed toward the heme in the human structure and away from the heme in the ancestral CYP1B1 complex. As a result, in the ancestral CYP1B1 complex α-naphthoflavone, atoms C8 and C9 on the fused ring are closest to the heme iron, but with distances of 7.4 and 7.2 Å, respectively. This orientation is consistent with these atoms as likely sites of metabolism, as opposed to the orientation found in the human CYP1B1 structure. However, this α-naphthoflavone molecule would need to move closer to the heme (to 4–5 Å) for metabolism. In both α-naphthoflavone complexes, this ligand forms a π–π stacking interaction with the conserved F helix Phe-231 (Fig. 2, A and B). This interaction was also key in α-naphthoflavone binding within human CYP1A1 (27) and CYP1A2 (28). In all three human structures, the positioning of this Phe helped to define the narrow and planar active-site architecture. Whereas this interaction is maintained in the ancestral enzyme, repositioning of the end of the F helix 5–8 Å toward the distal side means that Phe-231 in the F helix is located higher up in the active site, consistent with the ligand location further from the heme. An additional interaction is formed with this same copy of α-naphthoflavone in the N98_CYP1B1_Mammal structure. The F helix His-227 is a hydrogen...
bond donor to the α-naphthoflavone carbonyl oxygen. This interaction is not observed in the human CYP1B1 structure because the F helix break and repositioning of the C-terminal end of the F helix result in direction of this residue away from the active-site cavity.

In the ancestral CYP1B1 complex, the next two copies of α-naphthoflavone pack parallel with each other via π stacking. They are mostly confined by van der Waals and/or hydrophobic interactions between the F helix and sequences immediately following the K helix. Whereas the K helix side is largely unperturbed compared with the human structure, the ancestral CYP1B1 F helix repositioning 5–8 Å toward the distal side opens up the active site to bulk solvent. The fourth α-naphthoflavone ligand is at the mouth of this cleft or channel. It is pos-
Ancestral mammalian P450 1B1 structures

Figure 3. Comparison of ancestral CYP1B1 (blue) and extant human CYP1B1 (green) enzymes highlighting structural differences in helices F through G, the β4 loop, and helix I. Shown are the protein backbone (ribbons) and heme (black sticks). A, the active site for the extant human CYP1B1 (solid green) is much smaller than the active site and channel observed for the ancestral CYP1B1 (blue mesh). B, α-naphthoflavone ligands (colored sticks).

The striking differences in the conformations of the extant and ancestral CYP1B1 enzymes with α-naphthoflavone are the result of significant alterations in positioning of structural elements forming parts of the active-site roof. The most significant structural change is the conformation of the F helix (Fig. 3A). The RMSD for Cα in this region is 3.2 Å, compared with 1.43 Å overall. As described previously, the extant human CYP1B1 structure (and those of CYP1A1 and CYP1A2 with α-naphthoflavone) has a notable break in the F helix (Fig. 3B), which is not typically observed in other family 2 P450 enzymes and which contains a conserved Phe that projects into the active-site cavity (Fig. 3B). This potentially restricts the ligand α-naphthoflavone from obtaining closer proximity to the heme in the ancestral structure.

Ancestral CYP1B1 titrations with α-naphthoflavone

To further probe α-naphthoflavone binding and interactions near the heme, the effects of ligand binding on the heme coordination were monitored by changes in the Soret heme absorbance. The as-isolated ancestral CYP1B1 protein has a Soret peak absorbance maximum at 417 nm (Fig. 2D, black spectrum), consistent with water coordination to the heme iron, as is typical for isolated P450 enzymes in the oxidized state. Titration of this enzyme with α-naphthoflavone to concentrations up to 1.6 μM produced partial high-spin character with absorbance increases at 394 nm and decreases at 417 nm (Fig. 2D, gray spectra). This response is consistent with α-naphthoflavone binding in the active site and partially displacing coordinated water from the heme. Indeed, difference spectra showing these initial titrations are characteristic of these “type I” spectral changes (Fig. 2E, gray spectra). However, when higher concentrations of α-naphthoflavone were added, the high-spin component at 394 nm began to decrease, eventually returning to a Soret maximum at absorbance at 417 nm, indicating water binding to the heme (Fig. 2D, red spectra). This reversal in binding mode was also reflected in the difference spectra, as the trough returned to baseline at higher concentrations (Fig. 2E, red lines). The absorbance peak is overshadowed by intrinsic ligand absorbance at below ~375 nm at these high concentrations. With the reversal in binding mode halfway through the titration, the typical plot for the absorbance change versus ligand concentration cannot be fit to a simple two-state model. Even using only the data at the low ligand concentrations corresponding to the initial type I spectral shift reveals a sigmoidal trend (Fig. 2E, main panel), which is best fit using the Hill equation, resulting in a Hill coefficient of 1.6 (95% CI: 0.24–0.30) and S0.5 of 0.26 μM (95% CI: 1.4–1.9). Human CYP1B1 shows a “reverse type I” spectral change upon binding α-naphthoflavone (29), which is likely the result of ligand binding in such a way as to promote water ligation. Overall, the spectral changes observed for the ancestral CYP1B1 enzyme are consistent with α-naphthoflavone binding near the heme iron at lower concentrations where the ligand:P450 stoichiometry is 1:1 or lower and reverting to water-coordinated heme when additional molecules pack into the protein, as observed in the crystal structure.

