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Supplementary Information

Structure and Peroxidase Activity of Ferric *Streptomyces clavuligerus orf10*-encoded Protein P450CLA: UV-Visible, CD, MCD and EPR Spectroscopic Characterization

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Experimental

Cloning and construction of expression systems

S. clavuligerus ATCC 27064 cells were cultivated for 72 h at 28 °C in ISP2 medium for the isolation of genomic DNA. The cells were collected via centrifugation and washed with phosphate-buffered saline (50×10^{-3} mol L^{-1} sodium phosphate, 150×10^{-3} mol L^{-1} NaCl). Approximately 200 mg (wet weight) were used for DNA extraction, using the Wizard Genomic DNA extraction kit (Promega). Oligonucleotides were designed to incorporate an *Nde*I restriction site at the 5' end of the PCR product (ORF10_NdeI_FW: 5'GCAGCCATATGAACGAGGCAGCGCCTC AGTCCGACCAG3') and an *Xho*I site at the 3' end, right after the predicted *orf10* stop codon (ORF10_XhoI_RV: 5' GTGGTGCTCGAGTCACCAGGTCACCGGGAG GGCGCCGAGGCC3'). PCR was performed using a Mastercycler thermocycler (Eppendorf - Hamburg, Germany), programmed to execute a 98 °C initial 7 min denaturing step, followed by 40 cycles of 94 °C for 30 s, 75 °C for 1 min, 72 °C for 2.5 min, and a final elongation step of 10 min at 72 °C. *Orf10* was amplified using 500 ng of *S. clavuligerus* genomic DNA and 100 pmol of each primer in a 50 µL reaction containing reagents recommended by the supplier (*Taq*-HiFi, Fermentas), except for DMSO which was added up to 5% v/v. The amplification product was purified and cloned into pTZ57R/T (Fermentas) and the recombinant plasmid was transformed into *E. coli* DH5α for amplification and plasmid extraction. Plasmid DNA sequencing was performed using standard methodology,[1] using an ABI Prism 377 (Perkin Elmer, Waltham, MA), according to the standard manufacturer's recommendations. DNA encoding CYP105M1 was excised from pTZ57R/T using *Nde*I and *Xho*I (restriction enzymes) and subcloned into pET28a (Novagen, EMD Chemicals Inc., San Diego, CA). The resulting plasmid provides IPTG-induced expression of *orf10*, with an *N*-terminal fused hexahistidine-tag.

Recombinant expression and protein purification

The expression vector was transformed into *E. coli* Rosetta (DE3) (Novagen) and plated onto LB-agar with the proper antibiotics added. Expression was carried out overnight at 18 °C in LB containing 30 µg mL^{-1} kanamycin, 0.1×10^{-3} mol L^{-1} IPTG, 1×10^{-3} mol L^{-1} 5-aminolevulinic acid and 0.1 g L^{-1} $FeCl_3$. Except for the antibiotic, all additives were added to the culture during the mid-log *E. coli* growth phase (OD_{600} = 0.5), using filter-sterilized frozen stock solutions. Cells from 1 L of culture were collected by centrifugation and resuspended in 25 mL of 50×10^{-3} mol L^{-1} Tris-HCl, pH 8.0, containing 100×10^{-3} mol L^{-1} NaCl. The cells were lysed using 20 ultrasound pulses of 30 s intercalated by 1 min resting times in an ice bath. Insoluble cellular debris was separated by centrifugation and discarded. The soluble extract was loaded onto a 5 mL Ni-NTA column (Novagen) pre-equilibrated with the same running buffer, for IMAC purification. The column, with bound protein, was washed with 20 volumes of 20×10^{-3} mol L^{-1} imidazole in 50×10^{-3} mol L^{-1} Tris-HCl, pH 8.0 buffer, containing 100×10^{-3} mol L^{-1} NaCl, for the

