Expression, Purification, and Biochemical Characterization of the Flavocytochrome P450 CYP505A30 from *Myceliophthora thermophila*

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**ABSTRACT:** The cytochrome P450/P450 reductase fusion enzyme CYP505A30 from the thermophilic fungus *Myceliophthora thermophila* and its heme (P450) domain were expressed in *Escherichia coli* and purified using affinity, ion exchange, and size exclusion chromatography. CYP505A30 binds straight chain fatty acids (from ~C10 to C20), with highest affinity for tridecanoic acid ($K_d = 2.7$ μM). Reduced nicotinamide adenine dinucleotide phosphate is the preferred reductant for CYP505A30 ($K_M = 3.1$ μM compared to 330 μM for reduced nicotinamide adenine dinucleotide in cytochrome c reduction). Electron paramagnetic resonance confirmed cysteine thiolate coordination of heme iron in CYP505A30 and its heme domain. Redox potentiometry revealed an unusually positive midpoint potential for reduction of the flavin adenine dinucleotide and flavin mononucleotide cofactors ($E^{0'} = -118$ mV), and a large increase in the CYP505A30 heme domain Fe$^{III}$/Fe$^{II}$ redox couple (ca. 230 mV) on binding arachidonic acid substrate. This switch brings the ferric heme iron potential into the same range as that of the reductase flavins. Multangle laser light scattering analysis revealed CYP505A30’s ability to dimerize, whereas the heme domain is monomeric. These data suggest CYP505A30 may function catalytically as a dimer (as described for *Bacillus megaterium* P450 BM3), and that binding interactions between CYP505A30 heme domains are not required for dimer formation. CYP505A30 catalyzed hydroxylation of straight chain fatty acids at the ω-1 to ω-3 positions, with a strong preference for ω-1 over ω-3 hydroxylation in the oxidation of dodecanoic and tetradeconoic acids (88 vs 2% products and 63 vs 9% products, respectively). CYP505A30 has important structural and catalytic similarities to P450 BM3 but distinct regioselectivity of lipid substrate oxidation with potential biotechnological applications.

**INTRODUCTION**

The cytochromes P450 are a superfamily of heme-binding enzymes found in virtually all organisms. They are typically monoxygenases, catalyzing the oxidative scission of dioxygen (O$_2$) bound to their heme iron with the insertion of one atom of oxygen into a substrate bound in their active site and the other atom reduced to form a water molecule. Although often referred to as hydroxylases, P450s can also catalyze a wide range of other reactions, including N-dealkylation, demethylation, sulfoxidation, dehydrogenation, reduction, epoxidation, C=C bond formation, and decarboxylation.

Human P450s play key roles in the transformation of xenobiotics (e.g., pharmaceuticals and environmental toxins) to facilitate their detoxification and excretion, and also in the biosynthesis and interconversion of steroids. Microbial P450s have diverse roles, including the catabolism of organic compounds as energy sources and oxidative reactions in the synthesis of polyketides.

Most of the cytochromes P450 require electron transfer partners to provide them with electrons that are ultimately derived from reduced nicotinamide adenine dinucleotide phosphate (NADPH) or reduced nicotinamide adenine dinucleotide (NADH). Most P450s fall into one of two major classes, based on their redox partner systems. Class I P450s use a NAD(P)H-dependent, flavin-containing ferredoxin reductase and an iron–sulfur cluster binding ferredoxin. The class I P450s are usually membrane-bound in eukaryotes (e.g., adrenal mitochondrial P450s involved in steroid biosynthesis) but are soluble in prokaryotes (e.g., the camphor hydroxylase P450cam, CYP101A1). Class II P450s use a NADPH-dependent cytochrome P450 reductase (CPR) redox partner that binds both flavin adenine dinucleotide (FAD) and flavin...
Figure 1. continued

Bacillus | LSGQALHECERFQFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 111
Fusarium | VSNSLNKVECBEREFKFTKLYSLQCGFGLPFTDMEKHNKExABHLLLPSFGQ-A | 112
Neurospora | VTSQALHECERFQFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 118
Myceliophthora | VSQALHECERFQFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 119
Chaetomium | VSQALHECERFQFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 120

Bacillus | MGRKMNNVCAQVYLKNEKLENHEDEIVFEDMTQIL1TDGCFLNFWRYFVRFDQPH | 172
Fusarium | IRQHPDMHKVQCSLCMKFARQGFTPDTSISFMTRIL1TDLIALCMDFQ/story | 178
Neurospora | IQNMPTMEHIQAIQAXLHARKSFQPDIVMTDFDTRPIL1TDLIALCMDFQ/story | 177
Myceliophthora | IQNMPTMEHIQAIQAXLHARKSFQPDIVMTDFDTRPIL1TDLIALCMDFQ/story | 180
Chaetomium | IQNMPTMEHIQAIQAXLHARKSFQPDIVMTDFDTRPIL1TDLIALCMDFQ/story | 180

Bacillus | FFH55MVDAELMNKQHAPFO-DQATPAKMKQREUDQEPKZVNLKQIDRKKQAGESQ | 230
Fusarium | FFH55MVDAELMNKQHAPFO-DQATPAKMKQREUDQEPKZVNLKQIDRKKQAGESQ | 232
Neurospora | FFH55MVDAELMNKQHAPFO-DQATPAKMKQREUDQEPKZVNLKQIDRKKQAGESQ | 233
Myceliophthora | FFH55MVDAELMNKQHAPFO-DQATPAKMKQREUDQEPKZVNLKQIDRKKQAGESQ | 236
Chaetomium | FFH55MVDAELMNKQHAPFO-DQATPAKMKQREUDQEPKZVNLKQIDRKKQAGESQ | 239

Bacillus | VGDGKQNLKQLFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 290
Fusarium | KDLIANQASKLQKQFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 292
Neurospora | KDLIANQASKLQKQFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 296
Myceliophthora | KDLIANQASKLQKQFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 296
Chaetomium | KDLIANQASKLQKQFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 299

Bacillus | AEKAAEQLQVYLVKQVNQFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 350
Fusarium | QVEQDVQGKQVFGLQVENKQKQNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 352
Neurospora | QVEQDVQGKQVFGLQVENKQKQNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 357
Myceliophthora | QVEQDVQGKQVFGLQVENKQKQNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 358
Chaetomium | QVEQDVQGKQVFGLQVENKQKQNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 359

Bacillus | DELYVLVIQFLRLOKRT | 405
Fusarium | ETVALLSKQKQFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 412
Neurospora | QFALLQASKLQKQFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 417
Myceliophthora | QFALLQASKLQKQFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 419
Chaetomium | QFALLQASKLQKQFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 419

Bacillus | ALKIAVVLQIULZMODKDLQIUSKQKQFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 455
Fusarium | ANQHMIQALKWVQIUNQFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 472
Neurospora | ANQHMIQALKWVQIUNQFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 477
Myceliophthora | ANQHMIQALKWVQIUNQFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 478
Chaetomium | ANQHMIQALKWVQIUNQFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 479

Bacillus | GQH155FO7T | 510
Fusarium | GQH155FO7T | 529
Neurospora | GQH155FO7T | 529
Myceliophthora | GQH155FO7T | 536
Chaetomium | GQH155FO7T | 536

Bacillus | FO-VATLSGLQHDLQFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 563
Fusarium | ATTNGQALQKQKQFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 569
Neurospora | ATIDLQALQKQKQFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 590
Myceliophthora | AAEEVLQALQKQKQFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 596
Chaetomium | ATVDDLPDVQALQKQKQFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 596

Bacillus | CDRKMTAQYAF | 627
Fusarium | CDROAQQYAF | 652
Neurospora | CDROAQQYAF | 652
Myceliophthora | CDROAQQYAF | 656
Chaetomium | CDROAQQYAF | 656

Bacillus | FNLDI55SNFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 682
Fusarium | FNLDI55SNFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 698
Neurospora | FNLDI55SNFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 706
Myceliophthora | FNLDI55SNFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 711
Chaetomium | FNLDI55SNFQKNGLFQKLSLQKYFVRFAFGGLPFTDMEKHNKExABHLLLPSFGQ-A | 711

Bacillus | HILDFLEF | 741
Fusarium | HILDFLEF | 755
mononucleotide (FMN) cofactors. These are typified by mammalian systems in the liver, where both the CPR and the P450s are bound to the endoplasmic reticulum through a helical N-terminal transmembrane anchor. P450s are characterized by the proximal ligation of their heme iron through an invariant Cys thiolate, a residue essential for the monooxygenase activity of P450s. A water molecule typically occupies the distal site on the ferric heme iron in the resting state of a P450. However, when a substrate binds, the distal water is displaced, and it is replaced by dioxygen once the heme iron is reduced to the ferrous form. A further reduction step mediated by the redox partner converts the ferrous−oxo form to the ferric−peroxo state, which is then protonated in two consecutive steps, forming the reactive intermediates compound 0 (ferric−hydroperoxo) and then compound I (ferryl−oxo). Compound I is considered to be the major species responsible for substrate oxidation in P450 enzymes.

A particular group of P450 enzymes is exemplified by the Bacillus megaterium P450 BM3 (CYP102A1, BM3), in which the P450 (N-terminal) is fused to a CPR enzyme through a short peptide linker region. BM3 was first characterized by Armand Fulco’s group, who demonstrated that the enzyme was soluble and that it acted as a highly efficient and catalytically self-sufficient fatty acid hydroxylase, requiring only NADPH and a lipid substrate for activity. BM3 was reported to catalyze oxidation of arachidonic acid with a rate constant of >17 000 min⁻¹. BM3 and P450cam are probably the best characterized P450 enzymes, and both of these enzymes have...
been extensively engineered to explore their catalytic mechanism and in efforts to diversify their substrate selectivity. Recent protein engineering studies on BM3 have produced variants capable of oxidizing alkanes and steroids, and of producing metabolites of human drugs.\(^\text{19–21}\) The catalytic efficiency of the P450 BM3 enzyme has made it a popular enzyme for synthetic biology applications. However, \textit{B. megaterium} is a meso thermophilic organism that grows optimally at \(\sim 30 ~\text{°C}\), and a lack of thermostability of the BM3 enzyme may thus pose challenges for its application in synthetic biology. In particular, FMN binding is relatively weak in BM3 in comparison to that of mammalian CPR enzymes.\(^\text{22}\) In addition, a dimeric state of BM3 is the catalytically relevant form of the enzyme, and the dimer may also be destabilized at higher temperatures.\(^\text{23}\)

In this report, we describe the identification, expression, biochemical, spectroscopic, kinetic, and catalytic properties of a novel, eukaryotic P450–CPR fusion enzyme (CYP505A30) from the moderately thermophilic fungus \textit{Myceliophthora thermophila}, an organism that grows optimally at \(\sim 45–50 ~\text{°C}\).\(^\text{24}\) The spectroscopic, biophysical, thermodynamic, and kinetic properties of CYP505A30 are detailed to provide insights into the catalytic properties of this novel member of the eukaryotic CYP505 P450 family.