Ancestral CYP1B1 metabolism of α-naphthoflavone

Both the human CYP1B1 and N98_CYP1B1_Mammal enzymes convert α-naphthoflavone to three monoxygenated products (Fig. 4, M1, M2, and M3). M1 was the major product in both cases (12 ± 4 and 20 ± 6% relative yield for human CYP1B1 and N98_CYP1B1_Mammal, respectively), followed by M2 (~1%) and M3 (~<0.05%). Comparison with products formed by...
hCYP1A1 and hCYP1A2 run in the same system suggested that M1 was likely to be the 5,6-epoxide and M2 was the 7-hydroxy metabolite. The identity of M3 could not be clarified (possibly the 7,8-dihydrodiol, 5,6-dihydrodiol, 7,8-epoxide, or 6-hydroxy metabolite). Application of a two-tailed, unpaired Student’s t test suggested that there was no significant difference between the ratio of M1/M2 produced by the two P450 enzymes (however, N98_CYP1B1_Mammal had a slightly higher ratio of M2/M3 compared with human CYP1B1 (15:1 versus 13:1, p < 0.01)).

Human CYP1B1 has previously been shown to metabolize α-naphthoflavone to more polar, unidentified metabolites (30), but α-naphthoflavone is also a potent inhibitor of human CYP1B1 (4). In the current studies, each of the three products produced by both ancestral and human enzymes had a mass of 289, consistent with the addition of a single oxygen. Whereas these metabolites could not be identified unambiguously by LC-MS alone, and the quantities produced precluded identification by NMR, the fragmentation patterns of M1 and M2 are consistent with an oxide or hydroxyl group added to one of the terminal rings, in positions 5–10 (Fig. S1). The substrate specificity of the human CYP1 enzymes frequently overlaps, so M1, the major metabolite for both human CYP1B1 and N98_CYP1B1_Mammal, may be the 5,6-oxide reported for human CYP1A1 and CYP1A2 (Fig. S1) (31). Similar reasoning suggests that M2 may result from 7-hydroxylation and M3 from 6- or 9-hydroxylation, as observed for rat CYP1A1 (Fig. S2).

**Ancestral CYP1B1 structure with 17β-estradiol**

One of the key questions that arises from the ancestral CYP1B1 crystal structure with α-naphthoflavone is whether the open conformation of the protein was induced by the stacking of multiple ligand molecules within the active-site channel or whether this is an intrinsic feature of the ancestral CYP1B1 protein. To potentially answer this question, attempts were made to co-crystallize with several other ligands. 17β-Estradiol could be co-crystallized with the ancestral CYP1B1 by employing crystallization conditions similar to those yielding the α-naphthoflavone structure. The global structure of the mammalian ancestral CYP1B1 bound to 17β-estradiol was highly similar to the structure bound to α-naphthoflavone, with an RMSD of 0.57 Å. Thus, the 17β-estradiol–bound structure also had a large channel from the active site proper extending between the F helix and β4-loop to the protein surface. However, only one copy of 17β-estradiol was present, located directly adjacent to the heme in the active site proper (Fig. 2C). Despite the open conformation, density was not observed for ordered water molecules in the 17β-estradiol structure. From this, we can conclude that the overall open protein conformation observed is not dependent on the specific ligand or number of copies of the ligand. Thus, it is likely that the additional three α-naphthoflavone molecules take advantage of the open channel to bind fortuitously. As always, however, one cannot exclude the possibility that multiple protein conformations occur and that the specific conformation selected depends on its facility to pack into crystals for structure determination.