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removal of nonspecifically-bound contaminants. Purified recombinant CYP105M1 (P450CLA) was eluted in 5 volumes of 50×10^{-3} mol L^{-1} Tris-HCl, pH 8.0 buffer, containing 100×10^{-3} mol L^{-1} NaCl and 500×10^{-3} mol L^{-1} imidazole. The protein eluted from the Ni-NTA resin was dialyzed against 20×10^{-3} mol L^{-1} Tris-HCl, pH 8.0 buffer and loaded into a MonoQ 5/5 column (GE Healthcare, Piscataway, NJ) pre-equilibrated with the same buffer. Purified protein was eluted from the ion-exchanger in a linear gradient of 0-1 mol L^{-1} NaCl controlled by an Äkta purifier chromatograph (GE Healthcare), monitoring the absorbance of the eluate at 280 and 417 nm. The yield of purification was 1 L of culture results in approximately 13 mg of protein. P450CLA was stored at −20 °C in 50×10^{-3} mol L^{-1} Tris-HCl, pH 8.0, 100×10^{-3} mol L^{-1} NaCl, 10% v/v glycerol, for later experiments. Aliquots were reserved for SDS-PAGE analysis.

Results

Figure S1 compares the amino acids primary sequence of CYP105M1 with two others P450s: CYP105A1 and CYP101A1 (P450CAM).

Figure S2 shows the SDS PAGE of P450CLA to homogeneity using ion-exchange chromatography and the crude soluble cell lysate obtained by the IMAC procedure.

Figure S3 shows the far UV CD spectrum of P450CLA after purification.

Reference

1. Sanger, F.; Nicklen, S.; Coulson, A. R.; *Proc. Natl. Acad. Sci. U. S. A.* **1977**, *74*, 5463.

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CYP105M1--MNEAAPQ-SDQVAPAYPMHRVCPVDPPPQLAGLRSQK-AASRVTLWDGSQVWLVTSHAG  57
CYP105A1--MTDTATTPQTTDAPAFPSNRSCPYQLPDGYAQLRDTPGPLHRVTLYDGRQAWVVTKHEA  59
CYP101A1TTETIQSNANLAPLPPHVPEHLVFDFDMYN-PSNLSAGVQEAWAVLQESNVPDLVWTRCNG  60

CYP105M1ARAVLGDRRFTAVTSAPGFPML TRTSQLVRANPESASFIR--MDDPQHSRLRSMLTRDFLA 116
CYP105A1ARKLLGDPRLSSNRTDDNFPAT SPRFEAVRESPQ--AFIG--LDPPEHGTRRRMTISEFTV 116
CYP101A1GHWIATRGQLIREAYEDYRHFS SECPFIPREAGEAYDFIPTSMDPPEQRQFRALANQVVGM 121

CYP105M1RRAEALRPAVRELLDEILGGLVKGERPV DLVAGLTIPVPSRVITLLFGAGDDRRE FIEDRS 177
CYP105A1KRIKGMRPEVEEVVHGFLDEMLAAGPTA DLVSQFALPVPSMVICRLLGVPYADHE FFQDAS 177
CYP101A1PVVDKLENRIQELACSLIESLRPQGQ-CNFTEDYAEPFPIRIFMLLAGLPEEDIP HLKYLT 181

CYP105M1AVLIDRGYTPEQVAKARDELDGYLRELVEERIENPGTDLISRLVIDQVRPGHLRVEEMVPM 238
CYP105A1KRLVQS-TDAQSALTARNDLAGYLDGLITQFQTEPGAGLVGALVADQLANGEIDREELIST 237
CYP101A1DQMTRP-DGSMTFAEAKEALYDYLIPIIEQRRQKPGTDAISIVANGQVNGRPITSDEAKRM 241

CYP105M1CRLLLVAGHGTTTSQASLSLLSLLTDPELAGRLTEDPALLPKAVEELLRFHSIVQNGLARA 299
CYP105A1AMLLLIAGHETTASMTSLSVITLLDHPEQYAALRADRSLVPGAVEELLRYLAIADIAGGRV 298
CYP101A1CGLLLVGGLDTVVNFLSFSMEFLAKSPEHRQELIERPERIPAACEELLRRFSLVADG--RI 300