## RESULTS

### CYP505A30 Amino Acid Sequence and Phylogenetic Analysis

The \textit{M. thermophila} CYP505A30 amino acid sequence (Figure 1) reveals a protein of 1080 residues (including the initiator methionine) that is closely related to other fungal members of the CYP505 family, for example, the well characterized CYP505A1 (P450foxy from \textit{Fusarium oxysporum}, 57% identity),\(^\text{25}\) and CYP505 family orthologues in \textit{Neurospora crassa} OR74A (64% identity) and in the saprophytic fungus \textit{Chaetomium globosum} CBS 148.51 (79.5% identity). P450foxy was shown to catalyze the hydroxylation of decanoic acid (C10:0), undecanoic acid (C11:0), and dodecanoic acid (C12:0) at the \(\text{o}-1, \text{o}-2,\) and \(\text{o}-3\) positions, as was also reported for P450 BM3 by Fulco and co-workers for a number of fatty acid substrates.\(^\text{26,27}\) Figure 1 shows the amino acid alignment of CYP505A30 with the well-studied \textit{B. megaterium} P450 BM3 (BM3, CYP102A1) and with three other members of the eukaryotic CYP505 family, revealing conserved regions in these enzymes associated with functions including heme, FAD, and FMN binding. Important conserved residues in CYP505A30 include Phe93 (Phe87 in BM3) for regioselectivity of substrate oxidation; Glu273/Thr274 (Glu267/Thr268) for protonation of iron–oxo species in the P450 catalytic cycle; Cys411 (Cys400) as the heme iron proximal ligand with Phe404 (Phe393) as a regulator of heme iron potential; and Ser857/Cys1035/Asp1077 (Ser830/Thr829/Cys999/Asp1044) involved in NADPH binding/FAD cofactor reduction.\(^\text{28}\)

Figure 2 shows a phylogenetic tree describing the relatedness of CYP505A30 to other selected members of the CYP505 family, and to the \textit{B. megaterium} fatty acid hydroxylase BM3. Other bioinformatics studies used the genome mining tool antiSMASH to identify secondary metabolite biosynthesis gene clusters in \textit{M. thermophila}.\(^\text{28}\) This analysis revealed that the CYP505A30 gene on chromosome 3 of \textit{M. thermophila} is located directly adjacent to a type 1 polyketide synthase gene cluster that is predicted to encode proteins required for production of a monodictyphenone class (phenolic benzophe-

![Figure 2](image-url). Phylogenetic tree of \textit{M. thermophila} CYP505A30 and related CYP505 family P450–CPR fusion enzymes. The amino acid sequences of several selected members of the CYP505 family were aligned using the Clustal Omega program, and the image was made using the program FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

The CYP505A30 amino acid sequence aligns closely with CYP505A2 from \textit{N. crassa} OR74A (64.3% identity), and has 56.5% amino acid sequence identity with CYP505A5 from \textit{Magnaporthe grisea}. CYP505\(^\ast\) and CYP505\(^\ast\) proteins are P450 and P450–CPR fusions, respectively, identified in the genome of the \textit{Thielavia terrestris} fungus. CYP505\(^\ast\) protein has 73.5% identity with CYP505A30.\(^\text{60}\) CYP505A30’s orthologue from \textit{C. globosum} is 79.5% identical to CYP505A30.

none) molecule. A near-identical gene organization was found in \textit{Sclerotinia borealis}, a psychrophilic plant pathogen. However, there is no evidence that CYP505A30 is involved in polyketide synthesis in either organism.

### Expression and Purification of CYP505A30 and Its Heme Domain

CYP505A30 and its heme domain were expressed in \textit{Escherichia coli} and purified as described in the Experimental Methods section. Typical recovery yields were \(\sim 15 \text{ mg/L}\) of expression cell culture in the case of intact CYP505A30, and \(\sim 40 \text{ mg/L}\) for the heme domain. Figure 3 shows sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels demonstrating purification of both CYP505A30 and its heme domain. The apparent molecular masses, by comparison to protein standards, are \(\sim 120\) and \(\sim 55 \text{ kDa}\) for CYP505A30 and its heme domain, respectively. These are consistent with the predicted masses from the protein amino acid sequences of 120.22 (CYP505A30) and 53.23 kDa (heme domain), including the N-terminal hexahistidine tag region.

### UV–Visible Spectroscopic Properties of CYP505A30 and Its Heme Domain

Figure 4 shows the UV–visible spectra for (i) the ligand-free, oxidized (OX) CYP505A30, and for (ii) a sodium dithionite-reduced form of CYP505A30. In the OX, ligand-free state, CYP505A30 has a typical P450 absorption spectrum with the Soret maximum at 415 nm, and with \(\alpha\)– and \(\beta\)-bands in the visible region at \(\sim 567\) and \(\sim 533\) nm, respectively. There are also large CYP505A30 spectral
substrate-free, ferric CYP505A30 heme iron. Reduction potentials are considerably more positive than that of the ~360 nm. Addition of limited amounts of dithionite bleaches the flavin absorbance, indicating extensive reduction of the flavins. However, there is not a substantial effect on the wavelength maximum of the Soret band at ~415 nm, suggesting that the heme does not become significantly reduced under the conditions used and that the reduction potential of the low-spin (LS), ferric CYP505A30 heme iron is considerably more negative than those of the flavins. There is a small increase in spectral intensity between ~530 and 650 nm in the dithionite-reduced enzyme, likely due to a proportion of the flavins being in the neutral SQ state with an absorbance maximum at ~600 nm.29,30

Figure 4 shows the UV–visible spectra for (i) the ligand-free, OX CYP505A30 heme domain, (ii) the sodium dithionite-reduced heme domain, (iii) the heme domain bound to arachidonic acid and in a high-spin (HS) form, (iv) the nitric oxide (NO) complex of the OX heme domain, and (v) the ferrous/CO-bound form of the heme domain. The ferric heme domain has a Soret maximum at 415 nm and the α- and β-bands at 567 and 533 nm, respectively. The Reinheitszahl (Rz, $A_{340} / A_{280}$) value is ~1.45 for the purified heme domain. On reduction of the heme domain with a larger amount of dithionite than that which was used to reduce the flavins in intact CYP505A30, the heme iron was fully reduced and the Soret band shifted to 411 nm, with a single spectral feature in the Q-band region at approximately 546 nm. The arachidonic acid substrate-bound form has an absorbance maximum at 399 nm and is extensively HS. The HS character is further confirmed by the development of an absorbance band at ~650 nm that is characteristic of a ferric heme iron-to-Cys thiolate charge transfer (CT) complex. NO binding to the heme domain resulted in a Soret shift to 434 nm, along with the development of two prominent absorbance bands at 543 and 537 nm. The spectrum for the CYP505A30 heme domain Fe$^{III}$–NO complex is similar to that reported previously for the P450 BM3 heme domain.31 On binding CO to the ferrous heme domain, a characteristic shift of the Soret feature to 450 nm was seen, along with the development of a single band at ~550 nm in the Q-band region. This spectrum is characteristic of a P450 Fe$^{II}$–CO complex in which Cys thiolate is retained as the proximal ligand to the heme iron.2

Analysis of the Binding of the Flavin Cofactors to CYP505A30. The UV–visible spectrum of intact CYP505A30 (Figure 4) is representative of several preparations of this enzyme. The CYP505A30 flavin absorbance is relatively more intense (compared to its heme absorbance) than is the case for the well-studied flavocytochrome P450 BM3 enzyme, suggesting that heme incorporation is substoichiometric in CYP505A30. In contrast, the CYP505A30 heme domain showed a consistent level of heme incorporation, corresponding to at least 90% heme content in the highly purified samples. Previous studies on the P450 BM3 orthologues CYP102A2 and CYP102A3 from Bacillus subtilis also revealed substoichiometric heme incorporation in the case of the intact flavocytochrome CYP102A when expressed in E. coli cells.32 To compare heme content with those of the flavin cofactors, a 40 µM sample of CYP505A30, quantified based on a heme extinction coefficient of $e_{418} = 105 \text{mM}^{-1} \text{cm}^{-1}$,33 was incubated at 90 °C for 10 min in buffer B to release flavin cofactors from its reductase domain. The FAD and FMN cofactors released were then resolved using high-performance liquid chromatography (HPLC) and their concentrations determined using HPLC by reference to the peak areas of flavin (FAD and FMN) standards (Sigma-Aldrich), as described in the Experimental Methods section. Interpolation provided CYP505A30 flavin concentrations of 41.5 and 49 µM for FAD and FMN, respectively. These data are apparently confirmatory of the substoichiometric binding of heme to CYP505A30, particularly in view of the known tighter binding of FAD over that of FMN in CPR enzymes. For example, FMN is more readily dissociated from CPR by chaotropes than is FAD (e.g., by potassium bromide), and the

Figure 3. Purification of CYP505A30 and its heme domain. CYP505A30 and its heme domain were expressed in E. coli and purified as described in the Experimental Methods section. The SDS-PAGE gel on the left side shows molecular weight markers in the first lane (Bio-Rad Precision Plus Unstained Standards 10–250 kDa) and fractions of the purified CYP505A30 heme domain at ~55 kDa in the lanes marked 1 and 2. In the adjacent SDS-PAGE gel, the lanes marked 3 and 4 contain purified intact CYP505A30 at ~120 kDa, with the final lane containing the same molecular weight markers as before. The apparent masses of the two protein samples correlate well with their predicted masses (53.23 kDa for the heme domain and 120.22 kDa for intact CYP505A30).
FMN \( K_\text{D} \) values are 42 ± 7 and 86 ± 14 nM for the FMN (flavodoxin-like) domains of human and P450 BM3 reductases, compared to, for example, 2.4 nM for Desulfovibrio vulgaris flavodoxin.\(^{33-36} \) FAD binding to rat CPR was reported to be subnanomolar,\(^{37} \) and it is likely that FAD binding is near-stoichiometric in CYP505A30, as this is the larger of the flavins and has more interactions with the protein, compared to FMN, through its additional adenosine monophosphate (AMP) group. Thus, comparative analyses suggest that CYP505A30 heme binding occurs up to only ~80% stoichiometry, even with supplementation of the heme precursor delta-aminolevulinic acid (\( \Delta \text{ALA} \)) in the growth medium. FAD is likely to be stoichiometrically bound, whereas purified CYP505A30 retains ~85% FMN.