Within the active site, the positioning of side chains is also largely conserved compared with the same ancestral enzyme bound to α-naphthoflavone. 17β-Estradiol extends across the heme, with its flat, unsubstituted planar α-surface approximately orthogonal to the heme plane and the stereoidal A and B rings packing against the against the I helix Gly-329–Ala-330 peptide bond (Fig. 2C). Because 17β-estradiol is positioned closer to the heme than α-naphthoflavone, it does not interact with Phe-231 or other F helix residues, but 17β-estradiol does appear to make two hydrogen bonds with active-site residues. The 17β-estradiol C3 hydroxyl is a hydrogen bond donor to the backbone carbonyl of Leu-509 in the β4 loop, whereas the C17 hydroxyl hydrogen bonds with the side chain of Asp-326 in the I helix. The overall result is that 17β-estradiol carbons 7 (4.2 Å), 15 (4.9 Å), and 6 (5.1 Å) are closest to the heme iron (Fig. 2C).
Ancestral mammalian P450 1B1 structures

Ancestral CYP1B1 titrations with 17β-estradiol

Titration of the ancestral CYP1B1 protein with 17β-estradiol resulted in decreases in the Soret band at 417 nm associated with a hyperbolic response (Fig. 2G, main panel) best fit to a single-site binding equation with a $K_d$ of 2.75 μM (95% CI: 2.55–2.96). This is an order of magnitude larger than the α-naphthoflavone $K_d$ and with a 2-fold reduction in the maximal absorbance change ($\Delta A_{max} = 0.03$ for 17β-estradiol versus 0.07 for α-naphthoflavone). Overall, these observations correspond to positioning of 17β-estradiol in the ancestral CYP1B1 structure.

Ancestral CYP1B1 metabolism of 17β-estradiol

It has been observed previously that the ancestral CYP1B1 enzyme is ~9-fold less active toward 17β-estradiol than human CYP1B1 but also shows differences in the regioselectivity. Extant human CYP1B1 produces 4-hydroxy-17β-estradiol as the major metabolite, with smaller amounts of 2-hydroxyestradiol and trace amounts of a third hydroxy-17β-estradiol metabolite likely to be 15α-hydroxy-17β-estradiol. The ancestral CYP1B1 enzyme produces these same three metabolites but in approximately equimolar amounts. The orientation of 17β-estradiol observed in the crystallographic structure is consistent with the formation of 15α-hydroxy-17β-estradiol, but to form the 2- and 4-hydroxy metabolites, 17β-estradiol would need to reorient in the active site so that C2 and C4 were presented to the iron for catalysis at these positions.

The regioselectivity of extant CYP1B1 forms for 4- or 2-hydroxylation is known to vary among species; human, dog, and monkey isoforms preferentially produce 4-hydroxy-17β-estradiol, whereas rodent isoforms produce more 2-hydroxy-17β-estradiol (32). This preference has been attributed to the residue at position 395 (32). Forms containing Val at this position produced more 4-hydroxy-17β-estradiol, whereas those containing Leu produced more 2-hydroxy-17β-estradiol, and exchanging these two residues reversed the regioselectivity. The current results suggest that additional factors influence regioselectivity because whereas the ancestral CYP1B1 has Val at position 395, it does not exhibit a strong preference for either 2- or 4-hydroxylation. In the ancestral CYP1B1 structure with 17β-estradiol, the terminal methyl of Val-395 is 3.6 Å from C16.

Structural changes to the redox partner binding surface

One final notable structural difference between the extant and ancestral CYP1B1 structures occurs on the opposite side of the heme from the active site, on the proximal surface of the protein comprising part of the surface that binds the redox partner NADPH-cytochrome P450 reductase required for catalysis. In particular, the loop between the C and D helices contains three positively charged Arg residues that have been implicated in electrostatic interactions with the negatively charged surface of the reductase. This loop has different conformations in all three structures (Fig. 5A). In the extant human CYP1B1 structure, the C/D loop is positioned closer to the H helix (Fig. 5A, green), whereas in the ancestral CYP1B1 structure with α-naphthoflavone, it is displaced in the opposite direction, toward the L helix (Fig. 5A, blue). In the ancestral CYP1B1 structure with 17β-estradiol, the C/D loop adopts an intermediate conformation (Fig. 5A, purple). In the ancestral CYP1B1 structures, the C/D loop conformations are coincident with similar shifting of the H helix on one side and the loop preceding the J’ helix on the opposite side. As a result, the placement of the positively charged C/D loop guanidine groups is different for all three structures. These differences are reflected in the surface shape and exposed charge for this region (Fig. 5B–D), in which the ancestral CYP1B1 structures display a less prominent concavity and positive charge. The C/D loop region in P450 enzymes is thought to be a critical binding surface for reductase, so it is possible that changes in the charged features in this region affect reductase binding for the ancestral CYP1B1 enzyme. The ancestral CYP1B1 has similar catalytic activity for α-naphthoflavone as the human CYP1B1 enzyme, suggesting that its C/D-loop conformation is compatible with reductase binding. However, when the ancestral CYP1B1 enzyme is bound to 17β-estradiol, the enzyme has much lower activity toward production of all three metabolites than human CYP1B1. One possible explanation for this is that the conformation of the CD loop observed in this structure prevents effective reductase binding and/or electron transfer.