CYP105M1AVEDVQLDDVLIRAGEGVVLSLSAGNRDETVFPDPDRVDVDRDARRHLAFGHGMHQCLGQW 360
CYP105A1ATADIEVEGQLIRAGEGVIVVNSIANRDGTVYEDPDALDIHRSARHHLAFGFGVHQCLGQN 359
CYP101A1LTSDYEFHGVQLKKGDQILLPQMLSGLDERENACPMHVDFSRQKVSHTTFGHGSHLCLGQH 361

CYP105M1LARVELEEILAAVLRWMPGARLAVPFEELDFRHEVSSYGLGALPVTW--------  407
CYP105A1LARLELEVILNALMDRVPTLRLAVPVEQLVLRPGTTIQGVNELPVTW--------  406
CYP101A1LARREIIVTLKEWLTRIPDFSIAP--GAQIQHKSGIVSGVQALPLVWDPATTKAV  414
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Figure S1. Multiple alignment of CYP105M1 with other P450s. The predicted primary sequence for CYP105M1 was aligned to CYP105A1 and CYP101A1 (P450CAM) for a similarity search. CYP105M1 has shown 41% identity to CYP105A1 and 22% to CYP101A1. Identical residues are highlighted with a black background, conserved substitutions have dark gray backgrounds and semi conservative substitutions have light gray backgrounds. The identified heme coordination motif is underlined, and the conserved EXXR sequence is dot underlined in the CYP105M1 sequence.

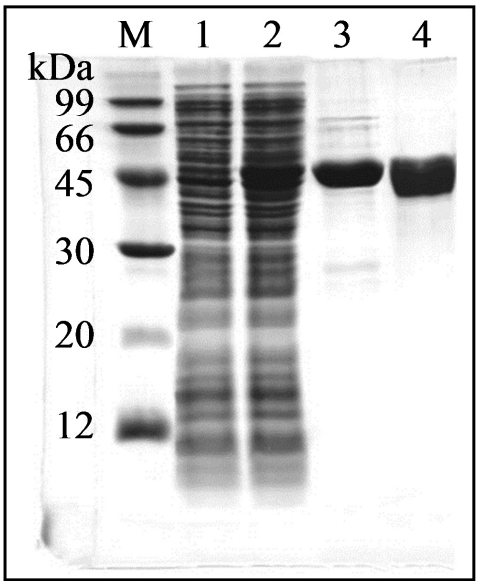


Figure S2. Expression and purification of P450CLA as analyzed using SDS-PAGE: (lane M) Molecular mass standards, (lanes 1 and 2) whole cell extracts from Rosetta (DE3) transformed with the P450CLA expression plasmid prior to IPTG induction and after overnight induction, respectively, (lane 3) IMAC purification sample, (lane 4) P450CLA after purification using ion exchange chromatography.

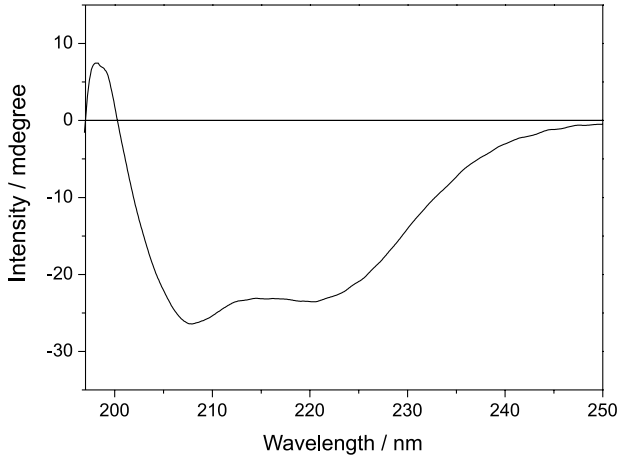


Figure S3. Far-UV CD of P450CLA. Far UV CD of purified P450CLA was observed in the wavelength range 250-195 nm. For secondary structure determination, a 0.17 mg mL^{-1} protein solution in 50×10^{-3} mol L^{-1} Tris-HCl pH 8.0, 50×10^{-3} mol L^{-1} NaCl, 10% v/v glycerol was measured in the far-UV wavelength range (250-195 nm) and recorded as an average of 8 scans at 100 nm min^{-1} using a 0.1 nm data pitch dump. Secondary and tertiary structural predictions were done using the CDPro software package.