**Substrate and Inhibitor Binding to CYP505A30 and Its Heme Domain.** As is consistent with the properties of its bacterial homologue P450 BM3 and its fungal orthologue P450oxo (CYP505A1), several medium to long chain length fatty acids bind to CYP505A30 and its heme domain, and in doing so displace the distal water ligand bound to the ferric heme iron. This causes a LS to HS shift in the ferric heme iron spin-state equilibrium, inducing a "type I" spectral shift characteristic of substrate binding to P450 enzymes. On the binding of fatty acid substrates to the CYP505A30/heme domain, the Soret maximum shifts from 415 to ~399 nm. There are alterations in the \( \alpha/\beta \)-band positions, as well as the development of a low intensity Cys thiolate-to-HS ferric heme iron CT absorbance band at ~651 nm. UV–visible spectral binding titrations were done using several lipid substrates and with both CYP505A30 and its heme domain. Figure SB shows a spectral titration for the binding of the substrate arachidonic acid to the CYP505A30 heme domain. The inset shows a plot of arachidonic acid-induced absorbance change against substrate concentration, with data fitted using Morrison’s quadratic function for tight-binding ligands to give \( K_\text{D} = 69.1 \pm 4.6 \) \( \mu \text{M} \). Similar modes of CYP505A30 inhibition through distal coordination of the heme iron were observed for other imidazole-based inhibitors, with the following \( K_\text{D} \) values: 723 ± 37 \( \mu \text{M} \) (imidazole); 3.4 ± 0.2 \( \mu \text{M} \) (1-phenylimidazole); 12.7 ± 0.8 \( \mu \text{M} \) (4-phenylimidazole); and 10.0 ± 0.9 \( \mu \text{M} \) (econazole).

**Redox Potentiometry Studies on CYP505A30 and Its Heme Domain.** CYP505A30 Diflavin Reductase Potentiometry. To establish the flavin midpoint reduction potentials for CYP505A30, spectroelectrochemical titrations of both the substrate-free and the arachidonic acid-bound forms of the intact CYP505A30 enzyme and its heme domain were carried out as described in the Experimental Methods section. Initial studies involved a titration of intact CYP505A30 with dithionite.
Table 1. Dissociation Constants for Substrate Binding to CYP505A30 and Its Heme Domain"c

| substrate                  | CYP505A30 | heme domain |
|----------------------------|-----------|-------------|
|                            | $K_d$ (µM) | HS (%)      | $K_d$ (µM) | HS (%)     |
| decanoic acid (C10:0)      | 21.1 ± 2.0 | 30          | N.D.       | N.D.       |
| dodecanoic acid (C12:0)    | 6.1 ± 0.4  | 10          | 9.0 ± 0.9  | 60         |
| tridecanoic acid (C13:0)   | 2.7 ± 0.3  | 65          | 4.8 ± 0.3  | 30         |
| tetradecanoic acid (C14:0) | 7.4 ± 0.2  | 80          | 3.9 ± 0.3  | 85         |
| pentadecanoic acid (C15:0) | 4.4 ± 0.6  | 85          | 1.1 ± 0.3  | 90         |
| hexadecanoic acid (C16:0) | 10.5 ± 0.2 | 80          | 1.0 ± 0.4  | 55         |
| heptadecanoic acid (C17:0) | 4.1 ± 0.7  | 10          | 8.7 ± 0.8  | 20         |
| octadecanoic acid (C18:0)  | 11.9 ± 1.0 | 15          | 5 ± 0.7    | 10         |
| arachidonic acid (C20:4)   | 1.7 ± 0.1  | 60          | 0.12 ± 0.01| 65         |
| N-palmitoylglycine (NPG)   | 5.4 ± 0.1  | 95          | 2.7 ± 0.8  | 75         |

“UV–visible titration data were collected and analyzed as described in the Experimental Methods section. The $K_d$ values for the binding of saturated fatty acids (C10:0–C18:0), the polyunsaturated fatty acid, arachidonic acid, and the N-acylamino acid, NPG, were determined by fitting of substrate-induced heme absorbance change versus applied substrate concentration using either a standard hyperbolic function or the Morrison equation for tight-binding ligands." The extent of HS heme iron accumulated is estimated to the nearest 5%. N.D. indicates that a $K_d$ could not be accurately determined due to weak spin-state shift.

reductant in the range from approximately +175 to −210 mV versus the normal hydrogen electrode (NHE). Essentially complete flavin reduction was observed with negligible heme reduction in the same potential range. The heme Soret maximum remained at 415 nm, with no evidence of change in the position of the α- and β-bands (Figure 6A). A plot of the absorbance change at 460 nm against the applied potential revealed an apparent single transition, without any obvious inflexions. This suggested that the reduction potentials for the FMN and FAD OX/semiquinone (SQ) and SQ/hydroquinone (HQ) transitions are in the same range. The data were fitted using the Nernst equation to give an apparent flavin (FAD and FMN) midpoint potential of $E^0 = −118 ± 2$ mV for the complete, four-electron reduction of both flavins that occurs in the range between −~50 and −180 mV versus NHE (Figure 6B). The near-stoichiometric binding of the flavins to CYP505A30 (~1:0.85, FAD/FMN) determined earlier in this study is also consistent with both FAD and FMN being bound and having similar redox potentials. Further examination of the spectra shown in Figure 6A revealed a flavin isosbestic point at ~525 nm and small increases in flavin absorbance from ~530 to 690 nm. Flavin absorbance in the 600 nm range is typical of blue (neutral) flavin SQs, suggesting that one or both of the flavins may form a blue SQ during the course of the redox titration. Figure 6C shows a plot of A$_{600}$ versus applied potential, showing a biphasic increase and then decrease in flavin absorbance in the range between −~50 and −200 mV versus NHE. Although the A$_{600}$ change is small, it is consistent with a proportion of the flavins undergoing single electron reduction to the blue SQ form, followed by their further reduction to a neutral HQ form at more negative potentials. Although the A$_{600}$ absorbance data are affected to an extent by small increases in solution turbidity toward the end of the titration, it is still possible to fit the data using a two-electron Nernst function, yielding a potential for the OX/SQ transition of $E^0 = −118 ± 8$ mV, whereas the potential for the incomplete SQ/HQ transition can be estimated at approximately −180 ± 15 mV versus NHE. Although the identity of the flavin or flavins populating the SQ state is not clear, it appears most likely that FAD should form this species. This is in view of the ability of the FAD cofactor in P450 BM3 to stabilize a blue SQ in this enzyme, whereas a transient red (anionic) SQ is formed by the BM3 FMN cofactor.40

CYP505A30 Heme Domain Potentiometry. The reduction potential for the heme iron was determined for the isolated heme domain of CYP505A30 using both the substrate-free and arachidonic acid-bound forms of the protein. On reductive titration of the substrate-free heme domain, the Soret maximum shifts from 415 to ~406 nm and broadens with a partial “merging” of the α- and β-bands, which shift to approximately 559 and 536 nm, respectively. A shoulder appears at ~430 nm for the Soret feature in its reduced form. These data are
Figure 7. Measurement of heme iron potentials in the substrate-bound and substrate-free forms of the CYP505A30 heme domain. Panel A (main image) shows selected UV–visible spectra collected during a redox titration of the arachidonic acid-bound form of the CYP505A30 heme domain. The thick solid line shows the spectrum of the OX, substrate-bound heme domain with a Soret maximum at 399 nm, and α- and β-bands at ~563 and 522 nm, respectively. The dashed line shows the spectrum for the reduced heme domain, with the Soret band shifted to 413 nm, a single feature in the Q-band region at ~550 nm, and the loss of an HS CT signal at ~650 nm. Selected spectra collected during the titration are shown in thin solid lines. Data fitting using the Nernst equation gives a FeIII/FeII heme iron midpoint potential of $E^\circ = -69 \pm 3$ mV (panel A, filled circles). Panel A (inset) shows selected spectra from a redox titration of the substrate-free heme domain. The spectra for the OX and reduced forms are shown as thick solid and dashed lines, respectively. Other selected spectra collected during the titration are shown as thin solid lines. The Soret band shifts from 415 to ~408 nm on heme iron reduction with partial merging of the α- and β-bands and small peak shifts to ~559 and 536 nm, respectively. Data fitting as above gives a midpoint potential of $E^\circ = -298 \pm 5$ mV for the substrate-free heme domain (panel B, filled circles).

consistent with the near-complete reduction of the heme iron, but suggest that the distal coordination of the heme iron may occur through both Cys thiol and Cys thiolate axial ligands. Data fitting for the substrate-free heme domain was done using the Nernst equation, giving the reduction potential for the heme FeIII/FeII couple as $-298 \pm 5$ mV versus NHE, as described in Experimental Methods (Figure 7A, inset).

In the case of the arachidonic acid-bound form of the heme domain, the spectrum of the ferric protein is clearly indicative of an extensively HS P450 with a Soret maximum at 399 nm, and with α- and β-bands at approximately 563 and 522 nm, respectively (Figure 7A, main panel). Reductive titration of the substrate-bound heme iron results in a Soret shift to 413 nm. Reduction of the substrate-bound heme iron also results in the loss of the signal originating from the CT complex observed at ~650 nm in the OX state, and in the development of a single Q-band feature at approximately 550 nm. The midpoint potential for the substrate-bound CYP505A30 heme domain was determined by fitting absorbance data at 390 nm using the Nernst equation. The data indicated a substantial increase in heme iron potential for the arachidonic acid-bound heme domain to $-69 \pm 3$ mV versus NHE. These data demonstrate a large elevation of the heme iron redox potential of 229 mV (from $-298$ mV) on binding to the substrate. The redox data plots for the substrate-free and arachidonic acid-bound forms of the CYP505A30 heme domain are shown in Figure 7B.