Structural elements and interactions proposed to confer thermostability

A key consideration in engineering P450 enzymes for biocatalysis is thermostability. In the previous literature, several structural features have been proposed to contribute to thermostability, especially when comparing mesophilic and thermophilic P450s. This information is based on a limited data set of four thermophilic P450 enzymes for which structures are available (33–36). A recent meta-analysis of these structures compared with their mesophilic P450 relatives (37) concluded that cytochrome P450 thermostability may correlate with 1) smaller, more compact enzymes, especially due to N-terminal truncation and shorter loops; 2) a preference for charged and hydrophobic residues versus noncharged, polar, and Ala residues; 3) increased salt bridges, particularly complex, extended salt-bridged networks; and perhaps 4) extended clusters of aromatic side chains. These enzymes were all soluble, microbial cytochrome P450 enzymes.

The advantages of using bacterial cytochrome P450 enzymes for biocatalysis often include better stability, but many of them are highly specific for their substrates and the products generated. If a particular soluble P450 can be identified that performs a commercially useful transformation (38) or can be engineered to do so (39–42), then this may be the most advantageous route. However, many of the less stable mammalian cytochrome P450 enzymes have evolved to intrinsically bind and metabolize a wide diversity of foreign compounds and may be more commercially useful if one could engineer in thermostability.

Although the difference in thermostability of the ancestral mammalian CYP1B1 enzyme compared with extant human CYP1B1 is not as dramatic as thermophilic versus mesophilic
bacterial P450 enzymes, it does provide one of the first real opportunities to determine whether some of the same four concepts can confer increased thermostability in eukaryotic microsomal P450s.

First, the overall protein size and lengths of loops and N and C termini were compared. The ancestral CYP1B1 and extant human CYP1B1 structures reveal loop regions of similar length (Fig. 1, A and B). Only small differences are observed in a few of the loops, and they are not always in the anticipated direction. The D/E loop is three residues longer in the ancestral CYP1B1 enzyme (Fig. 1B). Likewise, the H/I loop appears to be six residues longer in the ancestral enzyme, a region that is disordered in both enzymes. A three-residue helical segment occurs in the ancestral CYP1B1 enzyme corresponding to the human K/H11033/L loop, whereas a two-strand β-sheet (the β-4 sheet) occurs in the human enzyme (Fig. 1B). Although both enzymes were truncated to remove the N-terminal transmembrane helix for crystallization, there are obvious differences in the remaining N termini. In the ancestral CYP1B1 enzyme, residues 48–60 could be modeled but not residues 61–67 (due to insufficient density to trace the backbone). By comparison, the human CYP1B1 structure could only be modeled beginning at residue 68 where the A helix begins. Because they are present in the construct crystallized, the absence of electron density for residues 48–60 in the human 1B1 structure must be due to high flexibility. It is not obvious from the primary sequence why this part of the ancestor would be more stable, but this region forms a small β-strand (Phe-55–Trp-57) that interacts with β-sheet 1. Additionally Trp-57 forms a potential face-to-edge aromatic interaction with Phe-74 (A helix). At the C-terminal end of the protein, the ancestor is 16 residues shorter, reducing the overall size by 2 kDa. In human CYP1B1, only six residues in the extended C terminus could be modeled. However, these were packing against symmetry-related molecules (26) and may be more conformationally flexible in solution. This shorter C terminus in the ancestral CYP1B1 enzyme is perhaps comparable with the shorter N termini observed in soluble thermophilic P450 enzymes and may reduce conformational flexibility.