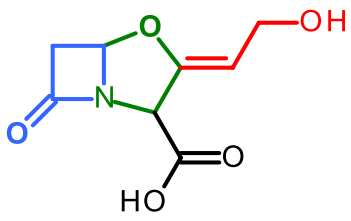
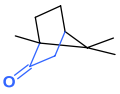
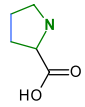
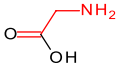
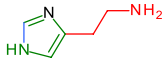
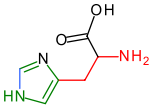
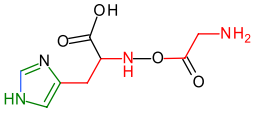
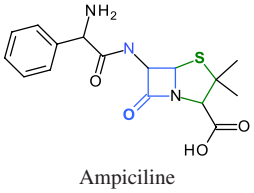


Figure S4. Chemical structure of clavulanic acid with the β-lactam, oxazole and alkyl amine-derived (alcohol) moieties highlighted as the respective blue, green and red components. The carboxylic acid moiety is represented as the black structural component.

Table S1. Spectral changes of P450CAM promoted by different exogenous ligands

Ligand	Mimicked structural moiety	Spectral changes	Comments
 Camphor	β-lactam ring	3 nm blue shift of the Soret band.[a] No significant change of Q bands. No significant spectral changes before dithionite addition.	Natural substrate of P450CAM from *Pseudomonas putida.*
 Imidazole	oxazole	6 nm red shift of Soret, Q_α and Q_β bands. ε decrease of Soret band.[b] 8 nm red shift of Soret band, 8 and 12 nm blue shift of respective Q_β and Q_α bands and inversion of $\varepsilon Q_\alpha/\varepsilon Q_\beta$ ratio.[b]	Oxazole with oxygen replaced by nitrogen. Similar to spectral changes observed for P450CAM.
 Proline	oxazole	No spectral change	Oxazole with oxygen replaced by carbon (pyrrolidine ring).
 Glycine	amino alkyl	2 nm red shift of the Soret band.[b]	Absence of a cyclic structure.
 3-amino-1,2,4-triazol	oxazole and amino alkyl	2 nm red shift of the Soret band without changes in Q bands.[b] No change in Soret band and 15 and 13 nm blue shift of Q_α and Q_β bands, respectively.[a]	Fungicide activity associated to P450 inhibition.
 Histamine	oxazole and amino alkyl	ε increase and 6 nm red shift of the Soret band. No significant change of Q bands.[b] 8 nm red shift of Soret band, 8 and 12 nm blue shift of respective Q_β and Q_α bands and inversion of $\varepsilon Q_\alpha/\varepsilon Q_\beta$ ratio.[a]	Amino alkyl associated to a cyclic structure.
 Histidine	oxazole and amino alkyl	4 nm red shift of the Soret and Q bands.[b] 2 nm red shift of Soret band, 10 and 14 nm blue shift of respective Q_β and Q_α bands without inversion of $\varepsilon Q_\alpha/\varepsilon Q_\beta$ ratio.[a]	Amino alkyl associated to a cyclic structure.
 Glycylhistidine	oxazole and amino alkyl	4 nm red shift of the Soret and Q bands.[b] Spectral changes reverted after dithionite addition.	Structure mimicking possible intermediates of the clavaldehyde synthesis: *N*-glycyl-clavaminic acid and N-acetyl-glycyl-clavaminic acid.
 Ampiciline	β-lactam and oxazole	4 nm red shift of the Soret without significant changes of Q bands.[b] 4 nm red shift of the Soret 4 and 10 nm blue shift of respective Q_β and Q_α bands without inversion of $\varepsilon Q_\alpha/\varepsilon Q_\beta$ ratio.[a]	Oxazole ring substituted by thiazole ring.

[a]Changes observed after heme iron reduction by dithionite; [b]changes observed in the resting form.