Substrate binding induces a much larger heme iron potential shift than was observed for the arachidonic acid-bound form of P450 BM3 (ca. 130 mV). In the case of the BM3 heme domain, arachidonic acid binding raises the heme iron reduction potential from $-368 \pm 6$ to $-239 \pm 6$ mV versus NHE. In so doing, it elevates the heme iron potential above that of the electron donor NADPH ($-320$ mV vs NHE) and into the range where electron transfer from the CPR partner FMN-binding domain can occur. Although the redox potentials in CYP505A30 are rather different from those in P450 BM3, the substantial elevation of the heme iron reduction potential that occurs in the arachidonic acid-bound CYP505A30 heme domain has a similar effect to that observed from its binding in BM3. The apparent overall midpoint potential for the flavins in CYP505A30 is $-120$ mV versus NHE, making electron transfer to the arachidonic acid-bound heme domain ($E^\circ = -69$ mV) thermodynamically favorable. Alterations to the CYP505A30 heme structure/environment likely contribute to the large change in CYP505A30 heme iron potential on arachidonic acid binding, and further insights may be obtained through structural analysis of the heme domain.

**Stopped-Flow Analysis of Flavin Reduction in CYP505A30.** Stopped-flow absorbance data were collected at 475 nm to follow the reduction of the flavins in CYP505A30 at 10 °C. The final NADH/NADPH concentrations used were in the range from 60 to 1200 μM. Flavin absorbance change versus time data were fitted accurately in both cases using a single exponential function. The kinetics of flavin reduction were faster with NADPH than that of those with NADH, as is also the case with BM3. In the case of NADPH, there was little variation in the rate constant across the [NADPH] range up to 1.2 mM ($k_{obs} = 82.4 \pm 9.1$ s$^{-1}$), suggesting a high affinity for NADPH. However, there was a hyperbolic dependence of $k_{obs}$ on [NADH], giving a limiting rate constant of $k_{lim} = 42.0 \pm 3.2$ s$^{-1}$ and an apparent $K_a$ for NADH of 816 $\pm 113$ μM when data were fitted using a hyperbolic function (Figure 8).

Compared to those of the B. megaterium BM3 enzyme, the kinetics of flavin reduction in CYP505A30 are much slower. Reduction of the P450 BM3 flavins was previously reported to be biphasic, with apparent rate constants of $k_1 = 758.4 \pm 5.9$ s$^{-1}$ and $k_2 = 117.6 \pm 2.4$ s$^{-1}$ at 25 °C. To examine the influence of temperature on flavin reduction in CYP505A30, further
CYP505A30 stopped-flow flavin reduction experiments were done at 5 °C temperature intervals between 10 and 35 °C, using 1 mM NADPH. Data were again fitted using a single exponential function. These data revealed an apparent linear dependence of the rate constants on NADH concentration (filled circles). These data were fitted using a hyperbolic (Michaelis–Menten) function to yield a limiting rate constant of \( k_{\text{lim}} = 42.0 \pm 3.2 \, \text{s}^{-1} \) and an apparent \( K_0 \) for NADH of 816 \( \pm 113 \, \mu\text{M} \).

**Spectroscopic Analysis of CYP505A30 and Its Heme Domain Using Electron Paramagnetic Resonance (EPR).**

**EPR Analysis of the CYP505A30 Ferric Heme Iron in Substrate-Free and Substrate-Bound States.** EPR spectra were collected as described in the Experimental Methods section. Figure 9 shows the EPR spectra for the substrate-free and substrate (arachidonic acid)-bound forms of CYP505A30 and its heme domain. The substrate-free and arachidonic acid-bound CYP505A30 (upper two spectra) show a derivative feature at 82.3 \( \pm 9.1 \, \text{s}^{-1} \). With NADH as the reductant, the rate constants were lower and there was a hyperbolic dependence of \( k_{\text{obs}} \) on NADH concentration (filled circles). These data were fitted using a hyperbolic (Michaelis–Menten) function to yield a limiting rate constant of \( k_{\text{lim}} = 42.0 \pm 3.2 \, \text{s}^{-1} \) and an apparent \( K_0 \) for NADH of 816 \( \pm 113 \, \mu\text{M} \).

**Figure 9.** EPR analysis of CYP505A30 and its heme domain. X-band EPR spectroscopy was used to characterize ferric heme iron coordination in CYP505A30 and its heme domain in both their substrate-free and arachidonic acid-bound forms. The data show rhombic signals typical for LS P450s in which Cys thiolate and water (or hydroxide) are the axial ligands to the ferric heme iron, with \( g \)-values of \( g_x = 2.41/2.24 \), \( g_y = 2.24/2.25 \), and \( g_z = 1.92 \). Minor features at \( g = 8.01/8.03 \) likely indicate small components of HS ferric heme iron retained in the CYP505A30 heme domain samples at the 10 K operating temperature. The small \( g = 3.50 \) signal is assigned to free ferric iron, with the \( g = 2.00 \) signal arising from a proportion of flavin SQ in the substrate-bound and substrate-free forms of intact CYP505A30.

The EPR spectra for the substrate-free and arachidonic acid-bound heme domains (lower two spectra) are also shown in Figure 9 for comparison to that of CYP505A30. These heme domains have similar \( g \)-values to the intact CYP505A30 enzyme samples (\( g_x = 2.42/2.41 \), \( g_y = 2.25 \), and \( g_z = 1.92 \)), though with some minor “splitting” of the \( g_x \) signal, suggesting subtle structural changes in the heme iron environment between the heme domain and the intact flavocytochrome CYP505A30. The absence of a minor flavosemiquinone signal in the heme domain samples is consistent with the absence of a reductase domain in this construct, and with the positive reduction potentials of the flavins reported earlier in this article. There is a small HS \( g_x \) signal at 8.01 (substrate-free heme domain) and a slightly larger \( g_x \) signal at 8.03 (arachidonic acid-bound heme domain), which may indicate that these heme domains retain a small proportion of HS, thiolate-coordinated heme iron at 10 K. The signal at \( g = 3.50 \) is assigned to free ferric iron, and the \( g = 2.00 \) signal in the intact CYP505A30 protein is assigned to a flavin SQ component (likely on the FAD cofactor).

**EPR Characterization of a Flavin SQ Species in CYP505A30.** Anionic and neutral flavin SQs have identical \( g \)-factors (2.004 in this case) but can be differentiated by their Gaussian split, which is the difference in the line width in G between the SQ maximum and the minimum in the first derivative presentation. A 15 G line width is characteristic of an anionic, red SQ, whereas a 19 G line width is characteristic of a neutral blue SQ. Intermediate values are usually indicative of a mixture of the two species. In studies of P450 BM3, Murataliev et al. demonstrated from EPR analysis that an NADPH-reduced, active form of P450 BM3 contained both anionic (FMN) and neutral (FAD) SQ species with an SQ line width of 15.7 G. An active form of BM3 in steady-state turnover with NADPH had a value of 16.8 G, again consistent with the presence of both anionic and neutral flavin SQs. However, an inactive form of NADPH-reduced BM3 had a value of 19.2 G, indicating that only a blue SQ (on the FAD) was present, whereas FMN was reduced to a HQ state.
inefficient in reducing the heme iron. Subsequent studies provided evidence for the role of a transient FMN anionic SQ in BM3 heme iron reduction using a stopped-flow kinetics approach.

To examine the formation of SQ species in CYP505A30, enzyme samples (190 μM) were incubated with NADPH (2 mM) for periods of 30 s, 5 min, and 10 min prior to freezing for X-band EPR analysis. Figure 10 shows the SQ species formed at these time intervals. After 30 s of incubation with NADPH the line width is 16.3 G, indicative of a mixture of blue/red SQ species. After 5 min of incubation a value of 16.8 G was obtained, and after 10 min of incubation the line width is 19.0 G. The transition with time from a mixture of blue/red SQ species in CYP505A30 to a form with only a blue SQ is similar to the data presented by Murataliev et al., and suggests that the mechanism of electron transfer through the flavins may be similar in BM3 and CYP505A30. Specifically, both enzymes may undergo a 0–2→1–0 cycle where the digits indicate the number of electrons on the CPR flavins. The reduction of FAD occurs by hydride transfer from NADPH and places two electrons on the cofactor. One of these is rapidly transferred to form an FMN anionic HQ, leaving a neutral SQ on FAD. The FMN HQ reduces the heme iron, and this event occurs again after a single electron transfer from FAD to FMN. The consecutive single electron transfers from FMN-to-heme enable (i) the binding of dioxygen to the ferrous/substrate-bound P450 heme iron, and (ii) the further single electron reduction of the resultant ferric–superoxide species in the P450 catalytic cycle, facilitating the later production of highly reactive iron–oxo species that oxidize the substrate.

**Steady-State Kinetic Analysis of Substrate Turnover by CYP505A30.**

Steady-state kinetic data for fatty acid oxidation were collected for a range of fatty acids. The kcat and K_M parameters for CYP505A30 in reactions with a range of fatty acids were determined by measuring substrate-dependent NADPH oxidation spectrophotometrically at 340 nm. These data are presented in Table 1, and Figure 11 shows examples of steady-state kinetic data for the CYP505A30-dependent oxidation of NADPH with tetradecanoic and pentadecanoic acid substrates. The kcat values determined range from 1.2 to 7.5 s⁻¹ (72–450 min⁻¹), with K_M values in the range from 7.7 to 21.2 μM. The second-order rate constants (kcat/K_M) reveal that catalytic efficiency is highest with pentadecanoic acid (C15:0, kcat/K_M = 0.96 μM⁻¹ s⁻¹). Arachidonic acid has a kcat of 2.0 ± 0.1 s⁻¹ and a K_M of 9.2 ± 0.5 μM (kcat/K_M = 0.21 μM⁻¹ s⁻¹).

**Figure 10.** EPR analysis of flavosemiquinone formation in CYP505A30 (190 μM) incubated with NADPH (2 mM) for periods of 30 s, 5 min, and 10 min prior to freezing in liquid nitrogen and X-band EPR analysis at 77 K. Flavosemiquinone signals occurred in each sample. The 30 s sample (thick line) has the highest SQ content and a SQ spectral line width of 16.3 G. The 5 min sample has a line width of 16.8 G and that of the 10 min sample was 19.0 G. These data are consistent with the presence of a mixture of anionic (red) and neutral (blue) SQs in the initial sample, but then a time-dependent progression toward the final sample, which is dominated by a neutral SQ signal. This most likely occurs due to the time-dependent reduction of an FMN anionic SQ to its HQ form, whereas the FAD cofactor retains a blue SQ species, as seen in the P450 BM3 enzyme.