Second, because thermophilic bacterial P450 enzymes seemed to have more charged and hydrophobic residues and fewer noncharged, polar, and Ala residues than related mesophilic P450 enzymes, the amino acid compositions of the ancestral CYP1B1 and extant human CYP1B1 were compared. The charged amino acids and noncharged but polar amino acids were very similar (0.5% different). The more thermostable ancestral CYP1B1 has 2% fewer hydrophobic amino acids, which is opposite to the trends in soluble P450 enzymes. Conversely, the ancestral enzyme also had 1% fewer Ala residues, which is consistent with the thermostable bacterial P450 trends. Of the 12 times that Ala in human CYP1B1 was replaced by another amino acid in the ancestral CYP1B1, 10 of these substitutions were to nonconserved amino acids (Pro (five), Glu (three), Arg (one), or Ser (one)).
Third, salt-bridge pairs and networks were compared between the two structures. In the extant human CYP1B1 structure, 13 two-residue salt bridges were found, whereas in the ancestor CYP1B1 structure, 14 were present. Evaluation of salt-bridge networks involving three or more residues revealed that human CYP1B1 had six, and the ancestral CYP1B1 had eight (Table S1). It is useful to examine two-residue salt bridges and networks that are unique to only one of the CYP1B1 enzymes (Fig. 6, A and B, spheres). In the extant human CYP1B1, five of the seven unique two-residue interactions and the one unique network occur within the same structural element, generally with residues found in close proximity within the primary sequence (Fig. 6A (black text) and Table S1 (red text but not boldface)). Two unique two-residue salt bridges occur between secondary structural elements (Fig. 6A (colored text) and Table S1 (red and boldface text)). In the mammalian ancestor, of the six unique two-residue salt-bridge interactions (Fig. 6B, gray spheres), three of them occur between different secondary structure elements (Fig. 6B (spheres with colored text) and Table S1 (red boldface text)). Additionally, of the three unique networks in the ancestor (Fig. 6B, black spheres), all three occur between secondary structure elements (Fig. 6B (colored text) and Table S1 (red, boldface text)). Such interactions likely help stabilize multiple regions of the protein and could be a contributor to the thermostability of the ancestor.

Finally, soluble thermophilic cytochrome P450 enzymes, such as CYP119A1 (33) and CYP119A2 (34) from archaea, have extended networks of aromatic residues. Analysis of aromatic and/or cation-π stacking interactions in the human and ancestral CYP1B1 proteins indicated some differences (Table S2). Human CYP1B1 contained six two-residue pairs, three of which had stacking interactions with a third aromatic residue. By comparison, the ancestral CYP1B1 structure had five unique two-residue aromatic interactions (Fig. 6D). Four of these interactions are likely to be involved in the thermostability of the protein.
action pairs occur between residues found in distinct secondary structure elements and thus are more likely to contribute to global protein structure.

**Conclusions**

The first structural evaluation of a reconstructed ancestral cytochrome P450 has reinforced several existing concepts about enzyme functionality and stability but also suggested some new ones. The ancestral CYP1B1 enzyme retains most of the active-site residues of the extant human CYP1B1 enzyme and retains some of the metabolic capabilities and metabolite stoichiometries for some substrates (in this case α-naphthoflavone) while showing very different metabolic capacity and metabolite ratios for others (e.g. 17β-estradiol). Subtle amino acid substitutions outside the active site seem to control overall protein conformation, in this case restoring the helical nature of the entire F helix and opening up a broad channel from the active site to the protein surface. Whereas ideas about shorter loops contributing to thermostability did not appear to apply here, a C-terminal truncation may be relevant. The somewhat more thermostable ancestral enzyme did show a few more electrostatic and aromatic interactions that could contribute to thermostability, but the locations of such interactions between distinct secondary structure elements, often far apart in the primary sequence, may be more critical. Additional structures will be required to determine whether these trends continue in even older reconstructions that have higher stability.

**Experimental procedures**

**Materials**

pCW plasmids containing CYP1B1, N98_CYP1B1_Mammal and human NADPH-cytochrome P450 reductase (hCPR) were constructed in previous studies (7, 26). Catalytic assays with human CYP1B1 used construct #3 from Ref. 7. All binding, catalytic assays, and crystallography for the ancestral CYP1B1 were done with the N-terminally truncated, C-terminally His-tagged version of N98_CYP1B1_Mammal, with the sequence shown in **Fig. 1B**. The chaperone co-expression plasmid, pGro7, was obtained from Prof. K. Nishihara (HSP Research Institute, Kyoto, Japan) (43) and from Takara Bio Inc. DH5aF’IQ *Escherichia coli* cells were purchased from Thermo Fisher Scientific (Scoresby, Australia).