**Figure 11.** Steady-state kinetics of CYP505A30. Steady-state kinetic data were collected for CYP505A30 with a number of lipid substrates, as described in the Experimental Methods section. The graphs in panels A and B show steady-state data collected for fatty acid substrate-dependent NADPH oxidation with tetradecanoic acid (C14:0) and pentadecanoic acid (C15:0) substrates. Rate constants were determined as the statistical average of three replicates at each concentration of the substrate, with measurements of initial rates done by monitoring NADPH consumption spectrophotometrically at 340 nm (ε340 = 6.21 mM⁻¹ cm⁻¹). Data points are shown with standard deviation error bars, and data were fitted using the Michaelis–Menten equation to give k_cat and K_M values of 1.21 ± 0.1 s⁻¹ and 7.7 ± 1.9 μM for tetradecanoic acid, and of 7.5 ± 0.7 s⁻¹ and 7.8 ± 2.1 μM for pentadecanoic acid, respectively.
Although there is good affinity for the fatty acids tested, the $k_{cat}$ values are much lower than those in P450 BM3 for the same substrates under similar conditions (e.g., $k_{cat} = 285 \pm 32 \text{s}^{-1}$ for arachidonic acid and $k_{cat} = 30.8 \pm 1.5 \text{s}^{-1}$ for myristic acid).\textsuperscript{45,18} Our stopped-flow data for the temperature-dependence of the CYP505A30 flavin reduction indicate that the relevant rate constant increases considerably as temperature is elevated up to 35 °C, and this may also result in stimulation of the rate of fatty acid oxidation. However, other data point to relatively modest thermostability in CYP505A30 (see 

**Thermostability of CYP505A30 and Its Heme Domain** section). Under the same conditions and in the absence of lipid substrate addition, CYP505A30 oxidizes NADPH with a rate constant of ~5 min\(^{-1}\).

**Steady-State Kinetics of CYP505A30-Catalyzed Electron Transfer to Cytochrome c and Ferricyanide.** The apparent $k_{cat}$ values for fatty acid-dependent NADPH oxidation in CYP505A30 are relatively slow at 25 °C ($<7.5 \text{s}^{-1}$, depending on the fatty acid) (Table 1). However, stopped-flow studies indicate that NADPH-dependent reduction of the CYP505A30 flavin cofactors is considerably faster ($k_{lim}$ is ~82 \text{s}^{-1} at 10 °C, rising to ~250 \text{s}^{-1} at 25 °C). To establish the efficiency of electron transfer from NADPH through the CYP505A30 flavins and onto electron acceptor molecules, we undertook steady-state kinetic studies using potassium ferricyanide (FeCN) and cytochrome c as substrates. Cytochrome c has been recognized as an excellent substrate for CPR enzymes, with electron transfer mediated through the FMN cofactor. FeCN reduction may occur through either flavin. However, the FAD/NADPH binding domain of BM3 was shown to be an excellent catalyst of FeCN reduction in its own right ($k_{cat} = 360 \pm 4 \text{s}^{-1}$ at 25 °C), indicating that electron transfer to FeCN through the FAD is likely to be the most efficient route.\textsuperscript{42} CYP505A30 catalyzed FeCN reduction with $k_{cat} = 38.4 \pm 1.8 \text{s}^{-1}$, $K_{M(\text{NADPH})} = 2.9 \pm 0.4 \text{\mu M}$, and $K_{M(\text{FeCN})} = 48.2 \pm 0.4 \text{\mu M}$. The comparable parameters for cytochrome c are $k_{cat} = 29.7 \pm 0.7 \text{s}^{-1}$, $K_{M(\text{NADPH})} = 3.1 \pm 0.4 \text{\mu M}$, and $K_{M(\text{cyt.c})} = 79.3 \pm 10.3 \text{\mu M}$. The $K_{M(\text{NADPH})}$ values in studies of the NADH-driven reduction of FeCN and cytochrome c by CYP505A30 were substantially higher than those for NADPH (265 ± 38 and 330 ± 56 \mu M, respectively), although the $k_{cat}$ values were very similar to those obtained with NADPH as the electron donor (34.3 ± 2.4 and 29.5 ± 2.4 \text{s}^{-1}, respectively). These data confirm that NADPH is the favored electron donor (the apparent binding constant [$K_M$] value is ~100-fold lower for NADPH than for NADH). However, the $k_{cat}$ values for CYP505A30-catalyzed cytochrome c and FeCN reduction are approximately 7.5-fold and 3.8-fold slower than the respective rate constants for the BM3 enzyme under similar conditions.\textsuperscript{46}

**Thermostability of CYP505A30 and Its Heme Domain.**

*M. thermophila* grows optimally in the temperature range from 45 to 50 °C. To probe the thermostability of the enzyme, differential scanning fluorimetry (DSF) was used, as described in the 

**Experimental Methods** section, and a diverse buffer set was used to identify conditions that best stabilized CYP505A30 and its heme domain. DSF was done using a SYPRO Orange dye as a reporter of protein unfolding, and by ramping the temperature from 20 to 90 °C in 0.2 °C increments following 5 s delays for signal stabilization at each temperature. In all cases, DSF analysis resulted in a sigmoidal (two-state) transition between folded and unfolded states with no evidence of any metastable intermediates during the process. For both CYP505A30 and the heme domain, the highest $T_m$ values were found using sodium/potassium phosphate at pH 7.0, at 58.0 and 48.0 °C, respectively (from $T_m$ values in the range 41.2–58.0 °C for CYP505A30 and 43.3–48.0 °C for the heme domain). These data indicate that the heme domain expressed in isolation is less thermostable than the intact CYP505A30 enzyme. Possible explanations for this phenomenon are that the reductase domain of CYP505A30 stabilizes the linked domain, or that CYP505A30 forms a dimer that stabilizes the heme domain. In the latter case, there is a precedent for the dimerization of the flavocytochrome P450 BM3 and for the BM3 dimer being the catalytically relevant state of this enzyme.\textsuperscript{45,46} In further studies of the stability of CYP505A30, the thermal stability of its Fe\(^{II}\)–CO complex was assessed by recording spectral changes associated with the conversion from the P450 (Cys thiolate-coordinated) to the P420 (Cys thiol-coordinated) form as temperature was ramped between 25 and 60 °C in 2.5 °C increments. The $T_m$ value for the P450-to-P420

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**Figure 12.** MALLS studies of CYP505A30 and its heme domain. MALLS was used to analyze the aggregation state of CYP505A30 and its heme domain. Panel A shows the MALLS data for the intact CYP505A30. Two major species are seen at elution volumes of ~11.4 and 12.4 mL, with corresponding masses of ~225 and 130 kDa, respectively. These correlate reasonably well with the predicted masses of the dimeric and monomeric forms of CYP505A30, and suggest that the CYP505A30 dimer may be the catalytically relevant form of the enzyme, as is seen for P450 BM3.\textsuperscript{23} A peak for the void volume of the column is seen at ~8 mL. Panel B shows the MALLS data for the heme domain with an elution volume of ~16.5 mL and a predicted mass of ~50 kDa. These data are consistent with the mass of the monomeric form of the heme domain, indicating that this domain does not self-aggregate and that the intact CYP505A30 dimer does form as a consequence of interactions between the heme domains of the enzyme.
transition occurred at \( \sim 43 \, ^\circ C \), with evidence of aggregation of the protein at temperatures above \( 55 \, ^\circ C \). These data again point to modest thermostability of CYP505A30, with formation of the P420 state associated with loss of catalytic activity in the P450 enzymes.
Analysis of the Quaternary Structure of CYP505A30 and Its Heme Domain Using Multilangle Laser Light Scattering (MALLS). Intact CYP505A30 has a predicted molecular mass of 119 377.4 Da (121 540.7 Da including the N-terminal His-tag), and the heme domain has a mass of 52 514.5 Da (54 677.8 Da including the His-tag). MALLS was used to ascertain the oligomerization status of both proteins. In the case of intact CYP505A30 (Figure 12A), the data reveal two major species at ∼11.4 and 12.4 mM with masses of approximately 225 and 130 kDa, respectively—suggesting that monomeric and dimeric forms of the flavocytochrome CYP505A30 exist in equilibrium under the conditions used, and that the dimer (as in the case for the P450 BM3 flavocytochrome) may be the catalytically relevant form of the enzyme. A minor species (approximately 80 kDa, eluting at ∼14 mM) may result from partial degradation of the enzyme, and the void volume of the column is at ∼8 mM. Figure 12B shows a rerun of a sample of the heme domain, eluting at ∼16.5 mM. The predicted mass is ∼50 kDa, consistent with the heme domain of CYP505A30 being monomeric, as also is the case for the P450 BM3 heme domain.

Gas Chromatography–Mass Spectrometry (GC–MS) Analysis of Fatty Acid Hydroxylation. CYP505A30-mediated oxidation reactions of lauric acid and myristic acid were done using a NADPH regeneration system to facilitate efficient enzymatic oxidation of these fatty acid substrates. Products were derivatized and resolved by GC–MS, as described in the Experimental Methods section. Figure 13A shows data from the reaction of CYP505A30 with lauric acid (C12:0), showing the resolution of three product peaks corresponding to the ω-1, ω-2, and ω-3 hydroxylated lauric acid products, with retention times (RTs) of 8.94, 8.87, and 8.76 min, respectively. A proportion of unconverted lauric acid is also seen at 7.65 min. The hydroxylated products are in the proportions of approximately 88% (ω-1), 10% (ω-2), and 2% (ω-3). Figure 13B,C shows product fragmentation data to identify the ω-1 and ω-2 hydroxylation products, respectively. A similar outcome was observed using myristic acid (C14:0) as the substrate, with proportions of hydroxylated product of approximately 63% (ω-1), 28% (ω-2), and 9% (ω-3). It is clear that hydroxylation is preferred at the ω-1 position for these saturated fatty acids. A similar behavior was reported for P450 BM3 with these substrates, with the ω-1 product (48%) being dominant over the ω-2 and ω-3 products (48, 26, and 26%, respectively) with lauric acid, and a similar outcome observed with myristic acid (58, 21, and 21%, respectively).

The most notable difference in the behaviors of CYP505A30 and P450 BM3 with these substrates is that there are substantially more ω-1 hydroxylated products formed by CYP505A30 compared to those by P450 BM3, and considerably less ω-3 hydroxylated products. Although further studies are needed to provide a more complete analysis of the fatty acid oxidation properties of CYP505A30, these initial data suggest important differences with P450 BM3. In particular, fatty acid hydroxylation at ω-1 appears to predominate for CYP505A30 with lauric and myristic acid, however there is relatively little hydroxylation at the ω-1 position. Coupling of NADPH oxidation to hydroxylated product formation was ∼80–85% for both substrates.