Bactotryptone, bactopeptone, and yeast extract were supplied by Becton Dickinson Pty. Ltd. (North Ryde, Australia). 17β-Estradiol metabolites were purchased from Steraloids Inc. (Newport, RI) and kindly made available for this work by Prof. F. Peter Guengerich (Vanderbilt University). Emulgen 913 and 911 were obtained from Kao Chemicals (Tokyo, Japan). All other materials, including α-naphthoflavone and 17β-estradiol, were purchased at the highest available grade from Sigma-Aldrich-Merck (Castle Hill, Australia) where possible or from a local supplier.

**Preparation of bacterial membranes for activity assays**

P450s and hCPR were expressed in *E. coli* and isolated in bacterial membranes as described previously (44, 45). P450 content was quantified by Fe(II).CO versus Fe(II) difference spectroscopy (46, 47). NADPH-cytochrome P450 reductase was quantified by the reduction of the surrogate electron acceptor, cytochrome c, as described previously (48).

**Metabolic assays**

Metabolic incubations were carried out as described previously with modifications (49). Bacterial membranes containing 0.5 μM P450 were incubated for 60 min at 37 °C in 100 mM potassium phosphate, pH 7.4, with 25 μM α-naphthoflavone in the presence and absence of an NADPH-generating system (100 mM glucose 6-phosphate, 25 μM NADP+, and 0.5 units/ml glucose-6-phosphate dehydrogenase). Progesterone (5 μM) was added as an internal standard for α-naphthoflavone assays, prior to quenching and extraction with 1 ml of ethyl acetate.

α-Naphthoflavone and its metabolites were identified using a Shimadzu Nexera Ultraperformance LC system coupled to an Orbitrap Elite mass spectrometer. Chromatography was performed on an Accucore™ C30 column (150 × 2.1 mm, 2.6 μm) at 0.4 ml/min at 50 °C. Buffer A comprised 1% acetonitrile, 0.1% formic acid, and buffer B comprised 90% acetonitrile, 0.1% formic acid. The gradient was developed as follows: 0 – 0.5 min, 50% B; 0.5–3 min, linear gradient to 98% B; 3–4.5 min, 98% B; 4.5–4.6 min, linear gradient to 50% B; 4.6–5 min, 50% B. The mass spectrometer was operated in positive mode. Source parameters included S lens 60; ion spray voltage 4.5 kV; capillary temperature 380 °C; heater 400 °C; sheath gas and auxiliary gases 50 and 16, respectively. Data-dependent acquisition was performed across 50–1500 m/z, and the top five precursors were selected. Higher-energy collisional dissociation fragmentation was optimized to use a normalized collision energy of 60. Data were processed using Xcalibur™ software. Spectra were searched against publicly available databases. α-Naphthoflavone metabolites were quantified using UPLC-APCI-MRM with the same chromatography conditions, but with a SCIEX 5500 QTRAP mass spectrometer operating in positive multiple-reaction-monitoring (MRM) mode. Source parameters included a nebulizer current of 3 μA, temperature at 500 °C, nebulizing gas (GS1) at 45, declustering potential at 120, and collision cell exit potential of 12. For each compound, the collision energy, entrance potential, and cell exit potential were optimized for maximum sensitivity by manual infusion. Data were processed using both Analyst™ 1.7 and Multiquant™ 3.0 (SCIEX) software. MRM transitions used for detection are listed in **Table S3**, and the recovery of α-naphthoflavone and its metabolites were estimated based on the relative intensity of MS/MS fragment 103.