DISCUSSION

In this study, we report the identification, expression, and characterization of CYP505A30, a member of the CYP505A family of cytochrome P450 enzymes for which CYP505A1 (P450foxy from the ascomycete fungus F. oxysporum) is the founding member. Studies presented in this article demonstrate that M. thermophila CYP505A30 is a fatty acid binding and hydroxylating enzyme, sharing similar catalytic properties to those exhibited by CYP505A1 and by the well characterized B. megaterium P450 BM3 (CYP102A1) enzyme. All three of these enzymes are natural fusions of a cytochrome P450 to a CPR, enabling them to be catalytically self-sufficient, requiring only NADPH and fatty acid substrates for catalytic activity. Both the intact CYP505A30 enzyme and its heme (P450) domain were expressed and purified using an E. coli expression system, mimicking work previously done for the P450 BM3 enzyme. Both constructs produced functional entities in terms of fatty acid substrate binding with associated HS ferric heme iron accumulation (Figure 5 and Table 1), and the intact CYP505A30 catalyzed efficient fatty acid substrate-dependent NADPH oxidation, in addition to reduction of cytochrome c and ferricyanide (Figures 11 and 13). MALLS analysis demonstrated that the heme domain was predominantly monomeric in solution, and the intact CYP505A30 showed the presence of both monomeric and dimeric forms (Figure 12). Previous studies showed that the related P450 BM3 enzyme dimerizes, and that its dimeric form is likely to be the catalytically active form, and we propose that the dimeric form is also the state relevant to fatty acid oxidation in CYP505A30. In the absence of addition of the heme precursor ΔALA, CYP505A30 is produced with substoichiometric heme content, typically resulting in preparations with a dark orange color. However, heme content is increased by expression cell growth in the presence of ΔALA and the protein color is red. It is possible that the heme-free form of CYP505A30 has lower propensity for dimerization, leading to an increased population of the monomeric state as seen in the MALLS studies. Other studies on the related F. oxysporum CYP505A1 (P450foxy) enzyme expressed in E. coli reported that FAD and FMN content in the reductase domain was low (15 and 65% occupancy, respectively). In contrast, flavin incorporation was considerably higher in the M. thermophila CYP505A30 (≈85% FMN and near-100% FAD incorporation).

Both intact CYP505A30 and its heme domain bind tightly to a range of different saturated fatty acids in the range from C10 to C20, in addition to arachidonic acid (C20:4) and NPG, in which palmitic acid (C16:0) is linked to the amino acid glycine through an amide bond. The binding constants for several lipids were determined by UV–visible absorbance titrations, revealing that all substrates had K_d values in the range from 2.7 to 21.1 μM for the intact CYP505A30, and from 0.12 to 9.0 μM for the heme domain. The most extensive HS shifts induced were with the C14:0–C16:0 fatty acids and NPG in intact CYP505A30 (80–95% HS heme iron accumulation), and with the C14:0 and C15:0 fatty acids and NPG with the heme domain (75–90% HS accumulation) (Table 1). Potentiometric studies of the flavins in the intact CYP505A30 revealed that the reduction potentials of the FAD and FMN cofactors are relatively positive and that their transition from the OX (quinone) to two-electron reduced (HQ) states occurs in the same potential range with a midpoint potential of −118 ± 2 mV for the apparent overall four-electron reduction of the two flavins. There is evidence of the formation of small amounts of the neutral flavin SQ during the redox titration, as seen by small changes (increase then decrease) in absorbance at ∼600 nm. These properties are consistent with the optical features of a
blue SQ, and EPR studies confirmed the formation of flavin SQ species in CYP505A30 (Figures 6, 9, and 10). A physiological basis for the positive potentials of the CYP505A30 flavins became clear through analysis of the redox potentials of the heme iron in the CYP505A30 heme domain in its substrate-free and arachidonic acid-bound forms. The midpoint reduction potential for the heme iron Fe\(^{III}/Fe^{II}\) transition in the substrate-free state is \(-298 \pm 5\) mV versus NHE, rising to \(-69 \pm 3\) mV versus NHE in the arachidonic acid-bound form (Figure 7). The heme iron redox potential thus undergoes a large increase on fatty acid binding, enabling its reduction by the FMN cofactor in the reductase domain. A similar phenomenon (albeit with flavin and heme potentials at more negative potentials) occurs in P450 BM3, resulting in heme iron a lower temperature than the apparent thiolate coordination of heme iron in both CYP505A30 and its substantial thermostability. DSF to monitor unfolding under different fatty acid) using the Michaelis–Menten equation.

### Table 2. Steady-State Kinetic Data for CYP505A30\(^a\)

| Substrate       | \(k_{cat}\) (s\(^{-1}\)) | \(K_{M}\) (\(\mu\)M) | \(k_{cat}/K_{M}\) (s\(^{-1}\) M\(^{-1}\)) |
|-----------------|--------------------------|----------------------|----------------------------------|
| Dodecanoic acid | 4.8 \(\pm 0.2\)          | 21.2 \(\pm 2.4\)    | 0.23                             |
| Tridecanoic acid| 5.0 \(\pm 0.3\)          | 14.6 \(\pm 2.5\)    | 0.34                             |
| Tetradecanoic acid | 1.2 \(\pm 0.1\)          | 7.7 \(\pm 1.9\)     | 0.16                             |
| Pentadecanoic acid | 7.5 \(\pm 0.7\)         | 7.8 \(\pm 2.1\)     | 0.96                             |
| Arachidonic acid | 2.0 \(\pm 0.1\)          | 9.2 \(\pm 0.5\)     | 0.22                             |

\(^a\)Steady-state kinetic assays were done as described in the Experimental Methods section. The \(k_{cat}\) and \(K_{M}\) values were determined by fitting fatty acid-induced NADPH oxidation rate constants (measured across a range of different concentrations of the particular fatty acid) using the Michaelis–Menten equation.

noic acids.\(^{25}\) Steady-state analysis of the CYP505A30-mediated reduction of cytochrome c and ferricyanide revealed \(k_{cat}\) values of 29.7 \(\pm 0.7\) s\(^{-1}\) for cytochrome c reduction and 38.4 \(\pm 1.8\) s\(^{-1}\) for ferricyanide reduction (using NADPH as the reductant in both cases). NADPH is the preferred cofactor, with a \(K_{M}\) value of 3.1 \(\pm 0.7\) \(\mu\)M in cytochrome c reduction compared to 330 \(\pm 56\) \(\mu\)M for NADH. These steady-state \(k_{cat}\) values are substantially higher than those for fatty acid oxidation, suggesting that the fatty acid reaction rate is limited by FMN-to-heme electron transfer(s) or, for example, by the rate of dissociation of the hydroxylated lipid product. In P450 BM3, the rate of FMN-to-heme electron transfer is \(~2\)-fold that of the overall turnover rate of the enzyme in steady-state with dodecanoic acid, suggesting that this event is important in steady-state rate limitation in BM3.\(^{18}\) Stopped-flow absorbance studies of flavin reduction in CYP505A30 provided further evidence for the much tighter binding of NADPH to the enzyme. There was negligible dependence of the apparent flavin reduction rate using NADPH, whereas there was a hyperbolic dependence observed with NADH as the electron donor and an apparent \(K_{M}\) of 816 \(\pm 113\) \(\mu\)M.

Following on from the steady-state kinetic and equilibrium binding studies of CYP505A30, substrate turnover studies were done using dodecanoic acid (C12:0) and tetradecanoic acid (C14:0) substrates and with product analysis done using GC–MS. The product outcomes were similar for both of these substrates, yielding a mixture of \(\omega-1\), \(\omega-2\), and \(\omega-3\) hydroxylated fatty acids in both cases. The formation of a similar set of products was reported in previous studies of P450 BM3-dependent oxidation of these substrates.\(^{47}\) However, a clear difference in the regioselectivity of oxidation of these substrates is evident between CYP505A30 and P450 BM3. Although BM3 favors \(\omega-1\) hydroxylation, it also produces a substantial amount of the \(\omega-2\) and \(\omega-3\) hydroxylated products (48% \(\omega-1\), 26% \(\omega-2\), and 26% \(\omega-3\) for dodecanoic acid). However, comparable data for CYP505A30 show that the \(\omega-3\) hydroxylated dodecanoic acid represents only a small amount of the final product mixture with there being a much larger component of the \(\omega-1\) hydroxylated dodecanoic acid (88% \(\omega-1\), 10% \(\omega-2\), and 2% \(\omega-3\) hydroxylated dodecanoic acid) (Figure 13). A similar phenomenon was observed using tetradecanoic acid as the CYP505A30 substrate (63% \(\omega-1\), 28% \(\omega-2\), and 9% \(\omega-3\) hydroxylated tetradecanoic acid). Kitaumae et al. also reported dodecanoic acid oxidation by CYP505A1.\(^{26}\) The products formed were \(~55\%\) \(\omega-1\), \(~37\%\) \(\omega-2\), and \(~8\%\) \(\omega-3\) hydroxylated dodecanoic acid. Although the order of the magnitude for the three hydroxylated products formed from dodecanoic acid is the same for CYP505A1/CYS05A30, there is a large difference in the ratio for the \(\omega-1/\omega-2\) hydroxylated products (1.5 for CYP505A1 and 8.8 for CYP505A30), highlighting greater specificity for \(\omega-1\) hydroxylation in CYP505A30.

In conclusion, a novel member of the CYP505 P450–CPR fusion enzyme family has been expressed and purified using an E. coli expression system. M. thermophila CYP505A30 has hydroxylase activity toward fatty acids, hydroxylating both dodecanoic acid and tetradecanoic acid predominantly at the \(\omega-1\) position. Further studies are required to characterize the full repertoire of lipid substrates for this P450, but its moderate thermostability may prove advantageous in biotechnological applications for production of oxygenated molecules. Ongoing work is being focused on identifying the hydroxylated products.
generated by CYP505A30 from a variety of other straight chain and branched chain lipids.

**EXPERIMENTAL METHODS**

**CYP505A30 Retrieval and Bioinformatics Analysis.** The CYP505A30 gene was identified as a potential orthologue of P450 BM3 (CYP102A1 from *B. megaterium*) using the conserved domain architecture retrieval tool (CDART) program.\(^49\) CDART was used to search for protein sequences of BM3 homologues from thermophilic organisms. The bioinformatics tool identifies functional domains from the amino acid sequence input through reverse position specific-BLAST before querying the National Center for Biotechnology Information database for protein sequences with similar domain architectures. The 1080 amino acid sequence of a hypothetical protein MYCTh_101224A from the fungus *M. thermophila* (UniProt: G2QDZ3 [G2QDZ3_MYCTh]) was selected from the resulting list based on the thermophilic nature of the host organism. This novel P450–CPR fusion gene was assigned as CYP505A30 in the cytochrome P450 P450 gene superfamily on the basis of its relatedness to other eukaryotic P450 homologues from thermophilic organisms. The correct cloning and sequence of the gene was verified by DNA sequencing (Source BioScience, Nottingham, U.K.).

**Cloning of the CYP505A30 Gene and Construction of Expression Plasmids for the Intact CYP505A30 and Its Heme Domain.** The CYP505A30 gene sequence from the thermophilic fungus *M. thermophila* was synthesized by GenScript (Cherwell, U.K.) in an *E. coli* codon optimized form and inserted into the pUC57 vector. The CYP505A30 gene was subcloned into the plTE15b expression vector by digestion of both plasmids with *Ndel* and *BamHI* (NEB, Hertfordshire, U.K.), and by ligation of the CYP505A30 gene-containing fragment into plTE15b using T4 DNA ligase (NEB). The correct cloning and sequence of the gene was verified by DNA sequencing (Source BioScience, Nottingham, U.K.).

A heme (P450) domain construct was generated from the intact wild-type CYP505A30 gene construct in pTE15b. A pairwise alignment of CYP505A30 and P450 BM3 (CYP102A1) facilitated the selection of D464 as an appropriate terminal residue of the CYP505A30 heme domain. An ochre stop codon (TAA) was introduced in place of the codon for amino acid Gly465 at the end of the heme domain (D464X mutant) using (TAA) was introduced in place of the codon for amino acid Gly465 at the end of the heme domain (D464X mutant) using a QuikChange Lightning site-directed mutagenesis kit (Stratagene-Agilent, U.K.). The oligonucleotide primer 5'-GTGCTATTCTGCGCGACGTGCTATTCTGCGCGAC-3' and its reverse complement were synthesized by Eurofins MWG Operon (Ebersberg, Germany) and used for polymerase chain reaction (PCR) mutagenesis. The D464X CYP505A30 heme domain gene construct in pTE15b was fully sequenced to ensure the presence of the stop codon at the correct location, and the absence of undesired mutations (Source BioScience, Nottingham, U.K.).

**Expression and Purification of Intact CYP505A30 and Its Heme Domain.** The genes for both the wild-type, intact CYP505A30 (amino acids 1–1080) and its heme domain (amino acids 1–464) were expressed in BL21-Gold (DE3) *E. coli* cells (Stratagene-Agilent, U.K.) using 500 mL of Luria–Bertani medium in 2 L shake flasks inoculated with 5 mL of an overnight culture of the respective transformant cells. The *E. coli* transformant cultures were incubated at 37 °C and with agitation at 200 rpm in an orbital incubator until an OD₆₀₀ of 0.6 was reached. Thereafter, P450 overexpression was induced with 0.8 mM isopropyl β-D-1-thiogalactopyranoside, and 0.4 mM ΔALA was added to the culture to promote heme incorporation into the intact CYP505A30 and its heme domain. The temperature was lowered to 25 °C, and cells were grown for a further 16–20 h. The bacterial cells were then recovered by centrifugation at 4 °C (6000g, 8 min) and resuspended in 25 mL of buffer A (50 mM potassium phosphate [KPi], pH 7.0) per litre of culture. DNase I (100 μg/mL, bovine pancreas; Sigma-Aldrich, Poole, U.K.) and lysozyme (100 μg/mL, hen egg white; Sigma-Aldrich) were added to degrade chromosomal DNA and to aid bacterial cell lysis. Ethylenediaminetetraacetic acid-free cComplete protease inhibitor cocktail tablets (Roche Applied Science, Mannheim, Germany) were used in all protein purification buffers. Cells were lysed by sonication on ice using a Bandelin Sonopuls sonicator (40% power, 45 pulses for 8 s with 40 s between pulses). The supernatant containing soluble intact CYP505A30 enzyme or its heme domain was separated from cell debris by centrifugation (20 000g, 40 min, 4 °C).

The intact CYP505A30 enzyme was partially purified from the clarified cell extract supernatant using an initial ammonium sulfate (30% w/v) mixing step on ice. Precipitated protein was removed by centrifugation (20 000g, 30 min, 4 °C). The supernatant was then mixed with 2’s,3’-adenosine 5’-diphosphate Sepharose resin in buffer A containing 10 mM S‘-AMP for 3 h at 4 °C on a rolling table. Thereafter, the resin was packed into a column (2 × 10 cm) and washed with a further 3 column volumes of buffer A plus 10 mM S‘-AMP to remove unbound contaminant proteins, followed by elution of CYP505A30 from the resin with 200 mM S‘-AMP in buffer A. The eluted protein fractions were pooled, dialyzed into 50 mM KPi (pH 8.0) containing 200 mM KCl (buffer B), and concentrated by ultrafiltration using a Vivaspin ultrafiltration device (100 kDa molecular weight cut-off [MWCO]) (Sartorius, Epsom, U.K.). The concentrated sample was loaded onto a Q-Sepharose ion exchange chromatography column. CYP505A30 elution was achieved by applying a linear gradient of 0–500 mM KCl in 50 mM Tris (pH 7.0) (buffer C). The fractions containing the purest CYP505A30 samples were pooled and concentrated by ultrafiltration as described above. A final purification step was done by size exclusion chromatography (SEC) in buffer A using a Sephacryl S-200 column (26 × 60 cm) on an ÄKTA purification system (GE Healthcare, Amersham, U.K.). Intact CYP505A30 fractions collected were checked for purity by SDS-PAGE, concentrated by ultrafiltration (as described above) to a final concentration of ~800 mM, and frozen in buffer A containing 10% glycerol at −80 °C. The flavoenzyme concentration was determined spectrophotometrically using an absorption coefficient of 23 000 M⁻¹ cm⁻¹ for the OX minus sodium dithionite-reduced difference spectrum, as described previously.\(^39\) The P450 heme concentration of CYP505A30 was determined as described previously, using a coefficient of 91 000 M⁻¹ cm⁻¹ for the absorption difference between 450 and 490 nm in the reduced/CO minus reduced P450 spectrum.\(^51\) The concentration of the CYP505A30 flavocytochrome in its OX form was estimated spectrophotometrically using a coefficient of 105 000 M⁻¹ cm⁻¹, whereas that for the OX heme domain was estimated using 418 = 95 000 M⁻¹ cm⁻¹.\(^39\)

The CYP505A30 heme domain was purified from its clarified cell extract by mixing with Ni–iminodiacetic acid nickel chromatographic resin (Generator, Maidenhead, U.K.) in 10 mM imidazole for 3 h on a rolling table at 4 °C, before elution
of the heme domain from the resin using 200 mM imidazole in buffer A. The eluted protein was then dialyzed into buffer A at 4 °C and concentrated by ultrafiltration (as described above, but using a 30 kDa MWCO Vivaspin) before a further purification step by SEC on an AKTA purifier using a Sephacryl S-200 column (as described above) in buffer A. Heme domain protein fractions were checked for purity by SDS-PAGE, concentrated by ultrafiltration, and frozen in buffer A at −80 °C.

**Identification and Quantification of Flavin Cofactors Using HPLC.** A 100 mM sample of intact CYP505A30 (40 mM) was incubated in a sealed Eppendorf tube in buffer A at 90 °C for 10 min to release the flavin cofactors from its reductase domain. Centrifugation at 20 000 g for 5 min separated the soluble flavins from the precipitated, denatured enzyme. A 60 mL sample of the flavin (FAD and FMN)-containing supernatant was then loaded onto a reverse phase C18 HPLC column (Ascentis, Sigma-Aldrich, U.K.) in 85% 5 mM ammonium acetate (pH 6.5) and 15% methanol. Chromatographic resolution of FAD and FMN was achieved using the same method, and integration of the peak areas of these flavin samples allowed a standard curve to be generated. In turn, this allowed for the quantities of the FAD and FMN samples released from denatured CYP505A30 to be determined by interpolation, as described previously.

**Fatty Acid and Inhibitor Binding Titrations with CYP505A30.** All P450 spectral binding measurements were carried out on a Cary 60 UV−visible spectrophotometer (Agilent). Spectral binding titrations of CYP505A30 and its heme domain with saturated linear chain fatty acids (C10−C18), arachidonic acid, and NPG (Sigma, Poole, U.K. and Cambridge Bioscience, Cambridge, U.K.) were performed at 25 °C in buffer A using 1 mL volume samples containing 4−6 μM enzyme in a 1 cm pathlength cuvette. Substrate stock solutions were made at 1−20 mM in 80% ethanol in H2O. Prior to the binding titrations, all enzyme samples in buffer A were passed through a Lipidex column of dimensions 5 × 1 cm2 (PerkinElmer, Cambridge, U.K.) to remove any residual lipid retained during protein purification. The CYP505A30 sample recovered from the column was in an extensively LS ferric state, as established by its UV−visible spectrum. Titrations were performed by stepwise additions of aliquots (0.1−1 μL) of the fatty acids to the CYP505A30/heme domain sample (substrate additions did not exceed 1% of the total sample volume). UV−visible spectra (750−250 nm) were recorded for the ligand-free enzyme and following each addition of substrate. Substrate additions were continued until no further shifts in the heme spectrum occurred. Difference spectra were generated at each stage in the titration by subtraction of the spectrum for the ligand-free enzyme from each subsequent ligand-bound form of CYP505A30/heme domain spectrum produced. The wavelengths of the absorbance minimum and maximum were identified from the difference spectra, and a maximal induced absorbance change (ΔA_{max}) at each point in the titration was determined by subtracting the absorbance at the wavelength minimum from that at the absorbance maximum, using the same wavelength pair throughout each titration. ΔA_{max} values were then plotted against ligand concentration, and data were fitted using either a hyperbolic (Michaelis−Menten) function or the Morrison function for tight-binding ligands, to determine dissociation constants (K_{D} values), as described previously. Morrison’s equation was used in preference when the K_{D} value was ≤5x the P450 concentration. All data fitting was done using Origin software (OriginLab, Northampton, MA).