**Protein expression and purification for ligand-binding assays and crystallization**

Expression and purification of the N98_CYP1B1_Mammal for crystallization were based on methods used for expression of human CYP1A1 for crystallography (50) but modified extensively, so a detailed expression protocol is described below for clarity. The expression plasmid pCW_N98_1B1_Mammal was transformed into *E. coli* DH5α chemically competent cells already containing the plasmid pGro7 for coexpression of the
molecular chaperones GroEL/GroES. Transformed cells were plated on lysogeny broth (LB)-agar supplemented with 100 µg/ml carbenicillin for selection of the P450 plasmid and 20 µg/ml chloramphenicol for selection of the pGro7 plasmid and grown for ~18 h at 37 °C. A single colony from this plate was inoculated into a 5-ml LB starter culture, supplemented with antibiotics described above, and grown for ~7 h at 37 °C and shaking at 250 rpm. This starter culture (50 µl) was then used to inoculate a 200-ml LB culture, supplemented with the above antibiotics, grown for ~16 h at 37 °C and shaking at 250 rpm. Finally, this overnight culture (10 ml) was used to inoculate 250-ml cultures of Terrific Broth in 1-liter Erlenmeyer flasks containing the above antibiotics. The culture volume grown for a single purification typically totaled 2 liters or eight flasks. These expression cultures were grown at 37 °C with 250 rpm shaking until they reached an optical density at 600 nm (OD600) of 0.3. At this point, chaperone expression was induced by the addition of 2 mg/ml arabinose, and δ-aminolevulinc acid was added to 1 mM as a heme precursor. The temperature and shaking of the cultures were then reduced to 25 °C and 225 rpm, respectively. At OD600 of 0.6, N98_CYP1B1_Mammal expression was induced by adding 1 mM isopropyl-1-thio-β-D-galactopyranoside. Cultures were grown for 48 h after induction.

Isolation of ancestral N98_CYP1B1_Mammal was performed using a purification method that was developed for human CYP1A1 engineered for crystallization as described (50) with only slight changes. Modifications included depletion of the detergent CHAPS during ion-exchange chromatography by using a carboxymethyl Sepharose wash buffer (10 mM potassium phosphate, 100 mM NaCl, 20% (v/v) glycerol, 1 mM EDTA, pH 7.4) for the wash step and carboxymethyl elution buffer (50 mM potassium phosphate, 500 mM NaCl, 20% (v/v) glycerol, 1 mM EDTA, pH 7.4) to elute the bound protein. Additionally, the final protein buffer used during gel filtration was a lower ionic size-exclusion chromatography buffer (20 mM potassium phosphate, 20% glycerol, 0.1 M NaCl, pH 7.4). Under these conditions, the main N98_CYP1B1_Mammal peak eluted from the size-exclusion column as a trimer, with a shoulder corresponding to a dimeric species. The major trimeric peak was pooled and used in ligand binding and crystallization experiments. The final purity of the protein was assessed by SDS-PAGE and the ratio of the Soret peak to the 280-nm peak. The protein appeared to be a single band on the SDS-polyacrylamide gel and typically had A416 nm/A280 nm = 1.2. The protein concentration was determined using the absorbance of the Soret band and an extinction coefficient of 100 mM⁻¹ cm⁻¹ in the size-exclusion chromatography buffer listed above. Finally, a reduced carbon monoxide-difference spectrum (47) revealed a peak exclusively at 450 nm, consistent with active enzyme, and no peak or shoulder at 420 nm.

**Ligand-binding assays**

α-naphthoflavone and 17β-estradiol binding to N98_CYP1B1_Mammal were monitored spectrophotometrically. Initial titrations in the absolute spectrum mode employed 1 µM P450 in buffer (50 mM potassium phosphate, 500 mM NaCl, 20% glycerol, 0.5% (w/v) CHAPS, pH 7.4) in 1-cm quartz cuvettes, with additions of ligand dissolved in DMSO being added to the sample cuvette and equal volumes of ligand added to the reference cuvette only containing buffer. Subsequent titrations accomplished in difference mode to focus on ligand-induced changes used 1 µM protein in 1-cm quartz cuvettes in the same buffer. Data were fit to a simple one-site hyperbolic binding equation for estradiol and to the Hill equation for α-naphthoflavone over the concentration ranges shown in the figures.

**Crystallization, data collection, structure determination, and analysis**

Purified N98_CYP1B1_Mammal was first saturated with either α-naphthoflavone or 17β-estradiol by the addition of 100 µM ligand during final concentration using centrifugal ultrafiltration (Amicon Ultra-15 filters with a molecular mass cut-off of 50 kDa), once for α-naphthoflavone and three times for estradiol. Crystals of the ancestral CYP1B1 protein with α-naphthoflavone were then grown at 20 °C using the sitting-drop vapor diffusion method in 96-well plates by mixing 0.5 µl of N98_CYP1B1_Mammal/α-naphthoflavone (protein at 290 µM as determined by absolute spectrum) with 0.5 µl of crystallization solution (900 mM sodium citrate tribasic, 90 mM Tris base/HCl, pH 7.0, 180 mM NaCl, 10% glycerol) and equilibrating against a 50-µl reservoir of the same crystallization solution. Cocryocristallization with estradiol was performed at 20 °C in sitting-drop 24-well plates by mixing 0.9 µl of N98_CYP1B1_Mammal/estradiol (290 µM) with 1.1 µl of crystallization solution (1 mM sodium citrate tribasic, 0.1 M Tris/HCl, pH 7.0, 0.2 M NaCl) and equilibrating against 250 µl of the same crystallization solution. Cubic crystals grew in 2–3 days. Harvested crystals were cryoprotected using the respective crystallization solution supplemented with either additional 20% glycerol (α-naphthoflavone) or 22% glycerol (estradiol) before being flash-cooled in liquid nitrogen. The N98_CYP1B1_Mammal/α-naphthoflavone diffraction data were collected on a single crystal at the Advanced Photon Source LS-CAT beamline 21-ID-G. The N98_CYP1B1_Mammal/estradiol diffraction data were collected on a single crystal on beamline 12-2 at the Stanford Synchrotron Radiation Lightsource.

Data processing for the N98_CYP1B1_Mammal/α-naphthoflavone data set was performed with HKL2000 (52). The N98_CYP1B1_Mammal/estradiol data set was processed in XDS (53) with scaling in AIMLESS (54). A molecular replacement solution for N98_CYP1B1_Mammal/α-naphthoflavone was initially obtained using MolRep (55) using the CYP1B1/α-naphthoflavone crystal structure (PDB: 3PM0) as a search model (contrast score of 9.9). Inspection of packing and electron density as well as initial statistics after one round of refinement (R = 0.34 and Rfree = 0.43) indicated a correct solution with two molecules in the asymmetric unit. A molecular replacement solution for the N98_CYP1B1_Mammal/estradiol structure was obtained using Phaser (56) and the N98_CYP1B1_Mammal/α-naphthoflavone (PDB: 6OYU) structure as a search model. The molecular replacement solution was successful with a log likelihood of 3,702 and translation function Z score of 58. For both structures, multiple rounds of model building and refinement were performed using COOT (57) and PHENIX.
(58). Torsion angle noncrystallographic symmetry (NCS) with automatic group NCS group determination was employed in Phenix for the slightly lower-resolution 17β-estradiol structure. Coordinates and restraints for α-naphthoflavone and estradiol were obtained from the CCD (Chemical ID: BHF and EST, respectively) and minimized through PHENIX eLBOW (59) using AM1 geometry optimization.

Structural comparisons with extant human P450 enzymes were executed using molecule B of N98_CYP1B1_Mammal complexed with α-naphthoflavone. Whereas the two molecules in the asymmetric unit of each structure with both ligands are all similar, the rationale was that the α-naphthoflavone structure had higher resolution than the estradiol structure, that all of the human CYP1 structures were obtained with α-naphthoflavone as the ligand, and that chain B of the α-naphthoflavone structure is most complete. By comparison, chain A of the α-ancestral CYP1B1 naphthoflavone structure has an additional disordered gap for amino acids 448–452 comprising the K’–K” loop. Calculation of the active-site void volumes for both N98_CYP1B1_Mammal/α-naphthoflavone and human CYP1B1 (PDB: 3PM0) (26) was performed using VOIDOO (60) with a probe radius of 1.4 Å and grid spacing of 1.0. For the N98_CYP1B1_Mammal/α-naphthoflavone structure, it was necessary to add a network of water molecules to close the large cleft on the surface of the protein for the purposes of active-site volume calculation. Structural alignments were performed using the super command in PyMOL (25). Sequence alignment was performed using CLC Genomics Workbench (61). Analysis of salt bridges was performed in COOT (57) using a definition of ≤4 Å between the oxygen of Glu or Asp side chains and the nitrogen group(s) of Arg, His, or Lys (36, 37). Aromatic and π-cation stacking interactions were defined by selection of aromatic residues with ring-to-ring or ring-to-arginine guanidinium group distances of ≤4 Å using Schrödinger Maestro (Schrödinger, LLC, New York). All salt-bridge and stacking interactions were visually inspected to ensure that residue positions were well-defined in the electron density. Structure figures were prepared using PyMOL (25) with electrostatic potential surfaces generated using APBS (24).

Data availability

The atomic coordinates and structure factors for the ancestral CYP1B1 structures have been deposited in the Protein Data Bank under accession codes 6OYU (complex with α-naphthoflavone) and 6OYV (complex with estradiol). For titrations in Fig. 2, not all intervening scans are shown, but they are available from the corresponding author. All other data are included in the article.

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