**Steady-State Enzyme Assays.** The steady-state activities of CYP505A30 were determined using cytochrome c and FeCN as electron acceptors, and NAD(P)H as electron donors. Experiments were done using a Cary 300 (Varian) dual-beam spectrophotometer. To account for low rates of nonenzyme-mediated (i.e., NAD(P)H-dependent) reduction of electron acceptors, assays were performed in the dual-beam spectrophotometer alongside a reference cuvette containing the same components but no enzyme, and by replacing the same volume of buffer in place of the enzyme. The reaction was initiated by the simultaneous addition of NAD(P)H to both cuvettes. Cytochrome c (horse heart; Sigma-Aldrich) reduction rate constants were measured at 550 nm (Δε_{550(red-ex)} = 22 640 M^−1 cm^−1) and ferricyanide reduction rate constants were measured at 420 nm (Δε_{420(red-ex)} = 1020 M^−1 cm^−1). Reactions were performed in 1 mL of buffer A at 25 °C using 20−50 nM CYP505A30. The k_{cat} Values for cytochrome c and ferricyanide were determined at a fixed and near-saturating concentration of NAD(P)H (200 μM for NADPH and 800 μM for NADH). The K_{M} values for NADH and NADPH were measured at near-saturating concentrations of cytochrome c and FeCN (400 μM for cytochrome c and 2 mM for FeCN). Steady-state kinetic assays were also done at 25 °C in buffer A using a series of saturated fatty acids (C12:0, C13:0, C14:0, and C15:0) and the polysaturated arachidonic acid (a good substrate for P450 BM3). Reactions were initiated by the addition of 200 μM NADPH to 1 mL of buffer A containing 20−50 nM CYP505A30 and various concentrations of lipid substrates. Rate constants at different substrate concentrations were determined using Δε_{440} = 6.210 M^−1 cm^−1 for NADPH oxidation. Origin software was used in data fitting to derive K_{M} and k_{cat} values from steady-state assays, using the Michaelis−Menten equation to fit the data (OriginLab, Northampton, MA).

**Stopped-Flow Kinetic Studies on CYP505A30.** Single-turnover stopped-flow kinetic studies were done to determine the kinetics of CYP505A30 flavin reduction using NAD(P)H, similarly to previous experiments done on P450 BM3. Reactions were performed using an Applied Photophysics SX.18 MVR stopped-flow spectrophotometer (Leatherhead, U.K.) contained within an anaerobic glovebox under a nitrogen environment, and with oxygen levels maintained below 2 ppm (Belle Technology, Weymouth, U.K.). Stopped-flow spectral data accumulation was done at single wavelengths and by using a photodiode array detector on the same instrument for full spectral accumulation. Kinetics of CYP505A30 flavin reduction by NADH and NADPH were monitored at 475 nm and at 10 °C in buffer A. The final CYP505A30 concentration in the stopped-flow mixture was 10 μM and final NADPH and NADH concentrations were in the range from 60 to 1200 μM. Subsequent studies of the NADPH-dependent flavin reduction of CYP505A30 were done at 5 °C intervals between 10 and 35 °C in buffer A, and with final enzyme and NADPH concentrations of 10 μM and 1 mM, respectively.

**Thermostability Studies on CYP505A30.** Thermal fluorescence studies were done using SYPRO Orange (SO) (Life Technologies, Carlsbad) to report on CYP505A30 thermal stability. SO used for thermal unfolding was supplied as a 5000× stock solution in anhydrous DMSO, and was
diluted to a 25× working concentration for all samples. Samples of CYP505A30 (1 mg/mL) were prepared in a range of 50 mM buffer solutions (containing various additives) from the JBS solubility screen kit (Jena Bioscience GmbH, Jena, Germany). Samples were analyzed in a 96 well thin-wall PCR plate (Bio-Rad) sealed with optical-quality sealing tape (Bio-Rad). The plates were heated in an iCycler iQ RT PCR detection system (Bio-Rad) from 20 to 90 °C in increments of 0.2 °C, and fluorescence emission intensities were monitored simultaneously with a charge-coupled device camera. The wavelengths used for excitation and emission of SO were 492 and 610 nm, respectively. Thermal unfolding was measured as a function of fluorescence emission at 610 nm, enabling the production of a fluorescence melt curve of relative fluorescence units against temperature. The melting temperature ($T_m$) in each case was determined from the peak of the first derivative plot of the fluorescence melt curve. Further studies were done to explore the thermostability of the CYP505A30 Fe$^{II}$–CO complex and its transition between the thiolate-coordinated P450 state and the thiol-coordinated P420 state. A solution of ∼2.5 μM CYP505A30 in buffer A containing 200 mM KCl was made under anaerobic conditions and a P450 complex at ∼450 nm was prepared through addition of a few grains of sodium dithionite and slow bubbling of the sample with CO. Thereafter, the temperature was increased in 2.5 °C increments between 25 and 60 °C with 30 s delays at each new temperature prior to spectral data collection. The extent of the P450-to-P420 transition was established by plotting the observed absorbance change ($\Delta A_{420} - \Delta A_{450}$) to obtain a midpoint ($T_m$) for the transition.

MALLS Analysis. MALLS data were collected using a DAWN EOS MALLS detector (Wyatt Technology, Santa Barbara) using 5 mg protein samples in buffer A containing 200 mM KCl, immediately following an integrated Superdex 200 gel filtration step (GE Healthcare). MALLS data were collected at a 1 s interval rate using a K5 cell type and a laser wavelength of 658 nm.

EPR Studies on CYP505A30 and Its Heme Domain. Continuous wave EPR spectra were recorded at the X-band (~9.4 GHz) using a Bruker ELEXYS ES50/ES80 EPR spectrophotometer (Bruker GmbH, Rheinstetten, Germany). Temperature control was effected using an Oxford Instruments ESR900 helium flow cryostat coupled to an ITC503 controller from the same manufacturer. EPR spectra for both CYP505A30 and its heme domain were recorded at 77 K with a microwave power of 2.08 mW. Samples contained enzyme at a concentration of 225 μM in buffer A containing 500 mM KCl. Fatty acid substrates were added to a concentration of 1 mM. EPR spectra for flavin SQ analysis were collected at 77 K using intact CYP505A30. To enable formation of the flavin SQ species, CYP505A30 samples were incubated with NADPH (2 mM) and incubated for 30 s, 5 min, and 10 min in each case at ambient temperature, prior to freezing of the samples in liquid nitrogen.

CYP505A30 Redox Potentiometry. Redox titrations were performed for both CYP505A30 and its heme domain in an anaerobic glovebox (Belle Technology) under a nitrogen atmosphere, with O$_2$ levels maintained below 2 ppm. All solutions were degassed by sparging with N$_2$ gas. The proteins were applied to an Econo-Pac 10DG desalting column (Bio-Rad, Hemel Hempstead, U.K.) in the anaerobic box, which was pre-equilibrated with degassed buffer A containing 200 mM KCl (redox buffer) enabling transfer of the enzymes into the anaerobic redox titration buffer. For the substrate-bound heme domain titration, the redox buffer also contained 10% glycerol (v/v) and 1 mM arachidonic acid. The proteins (∼10–20 μM in 5 mL of redox buffer) were titrated electrochemically according to the method of Dutton, using sodium dithionite as a reductant.$^{53}$ Dithionite was delivered in approximately 0.1–0.5 μL aliquots from concentrated stock solutions (typically ∼50 mM). Mediators were added to facilitate electrical communication between the redox cofactors in the enzyme and the electrode, prior to titration. 2 μM phenazine methosulfate, 5 μM 2-hydroxy-1,4-naphthoquinone, 0.5 μM methyl viologen, and 1 μM benzyl viologen were included to mediate in the range between +100 and −480 mV. The electrode was allowed to stabilize between each dithionite addition and spectra (250–750 nm) were recorded using a fiber optic probe linked to the Cary UV-50 Bio UV–visible scanning spectrophotometer and immersed in the redox protein solution. Data manipulation and analysis were performed using Origin software. For titration of intact CYP505A30, absorbance values at 475 nm were plotted against potential to follow flavin reduction. For the redox titration of the substrate-free heme domain, difference spectra were generated by subtraction of the spectrum for the OX heme domain from each subsequent heme domain spectrum collected during the heme iron reduction process. The overall absorbance change occurring during heme domain reduction was calculated by subtracting the absorbance minimum ($A_{\text{rough}}$) from the maximum ($A_{\text{peak}}$) to give $\Delta A_{\text{max}}$ using the same wavelength pair throughout. The $\Delta A_{\text{max}}$ was then plotted against the applied potential and data were fitted using the Nernst equation. For the arachidonic acid-bound heme domain redox titration, absorbance data at 390 nm (at the peak for the substrate-bound form) were plotted versus applied potential, and data were again fitted using the Nernst equation, as described previously.$^{3,39}$ A factor of +207 mV was used to correct for the difference in electrode potential reading between the Ag/AgCl electrode used in the titration and the NHE.

Fatty Acid Oxidation by CYP505A30, and Product Derivatization and Analysis Using GC–MS. Turnover reactions for fatty acid hydroxylation by WT CYP505A30 were carried out at 37 °C with shaking for 30 min. Reaction mixtures contained purified CYP505A30 (100 μM), fatty acid substrate (1 mM lauric acid or myristic acid), NADPH (500 μM), and an NADPH regeneration system (glucose 6-phosphate [7.76 mM], NADP$^+$ [0.6 mM], and glucose-6-phosphate dehydrogenase [0.75 units/mL]) in buffer A and in a final volume of 2 mL. Following completion of the reaction, the protein was precipitated by heating at 100 °C for 10 min and pelleted by centrifugation (4000 g, 25 min, 10 °C). Hydroxylated fatty acid products were isolated from the supernatant by fractionation through a StrataX SPE column (Phenomenex, Macclesfield, U.K.) into mass spectrometry vials. Samples were dried down before derivatization in a rotary evaporator. Derivatization was carried out by adding 0.5 mL of N$_2$O-bis(trimethyl)-trifluoroacetamide/0.1% trichloromethylsilane (Sigma, Poole, U.K.) to the dried sample and by incubating at 60 °C for 60 min. Product analysis was done using a Thermo Fisher DSQ II GC/MS instrument with a 30 m × 0.25 mm × 0.25 μm ZB5MS GC column (Phenomenex). Injection was cold on-column. The oven program was set so that an initial temperature of 50 °C was ramped at 23 °C/min to 310 °C post-injection. Electronic ionization was used, and ions in the range of 40–640 m/z were scanned at two scans per second.
Materials. Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (Poole, U.K.) and were of the highest grade available.

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Notes
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ABBREVIATIONS
BM3, Bacillus megaterium flavocytochrome P450 BM3 (CYP102A1); CT, charge-transfer; CPR, cytochrome P450 reductase; CYP505A30, M. thermophila P450–CPR fusion enzyme; EPR, electron paramagnetic resonance; FeCN, potassium ferricyanide; HS, high-spin; LS, low-spin; MALLS, Multiangle laser light scattering; NHE, normal hydrogen electrode; SQ, semiquinone

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